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Molecular pharmacology
of histamine H₃ receptor ligands
and their implications in neurogenetic disorders

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Dedicated to my brothers,

Niclas
&
Maximilian

and to Steven,
without whom this journey would have ended too early.

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Abstract

The histamine H₃ receptor (H₃R) regulates the release of histamine and various other central transmitters such as acetylcholine, norepinephrine, glutamate and γ-aminobutyric acid. Therefore, the receptor plays a part in numerous neurologic processes. Consequently, H₃R ligands have emerged as promising tools in neurogenetic disorders such as schizophrenia and Gilles de la Tourette syndrome (GTS), in the neurodegenerative conditions Alzheimer's disease (AD) and Parkinson's disease (PD), in autism-spectrum disorder (ASD) and the rare Prader–Willi syndrome (PWS). However, only two H₃R inverse agonists have been marketed to date of which only one emanated from dedicated drug development campaigns.

Firstly, this work aimed to identify novel strategies in the molecular pharmacological characterisation of H₃R ligands to elucidate their binding behaviour at the receptor. Secondly, novel H₃R ligands should be discovered, without or with strategical co-activity at relevant other targets to address neurogenetic disorders following the multitargeting ligand (MTDL) concept.

A novel homogeneous fluorescence polarisation approach revealed marked affinity differences between prominent receptor ligands, although, they are usually determined as highly-potent ligands by common reference methods. This study points towards differences in target binding modes of various receptor ligands and emphasises the importance of non-traditional methods and receptor-labelling tracers in molecular pharmacology of H₃R. Further studies revealed limits of the bioisosteric replacement according to the common pharmacophore blueprint for the medicinal chemistry of H₃R ligands and solutions for the design of potent H₃R drug candidates.

Furthermore, hit and lead compounds were discovered, but also drug candidates with co-activities at dopamine D₂/D₃ receptor (D₂R/D₃R) subtypes and enzymes involved in neurotransmitter degradation. Hit structures with combined H₃R/D₂R/D₃R activity showed novel drug design strategies for MTDLs against schizophrenia and GTS. New lead and drug candidates with combined activity at monoamine oxidase B and cholinesterases (ChE) emerged as potential tools against PD and AD. Furthermore, this study demonstrates the applicability of H₃R/ChE ligands in an *in vivo* model of ASD.

Finally, a novel MTDL approach to neurogenetic disorders was proposed, bridging to the recently identified involvement of the histone H3 methyltransferase G9a in AD, ASD and especially in PWS. Strategic manipulation of neurotransmitter levels, combined with the manipulation of cellular neuronal function, present multi-strategical perspectives to multifactorial disorders. Thus, combined H₃R antagonists/G9a inhibitors represent a single strategy against a plethora of neurogenetic disorders.

Zusammenfassung

Der Histamin-H₃-Rezeptor (H₃R) moduliert die Freisetzung von Histamin und weiteren zentralen Neurotransmittern, wie Acetylcholin, Noradrenalin, Glutamat und γ-Aminobuttersäure. Deshalb ist dieser Rezeptor in zahlreiche neurologische Prozesse involviert. Folglich haben sich H₃R Liganden als vielversprechender Ansatz in neurogenetischen Erkrankungen erwiesen, wie Schizophrenie, Gilles de la Tourette Syndrom (GTS), in den neurodegenerativen Erkrankungen wie dem Alzheimer und Parkinson Syndrom (AS und PS), in der Autismus-Spektrum-Störung (ASS) und dem seltenen Prader–Willi-Syndrom (PWS). Jedoch sind bis heute nur zwei inverse Agonisten des H₃R zugelassen, wovon nur einer aus gezielten Arzneistoffentwicklungsprogrammen stammt.

Als Erstes verfolgte diese Arbeit das Ziel, neue Strategien zur molekular-pharmakologischen Untersuchung des Rezeptorbindungsverhaltens von H₃R Liganden zu finden. Zweitens sollten neue H₃R Liganden entdeckt werden, um mit gezielter Koaktivität an relevanten weiteren Zielstrukturen im Rahmen des Multi-Targeting-Ligand (MTL) Konzeptes neurogenetische Erkrankungen zu adressieren.

Eine neue, kontinuierliche Fluoreszenzpolarisationsmethode zeigte deutliche Affinitätsunterschiede von Rezeptorliganden, die mittels Standardmethoden gewöhnlich als hoch potente Liganden charakterisiert werden. Diese Studie deutet auf Unterschiede im Rezeptorbindungsverhalten verschiedener Liganden hin und spricht für den Einsatz neuartiger Methoden in der Molekularpharmakologie des H₃R. Weitere Studien zeigten Grenzen des bioisosteren Ersatzes nach der Pharmakophor-Blaupause für die medizinische Chemie von H₃R Liganden auf und Lösungen für das Design potenter H₃R Arzneistoffkandidaten.

Ferner wurden Hit- und Leitstrukturen, aber auch Arzneistoffkandidaten mit Koaktivität an Dopamin D₂/D₃ Rezeptorsubtypen und Enzymen entdeckt, die für den Neurotransmitter-Abbau verantwortlich sind. Hitstrukturen mit kombinierter H₃R/D₂R/D₃R Aktivität zeigten neue Strategien für die Entwicklung von MTDLen gegen Schizophrenie und GTS auf. Neue Leitstrukturen und Arzneistoffkandidaten mit kombinierter Aktivität an Monoaminoxidase B und Cholinesterasen (ChE) traten als mögliche Ansätze gegen PS und AS hervor. Darüberhinaus konnte mittels *in vivo* Studien die Anwendbarkeit von H₃R/ChE-Liganden in der ASS aufgezeigt werden.

Abschließend wurde ein neuer MTDL Ansatz für neurogenetische Erkrankungen vorgestellt, als Brückenschlag zu den kürzlich identifizierten Verbindungen der Histon-H3-Methyltransferase G9a zu dem AS, der ASS und insbesondere, dem PWS. Die strategische Manipulation des Neurotransmitterhaushalts, kombiniert mit der Manipulation zellulärer, neuronaler Funktionen, eröffnet multistrategische Perspektiven für multifaktorielle Erkrankungen. Deshalb stehen H₃R Antagonisten/G9a Inhibitoren für eine einzige Strategie gegen zahlreiche neurogenetische Erkrankungen.

1. Introduction

1.1. Physiological implications of the histamine H$_3$ receptor

Histamine (HA) was discovered by Sir Henry Dale, whose findings date back to the year 1907 (Riley, 1965). However, these credits must be taken with care. While he admitted that HA was already known from a synthesis project in the same year, he was initiating the isolation of the compound from the *Wernich's* preparation - a putrefied extract of ergot-alkaloids in which he assumed the presence of a compound with unprecedented pharmacological effects. In the same year of publishing this procedure, it was another researcher who identified HA in putrefied samples of L-histidine. However, it was Dale who correlated the pharmacological effects of specific fractions from the *Wernich's* preparation with known effects of an endogenously released compound that exerts anaphylaxis and with the then-recent identification of HA (Riley, 1965). Several decades had passed before HA was established as a neurotransmitter in the late 1970s, although several other neuronally released compounds have manifested well before (Tiligada et al., 2020). In contrast to other neurotransmitters such as norepinephrine (NE), HA is a hormone that exerts both, endocrine and neurohumoral, actions by the same chemical entity (Tiligada et al., 2020).

HA exerts its actions among receptor subtypes of the class-A of G protein-coupled receptors (GPCRs). Thereof, four histaminergic ones are known to date (H$_1$R, H$_2$R, H$_3$R and H$_4$R). They differ in tissue distribution within the mammalian organism as well as in their coupling to G proteins and other signalling pathways (Table 1). The subtypes H$_1$R, H$_2$R and H$_3$R shows relevant participation in the signalling of brain HA. The role of the H$_4$R, which shows high structural similarity to H$_3$R, is considered without functional expression in neurons; however, this is still a topic of discussion (Schneider et al., 2016).

Table 1. Coupling and tissue distribution of HA receptor subtypes.

	H$_1$R	H$_2$R	H$_3$R	H$_4$R
Main coupling partners[0]	Gα_q/Gα_{11} β-arrestin2	Gα_s β-arrestin2	G$\alpha_{i/o}$	G$\alpha_{i/o}$ β-arrestin2
Tissue distribution[0]	Neuronal (CNS), lung, blood vessels	Neuronal (CNS), stomach, heart	Neuronal (CNS)	Bone marrow & haematopoietic cells

Abbreviations: CNS = central nervous system
References: (Panula et al., 2015; Southan et al., 2015; Bosma et al., 2016; Pandy-Szekeres et al., 2018)

As presynaptic receptors were found for other neurotransmitters before, the paradigm of an autoreceptor for HA has evolved concurrently with the discovery of neuronal HA. Therefore, the disclosure of H$_3$R and its characteristics was concluded from depolarisation-induced [^{3}H]HA release from cortical slices

of the rat and in the human brain (Arrang et al., 1983; Arrang et al., 1988). In detail, Arrang and co-workers found that

    i.   the release of [^{3}H]HA from histaminergic neurons was inhibited in the presence of HA,

    ii.   the effect was abolished by two competitive antagonists,

    iii.   there was no evidence for product repression of L-histidine decarboxylase (HDC) by HA in vitro, and thus, the observed actions were unlikely to be linked to this enzyme,

    iv.   the observed effects were saturable and thus, target mediated,

    v.   the results were indistinguishably linked with an inhibitory process that was not in line with stimulation (Gα_s-mediated or Gα_q-mediated) that is elicited by H₁R or H₂R,

    vi.   the potencies of a series of agonists differed significantly from such for H₁R and H₂R (Arrang et al., 1983).

Additionally, first hints for constitutive activity were revealed by the ability of the antagonists to increase the depletion of [^{3}H]HA, though, this was not fully understood before the emerging of recombinant experimental systems in H₃R pharmacology (Arrang et al., 1983; Morisset et al., 2000).

In contrast to various other GPCRs, for which coupling to various isoforms of G proteins have been described, it was assumed that the H₃R transduced intracellular actions are exclusively mediated by G$\alpha_{i/o}$ and the corresponding G$\beta\gamma$-proteins (Inoue et al., 2019). After previous experiments, direct evidence was provided by Clark & Hill, who found pertussis-toxin sensitivity of agonist-mediated stimulation of ^{35}S-labelled analogue of guanosine 5'-O-[gamma-thio]triphosphate (GTPγ[^{35}S]) binding (Clark et al., 1996). Interestingly, G$\alpha_{i/o}$ coupling is a shared feature of most auto- and heteroreceptors as they predominantly act via inhibition of neurotransmitter-release by a negative feedback mechanism (Hill, 1990).

After receptor activation, H₃R triggers various cell signalling pathways as depicted in **Figure 1**. The heterotrimeric G protein exchanges bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP) and dissociates into subunits G$\alpha_{i/o}$ and G$\beta\gamma$ (right panel, **Figure 1**). G$\alpha_{i/o}$ reduces the activity of adenylyl cyclase and thereby cyclic adenosine monophosphate (cAMP)-elicited pathways. Both G$\alpha_{i/o}$ and G$\beta\gamma$ seem to inhibit voltage-gated Ca^{2+} channels, which decrease Ca^{2+}-influx and vesicular exocytosis (Nieto-Alamilla et al., 2016). G$\beta\gamma$ elicited pathways comprise of mitogen-activated protein kinase (MAPK) and PI3K/PKB/GSK3β (phosphoinositide 3 kinase/protein kinase B/ glycogen synthase kinase 3β), which are essential for antiapoptosis, neuronal development, and it is a mechanism that is supposed to be involved in H₃R-mediated neuroprotection (Bongers et al., 2007).

Moreover, H₃R triggers the release of lipid mediators such as arachidonic acid and may thereby elicit a direct role in neuroinflammatory processes (Bongers et al., 2007)
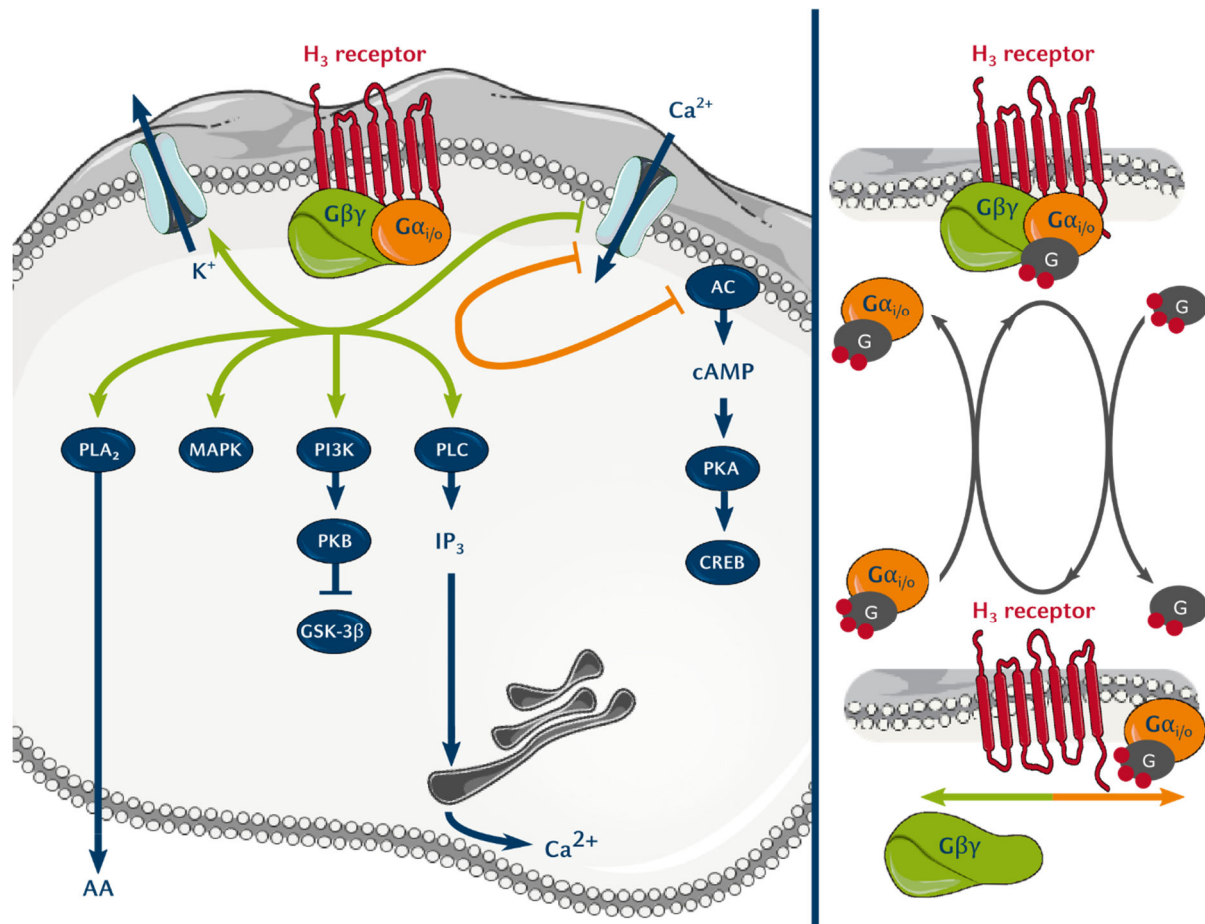
Figure 1. H₃R associated signalling pathways (left panel) and G protein activation (right panel), adapted from Bongers et al. (2007), Panula et al. (2015) and Nieto-Alamilla et al. (2016).
Left panel: green and orange arrows depict Gβγ and Gα-triggered pathways, respectively.
Abbreviations: PLA₂ = phospholipase A₂, AA = arachidonic acid, MAPK = mitogen-activated protein kinase,
PI3K = phosphatidylinositid 3-kinase, PKB = protein kinase B, GSK-3β = glycogen synthase kinase 3β, PLC = phospholipase C,
IP3 = inositol-1,4,5-trisphosphate, AC = adenylyl cyclase, cAMP = cyclic adenosine monophosphate, PKA = protein kinase A,
CREB = cAMP response element-binding protein.
Right panel: grey circles with two and three red dots depict GDP and GTP, respectively.

G protein-coupled inwardly rectifying potassium channels (GIRK) also belong to the Gβγ–associated pathway, mediate membrane hyperpolarisation and consequently, decrease in granular exocytosis (Hibino et al., 2010). Additionally, the typically $G\alpha_{q/11}$ coupled phospholipase C/inositol-1,4,5-trisphosphate (PLC/IP3)-pathway, which mediates Ca^{2+} influx, has been described within recombinant cell lines (Coge et al., 2001). While coupling of Gβγ to this pathway is known but usually less efficient than $G\alpha_{q/11}$ coupling, the relevance for signalling *in vivo* remains to be clarified (Nieto-Alamilla et al., 2016).

Besides G proteins, arrestins have emerged as signalling modulators, which mediate receptor internalisation after prolonged activation. However, this signalling pathway could not be linked to the H₃R yet, while internalisation was shown in some previous studies (Lozeva et al., 2003; Bongers et al., 2007; Riddy et al., 2017).

Histaminergic neurons have been defined as HA producing, storing and releasing neurons which in mammalian brain exclusively emanate from the hypothalamic tuberomammillary nucleus (TMN) (Panula et al., 1984) but show extensive projections to the cerebral cortex, midbrain, cerebellum and stem brain (Haas et al., 2008). TMN neurons receive inputs from areas to which, conversely, histaminergic neurons themselves have projections (Brown et al., 2001), highlighting the regulatory functions of HA in the CNS. This supervisory role becomes even more perspicuous, considering the fine-tuning between the histaminergic and other neurotransmitter systems. As summarised in **Table 2**, the tone of histaminergic neurons is modulated through various neurotransmitter systems. In several cases, TMN neurons are not activated or inhibited directly but through neuronal circuits of non-histaminergic fibres. For example, norepinephrine shows both excitatory and inhibitory modulation of HA release. While the inhibition is performed by activation of α_2 heteroreceptors on terminals of histaminergic neurons, the excitation follows an indirect pathway among γ-amino butyric acid (GABA)ergic terminals in TMN (Gulat-Marnay et al., 1989a).

Table 2. Modulation of TMN-histaminergic neuronal activity.

Afferent origin[1]	Neurotransmitter	Receptor[2]		Effect on histaminergic TMN neurons
DBB, VLPO, LHA	GABA	GABA$_A$R	postsyn.	↓ (direct)
		GABA$_B$R	presyn. ar.	↓ (indirect, via GABA)
VLPO	galanin (from GABAergic fibres)	GalR$_x$	presyn. hr.	↓ (indirect, via GABA)
LPO, LHA	glutamate	NMDAR	postsyn.	↑ (direct)
MPT	acetylcholine (ACh)	M$_1$R, M$_3$R	presyn. hr.	↓ (direct)
		nAChR	postsyn./ax.	↑ (direct)
NCA1/A2	norepinephrine (NE)	α$_2$AR	presyn. hr.	↑ (indirect, via GABA)
PAG (?)	enkephalins	κOR	postsyn.	↓ (direct)
ArcN (?)	nociceptin	ORL$_1$	postsyn.	↓ (direct)
PFA (LHA)	orexin-A/-B (ORX-A/ORX-B)	ORX$_2$R	postsyn.	↑ (direct)
Raphe nuclei	serotonin (5-HT)	5-HT$_{2C}$R	postsyn.	↑ (direct)

[1] <u>Abbreviations</u>: DBB = diagonal band of Broca; (V)LPO = (ventro)lateral preoptic area; LHA = lateral hypothalamic area; MPT = mesopontine tegmentum; NCA1/A2 = Noradrenergic cell group A1/A2 (formatio reticularis); PAG = periaqueductal grey;; ArcN = arcuate nucleus; PFA (LHA) = perifornical area (part of LHA).

[2] <u>Abbreviations</u>: postsyn. = postsynaptic, on histaminergic fibres; presyn. ar./hr. = presynaptic autoreceptor / heteroreceptor; ax. = axonal
<u>Data were extracted from references</u> (Ericson et al., 1989; Gulat-Marnay et al., 1989b; Gulat-Marnay et al., 1990; Arrang et al., 1991; Schönrock et al., 1991; Sherin et al., 1996; Uteshev et al., 1996; Yang et al., 1997; Stevens et al., 1999; Eriksson et al., 2000; Pillot et al., 2002; Yamanaka et al., 2002; Sergeeva et al., 2003; Stevens et al., 2004; Faucard et al., 2006)

The synthesis of HA starts from the naturally occurring semi-essential amino acid (aa) L-histidine, exclusively by HDC (Schwartz et al., 1970). This process is blocked upon H₃R activation (Arrang et al., 1987b). Ahead of cytosolic synthesis, L-amino acid-transporters mediate neuronal L-histidine resorption, and afterwards, the vesicular monoamine transporter 2 mediates the HA uptake into synaptic vesicles (Peter et al., 1994). Vesicular stored HA is released from depolarised histaminergic neurons in a $[Ca^{2+}]$-dependent manner (Arrang et al., 1985). This release is slightly reduced in the presence of exogenously added HA, suggesting an influence of H₃R on Ca^{2+} influx. Additional studies in a perfused system allowed to diminish the contribution of endogenously released HA. There, the potency of exogenously added HA to inhibit HA release was shifted with increased frequency of electric stimulus that raised the assumption for an H₃R reserve within this physiological environment (van der Vliet et al., 1988).

A specific mechanism for a neuronal re-uptake of depleted HA has been doubted as there is a rather slow HA influx (Arrang et al., 1983), despite of an inflicting demonstration of an antagonisable HA enrichment in synaptosomes (Sakurai et al., 2006).

After release of HA into the synaptic cleft, the neurotransmitter is inactivated by the membrane linked HNMT to teMH whose turnover serves as a common surrogate for HA neuronal activity due to prolonged metabolic stability (Schayer et al., 1973; Barnes et al., 2002). The metabolite, but not HA itself, is subsequently further catabolised by diamine oxidase (DAO) or monoaminoxidase B (MAO B), DAO only occurring in the periphery (Burkard et al., 1963; Elsworth et al., 1980; Lin et al., 1991).

H₃R cloning facilitated the disclosure of the **receptor expression** pattern on messenger ribose nucleic acid (mRNA)-level, supplementing previous knowledge from receptor staining studies with radiotracers. In human, the H₃R encoding sequence is localised on chromosomal loci 20q13.32-13.33 and consists of three exons flanked by two introns. Based on indirect mRNA *in situ* hybridisation experiments and upon direct labelling with [³H]NAMH, high receptor densities were determined in the putamen of the corpus striatum with elevated levels in the human brain from Parkinson's disease (PD) (Anichtchik et al., 2001). Interestingly, the corresponding H₃R was not labelled there, but in substantia nigra (SN), pars reticulata (SNr) indicating either the histaminergic innervation of SN or striatonigral projections from presumably GABAergic neurons that co-express H₃R (Anichtchik et al., 2001).

Based on HA-induced GTPγ[³⁵S] binding, slightly lower activity was determined in SNr of PD patients compared to healthy brains (Anichtchik et al., 2001). Low H₃R expression was found in SN, pars compacta (SNc) of healthy humans which produce nigrostriatal dopaminergic output (Pillot et al., 2002). In rat brain, high receptor expression was found in thalamus, caudate nucleus, cerebral cortex, hippocampus, olfactory

tubercle (Lovenberg et al., 1999; Lovenberg et al., 2000). Within the hypothalamus, high receptor expression was found in the TMN as well as in neurons of the ventrolateral preoptic nucleus (VLPO), the suprachiasmatic nucleus (SCN), the lateral hypothalamic area (LHA) and the arcuate nucleus (ArcN) (Pillot et al., 2002). Interestingly, most of such regions are the origin of GABAergic (VLPO, LHA) and glutamatergic (LHA) projections, again emphasising the central regulatory role of HA in the mammalian brain. In the caudal area as well as in many (sub-)thalamic nuclei and the zona incerta, high H_3R mRNA expression can be found in Raphe nuclei, locus caeruleus (LC) and Purkinje layer of the cerebellar cortex. However, in these cases, binding sites were located elsewhere, supporting H_3R expression on serotonergic, noradrenergic, enkephalinergic and melanin-concentrating hormone (MCH) neurons (Pillot et al. 2002).

However, not all of them are linked with modulation of HA release. There are several ways how HA influences neuronal activation, as well, via **H₃ heteroreceptors** that inhibit the release of other neurotransmitters. This mechanism was demonstrated for NE and 5-HT, for striatonigral and striatopallidal GABAergic neurons, as well as in hypothalamic GABAergic neurons (Schlicker et al., 1988; Schlicker et al., 1989; Smits et al., 1991; Yamamoto et al., 1997; Zhou et al., 2006; González-Sepúlveda et al., 2013). Moreover, Schlicker et al. could evidence heteroreceptors on mouse striatal dopamine (DA) neurons, which failed in various previous research projects, presumably due to only mild modulation of striatal DA release in the presence of HA (Schlicker et al., 1993; Chiavegatto et al., 1998). This group assumed that this might be due to a negative interaction between H_3R and DA D_2 receptor (D_2R) that, indeed, could be evidenced 15 years later by the discovery of H_3R/D_2R heteromers (cf. page 19)(Ferrada et al., 2008). Conclusively, striatal DA levels are not influenced by H_3R inverse agonists, but they are elevated in the frontal cortex (Fox et al., 2005; Ligneau et al., 2007a). Functional heteroreceptors exist on corticostriatal glutamatergic neurons (Doreulee et al., 2001; Molina-Hernández et al., 2001), such as in the dentate gyrus and hippocampus that contain relevant circuits for facilitating the consolidation of memory (Brown et al., 1996). Finally, acetylcholine release is modulated by H_3R heteroreceptors on cholinergic neurons and is increased upon H_3R inhibition. However, H_3R activation is not sufficient for a complete abolishment of ACh release (Clapham et al., 1992; Prast et al., 1994).

Besides presynaptic expression of H_3R, evidence for **postsynaptic H₃R** has been made. For example, postsynaptic H_3R were found on MCH neurons (Parks et al., 2014) and are believed to dominate in histaminergic transmission within the caudate nucleus and putamen (Ellenbroek et al., 2014).

The most central function of the brain HA system is the control of arousal which is due to the involvement of the TMN in the **ascending** (non-reticular) **arousal system** (**AAS**, Figure 2)(Lin et al., 1990). A key indicator of arousal is pronounced activity of the layers 2/3 of cerebral pyramidal cells that influenced by
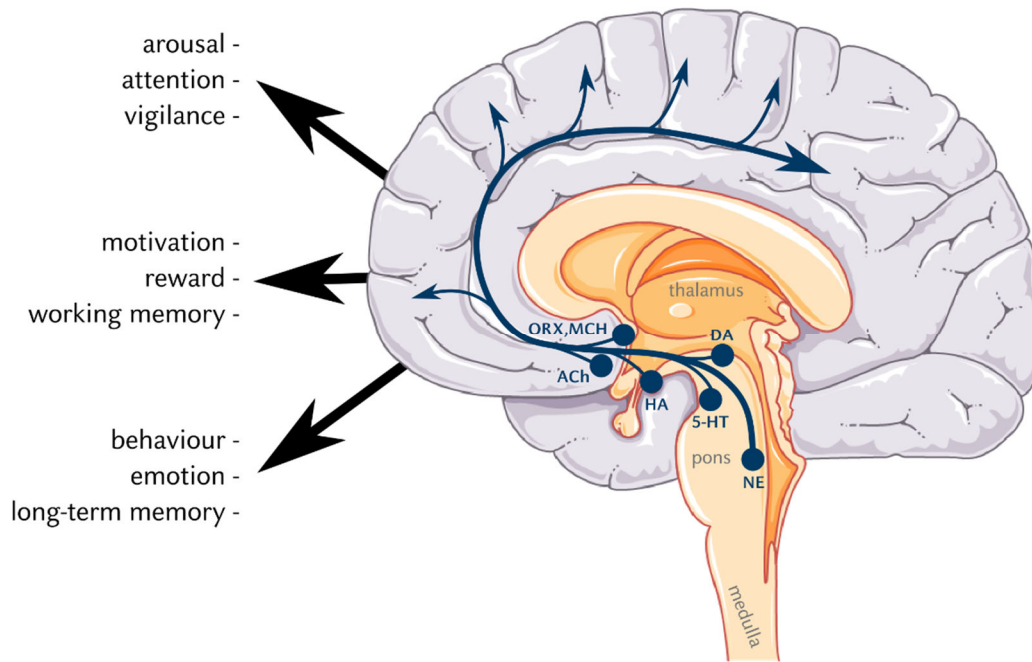
Figure 2. The brain HA system takes up an activator role in the ascending arousal system (AAS, blue arrows). Apart from histaminergic neurons, H$_3$R acts as a versatile regulator of the co-actors (ACh = acetylcholine, ORX = orexin, MCH = melanin-concentrating hormone, DA = dopamine, 5-HT = serotonin, NE = norepinephrine). Graphics was adopted and modified from (Saper et al., 2005; Wallis, 2019).

both the direct ascending reticular activation system and the bypassing AAS (Poulet et al., 2019). The latter makes use of cholinergic projections from pontine and tegmental nuclei to thalamocortical gatekeeper neurons (Saper et al. 2005, Haas et al. 2008). Within the AAS, the histaminergic neurons from TMN exert direct activation of lateral-, medial- and orbitofrontal cerebral cortex, in concerted action with excitatory input towards other neurotransmitter systems that ascend from the LHA (ORX), basal forebrain (ACh) and formation reticularis, in particular the PAG (DA), Raphe nuclei (5-HT) and LC (NE)(Saper et al., 2005). Apart from such activator circuits in AAS, this system is counteracted by inhibitory GABAergic inputs that emanate from the ventrolateral preoptic nucleus (VLPO) and MCH from LHA (Sherin et al., 1996; Jones et al., 2013). Conclusively, activation of AAS correlates with wakefulness, vigilance, memory, cognition, emotion and reward and motivation while deactivation contributes to wake-promotion (Saper et al., 2005; Sander et al., 2008; Edlow et al., 2012).

None of those neurotransmitters mentioned above contribute to the AAS in a simple, additive manner but rather in a comprehensive network of several fine-tuned circuitries. There, HA plays a central role as an activator for the AAS, as a mediator for the other neurotransmitter systems, but as well as a regulator for the feedback inhibition of neurotransmitter release. Consequently, reductions of HA levels, activity of tuberomammillary HA producing neurons and H$_1$R blockade are well known to alter arousal, vigilance, attention, cognition and wakefulness (Parmentier et al., 2016).

In more detail, the **orexinergic system** (hypocretin) which consists of two polypeptides (ORX-A/ORX-B) transmit their signals through two GPCRs, the orexin-1 and the orexin-2 receptor (ORX$_1$R and ORX$_2$R, respectively)(Gotter et al., 2012). Both receptors couple primarily, but not exclusively, to G$\alpha_{q/11}$ proteins through which they exert excitatory input (Pandy-Szekeres et al., 2018). It has been demonstrated that orexin-A liberation in the TMN leads to HA release in the frontal cortex as well as ventrolateral preoptic area, which is followed by an ORX$_2$R-mediated and H$_1$R-dependent boost in arousal (Huang et al. 2001)(Nishino et al., 2001; Yamanaka et al., 2002). ORX$^{-/-}$ mice have shown both reduced wakefulness and **narcolepsy**, and both can be reversed upon ORX administration (Chemelli et al. 1999). Additionally, overall brain HA levels are reduced under impaired ORX$_2$R signalling (Nishino et al., 2001). In contrast, it is noteworthy that H$_1$R knock-out mice show reductions in wakefulness but absence of narcoleptic attacks (Huang et al. 2001). In summary, histaminergic neurons in TMN serve as an interface for excitatory input from orexinergic neurons from the dorsal LHA (De Lecea et al., 1998).

H$_3$R inverse agonists can effectively increase histaminergic tone and thereby, bypass the missing activating input from orexinergic neurons and increase wakefulness under orexin-deficient conditions (De Lecea et al. 1998, Guo et al. 2009). Moreover, they have emerged to bypass orexin deficiency that commonly occurs in neurodegenerative diseases such as Alzheimer´s disease (AD) and PD (section 1.5) where HA neurons have appeared unaffected from the main degradation process (Shan et al., 2015b).

Once activated, the TMN inhibits sleep-promoting MCH neurons to maintain wakefulness (Parks et al., 2014). HA release in or injection into TMN causes strong suppression of the activity of GABAergic VLPO neurons (Lin et al. 1994, Williams et al. 2014). Vice versa, GABAergic interneurons inhibit ascending histaminergic neurons via GABA$_B$ receptors during sleep induction (Okakura-Mochizuki et al., 1996; Stevens et al., 1999).

The H$_3$R exercises its **role in cognition, learning & memory** as a modulator of other neurotransmitter levels acting as heteroreceptors. Among such neurotransmitters, ACh is of particular interest for its spacious cortical activation that leads to normalisation of impaired cognition as well as facilitated perception, association and attention (Sarter et al., 1997). The cholinergic and histaminergic activity is interdependent (Onodera et al., 1998). In particular, it has been shown that increased activity of histaminergic neurons can functionally antagonise memory deficits induced by the muscarinergic cholinergic receptor antagonist scopolamine (Miyazaki et al., 1997; Onodera et al., 1998). Moreover, it has been demonstrated that histaminergic activity is a prerequisite for cholinergic signalling (Munari et al., 2013; Provensi et al., 2016b). As a consequence, H$_3$R inverse agonists have shown to facilitate cholinergic neurotransmission (Abdul Majeed et al., 2017).

Additionally, it seems likely that H$_3$R has a direct contribution to the establishment and the consolidation of memory. This assumption is based on their expression as heteroreceptors on glutamatergic neurons in the dentate gyrus that contains relevant neuronal circuits that have been associated with these processes (Brown et al., 1996; Hainmueller et al., 2020). The pro-cognitive effects of H$_3$R inverse agonists rendered them attractive for application in neurodegenerative disorders and, recently, this strategy was attested effectiveness in paediatric patients suffering from the Prader–Willi **syndrome** which represents a neurogenetic disorder with low prevalence (section 1.5).

From current knowledge, H$_3$R has only a minor part in the modulation of striatal DA release (cf. page 10). However, several studies have raised at least a modulatory participation of H$_3$R **in neurotransmission of basal ganglia** for a good reason, as more than 85% of striatal D$_1$R and D$_2$R-expessing neurons express H$_3$Rs (Hu et al., 2017). Regarding the **role in the regulation of motor activity**, it has been demonstrated that antagonising H$_3$R potentiates locomotion upon DA D$_1$ receptor (D$_1$R) activation that has been attributed to a shifted signal transduction (cf. page 19).

Moreover, *i.c.v.* injections of HA shows a dynamic and complex impact on motor activity that strongly depends on the administration route and involves an H$_1$R-mediated increase and an H$_2$R or (postsynaptic) H$_3$R mediated decrease in motor activity (Bristow et al. 1988, Chiavegatto et al. 1998). A transient phase of decreased locomotion is followed by a period of increased locomotion that may be explained by differential receptor affinity profiles of HA (section 1.3). Additionally, such effects disappear upon H$_1$R$^{-/-}$, H$_2$R$^{-/-}$ or H$_3$R$^{-/-}$ or their blockade, emphasising the central dependence of HA in these regulatory circuitries (Huang et al. 2006, Haas et al. 2008).

In some studies, the H$_3$R inverse agonist thioperamide (cf. page 28) showed a slight increase in locomotor activation whereas the compound and several other H$_3$R inverse agonists failed to do so in other related models (Clapham et al. 1994, Ligneau et al. 2007b). Interestingly, they were able for an effective abrogation of methamphetamine-induced hyperlocomotion (Clapham et al. 1994, Fox et al. 2005, Ligneau et al. 2007b). On a cellular level, it was evidenced that H$_3$R activation results in inhibited GABA release within SNr, in reduced glutamate release from corticostriatal glutamatergic neurons, but not from thalamostriatal ones that are involved in motor symptoms of PD (Molina-Hernández et al., 2001; González-Sepúlveda et al., 2013). Such findings provide several hints for a potential role of H$_3$R in fine-tuning the deregulated glutamatergic and GABAergic system in basal ganglia in PD.

Additional support has been provided by physiological studies, demonstrating an important role of HA in gating sensorimotor circuitries in the striatum. For example, lacking prepulse inhibition (PPI) was observed H$_3$R$^{-/-}$ mice but not in H$_1$R and HDC-deficient mutants (Kononoff Vanhanen et al., 2016). PPI serves as an

in vivo surrogate for schizophrenia and has been validated in clinical tests (Braff et al. 1999). In this model, startle behaviour of a subject to two subsequent acoustic signals is measured with a first, soft signal (i.e., the "prepulse") followed by a louder one. In healthy subjects, startling response to the second signal is usually less than in, e.g., schizophrenia patients due to affected sensorimotor gating (Braff et al. 1999). Also, this model draws some evidence for impacted attention and perception associated with schizophrenia. To relate these differential observations, it seems likely that the H₃R mediated effects on locomotion do not rely on the modulation of the striatal dopaminergic system ("motor processor component") but the stimulation of the dopaminergic system in other brain areas such as the prefrontal cortex ("decision making/planning component"). While some roles of H₃R in basal ganglia and on the dopaminergic system in other brain areas have been revealed within the last years, the complete puzzle remains to be solved to evaluate the therapeutic role of H₃R ligands in PD and schizophrenia (section 1.5)(Ellenbroek et al., 2014; Hu et al., 2017).

1.2. Molecular identity of the histamine H_3 receptor

The H_3R belongs to the large class of GPCR, which represents a target class for about one-third of approved drugs (Hauser et al., 2018). Here, the receptor belongs to the subclass of class-A receptors (class-R, according to the GRAFS (glutamate, rhodopsin, adhesion, frizzled/Taste2 and secretin families) scheme (Fredriksson et al., 2003)), which represents the rhodopsin family and encompass olfactory, visual and taste receptors. Besides such receptors, 197 of them have a known ligand and play central roles in humoral function, immunomodulation and neurotransmission (Alexander et al., 2019a). As all GPCR reside as membrane proteins within phospholipid bilayer, elucidation of GPCR structure and signalling was obstructed for a long time and, after the extensive works by Brian Kobilka and Robert Lefkowitz, awarded with the 2012 Nobel prize in chemistry (Kobilka, 2013; Lefkowitz, 2013). Most recently, a receptor of the D_2R in a bio-membrane and interacting with a G protein heterotrimer was disclosed (Yin et al., 2020).

The H_3R receptor has been classified for detecting HA as endogenous transmitter based on findings from classical pharmacological experiments. Meanwhile, attempts to determine the receptor encoding sequence were unsuccessful for a long time. Upon intensive reverse transcriptase, polymerase chain reaction (rtPCR)-based screening with an expressed sequence tag (EST) of the previously known orphan receptor GPCR97 among a size-selected thalamic copy deoxyribose nucleic acid (cDNA) library, a nucleotide sequence encoding for a putative biogenic amine receptor was detected by Lovenberg and co-workers (1999; Bongers et al., 2007), showing low overall sequence similarity to other GPCRs for biogenic amines (20 – 27 %). This receptor sequence is localised on chromosome 20q13.33. Among them, low sequence similarity to H_3R is surprisingly shown by H_1R and H_2R sequences (22 % and 21.4 %, respectively), which was attributed as a reason of previous failure to identify the sequence (Lovenberg et al., 1999; Lovenberg et al., 2000).

Moreover, the low similarity was a reason that the H_3R sequence has been previously introduced as a novel muscarinergic receptor subtype within a patent application, due to higher sequence similarity to M_1R (Goodearl et al., 1999). Highest sequence similarity of 38 – 58 % is shared with H_4R (Sander et al., 2008). Subsequently, they could correlate their pharmacological findings with literature results for H_3R ligands, markedly, the ability of a novel HA receptor to couple $G\alpha_i$ proteins instead of $G\alpha_q$ or $G\alpha_s$ like the H_1R or H_2R, respectively (Lovenberg et al., 1999).

About relevant **receptor isoforms** on early drug discovery stage, the human isoform shows 93.5 % sequence similarity with rH_3R (Ligneau et al., 2000), which rises to 97 % considering the transmembrane (TM) region only (Lovenberg et al., 2000). In turn, the TM region of rH_3R is homologue to the one of mH_3R

(Palczewski et al., 2000). Overall sequence similarity to other species has been determined as > 92 % (Hancock et al., 2003).

Several **splice variants** of the receptor have been identified so far. At least nine different **human H₃R isoforms** (hH₃R) emanating from combinatorial splicing are known, of which two have been characterised in more detail. The predominant isoform consists of 445 aa (hH₃R$_{445aa}$). In comparison, another functional one lacks 80 aa within the third intracellular loop (ICL3, hH₃R$_{365aa}$), has high brain expression levels (in particular in hippocampus and hypothalamus), shows decreased agonist affinity, but improved functional potency that might be due to a more efficient transducer coupling (Wellendorph et al., 2002). Interestingly, the two introns in H₃R encoding deoxyribose nucleic acid (DNA) sequences are located at positions corresponding to the expressed TMH2 and ICL2. The hH₃R$_{365aa}$ variant with deletions in ICL3 is therefore not a product of exon splicing flanked by two introns but due to intra-exonic in-frame deletions on mRNA-level (Morisset et al. 2001). Recently, the H₃R$_{365aa}$ isoform has been subjected to detailed investigation in recombinant cell lines on the consequences for the signalling behaviour (Riddy et al., 2017). Depending on the observed signalling pathway and in comparison to the hH₃R$_{445aa}$, it appeared that the hH₃R$_{365aa}$ maintains or increases signalling performance among the $G\alpha_{i/o}$ pathway for agonists while signalling was abrogated among the $G\beta\gamma$ exerted pathway (Riddy et al., 2017). If not stated otherwise within this thesis, the hH₃R refers to the hH₃R$_{445aa}$.

The sequence of H₃R has enabled first efforts for drawing structural comparisons to resolved receptor structures such as the bovine rhodopsin model that was presented by Palczewski and co-workers in 2000 (Palczewski et al., 2000) and others that have been resolved within the last decade. Most recently, However, as no direct **structural information** is available for the H₃R to date, computational simulations have been widely applied to explain molecular features of the receptor (Schlegel et al., 2005; Axe et al., 2006; Dastmalchi et al., 2008; Roche et al., 2008; Ishikawa et al., 2010).

A spatial representation of the receptor structures is denoted in Figure 3. Conserved features within terminal regions of H₃R comprise of a glycosylation site at N11, as well as palmitoylation site at C428 that is believed to facilitate the spatial orientation of helix 8 parallel to the membrane (Qanbar et al., 2003)(Nieto-Alamilla et al. 2016). Additionally, it is supposed that C107 and C188 form a cystine bridge between the extracellular loop (ECL)1 and ECL2 (Tiligada et al., 2009). Structural features within the transmembrane segments have been drawn in analogy to conserved motifs from other GPCRs that is currently the only anchor point for the structural explanation of H₃R. In the rhodopsin receptor model, the conserved "DRY" motif (composed of D/E$^{3.49}$, R$^{3.50}$, F/Y$^{3.51}$) is responsible for keeping the receptor in the inactive state by an ionic interaction between the positively charged R$^{3.50}$ residue and the negatively

charged $E^{6.30}$ residue and is therefore referred to as the "ionic lock" (Rovati et al., 2007). In case of H₃R, this DRY motif consists of a DRF motif (Yao et al., 2003). Molecular dynamics simulations failed to reproduce this feature for H₃R, as the corresponding aa in TMH6 represents the shorter $D353^{6.30}$ (Jończyk et al., 2017; Pandy-Szekeres et al., 2018). Thus, the homology model may give one rationale for the high **constitutive activity** observed for H₃R (Rovati et al., 2007). The "3-7 lock" is located within the upper receptor hemisphere and describes the congregation of TMH3 and TMH7 can be seen in the rhodopsin crystal structure as well (Palczewski et al., 2000). In the case of H₃R, it is represented by $D114^{3.32}$ and $W402^{7.42}$ and is abrogated upon agonist binding (Jończyk et al., 2017). The CWxP motif, which is responsible for the agonist-induced reorganisation of TMHs 3, 5 and 6 during receptor activation ("transmission switch"), is represented by $C370^{6.47}$, $W371^{6.48}$, $A372^{6.49}$ and $P373^{6.50}$ (Deupi et al., 2011; Jończyk et al., 2017). However, it is difficult to derive details of the dynamic changes within this region during receptor transition, as this process diverges considerably among different GPCRs (Trzaskowski et al., 2012). The NPVLY motif ($N408^{7.49}$ - $Y412^{7.53}$, "Y-toggle switch") in TMH7 corresponds to the conserved NPxxY motif (Nieto-Alamilla et al., 2016) that plays a role in receptor interaction with intracellular signal transducers (Galés et al., 2000; Bouley et al., 2003).
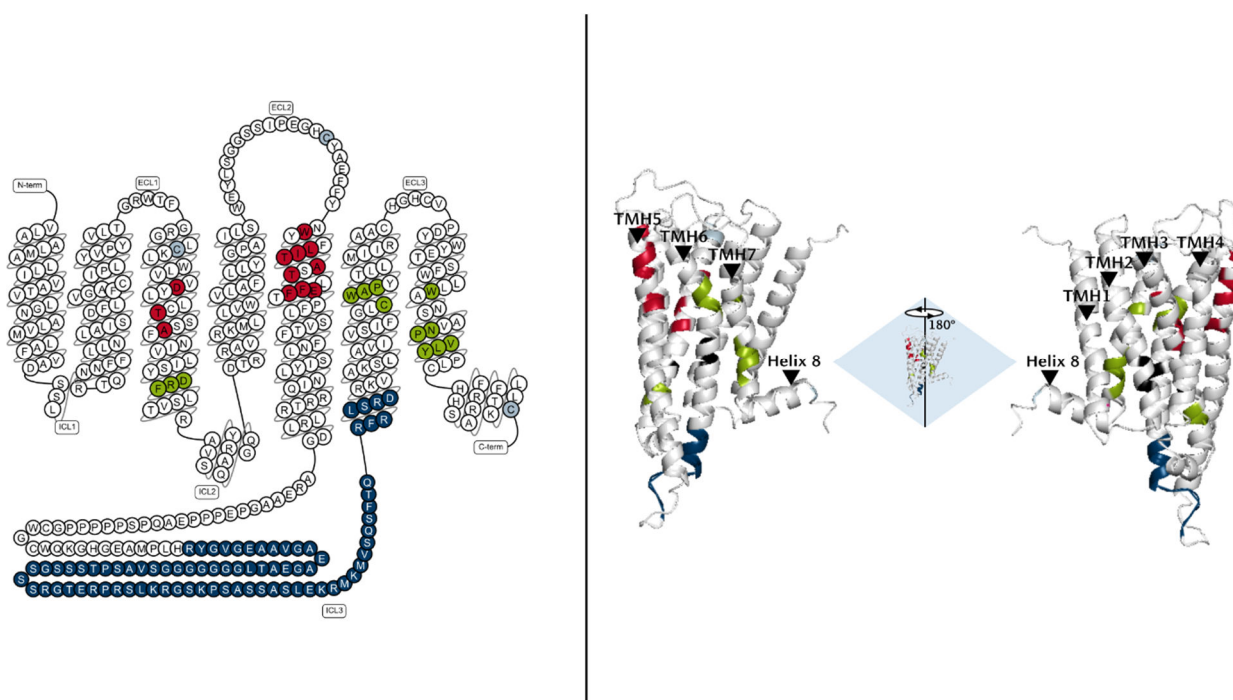

Figure 3. Receptor sequence given as snake-plot (left panel) and as a homology model with truncated ICL 3 (right panel). Light-blue and dark-blue residues denote post-translational modification sites (cystine bridge between TMH3 and ECL2, palmitoylation site in helix 8) and the lacking part in hH₃R₃₆₅ₐₐ, respectively. Residues in green depict conserved motifs which are important in receptor transition. Red highlighted residues are known to be involved in ligand binding, as determined from mutagenesis experiments. Snake-plot and H3R homology model of the inactive receptor state have been taken from GPCRdb (https://gpcrdb.org/, (Pandy-Szekeres et al. 2018)). Data are taken from references, cited within the main text.

ICL3 often handled in its truncated form (i.e., the 413aa H₃R) in *in silico* models as structural homologies to other GPCRs are hard to draw for this region (**Figure 3**)(Schlegel et al., 2005; Rai et al., 2010). The truncated isoforms did not differ in binding affinities for the H₃R antagonists [^{125}I]iodophenpropit, clobenpropit and thioperamide. However, they showed slightly improved affinities for the agonists HA, [^{3}H]NAMH and immepip at the rat receptor isoform (Drutel et al., 2001), whereas not at the human one (Nieto-Alamilla et al., 2018). Additionally, an A280V single-nucleotide polymorphism (SNP) within ICL3 was associated with reduced agonist-exhibited efficacy and reduced constitutive activity among the Gα$_{i/o}$ pathway. However, no reduced potency and no overall changes were observed among the Gβγ exhibited one (cf. **Figure 1**)(Flores-Clemente et al., 2013). These findings imply that the ICL3 is not involved in ligand binding, but can change the transducer coupling and thereby, the potency of agonists. This property was recently supported by two studies demonstrating an agonist-induced conformational change between a YFP-substituted or HaloTag®-substituted ICL3 and C-terminal fused CFP or NanoLuc®, respectively (Liu et al., 2018; Schihada et al., 2020).

Various molecular biological experiments have assisted in elucidating the **binding pocket of H₃R** (**Figure 4**). Working with h$_{1-144aa}$/r$_{145-445aa}$H₃R chimaera have localised the first 144 to be involved in ligand binding (Yao et al., 2003). E206$^{5.46}$ is supposed to interact with positively charged imidazole of HA as indicated by site-directed mutagenesis studies with E206$^{5.46}$A mutation (Uveges et al., 2002).
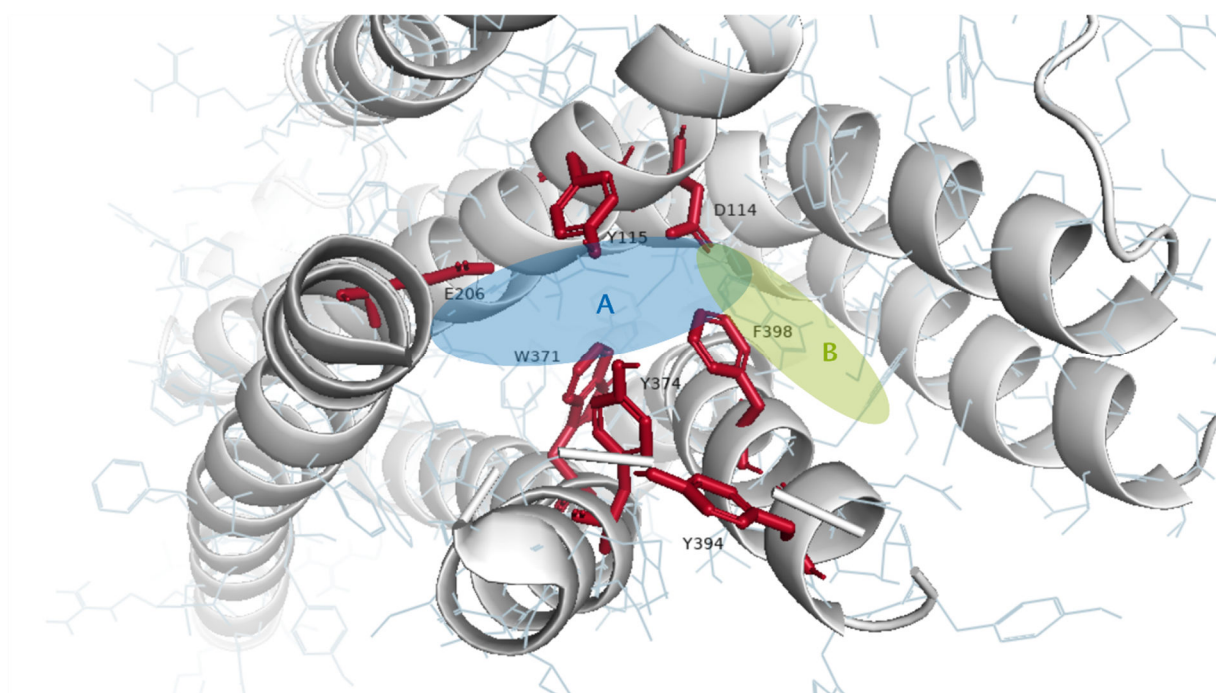

Figure 4. Binding pocket of H₃R with selected aa residues that are involved in ligand interaction (red), and highlighted 'A'-site of the binding pocket (blue) and 'B'-site (green). Modified from (Schaller et al., 2019). Homology model was taken from GPCRdb (https://gpcrdb.org/, (Pandy-Szekeres et al. 2018)).

D114$^{3.32}$, which is conserved among biogenic amine GPCRs, is assumed to interact with the positively charged amino-side chain of HA with resemblance to elucidated binding structures of other monoaminergic GPCRs. Interestingly, most agonists for H₃R possess imidazole as well as a basic side chain and thus, enabling interaction with both acidic amino residues (section 1.3). The H₃R binding pocket sub-divides into two hemispheres, stretching from D114$^{3.32}$ (Levoin et al., 2008). The bulky 'A'-site of approx. 225 Å^3 is formed between D114$^{3.32}$ and E206$^{5.46,}$ and a markedly smaller 'B'-site points out from D114$^{3.32}$ towards the opposing side (Levoin et al., 2008). Interestingly, while previously generated models have predicted that agonists can interact with the receptor within both cavities, the same has not been demonstrated for antagonists which seem to always interact with E206$^{5.46}$ within the 'A'-site (Levoin et al., 2008; Jończyk et al., 2017). Further residues within TMH5 involved in ligand binding have been identified by Uveges and co-workers (2002). W196$^{5.36}$A and T204$^{5.44}$A mutations have shown a 2 to 5-fold increase in potency of HA, the latter accompanied by an approximately 5-fold reduced affinity. In contrast, L199$^{5.39}$A and I200$^{5.40}$A reduced the affinity, with a depression of the maximum inhibitory effect in Gα$_s$ signalling exhibited by HA, an observation that was aligned with the role of K191$^{5.40}$ in H₁R in receptor activation (Uveges et al., 2002). The second-most pronounced affinity loss for the endogenous ligand was observed upon A202$^{5.42}$Q, causing 20-fold loss in potency due to a repulsive effect of the bulkier glutamine on E206$^{5.46}$ (Uveges et al., 2002). The latter residue has shown the highest impact on HA affinity towards H₃R that almost diminishes upon E206$^{5.46}$A mutation (affinity loss > 2,000-fold). Conclusively, E206$^{5.46}$ is an important residue for the detection of the endogenous ligand, while other ligands may not involve this residue and thus, not displace HA in a strictly competitive manner.

Additionally, sequence comparisons to **different species** have explicated binding residues for ligand recognition. Among them, the *Rattus norvegicus* isoform has been investigated most comprehensively. The residues A119$^{3.37}$ and V122$^{3.40}$ have been associated with discrepancies in affinity compared to the human isoform, which consists of T119$^{3.37}$ and A122$^{3.40}$ (Ligneau et al., 2000). Interestingly, such discrepancies have not been observed for H₃R agonists, including HA. In contrast, some inverse agonists showed either higher affinity (**ciproxifan**, **thioperamide**) or lower affinity (FUB349) at the rH₃R compared to the hH₃R (Ligneau et al., 2000; Lovenberg et al., 2000; Stark et al., 2001). Such observations enable for comparison with other species that share the same mutations, for which similar pharmacologic results are expectable. Similar to rH₃R, as well the *Mus musculus* isoform shows A119$^{3.37}$ and V122$^{3.40}$, while the *Cavia porcellus* (guinea pig) isoform shows T119$^{3.37}$ in analogy to the hH₃R.

Further evidence has been made that the H₃R may not mediate its actions as a monomolecular entity, but forming **heteromers with other receptors**, which has been investigated for D₁R, D₂R and A$_{2A}$R

(Ferrada et al., 2008; Ferrada et al., 2009; Márquez-Gómez et al., 2018). In each of such cases, the result of this interaction has been the inhibition of affinity towards the respective endogenous agonist (HA, DA or adenosine) and consequently, the inhibition of receptor function after activation of the other (Ferrada et al., 2008; Ferrada et al., 2009; Márquez-Gómez et al., 2018). Interestingly, each of such receptor dimers have been detected in striatal neurons, where predominantly postsynaptic expression of H_3R is assumed. Thus, such heteromers suggest a regulatory role and might be of importance to interpret the role of H_3R in neurotransmission of basal ganglia (cf. page 13). H_3R heteromers with D_1R have shown preferential coupling to $G\alpha_{i/o}$ proteins, while D_1R primarily couple to $G\alpha_s$ and sometimes to $G\alpha_{q/11}$ (Inoue et al., 2019). Therefore, D_1R can shift the transduction pattern upon heteromerisation with activated H_3R and thereby, eliciting inhibitory instead of excitatory features (Ferrada et al., 2009). Such heteromers do not seem to have relevance *in vitro* only, as less ERK-1/2 phosphorylation in striatonigral GABAergic neurons is seen in $D_1R^{-/-}$ mice (Moreno et al., 2011). Meanwhile, it is assumed that this mechanism requires as well σ_1R which modulate the functions of H_3R-D_1R complex (Moreno et al., 2014), which is an interesting finding as several H_3R ligands share the enigmatic σ_1R as co-target (Riddy et al., 2019).

H_3R shows a high degree of **constitutive activity**, a concept introduced in 1993 by Lefkowitz and co-workers (Lefkowitz et al., 1993; Morisset et al., 2000). This concept describes that a receptor spontaneously adopts active conformation(s) without priorly binding to an agonist. The discovery of this feature could not be observed before receptor cloning, as it is characteristic for overexpressed receptor systems that were introduced into molecular pharmacology after molecular biological techniques have emerged. This property is roughly detectable in native pharmacological systems (Morisset et al., 2000). The behaviour is extensively shown by H_3R along the $G\alpha_{i/o}$ coupled pathway (Figure 1)(Morisset et al., 2000; Rouleau et al., 2002; Ellenbroek et al., 2014). Therefore, the receptor is not only a target for throttling HA release and the release of other neurotransmitters, but inverse agonists can even reverse this activity.

Such findings are in line with the assumption that GPCRs do not act as simple on-off-switches. Meanwhile, this concept has been expanded by the assumption that GPCRs can as well adopt **multiple receptor conformations** (Baker et al., 2007). The model of multiple intermediate states between ligand binding and receptor activation has to be extended by several further intermediates, as the aa framework represents a flexible interactome of partially rigid strands that are linked together. Therefore, a model of sequential replacements of such strands from a completely inactive to a completely active receptor conformation gives rise for multiple intermediates within the ternary complex model (Deupi et al., 2011; Latorraca et al., 2017). Also, this model gives rise to the assumption that potentially each receptor-ligand differentially

impacts the arrangement of this framework. Furthermore, if different ligand affinities would represent such constitutively occurring conformational states, as seen with NAMH coupling to two distinct receptor conformations with different affinity (Witte et al., 2006), it would lead to the hypothesis that differentially targetable fractions of the receptor population will exist.

1.3. Medicinal chemistry of histamine H₃ receptor ligands

From the very beginning of H$_3$R pharmacology, the characterisation of the receptor was inextricably linked with pharmacological tools as it was a pharmacologically defined target (Hill et al., 1997). Therefore, they not only have importance for therapeutic exploitation of this target due to its versatile central functions (section 1.1). Additionally, they served as tools for revealing essential characteristics of the receptor and its function in the brain.

In the same experiments that led to the discovery of H$_3$R, the very first **agonists** of the receptor have been detected that mimicked the actions of HA (Arrang et al., 1987a). Likewise to the discovery of many blockbusters, the search for potent H$_3$R ligands has started with the orientation on a biological sample at hand. This procedure led to detailed insights into structural variations in order to obtain potent and selective H$_3$R agonists.

Intriguingly, the endogenous ligand HA has a remarkably higher affinity to H$_3$R than to H$_1$R and H$_2$R (Figure 5). This observation can be rationalised by the interactions of HA within the receptor binding pocket (Figure 4). In contrast to H$_1$R and H$_2$R, where the ionisable E206$^{5.46}$ is represented by asparagine or threonine, respectively, the H$_3$R binding pocket facilitates polar/ionic interactions with the imidazole moiety of HA (Ishikawa et al., 2010). This selectivity profile can be maintained in further methylated HA derivatives. Unfortunately, some H$_3$R agonists display comparable affinity at the H$_4$R as the E206$^{5.46}$ residue is conserved between both receptors. Methylation at distinct key positions of HA leads to alterations in this selectivity profile: While methylation of the amino group or an α-methylene group leads to a slight increase in H$_3$R preference over H$_4$R, the selectivity profile reverses upon 5-methylation of the imidazole moiety (Figure 5). The receptor affinity diminishes upon extensive N^τ methylation that resembles the endogenous HA metabolite in the CNS and has a high affinity towards monoaminoxidase B (Elsworth et al. 1980, Rouleau et al. 1997, Labeeuw et al. 2016).

RAMH is one of the H$_3$R agonists that have been most extensively studied *in vivo*, representing a chiral, methylated HA derivative (Table 3). Azomethine analogues of RAMH were developed as prodrugs to overcome the susceptibility for extensive inactivation by HNMT and the subsequently low bioavailability in human species (e.g., BP2.94, Table 3)(Rouleau et al., 1997).

Extension of side chain has been investigated regarding the influence on H$_3$R affinity. Imetit, imbutamine, immepip and methimmepip maintain a basic moiety, which is shifted by two methylene units from the imidazole moiety compared to HA (Table 3). In contrast to RAMH and HA, imetit does not serve as a substrate for HNMT (Garbarg et al., 1992). Various structural variations have been undertaken to derive potent and selective agonists. Direct derivatives of HA with increased alkyl linker between the imidazole
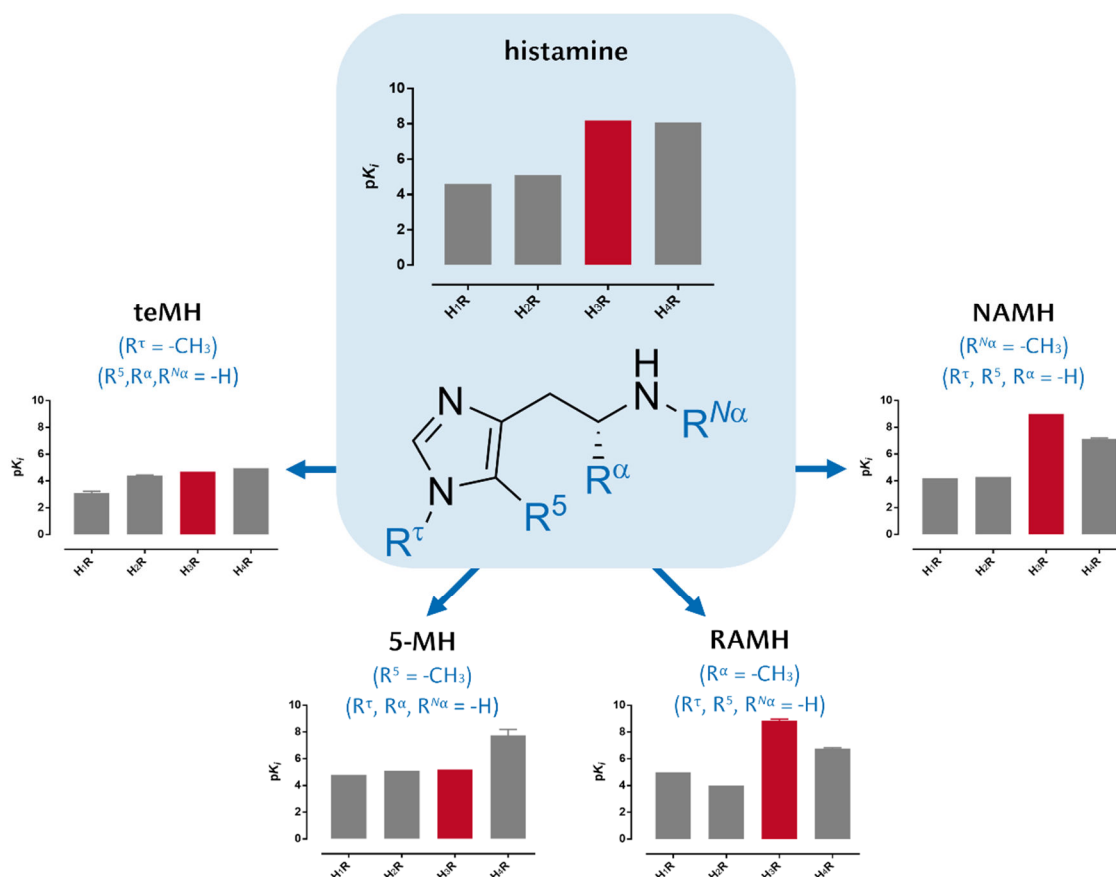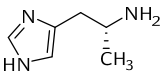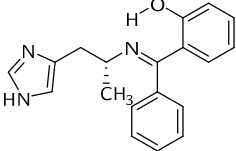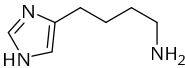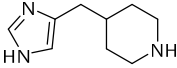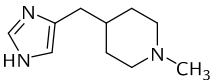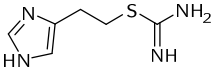
Figure 5. Differential methylation of HA and corresponding affinity profiles at HA receptor subtypes. Data were taken from Panula et al. (2015), Gaulton et al. (2016) and Chazot et al. (2019), except for teMH, which were taken from elsewhere (Driver et al., 1987; Baker, 2009; Labeeuw et al., 2016). Data were stated as means and s.e.m.
Abbreviations: teMH = N^τ-methylhistamine, 5-MH = 5-methylhistamine, RAMH = *(R)*-α-methylhistamine, NAMH = N^α-methylhistamine.

and primary amine encompass VUF-8326 (n=3), **imbutamine** (n=4), impentamine (n=5) and VUF4732 (n=6, with n depicting the number of methylene units between the imidazole and primary amine).

The propylene and ethylene (i.e., HA) analogues do not distinguish between H3R and H4R, both having affinities in low nanomolar concentration ranges (Kitbunnadaj et al., 2003). Upon side chain extension, however, selectivity increases progressively, but accompanied by a loss in their intrinsic efficacy as determined in CRE-driven β-galactosidase reporter gene assays in recombinant H3R-SK-N-MC cells (Kitbunnadaj et al., 2003). Thus, one could conclude, that the efficiency for stabilisation of the active receptor conformation inversely correlates with the spacer length between the amino moieties interacting with E206$^{5.46}$ and D114$^{3.32}$. **Immepip** resembles a structurally constrained rotamer of imbutamine that has an 8-fold increased H3R affinity (Table 3). After the discovery of H4R, imetit and immepip were re-characterised as potent agonists thereof which revitalised efforts for design of selective H3R agonists. The latter resulted in **methimmepip** that has a > 2,000-fold selectivity for H3R over H4R.

Table 3. H₃R agonists with side chain variations.

Structure	Compound / Data
	RAMH ➤ hH₃R: $K_i = 2.5$ nM ➤ <u>off-targets</u>: hH₄R: $K_i = 160$ nM HNMT: $K_M = 3,000$ nM
	BP2.94 ➤ hH₃R: $EC_{50} = 10$ nM
	imbutamine ➤ hH₃R: $K_i = 4.1$ nM ➤ <u>off-targets</u>: hH₄R: $K_i = 15$ nM
	immepip ➤ hH₃R: $K_i = 0.5$ nM ➤ <u>off-targets</u>: hH₄R: $K_i = 22$ nM hH$_{1/2}$R: $K_i > 16,000$ nM
	methimmepip ➤ hH₃R: $K_i = 1.0$ nM ➤ <u>off-targets</u>: hH₄R: $K_i = 2,000$ nM hH$_{1/2}$R: $K_i > 10,000$ nM
	imetit ➤ hH₃R: $K_i = 0.5$ nM ➤ <u>off-targets</u>: hH₄R: $K_i = 3.3$ nM hH$_{1/2}$R: $K_i > 7,900$ nM

<u>References:</u> (Vollinga et al., 1994; Rouleau et al., 1997; Kitbunnadaj et al., 2003; Kitbunnadaj et al., 2005; Panula et al., 2015; Chazot et al., 2019)

Side-directed mutagenesis experiments of H₃R suggest that RAMH, imetit and impentamine show similar receptor interactions like HA. However, these agonists differ in their susceptibility for alterations in receptor binding and activation upon mutation in distinct key positions as studied by Uveges et al. (2002).
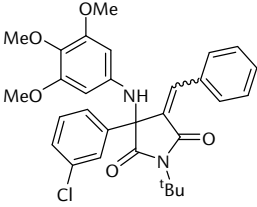
Here, the long-chain analogue impentamine shows only a slight loss in potency and no alterations in G protein coupling upon E206$^{5.46}$A mutation. On the contrary, this agonist is more sensitive to mutations of F208A and completely diminishes receptor binding upon L199$^{5.40}$A shift, where the others still excite the receptor in a nanomolar concentration range.

In 2018, putative H₃R agonists with almost no structural resemblance to canonical structures for H₃R ligands were reported, which consisted of either β-lactams or pyrrolidine-2,5-diones and were presented to the scientific community as potent H₃R agonists with selectivity over HA receptor subtypes and 5-HT$_{2C}$R (Ghoshal et al., 2018a; b). Compound **6-k** as the lead structure of this project will be subjected for profound pharmacological characterisation within this work as it exerted its action in subnanomolar concentration range and showed promising *in vivo* effects concerning food intake in obese mice. The latter might be beneficial as the previous question whether agonists or inverse agonists of the H₃R are more beneficial in controlling food intake remains a highly debated topic.

VUF16839 was presented as another potent non-imidazole-based agonist. It has potency in subnanomolar concentration range and showed an amnestic effect after i.p. application *in vivo*. However, usage as a pharmacological tool to investigate H₃R function may be limited by two factors (Wágner et al., 2019).

Table 4. Novel non-imidazole agonists.

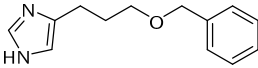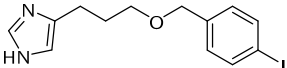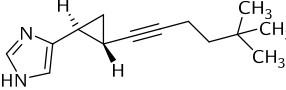| Structure	Compound	Data
	6-k ➤ hH₃R:	EC_{50} = 0.1 nM
	VUF16839 ➤ hH₃R: ➤ <u>off-targets</u>: hH₄R:	K_i = 3.2 nM K_i = 7.9 nM

<u>References:</u> (Ghoshal et al., 2018b; a; Wágner et al., 2019)

Due to the aminopyrimidine moiety, the compound shows substantial activity at H₄R as well, and data for blood-brain barrier permeability have not been disclosed yet despite a highly polar structure.

After cloning of H₃R and concomitant expression in recombinant cell lines with different receptor-effector coupling efficiency (i.e., an introduced "system bias"), systematic re-investigation of previously developed ligands was enabled due to novel opportunities in *in vitro* screening (Gbahou et al., 2003; Krueger et al., 2005; Kenakin et al., 2013). Occasionally, agonist properties have been measured for numerous ligands though they were initially identified as inverse agonists at the same receptor isoform (Krueger et al., 2005). This phenomenon, which was poorly understood in the early 2000s, has been termed '**protean**' (Kenakin, 2001). Meanwhile, the most common terminology for such phenomena encompass the terms "biased agonism/signalling" or "functional selectivity" (Kenakin et al., 2013).

Table 5. H₃R ligands with protean agonist behaviour.

proxyfan	iodoproxyfan	cipralisant
hH₃R: K_i = 5.0 nM	hH₃R: K_i = 0.10 nM	hH₃R: : K_i = 2.4 nM

Affinity data were taken from Wulff et al. (2002)

For H₃R and in contrast to other GPCRs, biased signalling could not be subdivided into a G protein-dependent and an independent (e.g., arrestin-mediated) component. So far, all observations for functional selectivity at H₃R have been linked with Gαi proteins and the corresponding Gβγ subunits (Riddy et al., 2017) and therefore, emphasising multiple receptor conformations with different active GPCR conformations (Kenakin, 2001; Krueger et al., 2005). The latter is supported by [¹²⁵I]iodoproxyfan binding

studies to E206$^{5.46}$A receptor mutants, in which HA completely loses competitiveness, while the receptor affinity of iodoproxyfan remains unaltered (Uveges et al., 2002).

This protean behaviour has been extensively investigated for proxyfan, iodoproxyfan and cipralisant (Table 5), which exert submaximal Gα$_{i/o}$-protein activation in recombinant cell systems but not in rat cerebral cortex membranes. In contrast, a pronounced bias towards Gβγ coupled pathways (GSK-3β, MAPK, PLA₂) was found for proxyfan and its iodinated analogue, in comparison to Gα$_{i/o}$ mediated cAMP-accumulation and to RAMH-induced signalling (Riddy et al., 2017). Moreover, this study evidenced that this behaviour is abolished in the shorter hH₃R$_{365aa}$ isoforms with truncation in ICL3 (Riddy et al., 2017).

H₃R inverse agonists have been developed as agents to increase the activity of histaminergic neurotransmission by enhanced release of HA and as well to influence the signalling of other neurotransmitters upon enhanced release thereof, mediated by heteroreceptors (cf. page 10).
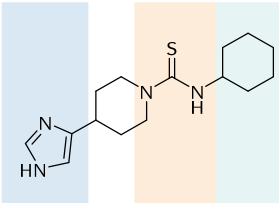

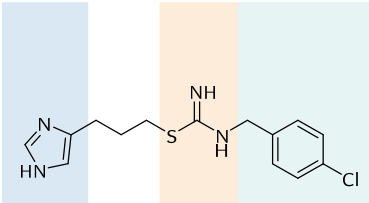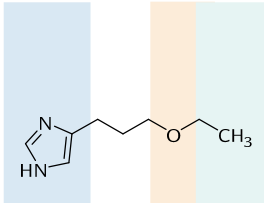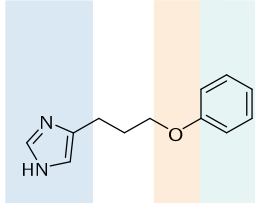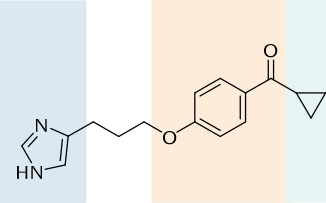**Figure 6.** Pharmacophore blueprint of imidazole-based H₃R ligands, adapted from Celanire et al. (2005).
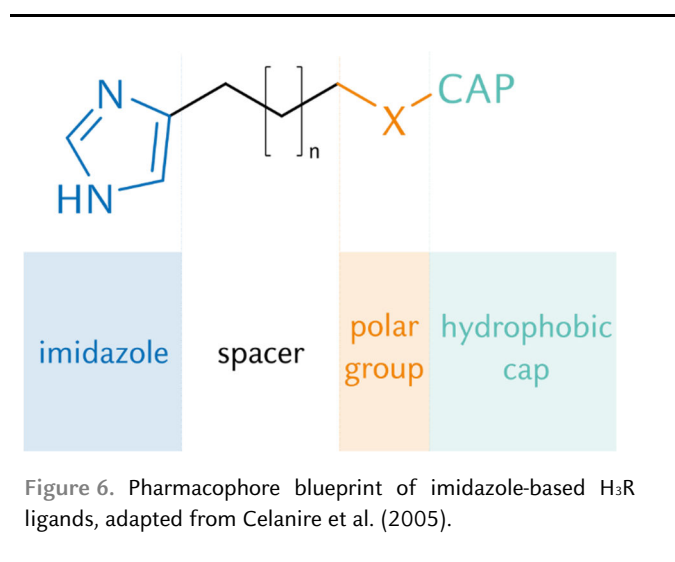
With orientation on the structure of HA, first rationally designed H₃R inverse agonists included an imidazole moiety for which this first-generation is referred to as the class of **imidazole-based ligands** (Figure 6)(Celanire et al., 2005). **Thioperamide** (Table 6) was one of the very first potent H3R antagonists/inverse agonists and has contributed as a pharmacological tool to many insights into H3R pharmacology. Clinical development was, however, hindered due to a marked hepatotoxicity of the compound that has been attributed to the incorporated thiourea moiety, and also poor pharmacokinetic properties such as relevant cytochrome P450 (CYP) inhibition and slow blood-brain barrier (BBB) permeability, associated with the imidazole moiety (Ganellin et al., 1996; Schwartz, 2011). Another standard H₃R ligand with the antagonist or inverse agonist efficacy is given with clobenpropit. The development of this ligand had the agonist imetit as a starting point for which it serves as a chlorobenzyl-substituted congener. This design strategy led to the working hypothesis that the introduction of a large hydrophobic cap like an aromatic moiety inverses efficacy of H₃R agonists (Celanire et al., 2005). While clobenpropit and thioperamide appeared selective before the early 2000s, both tool compounds must have been re-classified being potent inverse agonists at the H₄R as well, after this receptor was discovered (Table 6).

In analogy to the design strategy of clobenpropit to modify the side chain upon introduction of a

Table 6. Imidazole-based H₃R ligands with antagonist or inverse agonist efficacy.

thioperamide	clobenpropit	FUB 465

thioperamide
- hH₃R: K_i = 6.5 – 600 nM
- off-targets:
  hH₄R: K_i = 9.1 nM
  α_{2A}AR: K_i = 130 nM
  α_{2C}AR: K_i = 320 nM
  5-HT₃R: K_i = 2,500 nM

clobenpropit
- hH₃R: K_i = 0.34 – 3.2 nM
- off-targets:
  hH₄R: K_i = 10.5 nM
  hH₁/₂R: K_i > 3,400 nM
  $\alpha_{2A/2C}$AR: K_i = 160 nM
  5-HT₃R: K_i = 7.9 nM

FUB 465
- hH₃R: K_i = 190 nM
- off-targets:
  hH₄R: K_i = 700 nM

4-(3-(phenoxy)propyl)-1*H*-imidazole
- hH₃R: K_i = 27 nM

ciproxifan
- hH₃R: K_i = 50 – 280 nM
- off-targets:
  α_{2A}AR: K_i = 40 nM
  α_{2C}AR: K_i = 63 nM
  5-HT₃R: K_i = 320 nM
  hH₄R: K_i = 1,900 nM

References: (Ligneau et al., 2000; Meier et al., 2001; Morisset et al., 2001; Stark et al., 2001; Meier et al., 2002; Esbenshade et al., 2003; Liedtke et al., 2003; Lippert et al., 2004; Mikó et al., 2004; Gbahou et al., 2006; Kottke et al., 2011; Panula et al., 2015; Hagenow et al., 2017; Affini et al., 2018; Chazot et al., 2019)

hydrophobic cap (**Figure 6**), but now with HA as starting point instead of imetit, pointed out prospects for the development of further antagonists that are devoid of (iso)thiourea moieties (Ganellin et al., 1995). Consequently, the introduction of a second aryl moiety resulted in numerous potent antagonists/inverse agonists that avoid (iso)thiourea scaffolds and of which some have been exemplified in **Table 6**.

Introduction of electron-withdrawing groups at the aryl moiety increases the potency (Ganellin et al., 1996; Krause et al., 1998). This strategy has paved the path towards enlargement of the class of potent first-generation H₃R antagonists. Retrospectively, the exact spatial position of the aryl moiety apparently seems to play a role for the susceptibility for **protean** agonist behaviour (cf. page 26), which was observed for proxyfan and its iodinated analogue but not for others, e.g., **ciproxifan** or **clobenpropit** (Krueger et al., 2005).

Unfortunately, clinical investigation of H₃R ligands within the first two decades after H₃R discovery was without successful results, that has been retrospectively attributed to two reasons: First, the

pharmacological characterisation initially relied on models employing H₃R isoforms other than the human one (cf. page 19)(Sander et al., 2008). This issue was resolved after cloning of the hH₃R and facilitated the introduction of hH₃R already in *in vitro* studies. Secondly, the first representatives of H₃R inverse agonists relied on incorporated imidazoles (Table 6). Such scaffolds have higher liability to detrimental pharmacokinetic properties, as imidazoles tend to bind and inhibit isoforms of cytochrome-P450 upon coordination to the hem-iron of such enzymes (Alves-Rodrigues et al., 1996; Davenas et al., 2008). As exemplified above, such imidazole-based ligands often represent substrates of HNMT as degrading enzyme (Rouleau et al., 1997) Furthermore, tolerance to *in vivo* effects after repeated dosage was more often correlated with imidazole-based H₃R ligands than with non-imidazole ones (Guo et al., 2009). Probing for a potential bioisosteric replacement of imidazole by basic alicyclic amines in the late 90's led to a paradigmatic change in the design of H₃R antagonists and inverse agonist (Ganellin et al., 1991; Ganellin et al., 1998; Meier et al., 2001).

Thereby, a new, **second-generation of H₃R ligands** was proclaimed. However, not only disadvantages were associated with the inherent imidazole-motif. The beneficial potency that is attributed to them is
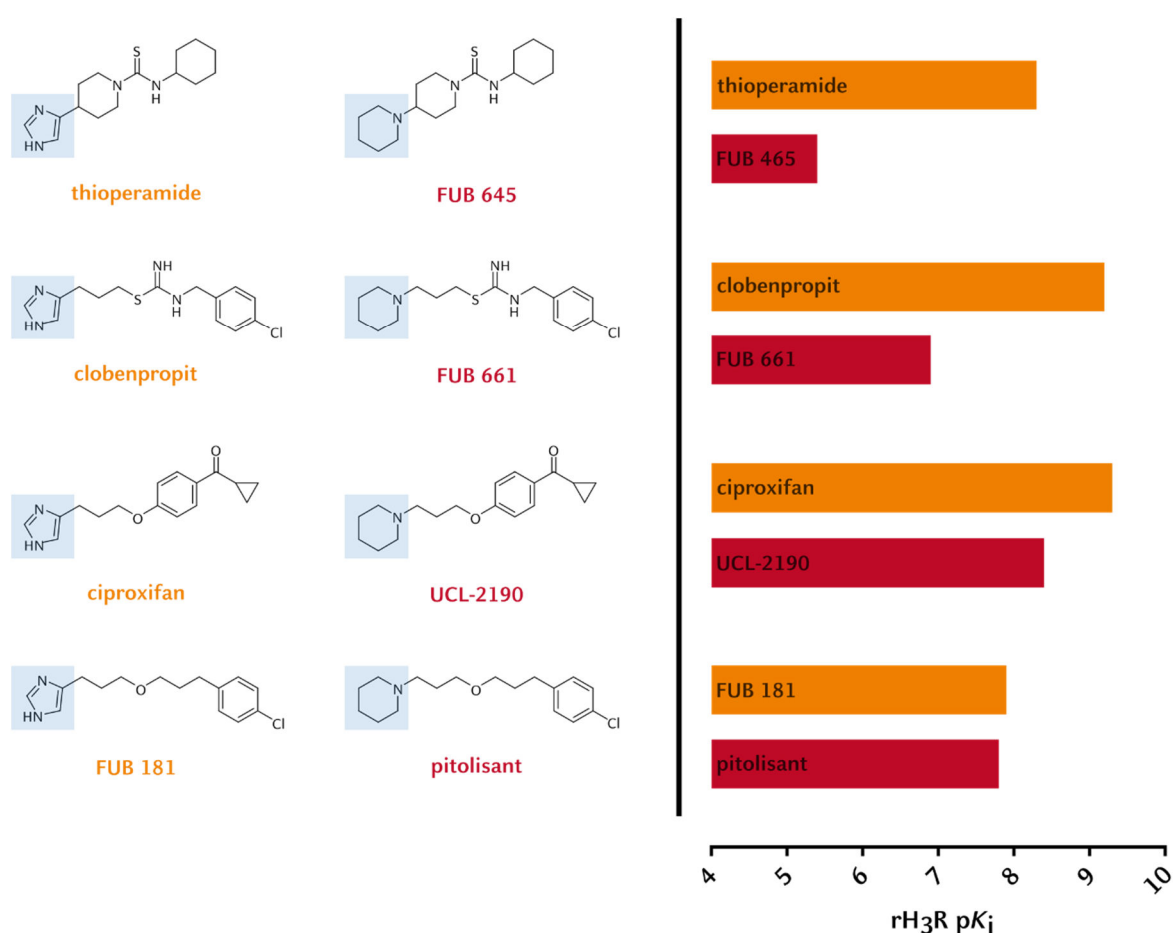

Figure 7. Bioisosteric replacement approach towards non-imidazole H3R inverse agonists with affinity scales at rH₃R. Data were taken from (Meier et al., 2001; Liedtke et al., 2003).

accompanied by selectivity towards H_1 and H_2 receptor subtypes (Gemkow et al., 2009). Therefore, the imidazole replacement came with new obstacles, as it was accompanied by a significant drop in receptor affinity, which might impact their selectivity profile. This behaviour was shown fo r **thioperamide** and **clobenpropit** of which the piperidine congeners (FUB 645 and FUB 661, respectively) showed reduced potencies in models of rH₃R in rat cerebral cortex (**Figure 7**)(Meier et al., 2001; Liedtke et al., 2003).

In the same models, it was demonstrated that the potency of **pitolisant** was considerably less affected compared to its imidazole congener FUB 181 (**Figure 7**). This observation may be a reason for a good correlation of its preclinical effects *in vivo* and the clinical performance of pitolisant, which was granted with marked approval in the EU and the U.S.A for the treatment of excessive daytime sleepiness in narcoleptic patients with or without cataplexy (Syed, 2016; Kumar et al., 2019). Preclinical characterisation demonstrated lack of off-target interaction among approx. 100 known human targets (Ligneau et al., 2007b). In contrast, pitolisant showed potent inhibition of [^{125}I]iodoproxyfan and [^{35}S]GTPγS binding to recombinantly and natively expressed hH₃R, synaptosomal release of [^{3}H]HA mediated by rH₃R, and inhibition of histamine-elicited twitches of isolated guinea pig ileum (Ligneau et al., 2007b). Despite previous reports of hERG binding liability associated with the non-imidazole strategy, this issue was not observed for pitolisant (Levoin et al., 2011; Shah et al., 2016). Consistent with previous results of H₃R inverse agonists, the pharmacological tool showed increased teMH levels in the cerebral cortex as well as in striatum and hypothalamus (Ligneau et al., 2007a). The observed elevated DA turnover in the prefrontal cortex, but unaffected one in striatum, raised the hope for pitolisant and other H₃R agonists as drug candidates against negative symptoms in **schizophrenia** for which effective compounds have been overdue until today (Ligneau et al., 2007a). Previous clinical studies have demonstrated the effectiveness and tolerability of pitolisant in **narcolepsy** compared to placebo, and a non-inferiority to the well-established modafinil (Dauvilliers et al., 2013; Kumar et al., 2019).

Another compound derived from bioisosteric replacement efforts has been **UCL-2190** (**Figure 7**). Although this compound displayed a slightly decreased affinity at rH₃R compared to **ciproxifan**, this behaviour reverses at the human isoform, where it shows an even increased affinity compared to the imidazole congener **ciproxifan** (**Figure 7**)(Mikó et al., 2004). However, ciproxifan is still highly rated as a
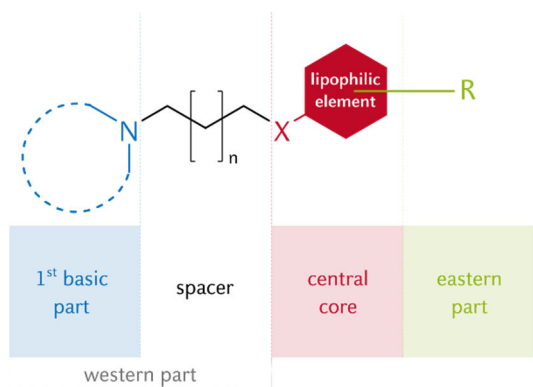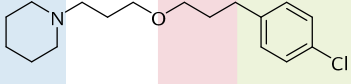
Figure 8. Pharmacophore blueprint of second-generation / non-imidazole H₃R antagonists, modified from (Celanire et al., 2005; Walter et al., 2010).

Table 7. Selected non-imidazole-based ligands, derived from the pharmacophore blueprint (**Figure 8**).

pitolisant
- hH$_3$R: K_i = 8.7 nM
- off-targets:
  σ$_1$R: K_i = 10 nM
  hH$_1$R: K_i = 1,700 nM
  CYP: IC_{50} = 2,600 nM

1-(3-phenoxypropyl)piperidine
- hH$_3$R: K_i = 310 nM

1-(3-([1,1'-biphenyl]-3-yloxy)propyl)piperidine
- hH$_3$R: K_i = 76 nM

DL76
- hH$_3$R: K_i = 22 nM

DL77
- hH$_3$R: K_i = 8.4-37 nM
- off-targets:
  hMAO B: K_i = 19 nM

References: (Lazewska et al., 2006; Sander et al., 2010; Panula et al., 2015; Calik, 2017; Łażewska et al., 2017; Lazewska et al., 2018; Riddy et al., 2019)

pharmacological tool for *in vivo* investigations (Hagenow et al., 2017).

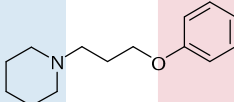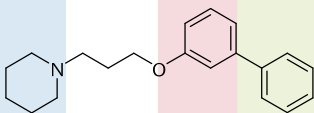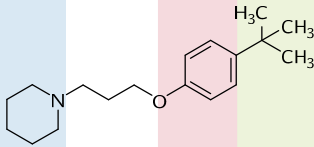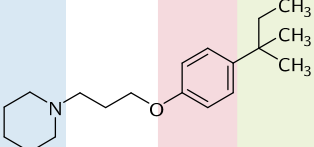Today, most second-generation inverse agonists follow the pharmacophore blueprint depicted in **Figure 8** (Celanire et al., 2005; Walter et al., 2010). This guide has led to a plethora of H$_3$R inverse agonists that have been developed to the present. The most prototypical second-generation ligand is represented by **1-(3-phenoxypropyl)piperidine** which consists of a benzene ring as an aromatic core scaffold and shows a more than tenfold reduced receptor affinity compared to its first-generation analogue. As hinted in **Table 7**, the receptor affinity can be modulated upon modifications within the eastern part of the molecule, where not only lipophilic residues can be placed as a strategy to a probably entropy-driven affinity increase.

This strategy is inherent in the tert-butyl and tert-pentyl substituted analogues **DL76** and **DL77**, as well as in the biphenyl-derivative **1-(3-([1,1'-biphenyl]-3-yloxy)propyl)piperidine**. Besides, polar groups, basic moieties and acidic moieties are tolerated within this region as will be shown in several studies throughout this work (Celanire et al., 2005; Sander et al., 2008; Walter et al., 2010; Ghamari et al., 2019a).
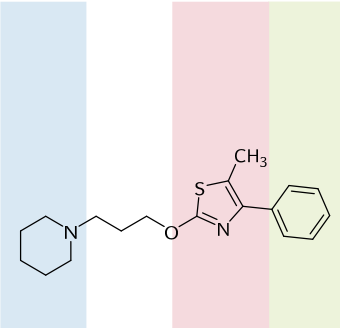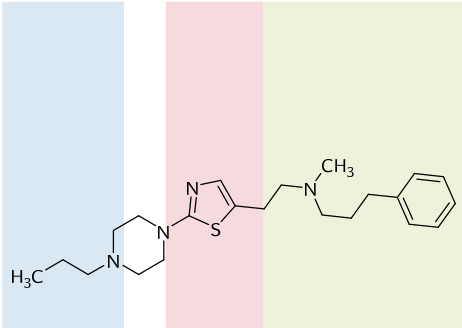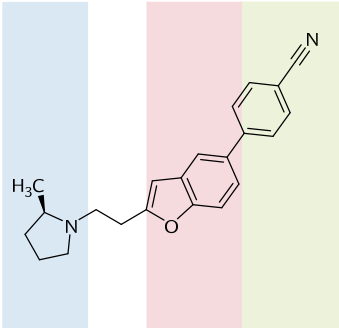
DL76 and DL77 have both already been profiled on preclinical investigation stage. As DL76 belongs to the first H$_3$R inverse agonists from the second-generation and was presented almost 15 years ago (Lazewska et al., 2006), pharmacokinetic parameters have already been determined (Szymura-Oleksiak et al., 2012; Szafarz et al., 2015). Therefore, this drug candidate has already be applied in *in vivo* model of non-neurogenetic disorders such as vascular dementia and, recently, glaucoma (Stasiak et al., 2011; Lanzi et al.,

2019). Besides, the more recent *tert*-pentyl analogue has shown anticonvulsant and pro-cognitive effects in rats, reduced ethanol intake in conditioned mice and alleviation of deficits associated with a mouse model of autism-spectrum disorder (Bahi et al., 2015; Sadek et al., 2016a; Eissa et al., 2018).

In contrast, the prototypic ligand 1-(3-phenoxypropyl)piperidine and its biphenyl congener have so far only got importance as *in vitro* tool compounds and can be ruled out as promising drug candidates due to their unsubstituted phenyl residues which are prone to extensive metabolism (Sharma et al., 2009).

As the H$_3$R pharmacophore shows as well tolerability for modifications at the central moiety, one approach towards increased drug-likeness has been the application of heterocyclic cores. This idea is based on the assumption that containing heteroatoms enable specific interactions within binding pockets of targets, and heterocyclic cores show increased metabolic stability and pharmacokinetic properties (Gomtsyan 2012). Another rationale is that a decreased lipophilicity may lead to decreased entropy-driven, unspecific interactions with off-targets and therefore, a reduced risk of toxicity and side-effects (Raymer et al., 2018). Several of H$_3$R inverse agonists that were designed upon this assumption have been investigated *in vivo* (ADS-531) or have already reached out for clinics, such as ABT-239 (Sadek et al., 2016b; Ghamari et al., 2019a). ADS-531 represents a 1,3-thiazole-based congener which shows high affinity towards H$_3$R and selectivity over H$_1$R. In a recent study, this tool compound was characterised as multitarget-directed agent with combined activity at cholinesterases and revealed pro-cognitive effects in *in vivo* studies (Jończyk et al., 2019). From docking studies, it was elucidated that the thiazole ring facilitates specific interactions with the cholinesterases and therefore, supporting the general hypothesis for employing heterocycles within drug development campaigns.

Table 8. Non-imidazole H$_3$R inverse agonists with heterocycles as central cores.

ST-979	ADS-531	ABT-239

➤ hH$_3$R: K_i = 41 nM	➤ hH$_3$R: K_i = 3.2 nM
	➤ off-targets:
	  eeAChE: IC_{50} = 14,000 nM
	  eqBuChE: IC_{50} = 15,000 nM
		➤ hH$_3$R: K_i = 0.45 nM
		➤ off-targets:
		  σ$_1$R: K_i = 6.3 nM
		  α$_{2C}$R: K_i = 63 nM
		  α$_{2A}$R: K_i = 250 nM
		  hH$_{1/2/4}$R: K_i > 1,000 nM

References: (Cowart et al., 2004; Frymarkiewicz et al., 2009; Walter et al., 2010; Riddy et al., 2019)

1.4. Progress in molecular pharmacology of histamine H₃ receptor ligands

Given the various implications of H₃R, several pharmacological concepts have emerged and are of current interest to reveal pharmacological secrets of H₃R and to screen and characterise potent H₃R ligands with improved clinical profile.

More than one decade ago, it was suggested that **receptor binding kinetics** might be more relevant in future screening campaigns. The parameter "drug-target residence time" (τ) was postulated as an improved predictor for clinical effectiveness in screening campaigns than affinity measures (only) (Copeland et al., 2006). As introduced by Robert Copeland (2006), this parameter has been defined as

$$\tau = \frac{1}{k_{\text{off}}},$$
(1)

that is proportional to the dissociation half-life, and with k_{off} being the dissociation rate constant for the given drug-target interaction:

$$RL \rightarrow R + L,$$
(2)

where R is the receptor and L ligand that together form the receptor-ligand complex RL. For the dissociation of L from LR under sink conditions, the empiric rate law is defined as

$$-\frac{d[RL]}{dt} = k_{\text{off}} * [RL],$$
(3)

that leads to the solution for $[RL]_t$:

$$[RL]_t = [RL]_0 * e^{-k_{\text{off}}*t}.$$
(4)

The relationship between affinity and kinetic rate constants is given by

$$K_D = \frac{[L]*[R]}{[LR]} = \frac{k_{\text{off}}}{k_{\text{on}}},$$
(5)

for the equilibration of L with R,

$$LR \rightleftharpoons L + R.$$
(6)

In equation (5), k_{on} depicts the association rate constant of a ligand according to equation (6). For the latter, the rate for the equilibrium reaction is defined by the reversible, second-order rate equation

$$\frac{d[RL]}{dt} = k_{\text{on}} * [R] * [L] - k_{\text{off}} * [RL].$$
(7)

Substituting experimental conditions $[L] >> [R]$, so that $[L] \approx [L]_{t=0}$, and $[R]_{t=0} = [R]+[RL]$ into (7), integration and solving for $[RL]_t$ leads to the expression for

$$[RL]_t = [RL]_{t\rightarrow\infty} * (1 - e^{-(k_{\text{on}}*[L]_{t=0}+k_{\text{off}})*t}).$$
(8)

The idea to focus on ligands with slow receptor dissociation rate has been derived from the pharmacological concept of insurmountability, which describes the inability of a ligand to displace from the receptor in an appropriate time and which, in turn, has similar effects as pseudo-irreversibility. It was alleged, τ being a better predictor for drug-effectiveness as it leads to a longer duration of target-occupancy and therefore, to sustained action in the organism (Copeland et al., 2006). This was justified referring to the organism as an open system, where equilibrium conditions do not apply, and therefore, kinetic descriptions may be more suitable to approximate the situation in organisms. Simultaneously, it was justified as a strategy to achieve kinetic selectivity and, conclusively, reducing off-target associated side-effects (Copeland, 2010). However, this claim is far from conclusions and needs more profound investigation, not only at the H₃R (Schuetz et al., 2017). In particular, this idea has been limited for the following reasons:

- Despite the aim of approximating the open system situation *in vivo*, most of the applied models utilise derivations from steady-state kinetics such as the derivations of Colquhoun (1968) and Motulsky & Mahan (1984) for competitive binding kinetics.

- Comparisons between τ and *in vivo* effectiveness are not always feasible as shown for tiotropium that shows a long residence time towards M₃R as well as kinetic selectivity over M₂R (Sykes et al., 2012). On the other hand, it is often not rationalisable, which *in vivo* parameters to correlate with (Folmer, 2018).

- If $t_{½,dissociation} < t_{½,elimination}$, then estimation of τ would be useless, meaning the drug would enable re-association as long as it is not cleared from the organism (Dahl et al., 2013). In this case, persisting drug action would emanate from its pharmacokinetic, not from pharmacodynamic, properties (Sykes et al., 2012). Additionally, $t_{½,elimination}$ is a mostly unknown parameter on the screening stage of drug discovery.

- Ranking based on dissociation kinetics only may be misleading for ligands with similar association rate constants as k_{off} then correlates with K_D, both becoming interchangeable parameters for drug screening (Copeland, 2016; de Witte et al., 2018; Folmer, 2018).

Taking account of the limitations mentioned above, the drug-target residence time concept was expanded by prompting investigation of association kinetics and reflecting τ together with the temporal receptor occupancy (de Witte et al., 2016; 2018). For example, if K_D is similar for two compounds, but they would differ in k_{off}, k_{on} will change consequently. In turn, this will affect receptor occupancy.

Albeit the unsolved question for medicinal chemistry, whether τ serves as a better surrogate for clinical effectiveness of leads, receptor binding kinetics are of general interest in molecular pharmacology. Given

limited approaches for systematic manipulation for k_{on} so far, this parameter plays a subordinate role for a medicinal chemist in lead optimisation (Copeland et al., 2006), while providing hints for the binding mechanism (Sykes et al., 2019). This rate constant consists of several micro-constants, characterising the 'journey' of a drug towards its target, such as diffusion towards the target, isomerisation processes, concentration next to and subsequent diffusion into the binding pocket and, maybe, conformational changes that reduce the probability of unbinding (i.e., induced fit). Considering a simple one-step binding mechanism, k_{on} is often limited by the diffusion rate, which is around 10^8 to 10^9 M^{-1} min^{-1} (Copeland et al., 2006).

Radioligand-based **methods** to investigate target binding kinetics have so far been of choice if target manipulation is not desired (Schuetz et al., 2017), with the accompanying disadvantage of low-throughput. The requirement of a labelled ligands puts further limits so far as direct monitoring is desired. Therefore, indirect models have been developed, such as the model of Motulsky & Mahan (1984). This model serves as a mathematical solution for the kinetics of an unlabelled ligand in competition with a labelled ligand, of which the association and dissociation rates are known. As it is derived from the law of mass action and the corresponding rate laws, this model can be described as a temporal resolution of a traditional displacement curve of a three parametric logistic fit (**Figure 9**). Previous efforts towards H₃R binding kinetics have relied on determining ligand binding by NanoBRET sensors attached to the receptor (Mocking et al., 2018), or functional [Ca²⁺]-mobilisation assays under hemi-equilibrium conditions (Riddy et al., 2019).

For labelling the receptor, some H₃R agonists and inverse agonists have been designed as radio- and fluorescence-labelled tracers. Working with commonly used and commercially available tracers in binding studies establishes comparability of data within the scientific community. However, working with radiolabelled ligands always puts constraints on the observable space of the targeted receptor and may,
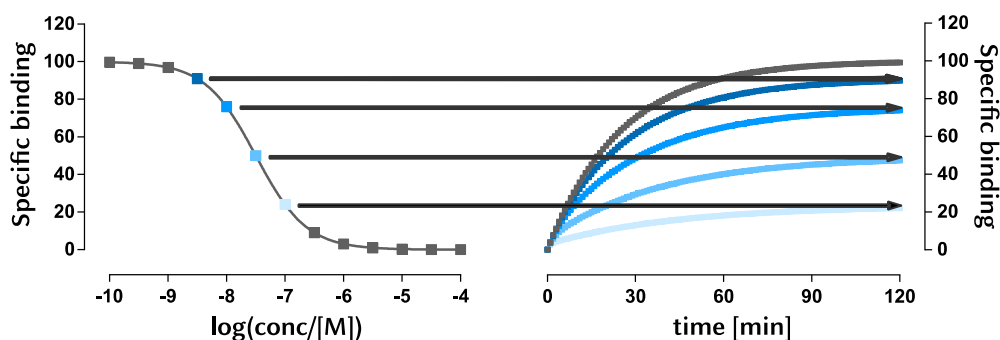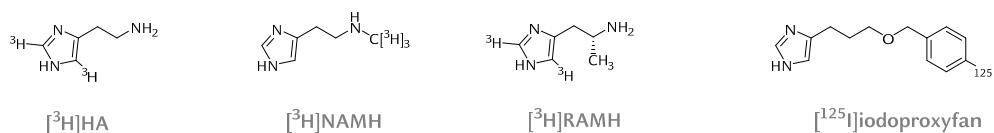

Figure 9. Relation of equilibrium and kinetics of competitive binding, simulated according to the model of Motulsky & Mahan (1984). Simulations were made with data for a labelled ligand (k_{on} = 1 10⁷ M⁻¹ min⁻¹, k_{off} = 0.02 min⁻¹, K_D = 2 nM [L] = 2 nM) and unlabelled ligand (k_{on} = 1 10⁷ M⁻¹ min⁻¹, k_{off} = 0.2 min⁻¹).

therefore, hide its essential characteristics (Kenakin, 2010). Radiolabelled tracers with widespread use in H₃R pharmacology are depicted in **Figure 10**. To date, only the presented agonist probes are commercially available for screening purposes even though interesting molecular pharmacological findings have been reported for the antagonist ones. For example, binding to a larger fraction of the receptor population was shown for both [³H]A-349821 and [³H]-thioperamide compared to agonists such as [³H]NAMH or RAMH, as well as they were able to label multiple receptor conformations with distinct affinities (Alves-Rodrigues et al., 1996; Witte et al., 2006). As introduced previously, [¹²⁵I]iodoproxyfan, which has been widely used to characterise H₃R antagonists earlier (Panula et al., 2015), despite its protean agonist behaviour and apparent insensitivity to E206^5.46 mutation that both indicate different binding modes of this chemical probe (cf. page 18).

Careful choice of a tracer for such heterologous experiments has been suggested as crucial for accurate resolution of receptor kinetics (van der Velden et al., 2020). In particular, selection of agonists may compromise the performance of kinetic experiments due to their marked biphasic binding kinetics (West et al., 1990).

full / protean agonists:

[³H]HA   [³H]NAMH   [³H]RAMH   [¹²⁵I]iodoproxyfan

inverse agonists / antagonists:

[³H]UR-PI294   [³H]thioperamide   [¹¹C]AZD5213

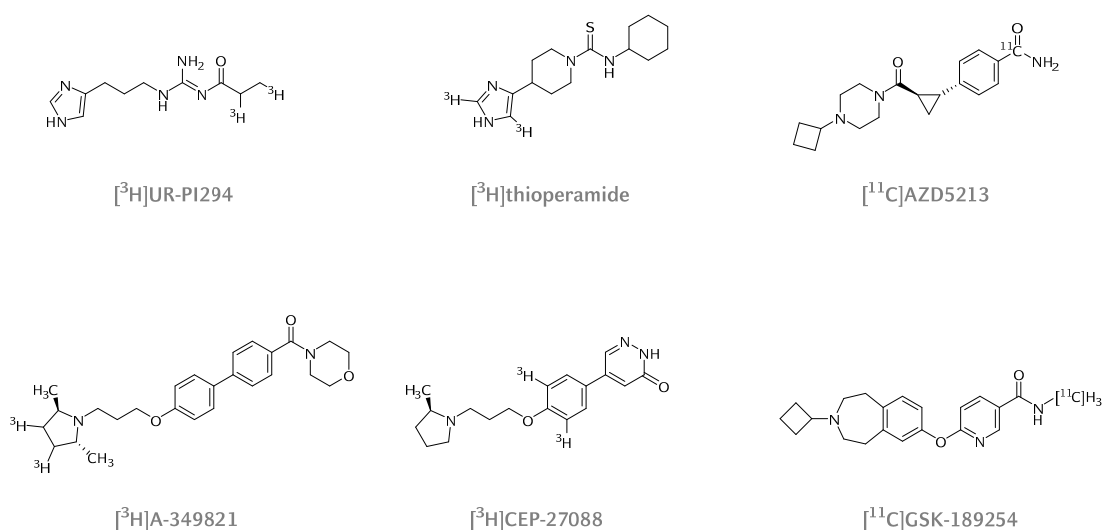[³H]A-349821   [³H]CEP-27088   [¹¹C]GSK-189254

Figure 10. Selected radiolabelled tracers for pharmacological characterisation of H₃R taken from (Arrang et al., 1987a; Alves-Rodrigues et al., 1996; Stark et al., 1996; Hamill et al., 2009; Igel et al., 2009; Andrews et al., 2012; Panula et al., 2015; Dahl et al., 2018).

Fluorescently labelled tracers are of high interest in modern molecular pharmacology due to their wide applicability in fluorescence-imaging and (fluorescence/bioluminescence) resonance energy transfer ((F/B)RET) techniques (Stoddart et al., 2016). Compared to radiolabelled ligands, they reduce the regulatory efforts and safety concerns (Sridharan et al., 2014). However, only a few attempts have been made in the application of fluorescently labelled ligands to elucidate further features of H₃R pharmacology, despite of commercial availability of several potent and selective tools such as mirisant-405 and bodilisant that are depicted in Figure 11. In addition to them, a BODIPY-labelled analogue of clobenpropit is commercially available, without disclosed structure but probably belonging to the imidazole-based type (Mocking et al., 2018). Additionally, a BODIPY-labelled histamine analogue shows mediocre binding at H₃R (Mocking et al., 2018). UR-DEBa242 represents the most recently published fluorescent tracer for H₃R (Bartole et al., 2020). However, as BODIPY-histamine and BODIPY-clobenpropit, the novel probe is not selective over other histamine receptor subtypes (Mirzahosseini et al., 2015; Mocking et al., 2018; Bartole et al., 2020). In contrast to the imidazole-based analogues, bodilisant resembles a selective H₃R ligand which is suitable for *ex vivo* receptor staining in tissues (Tomasch et al., 2013). Conclusively, this chemical probe permits novel approaches to H₃R binding behaviour with satisfactory properties for replacing radiolabelled tracers in the molecular pharmacology of the receptor.

imidazole-based fluorescence tracers

BODIPY-histamine   UR-DEBa242

non-imidazole-based fluorescence tracers

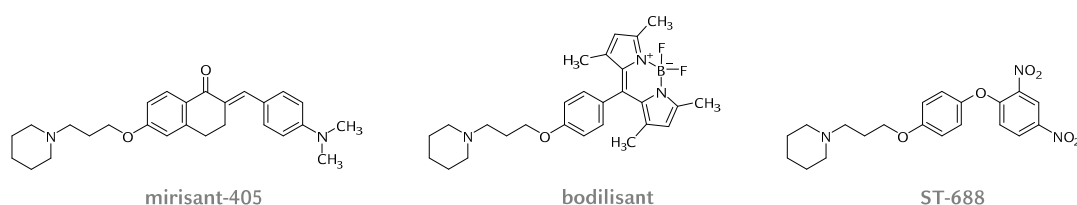mirisant-405   bodilisant   ST-688
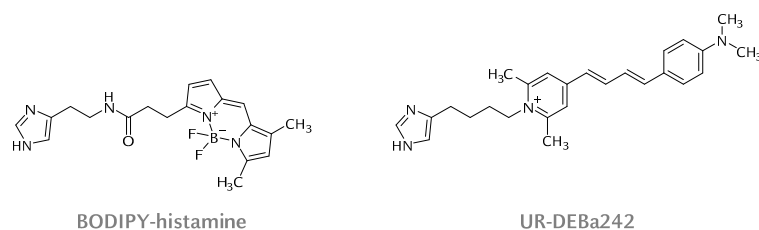
Figure 11. Fluorescence tracers for H₃R, taken from (Amon et al., 2006; Tomasch et al., 2012; Tomasch et al., 2013; Mirzahosseini et al., 2015; Bartole et al., 2020).

1.5. Histamine H₃ receptor ligands in neurogenetic disorders

Many diseases for which H₃R ligands may provide beneficial effects accompany disorders that are linked with alterations on gene expression level. Such diseases termed neurogenetic disorders have been defined as clinical diseases that are based on genetical defects with alterations in "differentiation and function of the neuroectoderm and its derivatives" (Müller et al., 1994). This large group of disorders can be subdivided into four major classes according to their genetic aetiology (Bird, 2009):

Chromosomal disorders are characterised by chromosomal deletions, inversions, translations and multiplications as well as *de novo* genetic mutations with strong selective pressure. **Mendelian disorders** consist of an inheritable component due to genetic mutations or copy-number variations. **Mitochondrial**ly denoted neurogenetic diseases show deficits in electron transport chain components and are as well inheritable, either maternally from mitochondrial DNA or autosomal from nuclear DNA. A fourth group encompass diseases which combine genetic disposition (polygenic) with environmental factors (**multifactorial**) and, thus, represent diseases of heterogenous aetiology, which

Figure 12. Classification of neurogenetic disorders with selected indications for H₃R ligands, according to literature (Bird, 2009; Ghamari et al., 2019a).

usually manifests after infancy (Müller et al., 1994; Bird, 2009). Various neurogenetic diseases where histamine H₃ receptor ligands have been under clinical evaluation are depicted within this classification in Figure 12. Of such, relevant conditions for this work are elaborated below in more detail.

Neurogenetic disorders often share **co-morbidities** of other mental diseases. Vice versa, candidate genes for several disorders often overlap with such for others, as seen from recent genome-wide association studies (GWAS). Such shared co-morbidities encompass

- intellectual disability,
- behavioural conspicuousness,
- impacted attention and perception (Ornitz, 1969),
- sleep disorders (Dosier et al., 2017),

- schizophrenia, 1 in 100-200 individuals with schizophrenia are linked with 22q11.2 deletion syndrome (Gur et al., 2017),

- autism-spectrum disorders (American Psychiatric Association, 2013a) and

- compulsive-obsessive disorders (Grove et al., 2019).

Additionally, many syndromes belonging to mental disorders show remarkable inheritability, although the molecular genetic nature is in most cases as elusive as the neuropathology. However, major advances have been made within the last two decades upon increasing cohorts of GWAS that allow for correlation with rare genetic variants (Hirschhorn et al., 2005). In all neurogenetic diseases, alterations on genomic level lead to alterations in distinct neuron populations and therefore, in different neurotransmitter systems. Two central theories have evolved to explain how ubiquitous genetic alterations can impact the function and even degeneration of specific cell-types: The theory of **selective vulnerability** rationalises that variances in a broadly/ubiquitously expressed gene makes one specific cell-type vulnerable (Fu et al., 2018). In contrast, that of **selective expression** describes a defect cell-type as consequence of perturbated gene expression that is usually expressed from this one cell-type only.

The sleep disorder **narcolepsy** is characterised by severe daytime sleepiness during phases where vigilance is required, and REM sleep during the wake period, despite adequate sleep during the night (Scammell, 2015). The disorder can be accompanied by cataplectic attacks which are characterised by sudden loss of muscular tone. According to the aetiology, narcolepsy is classified as type 1 that is characterised by loss of ORX-A or ORX-B and as an idiopathic type 2 (Scammell, 2015). It has been hypothesised that the loss of orexin producing neurons is linked to an autoimmune mechanism as a specific human leukocyte antigene (HLA) expression pattern has been associated with 95 % of the cases (HLA class II allele: HLA-DQB1*0602) (Mignot et al., 1997). However, previous descriptions of an infantile case of narcolepsy has been attributed to a *de novo* non-functional L16A mutation in the signal domain of the ORX precursor prepro-orexin (Peyron et al., 2000; Kornum et al., 2017). Given the multifactorial explanation of pathogenesis, the disorder is classified as a disorder of multifactorial genesis (Figure 12)(Yamasaki et al., 2016). The neuronal circuits of orexigenic neurons are directly linked to the pharmacotherapeutic strategy for H₃R ligands. Their application aim to compensate the missing orexinergic excitatory input to TMN histaminergic neurons by H₃ auto- and **heteroreceptors**. Thus, **pitolisant** has been granted market authorisation by the U.S. Food and Drug Administration (FDA, 2016) and the European Medicines Agency (EMA, 2019) while some other H₃R inverse agonists are on clinical development state (Syed, 2016; Ghamari et al., 2019a; Kumar et al., 2019).

Schizophrenia is a psychiatric syndrome characterised by episodic (> 1 months) hallucinations, delusions or disorganised speech, while each of such symptoms presents in complex forms and can appear in combination with each other (American Psychiatric Association, 2013d). During and in between of such episodes, patients appear as well with negative symptoms such as social withdrawal, cognitive impairments, lacking motivation as well as deficits in executive function and memory (McCutcheon et al., 2020). The still supported molecular pathological theory focussing on imbalanced dopaminergic, and subsequent glutamatergic and GABAergic neurotransmission, relies on the consistency with clinical effects of applied D_2R/D_3R antagonists (Lau et al., 2013). However, only the hypothesis of GABAergic hypofunction remains irrefutable up to post-mortem analysis (Birnbaum et al., 2017). The difficulties in the alignment of results from *in vivo* and post-mortem studies is mostly due to that examined tissues have persisted in pathological environment for up to several decades and may have undergone compensatory mechanisms (Birnbaum et al., 2017). Typically, the disease manifests in early maturity and has a long-since known inheritable component (Kallmann, 1938), which is estimated to be about 80 % (Cardno et al., 2000). Theories for attributing positive and negative symptoms to specific alterations in specific neuronal circuits dominate current discussion as will for the molecular genetic trigger. Despite remarkable inheritability and several identified candidate genes from genome-wide association studies (GWASs)(Hirschhorn et al., 2005), unambiguous ramifications with the aetiology of schizophrenia remains impossible as long as comprehensive pathophysiological explanations are overdue (Birnbaum et al., 2017). Thus, and due to non-excludable environmental factors contributing to schizophrenia, the disorder is best classified as of multi-factorial and polygenic origin, as done in Figure 12. Relying on the dopaminergic hypothesis, which is based in dopaminergic hyperactivity in the mesolimbic system but as well in caudate nucleus, H₃R inverse agonists have emerged as potential treatment of schizophrenia. Even if an effect of H₃R inverse agonists on psychosis is without evidence, they emerged due to their pro-cognitive properties and their ability to ameliorate glutamatergic hypoactivity seen in basal ganglia of many patients. The latter is a feature of H₃R inverse agonists that D_2R antagonists do not exert (Faucard et al., 2006; Moghaddam et al., 2012; Ellenbroek et al., 2014).

Gilles de la Tourette syndrome (GTS) is a disease belonging to tic disorders with involuntary, stereotyped motoric and vocal movements. It has an unknown genetic cause but a strong heritable component (Albin et al., 2006). However, some accordance with chromosomal translocations and genetic polymorphisms with impacts on neuronal growth and dopaminergic neurotransmission was found (O'Rourke et al., 2009). So far, mainly D_2R and D_3R antagonists have been used in the pharmacotherapy of GTS (Thomas et al., 2013). The linkage to H₃R in the disease is attributed to its role in basal ganglia,

where increased inhibitory striatonigral and striatopallidal input leads to insufficient curbing of motoric events (Albin et al., 2006; Rapanelli et al., 2016). From a genetic perspective, interestingly, an association to histaminergic neurotransmission seems likely for frequently observed SNP on the *hdc* gene on chromosome 15q21.2 (Karagiannidis et al., 2013; Udvardi et al., 2013). Interestingly, upregulated H₃R expression in striatum was reported for HDC$^{-/-}$ mice (Pittenger, 2020) as well as affected gene expression in H₁R and H₂R signalling pathways (Fernandez et al., 2012). Finally, promising effects from a phase II clinical trial in GTS patients have been reported for the H₃R inverse agonist AZD5213 (Rizzo et al., 2019). However, no follow-up has been communicated so far. Common co-morbidities with GTS are attention-deficit disorders, for which H₃R inverse agonists have been investigated clinically (Ghamari et al., 2019a), as well as compulsive-obsessive disorders which are shown by 52-75 % of GTS patients (McDougle et al., 1993).

Alzheimer´s disease (AD) belongs to the neurodegenerative disorders and represents a dementia syndrome, characterised by a loss of acetylcholine producing neurons. It is either diagnosed upon a decline in memory and learning, with a steadily progressive decline in cognitive functions, which is without transient stagnation and other cause (e.g., cerebrovascular events), or upon familial history associated with causative genetic basis (American Psychiatric Association, 2013b). In 2016, this disease affected around 43 Mio people globally, which was more than twice as much as in 1990, and this number is expected to double within another 15 years (Nichols et al., 2019). With about 2.4 Mio deaths within that year, AD became the fifth leading death cause in 2016 (Nichols et al., 2019). Slightly more women (1.17-fold, after standardisation for generally longer lifetime of women) suffered from the disease compared to men (Nichols et al., 2019). While most of the AD patients become symptomatic above the age of 65, it does not preclude events of earlier onset (Tellechea et al., 2018). Overall, a disease heritability of 80 % have been estimated while environmental factors contribute to the aetiology. Thus, AD also belongs to polygenic/multifactorial neurogenetic disorders (Figure 12)(Bettens et al., 2013; Van Cauwenberghe et al., 2016).

Progress has been made in delineating genetic predisposition of AD, which has found to attribute to 5-10 % of early-onset AD cases and show high degrees of inheritability (Van Cauwenberghe et al., 2016). Such approaches have focussed on neurotoxic β-amyloid plaques presented in the pathophysiology. First, autosomal-dominant mutations in genes encoding for amyloid precursor protein (APP, gene-locus 21q21.3) were ascribed responsible for driving the formation of the fibril precursor β-amyloid fragment, aa sequence 1-42 (Aβ$_{1-42}$)(Van Cauwenberghe et al., 2016). Secondly, mutations in components of the γ-secretase complex, which is responsible for Aβ$_{1-42}$ cleavage (presenilin 1 and 2, loci 14q24.2 and 1q42.13,

respectively), were correlated with several cases (Van Cauwenberghe et al., 2016). Thirdly, the high-density lipoprotein component apolipoprotein-E (ApoE), which has been found present in amyloid plaques, appears in different isoforms from allelic variants (ε2, ε3, ε4) on locus 19q13.32, of which ε3 is most common (Weisgraber et al., 1996). However, ε4 has been associated with increased susceptibility for AD with a positive correlation to ε4-concentration (Corder et al., 1993). At the age of 85, the ε4-allele accounts for an increased lifetime risk (LTR) for AD in heterozygous carriers of the ε4-allele (female: LTR = 30 %, male: LTR = 23 %) which duplicates again for homozygous ones (female: LTR = 60 %, male: LTR = 51 %), compared to people with homozygous ε3-allele (female: LTR = 14 %, male: LTR = 11 %). By contrast, a protective value was attributed to the second isoform (ε2) (Corder et al., 1994). A large GWAS among approx. 74,000 individuals was conducted and resulted in several loci, of which SNPs could be correlated with late-onset of AD (Lambert et al., 2013). Further GWASs revealed further genetic polymorphisms in the context of AD, and raised implications for intracellular deposited hyperphosphorylated tau protein-aggregates which represents another neurotoxic process in AD (Van Cauwenberghe et al., 2016).

AD has been associated with decreased activities of HA and ACh synthesising enzymes (HDC and choline acetyltransferase, respectively) that contributes to the overall cholinergic deficits in patients (Schneider et al., 1997). In addition to reductions in norepinephrine, 5-HT and DA brain levels as a result of a progredient global neurodegeneration, the orexinergic system has evolved as putative co-actor as it triggers generation of amyloid plaques in AD (Wang et al., 2018). H₃R ligands are clinically examined for their symptomatic approach to AD due to their role in cognition, learning & memory, such as GSK-239512 and AZD5213 (cf. Figure 13)(Nathan et al., 2013; Ghamari et al., 2019a).

After AD, **Parkinson´s disease (PD)** represents the second-most prevalent neurodegenerative disorder with 6.1 Mio persons affected in 2016. Similar to AD, this number has doubled from1990 and is assumed to double once again until 2040 as a result of higher life expectancy (Dorsey et al., 2018; Rocca, 2018). Also, this disease has an onset after the 50th year of age, but, in contrast to AD, affects 1.4 times more males than females. PD is a clinical diagnosis made upon loss of motoric movement (bradykinesia) with rest tremor, lead-pipe rigidity and assessment of selective or supportive criteria (such as response to levodopa or olfactory loss) (Kalia et al., 2016). Patients suffering from PD can show neurocognitive decline, anxiety, depressive and sleep-wake disorders during the progression of the disease (American Psychiatric Association, 2013c). Hence, pitolisant as well as bavisant (cf. Figure 13) were examined in phase 3 and phase 2 clinical trials concerning excessive daytime sleepiness (EDS) in PD (clinicaltrials.gov identifiers: NCT01066442, NCT01036139 and NCT03194217)(Ghamari et al., 2019a). Based on a case series, marked changes in the Epworth sleepiness scale-score were reported for which the community waits for

the pending results from the interventional study (Liguori et al., 2020). From the neuropathologic point of view, PD is based on the degeneration of dopaminergic neurons in SN well before the onset of symptoms, which has decoupling of motoric regulation between frontal motor-cortex, subthalamus and basal ganglia as common consequence (Poewe et al., 2017). On a molecular level, the detection of intraneuronal oligomeric aggregates consisting of α-synuclein (SNCA), ubiquitin and other protein filaments (termed "Lewy bodies") have been found as neurotoxic agent (Poewe et al., 2017). Recent progress on a molecular level has pointed towards a prion-like behaviour due to an apparent transferability of SNCA aggregates between different neurons, the ability of such aggregates to recruit additional SNCA in unaffected cells, and the potential of SNCA aggregates to cross the BBB (Kalia et al., 2015). However, the point of origin of the aggregation process is elusive in most of the PD cases, despite some cases of genetically determined forms (Blauwendraat et al., 2020). Furthermore, a potentially environmental intake of SNCA aggregates which trigger the pathophysiological process is under current discussion (Scialò et al., 2020).

More than 20 rare variants are known to predispose PD, and despite the increasing assumption of a genetic background for most cases, not all of the rare variants are unambiguously associated with the disease; still, larger cohorts with more rare or very rare variants in GWAS are needed to complete evidence (Blauwendraat et al., 2020). Prominent examples with reasonable relation to PD include autosomal-recessively inherited parkin (PRKN/PARK2, gene-locus: 6q26), PTEN-induced kinase (PINK/PARK6, gene-locus: 1p36) and DJ-1 (PARK7, locus: 1p36.23), all of which being essential for adequate mitochondrial function, for which genetic alterations can cause early-onset PD (Canet-Avilés et al., 2004; Valente, 2004). Besides such mitochondrial diseases, prominent monogenic variants are found for the lysosomal leucine-rich repeat kinase 2 (LRRK2/PARK8, locus: 12p11.2-q13.1) and SNCA encoding genes with autosomal-dominant inheritance and with either late- or early-onset characteristics, respectively (Deng et al., 2018).

Autism-spectrum disorder (ASD) is defined by deficits in social interaction, in communication to a verbal and non-verbal extent, and includes restriction or repetition in behaviour and motoric movements, all with typical onset in infancy (American Psychiatric Association, 2013a). It is a heterogeneous disease spectrum, based on the clinical appearance between several patients but as well on intra-individual differences in autistic behaviour throughout development, and it frequently occurs with a cognitive component (Lord et al., 2000). While a genetic cause of ASD seems confirmed based on correlations between twin pairs suffering from the disorder (Lichtenstein et al., 2010; Hallmayer, 2011), no pertinent link to one specific genetic aberration could be drawn yet. For example, ASD has been associated with partial tetrasomy on chromosome 15q11-13, together with various other chromosomal aberrations in some individuals (Gillberg, 1998). Conversely, *de novo* events have been put as a cause in the last years based

on the observation that ASD is often accompanied by fertility and, thus, genetic marks being mostly noninheritable (Castellani et al., 2020). Such missense and nonsense variants are found approximately in half (46.3 %) of the patients (Neale et al., 2012). Recently, some correlations between missense variants and ASD could be made in a whole-exome association study among approx. 12,000 patients, in which gain-of-function mutations were described, e.g., in the kcnq3 gene for $K_v7.3$ channel or *scn1a* gene for $Na_v1.1$. The first finding is an intriguing one as HA, through H_1Rs, can decrease the activity of K_v7-type channels (Obara et al., 2020). Besides possibly direct links between ASD and the brain HA system, recent findings point towards decreased acetylcholine levels in mouse models with autism-like characteristics (Karvat et al., 2014). Therefore, this may be a hint for the molecular action of the H₃R inverse agonist DL77 that provided promising effects in previous *in vivo* studies with ASD mice (Eissa et al., 2018). A direct effect of DL77 on H_1R cannot be precluded as the compound appeared selective towards H₃R but still with remarkable activity at H_1R in submicromolar concentration range (Eissa et al., 2018). Nevertheless, application of H₃R inverse agonists for the treatment of ASD represents an indication on preclinical investigation stage (Ghamari et al., 2019a).

Compared to other genetic disorders introduced above, **Prader–Willi syndrome (PWS)** represents a neurogenetic disorder with well-characterised genetic determinism which is long-since known and, thus, forms part of diagnosis (Buiting, 2010; Beygo et al., 2019). In more detail, PWS is an imprinting disorder that is caused by a loss of genes from paternal chromosome 15q11.2-13 by either *de novo* deletion, uniparental disomy, microdeletions or imprinting defects. Additionally, the corresponding genes on the maternal chromosome are epigenetically silenced under physiological conditions (Beygo et al., 2019). Within this imprinted domain, several genes encoding for proteins and small nucleolar RNA (snoRNA) clusters (SNORD) are located, the latter being involved in RNA editing processes (Mehler, 2008; Beygo et al., 2019). While the unique lack of several proteins (MKRN3, MAGEL2, NDN) have demonstrated not to induce PWS in full extent (Kanber et al., 2008), lacking the protein SNURF-SNRPN and SNORD116 cluster is strongly correlated with the full phenotype. However, no functional role thereof is known to date (Bieth et al., 2015; Beygo et al., 2019).

Langdon Down made the earliest known documentation of a PWS patient in 1864, but Andrea Prader, Alexis Labhart and Heinrich Willi provided a detailed clinical description about 90 years later (Prader et al., 1956; Ward, 1997). The disorder is in its initial prenatal and infantile stage characterised by markedly reduced muscular tone, leading to reduced foetal movements. After birth, complications in feeding and metabolism leads to a predisposition for increased mortality due to decreased energy intake and aspiration risk (Holm et al., 1993). Following normalisation of this hypotonia, which has an onset between the second

and mandatorily, before the sixth year of age, the situation reverses with weight gain and hyperphagia accompanied by delayed development, which leads to a reduced foetal movement (Cassidy, 1997; Cassidy et al., 2009). Mortality during adulthood is increased due to the occurrence of metabolic disorders, without accounting for an additional risk component due to mild intellectual disability (Einfeld et al., 2006). Besides, additional behavioural characteristics of PWS patients can be compulsivity, obsessiveness, manipulative and argumentative behaviour and problems with changes in daily routine (Cassidy et al., 2012).

Similar to other neurogenetic disorders, PWS shares many facultative co-morbidities such as sleep disorders/EDS, cognitive deficits, autism, attention-deficit/hyperactivity disorder and psychosis (Cassidy et al., 2012) for which H₃R inverse agonists have emerged as pharmacotherapeutic option. Interestingly, the long-since known betahistine emerged as a potential drug for PWS with market-approval for more than 50 years (cf. **Figure 13**). While it has been tried to exploit its H₃R antagonist and H₁R (partial) agonist mode of actions on improved vestibulocochlear blood flow in preclinical studies, these effects have not been sufficiently evidenced in clinics yet as elaborated by the Cochrane collaboration (Murdin et al., 2016). These drawbacks and the low interest in betahistine as combined H₁R (partial) agonist / H₃R antagonist are for one thing attributed to its low potency at H₁R but, maybe more relevant, to its poor bioavailability and BBB permeability. To overcome these obstacles, an intranasal formulation of this drug was developed and subjected to clinical trials. Recently, it was granted an orphan drug status by the FDA for the therapy of obesity that is associated with PWS (Provensi et al., 2016a; Timmins, 2019). Furthermore, **pitolisant** has emerged as a potential drug in the treatment of PWS as improvements in cognitive performance and mental clarity, decreased sleepiness, as well as moderate amelioration in behavioural problems, have been reported on case series (Pullen et al., 2019a; Pullen et al., 2019b; c).
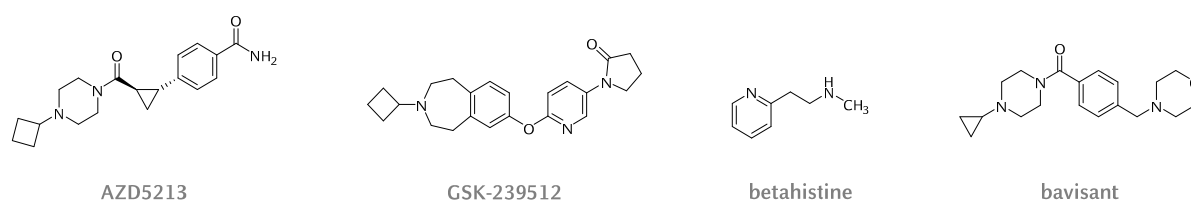

AZD5213   GSK-239512   betahistine   bavisant

Figure 13. Selected H₃R antagonists as current clinical candidates against neurogenetic disorders.

1.6. Multitarget-directed histamine H₃ receptor ligands in neurogenetic disorders

The variety of H₃R in neurological processes as outlined in section 1.1 in addition to the traditional role of H₃R in neurogenetic diseases (section 1.5) prompt for novel strategies to improve the effectiveness of H₃R ligands on clinical investigation stage. Studiously designed ligands that combine several activities among relevant neurological circuits have emerged, promising to achieve additive effects, while reducing side-effects due to off-targets (Proschak et al., 2018). In contrast to dirty drugs, a perfect multitarget-directed ligand (MTDL) would elicit its actions only on the desired targets while maintaining selectivity over off-targets (Dessalew et al., 2008). Besides, the application of such agents can reduce the number of administered drugs and thereby, reduce the metabolic work-load (Proschak et al., 2018).

The principle of H₃R-MTDLs is as old as the discovery of H₃R, with the early finding of potent H₃R antagonism exerted by a previously known H₁R partial agonist (betahistine, Figure 13). Thereby, this drug became the first marketed H₃R antagonist and first MTDL with H₃R potency. While clinical effectiveness in Menière´s disease is questioned based on a recent Cochrane review (Murdin et al., 2016), it has been subjected for clinical investigation proving for its effectiveness in CNS-mediated diseases such as primary and secondary obesity, associated with administration of antipsychotics and in the PWS (Nelson et al., 2012; Kang et al., 2018; Ghamari et al., 2019a).

From a drug design perspective, MTDL approaches can be subdivided in linking, fusing or merging pharmacophores and pharmacophore fragments (Morphy et al., 2005). Of such, the latter one is preferred due to the avoidance of decreased drug-likeness as a consequence of increased molecular size (Proschak et al., 2018).

MTDL approaches involving the H₃R can be subdivided among several co-targets, such as other (GPC)Rs, enzymes, transporters or releasing mediators for signalling (Khanfar et al., 2016). Various have been subject matter of successful drug design campaigns, but the only evidence for almost all of such drug candidates is of preclinical nature (Ghamari et al., 2019a). In most of such examples, strategic coactivity not only follows the purpose to address several neurotransmitter systems involved in a given disease, e.g., by addressing their receptors or inhibiting their degradation. Due to the widespread involvement of **heteroreceptors**, the major driver of MTDL approaches has been the purpose of achieving synergistic effects in specific neurotransmitter systems upon activation of their release, combined with inhibiting their catabolism (Bautista-Aguilera et al., 2018). Furthermore, several neurogenetic disorders share psychiatric co-morbidities, such as attention deficit ADHD, schizophrenia, sleep-wake disorders. Thus, these disorders may be best addressed in a multi-strategical approach.
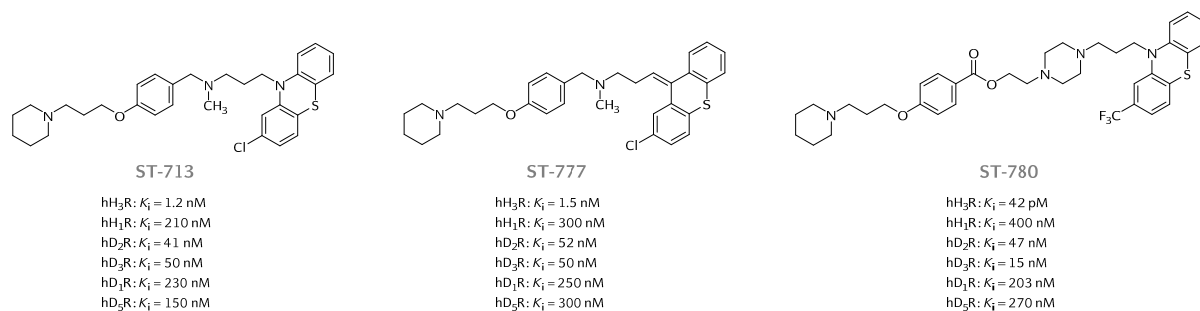
ST-713

hH₃R: K_i = 1.2 nM
hH₁R: K_i = 210 nM
hD₂R: K_i = 41 nM
hD₃R: K_i = 50 nM
hD₁R: K_i = 230 nM
hD₅R: K_i = 150 nM

ST-777

hH₃R: K_i = 1.5 nM
hH₁R: K_i = 300 nM
hD₂R: K_i = 52 nM
hD₃R: K_i = 50 nM
hD₁R: K_i = 250 nM
hD₅R: K_i = 300 nM

ST-780

hH₃R: K_i = 42 pM
hH₁R: K_i = 400 nM
hD₂R: K_i = 47 nM
hD₃R: K_i = 15 nM
hD₁R: K_i = 203 nM
hD₅R: K_i = 270 nM

Figure 14. H₃R-MTDLs with co-target activity at DA receptor subtypes, taken from von Coburg et al. (2009).

Besides the previously outlined example of the combined H₁R/H₃R activity exerted by betahistine (cf. page 46), DA and 5-HT receptors have emerged as reasonable co-targets among neurological GPCRs (Butini et al., 2016). Among them, **dopamine receptor subtypes** are potential co-targets to H₃R for antagonists applied in schizophrenia. However, they may have their implications for GTS and ASD as well, where antipsychotics have shown symptomatic amelioration (Posey et al., 2008; Roessner et al., 2011). Albeit the strategy to address striatal glutamatergic activity in patients suffering from schizophrenia and GTS, selective H₃R antagonists have appeared without consistent effectivity in models for schizophrenia (Ligneau et al., 2007a; Ellenbroek et al., 2014), for which H₃R/D₂R/D₃R antagonists/inverse agonists may be useful. Their implications in GTS is linked to the reduced HA synthesis, but as well to elevated striatal DA levels that have been found upon impairment of HDC function (Rapanelli et al., 2014). Figure 14 exemplifies several ligands with combined affinity at H₃R and dopaminergic receptor subtypes. Such were derived by fusing the H₃R pharmacophore with chlorpromazine (ST-713), chlorprothixene (ST-777) or fluphenazine (ST-780), a strategy making them appear with relatively large molecular size (M_r = 530 to 680 Da) (von Coburg et al., 2009). Hence, pharmacophores with decreased molecular size are of current interest.

Acetyl- and butyrylcholinesterase (AChE/BuChE) are enzymes, which belong to serin hydrolases and are responsible for the degradation of ACh in order to abrogate cholinergic neurotransmission (Alexander et al., 2019b). In turn, ACh is a crucial neurotransmitter in the parasympathetic nervous system, where it mediates vegetative functions among nicotinic and muscarinergic receptor subtypes, but as well in neuromuscular excitation upon activation of nicotinic receptors. In the central nervous system, where ACh elicits actions among both receptor types, this neurotransmitter is responsible for (re)cognition processes and consolidation of memory (Hasselmo, 2006). Thus, ACh catabolic enzymes are targets for the treatment of AD (cf. page 42). However, among several approved AChE/BuChE inhibitors, only one selective ligand with sufficient blood-brain-barrier permeability has been marketed so far (donepezil, Figure 15). While a recent review by the Cochrane collaboration ascribes some benefits in cognition, learning and memory for

physostigmine

hAchE: IC_{50} = 20 nM
hBuChE: IC_{50} = 20 nM

donepezil

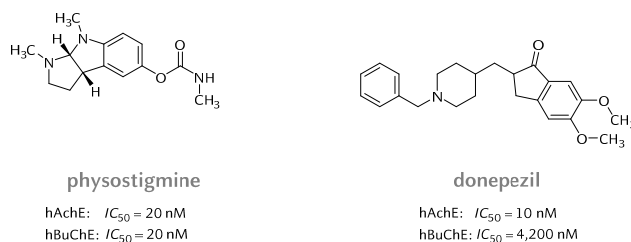hAchE: IC_{50} = 10 nM
hBuChE: IC_{50} = 4,200 nM

Figure 15. Structures of approved non-selective and selective AChE/BuChE inhibitors with activities (Luo et al., 2006; Alexander et al., 2019b).

donepezil, it did not markedly change the quality of life for patients suffering from AD (Birks et al., 2018). The engagement of H₃R in cholinergic transmission and its role in cognition, learning and memory conveyed its targetability in diseases such as Alzheimer´s (Esbenshade et al., 2008; Reitz et al., 2011; Shan et al., 2015a; Zlomuzica et al., 2016).

Even if H₃R antagonists still do not provide a causal cure to the disease, they have emerged as promising candidates being on clinical investigation stage, however, without relevant results (Ghamari et al., 2019a). Various demonstrations of increased acetylcholine levels in hippocampus and prefrontal cortex upon administration of H₃R inverse agonists (Panayi et al., 2017), together with retardation of acetylcholine degradation leads to additive synergism (Schwartz et al., 2016). Therefore, MTDLs involving H₃R, AChE and BuChE have been the subject matter of several research campaigns for exerting direct and indirect stimulation of cholinergic neurotransmission and thereby, increased cognitive performance.

Facilitating cholinergic signalling is not only valuable in AD. Moreover, it has recently found relevance for **ASD** (cf. page 44) as cholinergic deficits were observed in the BTBR T⁺tf/J mouse strain, which fulfils all behavioural symptoms of autism (McFarlane et al., 2008; Karvat et al., 2014). However, studies with rivastigmine and donepezil have shown inconsistent effectiveness towards behavioural and articulatory features of ASD (Handen et al., 2011; Lee et al., 2014). Besides, amelioration in glutamatergic signalling showed to improve sociability deficits in BTBR T⁺tf/J mice (Silverman et al., 2010; Meyza et al., 2013). Finally, cognitive improvements upon administration of AChE inhibitors have been suggested to treat Lewy body dementia in **PD** (Hutchinson et al., 1996).

Ladostigil represents an MTDL drug candidate on clinical investigation stage for the treatment of AD and Lewy body dementia (**Figure 16**). From a structural point of view, it consists of a rasagiline fragment,

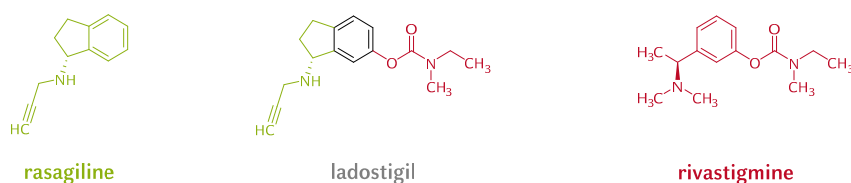
rasagiline

ladostigil

rivastigmine

Figure 16. Rational design of ladostigil as merged rasagiline and rivastigmine pharmacophore fragments and with combined MAO & cholinesterase affinity (Weinreb et al., 2012).

merged with the pharmacophore of rivastigmine to combine inhibition of ChE with MAO (Weinstock et al., 2000; Weinstock et al., 2003; Weinreb et al., 2012). However, and following rasagiline, ladostigil exerts inhibition of **MAO B** as 6-hydroxyrasagiline, which is formed as metabolite after carbamate cleavage (Sterling et al., 2002). The latter enzyme exists in two therapeutically exploited isoforms for which reversible and irreversible, and as well some subtype-selective inhibitors have been granted market authorisation for the treatment of PD (cf. page 43) and depression (Youdim et al., 2006).

The underlying pharmacodynamics is a diminished degradation of neurotransmitters DA, 5-HT and norepinephrine that results in increased availability of such within the synaptic cleft. For ladostigil, however, introduction of a propargylamine in order to irreversibly inhibit MAO was neither rationalised on increasing neurotransmitter levels nor as MAO being an established target of classic antidementives. The strategy for the design of ladostigil was based on neuroprotective effects associated with propargylamine. Such effects are a consequence of specific mechanisms in APP secretion (Weinreb et al., 2012). First, rasagiline triggered the secretion of soluble non-amyloidogenic APP via α-secretase and PKC/MAPK-dependent mechanisms. The same was shown by ladostigil, the distomer of rasagiline and even propargylamine alone (Yogev-Falach et al., 2002; Yogev-Falach et al., 2003). Secondly, all of such compounds showed a neuroprotective effect against Aβ$_{1-42}$ due to antiapoptotic effects (Yogev-Falach et al., 2003; Yogev-Falach et al., 2006). Unfortunately, the expectations of almost 20 years research about ladostigil failed the primary endpoint in phase 2 clinical trial, which was the delay of progression from mild cognitive impairment to AD (Schneider et al., 2019).

Combined H₃R antagonist/ChE inhibitors have been proposed and designed for more than ten years and emerged as potential lead compounds for cognitive impairment in dementia and AD. Such ligands consisted of a fused tacrine pharmacophore or donepezil fragment (Apelt et al., 2002; Petroianu et al., 2006; Morini et al., 2008), but still bearing a molecular weight below 480 Da (Figure 17). Recent developments in H₃R MTDLs have been the discovery of inherent reversible MAO-inhibiting properties of prominent H₃R ligands such as ciproxifan and UCL-2190 (cf. page 28 and 30), and the subsequent design of reversible **H₃R ligands/MAO B inhibitors** with the purpose to obtain leads for preclinical investigation in models of PD (Hagenow et al., 2017; Affini et al., 2018). Similar inhibition of MAO B elicited by ciproxifan, and its non-imidazole congener UCL-2190 raised the assumption that the pharmacophore for MAO B consist of the cyclopropylcarbonyl substituent (Bautista-Aguilera et al., 2017). As a combination of both strategies, such efforts were applied to the systematic design of ASS234 and contilisant that represent small-molecule H₃R inverse agonists (Bautista-Aguilera et al., 2017). Whereas ASS234 consists of a merged H₃R/ChE motif fused with a donepezil fragment, contilisant represents an MTDL with
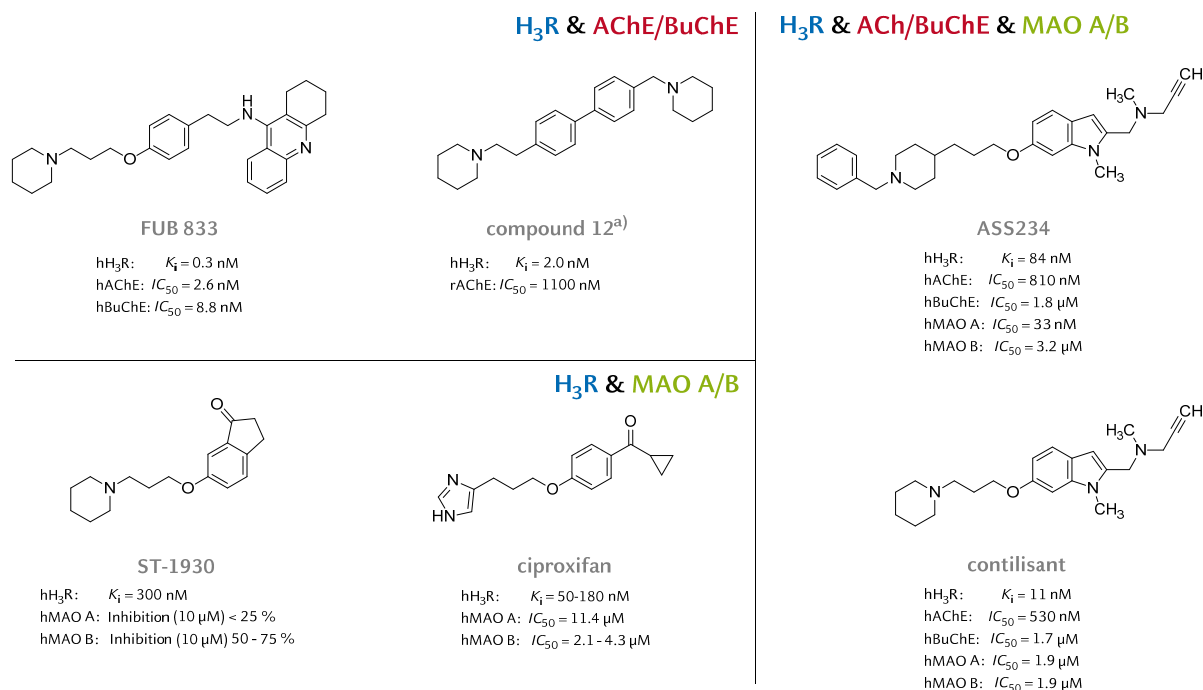
Figure 17. H$_3$R with inhibitory activity at either ChEs (upper left) or MAOs (lower left), or both (right panel) with pharmacological data taken from (Petroianu et al., 2006; Bautista-Aguilera et al., 2017; Hagenow et al., 2017). [a]Ref. (Morini et al., 2008)

completely merged pharmacophores and tetra-target activity among the former targets and towards σ$_1$Rs (Bautista-Aguilera et al., 2018). Finally, contilisant has appeared with neuroprotective properties that, together with previous results, have elicited promising *in vivo* effects in models of impaired cognition elicited by lipopolysaccharides and Aβ$_{1-42}$ (Bautista-Aguilera et al., 2017; Bautista-Aguilera et al., 2018).

Apart from the strategies mentioned above to facilitate neurotransmission, strategies focussing on the genetic aetiology are emerging in neurogenetic disorders. The most straightforward methods rely on genetic intervention such as vectorial substitution of missing or defective genes, which have already progressed into clinics for the treatment of AD or PD (Eberling et al., 2008; Malkki, 2015). However, the associated costs (5 to 6-digit U.S.$) will put an inconceivable financial burden on patients and will be intolerable by any health insurance system working in an intact ethical culture (Johnson et al., 2015). Thus, the development of small molecules to intervene on a genetic level by modulating physiological mechanisms that are involved in gene organisation (epigenetics), has resulted in remarkable progress within last years and will dominate future pharmacotherapeutic interventions. In neurogenetic disorders, epigenetic marks and the corresponding editing enzymes seem a promising mechanism of intervention.

Several of genes that are correlated with previously described pathologies are under the control of **epigenetic** regulatory mechanisms. Together, all distinct mechanisms interplay in a non-isolated, orchestrated manner in order to regulate cell cycle regulation, chromatin organisation, gene transcription,

post-translational modifications, translation of mRNA and DNA repair (Mehler, 2008). Such complex mechanisms can be subdivided upon their molecular nature (Mehler, 2008), into

- **histone modifications** acting by introduction and removal of acetylation and methylation marks by a plethora of residue-specific modifying enzymes,

- **DNA methylation**,

- regulation of **non-coding RNAs** (such as micro RNAs, spliceosomal RNAs and snoRNAs), and

- enzymes that are responsible for **RNA editing**.

Finally, such mechanisms provide a broad range of chances for pharmacological intervention. Therefore, several drugs or drug candidates have been investigated on preclinical or clinical investigation stage, or have already been granted market authorisation (Teijido et al., 2018; Peedicayil, 2019):

- inhibitors of histone acetylation (nicotinamide, vorinostat, panobinostat; all approved) or methylation (A-366, UNC-0642; on preclinical investigation stage),

- nucleoside analogues to be incorporated as demethylated DNA (decitabine; approved),

- non-nucleoside DNA methyltransferase inhibitors of which several have been discovered among approved drugs and natural compounds (procain, epigallocatechingallate; both approved), and

- non-nucleoside analogues acting as methyl donors (L-methyl folate, *S*-adenosylmethionine).

While most of the interventions mentioned above were aiming for the treatment of cancer, some preclinical progress in treatment of neurogenetic disorders have been made within recent years.

Among previously mentioned drugs and candidate drugs, inhibitors of the **histone H3 methyltransferase G9a** have emerged as potential tools to intervene at the basis of neurogenetic disorders, and several of them have been linked with this target. Interestingly, recent reports indicated the restoration of SNURF-SNRPN, SNORD116 and other candidate genes from the maternal copy by inhibition of the histone H3 methyltransferase G9a (syn. EHMT2/SU(VAR)3-9) and associated the findings with improved survival in a mouse PWS-model (Kim et al., 2017; Kim et al., 2019). Additionally, G9a inhibitors have shown to ameliorate autism-like social deficits in Shank3-deficient mice (Kim et al., 2017; Kim et al., 2019; Wang et al., 2019). Moreover, G9a inhibition recently showed ameliorated cognitive performance and reduced molecular risk-factors such as beta-amyloid plaques and consecutive neuroinflammation in mouse models of early-onset AD (Griñán-Ferré et al., 2019). Without pharmacological effects, G9a was associated in transcriptional repression of a factor involved in DA neuron maintenance am involvement in PD (Habibi et al., 2011).

Interestingly, it is especially this target that shows multiple implications in multifactorial diseases, while meanwhile, as well the H₃R has emerged as a therapeutic approach. Thus, from a MTDL perspective, G9a appears as an attractive co-target for H₃R ligands. However, studies focussing on MTDLs with binding towards H₃R and the histone H3 methyltransferase have not been conducted yet. Ultimately, the genetic and epigenetic basis of neurogenetic disorders is now progressively elucidated and thereby, accompanied by novel pharmacotherapeutic approaches.

Together with the multi-faceted neurological roles of H₃R that were outlined within this introduction, both approaches will be alongside one another for combined symptomatic and causal treatments.

2. Objectives & scope of this research

Preclinical insights that pinned hopes on H_3R inverse agonists have often been counteracted within clinical campaigns, leading to high attrition risk in development campaigns of H_3R ligands, and despite almost 40 years of H_3R research, only one progressed into the market (Schwartz et al., 2016; Ghamari et al., 2019a). Given the various physiological implications of H_3R that were outlined previously (section 1.1), the high failure rate of potent H_3R ligands in clinics puts several questions to molecular pharmacology as the proximal part of the drug discovery process.

Dedication of my work will be given to the development and application of molecular pharmacological methods to H_3R ligands, for profiling novel hit and lead compounds with improved pharmacological profile for investigation within *in vivo* models.

The section 3 starts with the development of a novel fluorescence polarisation (FP)-based method for the objective, to assess novel tracers in binding studies, to shift equilibrium read-outs towards a kinetic, and to increase the throughput upon working in a homogeneous environment. Thereby, this method will be tested among imidazole-based and non-imidazole-based inverse agonists, but also agonists. The desired increase in throughput will be required for the further purposes of my studies. There, the applicability of the pharmacophore blueprint of non-imidazole-based H_3R inverse agonists will be analysed within a bioisosteric replacement approach towards heterocyclic receptor ligands with improved drug-likeness.

Within the projects in section 4, I aim for the discovery of novel H_3R ligands with or without activities at promising co-targets, as a strategy to improve existing pharmacological approaches to neurogenetic disorders such as schizophrenia and GTS, the highly prevalent neurodegenerative diseases PD and AD, and the less prevalent ASD and PWS. This search will be conducted among novel hit and lead structures from medicinal chemistry or *in silico* screening efforts, but will as well encompass well-characterised drug candidates. As potential co-targets, dopaminergic GPCRs, MAO B, AChE and BuChE enzymes appear as the most attractive ones from current knowledge. Some of such MTDLs will be explored in *in vivo* models of PD and ASD, for which detailed knowledge of their molecular pharmacological profile is essential.

Final queries will be made to expand the concept of H_3R-MTDLs on novel candidate structures which combine H_3R antagonism with epigenetic modulatory function. Such novel MTDLs could be promising tools against neurogenetic disorders, which often are multifactorial and that often share co-morbidities. Therefore the pharmacological manipulation of neurotransmitter levels, as well as re-programming cellular function, may open novel perspectives towards this class of neurological conditions.

3.   Novel implications in molecular pharmacology of histamine H$_3$ receptor ligands

3.1. Ligand binding kinetics at histamine H₃ receptors by fluorescence polarization with real-time monitoring

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Contribution to research: DR developed the novel assay system, designed, planned, prepared and conducted the pre- and main-experiments. He analysed the data, wrote the manuscript and processed the revision.

Abstract:

Growing evidence recommends incorporating the concept of drug-target residence times within drug development and screening programmes. For many targets, systematic research for binding kinetics is emerging and reported, as in case of the histamine H₃ receptor. Alternatively, fluorescent methods based on Foerster resonance energy transfer have been reported recently but application of fluorescence polarization to kinetics of unlabeled ligands is not known to us. Thus, we established a radiolabel-free, real-time resolving method that is compatible to high-throughput-screening programmes with the objective to explore the underlying binding kinetics. This method takes benefit of bodilisant as H₃ receptor ligand. Thereby, we detected short residence times around 5 min for the H₃ receptor ligands ciproxifan, clobenpropit, thioperamide as well as pitolisant. Monitoring association rates, remarkably slower association rate constants were examined for ciproxifan and thioperamide when compared to those of pitolisant or clobenpropit. The affinities for the ligands derived by the kinetic approach differ from affinity estimates in literature using radiolabeled agonists in displacement assays. Further investigation raised exceptional pharmacological properties, consistent with occurrence of secondary binding sites at the H₃ receptor. Validation of resulting affinity constants was successfully performed by displacement assays based on fluorescence polarization with bodilisant.

Molecular and cellular pharmacology

Ligand binding kinetics at histamine H_3 receptors by fluorescence-polarization with real-time monitoring

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ABSTRACT

Growing evidence recommends incorporating the concept of drug-target residence times within drug development and screening programs. For many targets, systematic research for binding kinetics is emerging and reported, as in case of the histamine H_3 receptor. Alternatively, fluorescent methods based on Foerster resonance energy transfer have been reported recently but application of fluorescence polarization to kinetics of unlabeled ligands is not known to us. Thus, we established a radiolabel-free, real-time resolving method that is compatible to high-throughput-screening programs with the objective to explore the underlying binding kinetics. This method takes benefit of bodilisant as H_3 receptor ligand. Thereby, we detected short residence times around 5 min for the H_3 receptor ligands ciproxifan, clobenpropit, thioperamide as well as pitolisant. Monitoring association rates, remarkably slower association rate constants were examined for ciproxifan and thioperamide when compared to those of pitolisant or clobenpropit. The affinities for the ligands derived by the kinetic approach differ from affinity estimates in literature using radiolabeled agonists in displacement assays. Further investigation raised exceptional pharmacological properties, consistent with occurrence of secondary binding sites at the H_3 receptor. Validation of resulting affinity constants was successfully performed by displacement assays based on fluorescence polarization with bodilisant.

1. Introduction

In vitro screening of compounds for affinities and selectivity to a respective target has been the basis in pharmacological research over decades. Those methods investigate the attainment to Ehrlich´s prerequisite for any drug to take effect in vivo even if it is not a sufficient condition to prove effectiveness of drugs. Copeland et al. (2006) presented the drug-target residence time concept (hereinafter also termed 'residence time', τ, Eq. (1)) about 10 years ago to take further assumptions into account, namely (i) to regard the dynamic situation beyond target binding that is rather an estimate to describe the open-system physiological conditions, (ii) to correlate the duration of an effect to the time a drug resides at the receptor, (iii) to use this information to systematically improve in vivo effectiveness and (iv) finally, to reduce attrition risk on late-stage drug development (Lu and Tonge, 2010; Guo et al., 2014; Hoffmann et al., 2015; Copeland, 2016; Schuetz et al., 2017).

$$\tau = 1/k_{off} \tag{1}$$

Despite drug-target residence time emerges as new concept, the theoretical background for experimental procedures has already been set up for enzymes by Colquhoun in 1968 and refined by Motulsky and Mahan (1984) who delivered an analytical solution of the differential equations of a radioligand competing with an unlabeled ligand for one receptor site simultaneously. Applicability to radiolabel-free approaches as Foerster/bioluminescence resonance energy transfer (FRET/BRET) has been demonstrated at the histamine H_1, H_3 and H_4 receptor (Bosma et al., 2016; Liu et al., 2018; Stoddart et al., 2018; Mocking et al., 2018; Riddy et al., 2019).

One major disadvantage of the latter methods is the requirement of labeling two components attributing to the binding reaction, likewise the target itself that pharmacologically manipulates the target of investigation. Thus, we decided to use a method based on fluorescence polarization as it requires only one partner to be labeled (Banks et al., 2000), works with native receptors and as it was proposed by Copeland et al. (2006) that it might be applicable in examining residence times. This technique is based on the polarization of emitted light by a molecule excited with linearly polarized light (Perrin, 1926). When regarding those molecules as spinning dipoles that radiate with a different intensity at each angle of observation, high polarization will be seen at relatively slow rotation as caused by binding of this compound to a target (Lea and Simeonov, 2011; Rossi and Taylor, 2011).

The considerations above motivated us to set up a new method to investigate drug-target kinetics at the H_3 receptor (for review, see Panula et al. (2015)). The choice of a measurement technique based on fluorescence polarization is driven by its time resolving competence, its compatibility for high-throughput screening, the reduced hands-on work required compared to radioligand binding and functional studies, the yet successful application to classical competition studies at G-protein coupled receptors and finally, to extend this simple approach to kinetic investigations (Gagne et al., 2002; Kecskes et al., 2010; Stoddart et al., 2016).

2. Materials and methods

2.1. Materials and chemicals

HEK-293 cells stably expressing the 445-amino acid encoding gene of the human H_3 receptor were kindly gifted by Prof. Dr. Jean-Charles Schwartz (Bioprojet, France). 25 cm² cell-culture flasks (CELLSTAR® Tissue culture flasks) and 96-well, black microtiterplates (polystyrene, half-area, medium binding) were from Greiner bio-one (Frickenhausen, Germany). 75 cm² and 175 cm² cell-culture flasks (NUNC® EasYFlask), Dulbecco´s Modified Eagle Medium, Penicillin (10,000 U)/Streptomycin (10 mg/ml) solution, L-Glutamine-solution (200 mM), HEPES solution (1 M, pH: 7.0–7.6), Pluronic® F-127 as well as thioperamide maleate were purchased from Sigma-Aldrich (Taufkirchen, Germany). Fetal bovine serum albumin (FBS Good-Forte®) and Dulbecco's phosphate buffered saline (hereinafter termed 'phosphate buffer') were gained from PAN-Biotech (Aidenbach, Germany). Clobenpropit hydrobromide was purchased from Biotrend (Zurich, Switzerland), TRIS, sodium chloride and magnesium chloride were from Carl Roth (Karlsruhe, Germany). Bodilisant was obtained from our own synthesis as described by Tomasch et al. (2013), as well as ciproxifan, pitolisant oxalate and N^α-methylhistamine dihydrochloride (Stark, 2000; Meier et al., 2001; Grassmann et al., 2003).

2.2. Cell culture and membrane preparation

The above-mentioned cells were cultured in Dulbecco´s Modified Eagle Medium supplemented with 10% fetal serum albumin (FBS), 2 mM L-Glutamine, 10 mM HEPES, 100 units benzylpenicillin and 0.1 mg/ml streptomycin. Flasks were stored in an incubator (Binder, Tuttlingen, Germany) at 37 °C with 5.0% CO_2 saturation. Upon confluence, the medium was discarded, and cells were detached mechanically through aspiration in phosphate buffer. After centrifugation (Hettich, Tuttlingen, Germany) at approx. $1400 \times g$, 4 °C for 10 min, the pellet was resuspended in phosphate buffer and centrifugated repeatedly. The resulting pellet was taken into membrane buffer (75 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$; pH = 7.4) and homogenized using an UltraTurrax® T 25 (IKA®-Werke, Staufen, Germany) at 10,000 rpm. Two further centrifugation steps followed at approx. $50,000 \times g$ for 20 min each with a wash step using membrane buffer in between. The resultant pellet was resuspended in membrane buffer and aliquots were prepared for storage at −80 °C until further use. Protein content was quantified by the method of Bradford (1976). Ahead of all experiments, membrane preparations were thawed, diluted with membrane suspension and sonicated 3 times keeping the preparation on ice. Subsequently, the mixture was tempered on a water bath to 28 °C.

2.3. [³H]-N^α-methylhistamine in saturation binding and radioligand displacement assays

H_3 receptor expression was determined by saturation binding experiments using duplicates of eight concentrations of [³H]-N^α-methylhistamine titrated against 20 μg/well of receptor containing membrane preparations (volume = 200 μl), including samples with 10 μM of pitolisant for quantification of non-specific binding (same buffer as described above, supplemented with 0.01% Pluronic® F-127). To determine the competitiveness of bodilisant interacting with H_3 receptor, similar samples were prepared in presence of different concentrations of bodilisant (0.1, 1, 10 and 100 nM) to yield apparent K_D values of [³H]-N^α-methylhistamine. Displacement assays were carried out as described by Kottke et al. (2011) to determine affinities of test-ligands under experimental conditions close to those of the following procedures, using a titration pattern of seven competitor concentrations against [³H]-N^α-methylhistamine (2 nM, final concentration) and the same receptor concentration as above. For all radioligand experiments, the incubation was terminated after 90 min using the standard procedure for vacuum-filtration, filter mat-preparation and scintillation counting as described previously (Khanfar et al., 2018).

2.4. Optimization of assay conditions

All samples were prepared on microtiterplates and prewarmed to 28 °C in a TECAN® Infinite 1000 Pro multi-use-reader for 15 min, before starting the binding reaction. Measurements were carried out using the fluorescence polarization mode (excitation wavelength: 470 ± 5 nm, emission wavelength: 520 ± 20 nm, gain: 60, flashes: $50\,s^{-1}$, Z-level: 26,008 μm and 24,467 μm for 150 μl and 75 μl samples, respectively). All experiments were carried out at least in duplicates and contained buffer blanks solely consisting of buffer, protein blanks with H_3 receptor carrying membrane suspension, and samples for determination of anisotropy of free ligand. For determination of non-specific binding, one sample was prepared with 10 μM of pitolisant as competitor solution. The first step of optimization for all measurements within this assay was the selection of suitable concentrations of protein and fluorescence ligand within the standard pattern described above. First, different concentrations of bodilisant corresponding to 1, 2, 4, 8 and 10 times of its K_i value, determined by radioligand displacement assays (Tomasch et al., 2013) were titrated against a fixed amount of membrane preparation (120 μg/well) in membrane buffer (without detergents). Measurements were performed repeatedly after 45, 60 and 75 min. Secondly, as 12 nM provided a sufficient signal to noise-ratio based on Z′-level, this concentration was investigated against various concentrations of membrane preparation. Thirdly, the membrane buffer used for membrane preparation was modified subsequently by serial dilution of a solution (10%, m/m) of Pluronic® F-127 with membrane buffer. For determination of signal stability over time, the binding reaction was monitored every 10 min up to 12 h. For all further experiments in the sections below, 0.01% Pluronic® F-127 was added to the membrane buffer (hereinafter termed as "assay buffer").

2.5. Fluorescence polarization-based estimation of kinetics of bodilisant

Kinetics of bodilisant binding to the H_3 receptor was examined by measuring association and dissociation. For starting the association reaction, 50 μl of protein were pipetted to 100 μl of prewarmed bodilisant (12 nM, final) and polarization was measured every min for at least 60 min. Dissociation was initiated by addition of 5 μl of pitolisant (10 μM, final) to the same samples using an injector system integrated into the reader, shaking for 10 s and another read-out period of at least 75 min (minimum 5-fold k_{off}) pursued.

2.6. Fluorescence polarization-based kinetics of unlabeled ligands

To determine the kinetics of unlabeled ligands, 50 μl of bodilisant (12 nM, final conc.) and 50 μl of competitors at various concentrations were prewarmed inside the multi-use reader (10 min, 28 °C). Simultaneous incubation of competitor and labeled ligand was initiated through addition of 50 μl protein solution (100 μg/well) by means of automated pipetting. Plate transfer was carried out exclusively automated to maintain a reproducible lag-time between pipetting and the first measurement point t_1. Competitors used in the presented

investigations were the H_3 receptor inverse agonists pitolisant, ciproxifan, thioperamide clobenpropit and the agonist N^α-methylhistamine.

2.7. Fluorescence polarization-based displacement assay

For displacement assays the procedure above was modified to yield dose-inhibition curves. Serial dilution of 10 µM stock solutions by assay buffer were performed either manually or by using an automated pipetting robot Freedom Evo (Tecan, Maennedorf, Switzerland) connected to the previously mentioned reader. 25 µl of membrane preparations (50 µg/well) were added to 50 µl solutions of competitors and bodilisant (12 nM, final concentration). Maximum anisotropy of bound ligand (r_{max}) was determined by including a sample containing an excess of protein (150 µg/well). Reading was performed after an incubation period of 90 min at 28 °C as indicated above. Experiments were carried out using ten different concentrations per competitor in duplicates.

2.8. Data handling, calculations and curve fitting procedures

Fluorescence polarization was measured as parallel and perpendicular fluorescence intensity ($I_\parallel$ and $I_\perp$, respectively) and transformed into anisotropy (r) according to Eq. (2). The included G-factor (G) is a system property accounting for differences in sensitivity of emission filters to parallel and perpendicular polarized light, respectively (Note that G is most commonly the multiplicative of perpendicular intensity ($I_\perp$) but may be shifted, being the multiplicative of parallel intensity ($I_\parallel$), according to the manufacturers' instructions of the reader in use). G-factor ($G = 1.187$) of the reader was determined according to the instructions of the manufacturer.

$$r = (G \times I_\parallel - I_\perp)/(G \times I_\parallel + 2 \times I_\perp) \tag{2}$$

The measured anisotropy (r_M) is a ratiometric value that consists of the weighted sum of the anisotropy of free and bound ligand. The procedure to convert the r_M values of each sample into the fraction of labeled ligand specifically bound to the receptor (F_{SB}) was adopted from Roehrl et al. (2004) (Eqs. (3)–(6)). First, the fraction of labeled ligand bound to the receptor (F_B) of each sample was calculated by Eq. (3) (r_D denotes the anisotropy of free ligand, r_{max} the anisotropy of maximally bound ligand):

$$F_B = (r_M - r_D)/(r_{max} - r_D) \tag{3}$$

The bound fraction within the probe to determine non-specific binding $F_B{}^{NSB}$ and the estimate of non-specific binding N_B were derived by Eqs. (4) and (5), respectively, with r_I denoting anisotropy measured at large excess of unlabeled competitor:

$$F_B{}^{NSB} = (r_I - r_D)/(r_{max} - r_D) \tag{4}$$

$$N_B = F_B{}^{NSB}/(1 - F_B{}^{NSB}) \tag{5}$$

Finally, F_{SB} (in % of total binding) of each sample was derived by:

$$F_{SB} = [(1 + N_B) \times F_B - N_B] \times 100\% \tag{6}$$

The resultant F_{SB} were analyzed by non-linear least-squares fit using the software GraphPad Prism™ (2012, vers. 6.01, La Jolla, CA, USA).

For determination of dissociation rate constants k_{off}, data of dissociation reactions were fitted to the following expression accounting for monophasic dissociation (Eq. (7), $F_{SB,t=0}$ depicts the Fraction of receptor bound labeled species before initiating the dissociation):

$$F_{SB} = F_{SB,t=0} \times exp^{(-koff \times t)} \tag{7}$$

k_{off} values were used to determine k_{on} values from Eq. (8) ($F_{SB,max}$ and [L] accounts for bound fraction after equilibrium and concentration of labeled species, respectively):

$$F_{SB} = F_{SB,max} \times (1 - exp^{-(kon \times [L] + koff) \times t}) \tag{8}$$

Knowing these parameters, substitution into the following expressions can be used for determination of [C], $k_{on,C}$ and $k_{off,C}$ being the concentration, the association and dissociation rate constants of the unlabeled species, respectively (Eq. (9a)–(9f), (Motulsky and Mahan, 1984)):

$$k_A = k_{on} \times [L] + k_{off} \tag{9a}$$

$$k_B = k_{on,C} \times [C] + k_{off,C} \tag{9b}$$

$$S = sqrt((k_A - k_B)^2 + 4 \times k_{on} \times k_{on,C} \times [L] \times [C]) \tag{9c}$$

$$K_F = 0.5 \times (k_A + k_B + S) \tag{9d}$$

$$K_S = 0.5 \times (k_A + k_B - S) \tag{9e}$$

$$F_{SB} = (B_{max} \times k_{on} \times [L])/(K_F - K_S) \times [(k_{off,C} \times (K_F - K_S))/(K_F \times K_S)$$
$$+ (k_{off,C} - K_F)/K_F \times exp^{(-KF \times t)} - (k_{off,C} - K_S)/K_S \times exp^{(-KS \times t)}] \tag{9f}$$

Competitive binding data were fit to Eq. (10) to yield IC_{50} values.

$$F_{SB} = F_{SB,max}/(1 + 10^{([C] - log(IC50))}) \tag{10}$$

Since concentrations of free ligand, free receptor and ligand-receptor-complex do not change at equilibrium and the total association rate approaches zero, there is a direct link between receptor kinetics enabling the calculation of K_D by association (k_{on}) and dissociation rate constant (k_{off}, Eq. (11)).

$$K_D = [L] \times [R]/[RL] = k_{off}/k_{on} \tag{11}$$

K_i values were calculated via the correction derived by Cheng and Prusoff (1973) as stated in Eq. (12), where K_D represents the dissociation constant of the labeled ligand as obtained by Eq. (11). Munson and Rodbard (1988) derived an exact solution to Eq. (12) that accounts for errors due to ligand depletion. $K_{i,ex}$ values derived by the exact correction (Eq. (13)) were compared to K_i values of the first solution.

$$K_i = IC_{50}/(1 + [L]/K_D) \tag{12}$$

$$K_{i,ex} = IC_{50}/(1 + y_0 + [L] \times (y\_0 + 2)/(2 \times K_D \times (y_0 + 1)))$$
$$+ K_D \times y_0/(y_0 + 2) \tag{13}$$

Z′ factors were calculated as 1 minus the third ratio between the sum of the standard deviations σ_m, σ_{NSB} and the difference of the corresponding means of measured anisotropy r_m and r_i, used as a commonly used signal-to-noise substitute in fluorescence polarization (Eq. (14)) (Banks and Harvey, 2002).

$$Z' = 1 - 3 \times (\sigma_m + \sigma_{NSB})/(\hat{r}_M - \hat{r}_I) \tag{14}$$

For saturation binding experiments using [³H]-N^α-methylhistamine, specific binding was fit to Eq. (15), where $[L_R]$ and B_{max} stand for concentration of radioligand and maximum number of binding sites, respectively, and Y depicts specific binding to the receptor [c.p.m.].

$$Y = B_{max} \times [L_R]/([L_R] + K_D) \tag{15}$$

Same fitting was conducted for saturation binding experiments in presence of bodilisant, yielding apparent dissociation constants ($K_{D,app}$). The latter were converted to log ($K_{D,app}/K_D - 1$), plotted against logarithmic bodilisant concentration and inspected as Schild-type plots for competitiveness by linear regression (Ehlert, 1988; Christopoulos and Kenakin, 2002).

Where applicable, Kolmogorov-Smirnov test was conducted to assess significance while considering a limit of $p < 0.05$ and correlations are presented as Pearson correlation coefficient r_s.
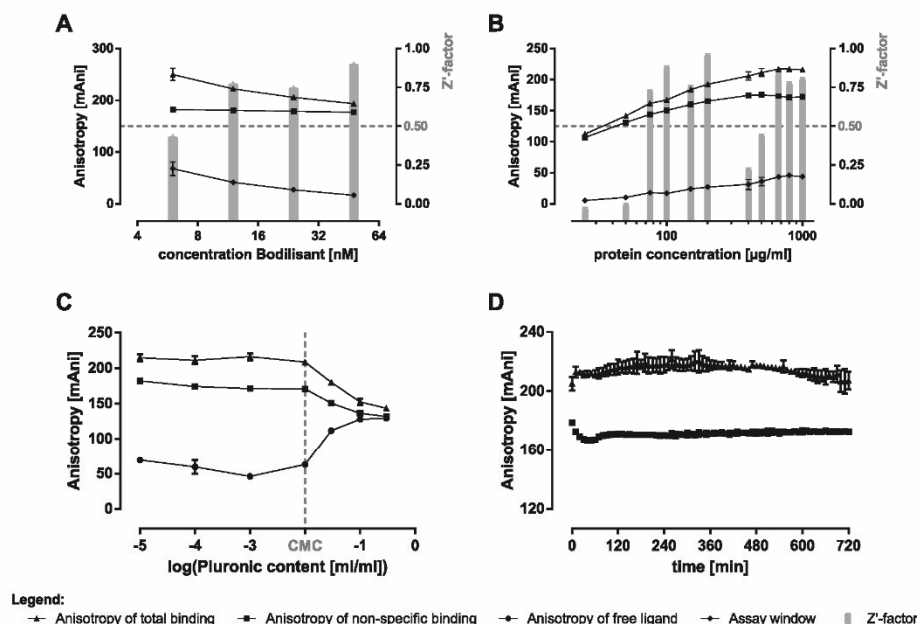
Fig. 1. Optimization of anisotropy measurements of bodilisant at H3 receptor with (■ = signal of non-specific binding) and without (▲ = signal of total binding) large excess of pitolisant (10 μM), plotted against left ordinate. The values represent means ± S.D. from a representative of three independent experiments performed in duplicate each. (**A**) Four concentrations of bodilisant (6 nM, 12 nM, 24 nM and 48 nM; abscissa) were incubated with 800 μg/ml of protein for 60 min. Grey bars plotted against the right ordinate indicate the calculated Z'-factor with Z' > 0.5 (dashed line) indicating high-throughput screening compatibility. (**B**) Various protein concentrations were titrated against 12 nM of bodilisant and incubated for 60 min ♦ indicate the difference between anisotropy of total and non-specific binding whereas grey bars indicate the calculated Z'-factors plotted against the right scale. (**C**) Effect of supplementation of buffer with a detergent. Various concentrations of a Pluronic®-F127 solution (10%) were added to the buffer. The dashed line indicates the critical micellar concentration of detergent (approx. 1000 ppm) and · denotes the anisotropy of free ligand. (**D**) Signal stability of total and non-specific binding of bodilisant up to 12 h. Concentrations of fluorescent ligand and membrane protein were 12 nM and 100 μg/well, respectively in buffer supplemented with Pluronic®-F127 (0.01%).

3. Results

3.1. Optimization of measurements

Selection of suitable concentrations of the reaction partners, bodilisant as fluorescently labeled ligand and the H3 receptor, was the first crucial step for obtaining a signal in fluorescence polarization. A sufficient concentration of bodilisant at a fixed concentration of receptor was found to be 12 nM (approx. 2 times K_i, derived by radioligand displacement curves), providing satisfactory signal to noise ratio and assay-window (Fig. 1A and Fig. S1.1A, Supplementary material). Higher concentrations lowered r_M due to the contribution of signal of unbound ligand, whereas lower concentrations did not provide sufficient fluorescence intensity signal compared to background, resulting in imprecision of measurement and Z'-factor below 0.5. Subsequently, different concentrations of receptor were titrated against bodilisant (12 nM), whereas the receptor content corresponded to 0.73 pmol/mg protein as determined by saturation binding curves (pK_D = 8.44 ± 0.08 nM). As higher protein concentration causes elevated binding of labeled ligand, assay windows have correlated with increasing protein content in contrast to Z'-factors. That might be rationalized as Z'-factors are influenced by both, assay window and precision of measurement, the latter being higher at decreasing background fluorescence. However, 100 μg/well (corresponding to approx. 666 μg/ml) provided a sufficient assay window at lowest receptor concentration and Z'-factor exceeding 0.5 (Fig. 1, panel B and Fig. S1.1, panel B, Supplementary material), indicating excellent assay conditions for

high-throughput screening assays (Zhang et al., 1999). As tentative addition of Pluronic® F-127 to the binding buffer resulted in less fluctuation of the anisotropy signals over time, we figured out the optimal concentration being 0.01%. At concentrations above the critical micellar concentration (c > 0.1% as stated in the analytical supplements of the manufacturer), anisotropy values of bound ligand decreased and increased for free ligand (Fig. 1, panel C). Given this standard set-up, signal stability was tracked up to twelve h showing no significant changes (Fig. 1, panel D).
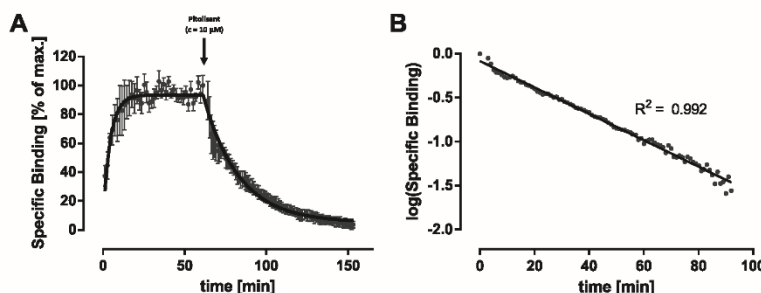
3.2. Kinetic characterization of bodilisant

The kinetic parameters of the labeled probe were determined as they are prerequisite in mathematical models for determination of the kinetics of unlabeled ligands and to examine the corresponding affinity to the receptor. In contrast to radioligand binding experiments where the binding reaction is terminated upon filtration to separate bound from unbound ligands, fluorescence polarization experiments allow homogeneous, multiple measurements in real-time resolution and reuse of the reaction mixture for further examinations. Therefore, we monitored the association reaction after assembling bodilisant (12 nM) with membrane preparations carrying the H3 receptor (100 μg/well) and using the same samples to examine the dissociation after addition of a large excess of the inverse agonist pitolisant (10 μM). After conversion of the measured anisotropy values to F_{SB} association/dissociation curves were plotted and fitted as shown in Fig. 2. This procedure delivers k_{on} = 22.5 ± 8.7·10⁶ M⁻¹ min⁻¹ and k_{off}

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A

B

Fig. 2. Kinetics of bodilisant binding to H₃ receptor. **(A)** Percentage plot of fraction of labeled ligand specifically binding to the receptor (n = 3, data showing mean ± S.E.M.) against a time-scale, showing association and dissociation. Fluorescent ligand (12 nM) and protein suspension were mixed and the association reaction was monitored with a fluorescence polarization reader every 1.5 min over 60 min. Subsequently, dissociation was initiated by addition of an excess of H₃ receptor inverse agonist pitolisant (10 µM), followed by another read-out period over 75 min. Non-linear least-squares regression yielded association and dissociation rate constants: k_{on}

$= 22 \pm 9 \cdot 10^6$ M^{-1} min^{-1} and $k_{off} = 0.034 \pm 0.010$ min^{-1} were calculated from 7 independent experiments (mean ± S.E.M.). **(B)** Semilogarithmic transformation of dissociation data from panel A (dissociation start was set as $t = 0$) and linear regression of the corresponding means obtains a straight line (R^2 = 0.992), indicating that bodilisant binds to a single class of binding site.

$= 0.034 \pm 0.010$ min^{-1}. Furthermore, we calculated pK_D $= 8.72 \pm 0.19$ as affinity measures not differing from the pK_i obtained by radioligand displacement assays that were performed and published earlier by our research group (p$K_i = 8.37 \pm 0.16$; $p = 0.07$; Tomasch et al., 2013). A useful method to check for numbers and interactions of different binding sites is the semilogarithmic conversion of dissociation-time curve that returned a straight line (R^2 = 0.992) indicating a single class of binding sites (Fig. 2, panel B).

3.3. Kinetic characterization of unlabeled ligands

Through investigation of the changes in the kinetic behavior of a labeled ligand in presence of an unlabeled ligand, the kinetics of the latter can be determined through the aforementioned mathematical framework of Motulsky and Mahan (1984) and Colquhoun (1968). Different concentrations of unlabeled competitor in presence of bodilisant were incubated with H₃ receptor simultaneously and the binding reaction was followed for about 90 min (Fig. 3). The kinetics of some

Table 1

Kinetic parameters of prominent histamine H₃ receptor ligands, obtained by a fluorescence polarization method.[a]

	k_{on} [10⁶ M⁻¹ min⁻¹]	k_{off} [min⁻¹]	τ [min]	n
Pitolisant	19 ± 6	0.22 ± 0.05	4.6 ± 1.0	6
Ciproxifan	0.072 ± 0.011	0.21 ± 0.04	4.7 ± 0.9	5
Thioperamide	0.10 ± 0.04	0.16 ± 0.02	6.3 ± 0.6	6
Clobenpropit	0.42 ± 0.02	0.19 ± 0.05	5.3 ± 1.5	4

[a] Fluorescence polarization was monitored over time after addition of membrane-preparations of H₃ receptor expressing HEK-293 cells to the fluorescent ligand bodilisant (12 nM) and different concentrations of competitor. Data were converted to fraction of bodilisant bound specifically to the receptor (F_{SB}) and fit to the model of Motulsky and Mahan (1984) to derive association rate constants (k_{on}) and dissociation rate constants (k_{off}). Drug-target residence times (τ) were calculated as reciprocal of means of k_{off}. All data represent means ± S.E.M. from the indicated number of experiments (n) performed in triplicate.
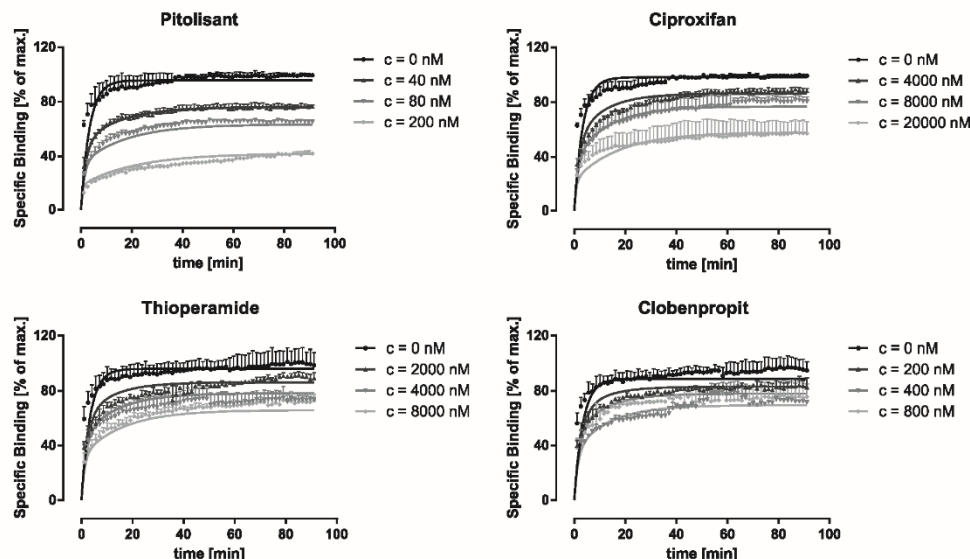

Fig. 3. Binding kinetics of representative ligands at the H₃ receptor. Three different concentrations of competitor were incubated simultaneously with bodilisant (12 nM) and receptor containing membrane preparations. Anisotropy values were converted to fraction of ligand specifically bound to the receptor (normalized as % of maximum) and plotted against a time-scaled abscissa.

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Table 2
Affinities of histamine H₃ receptor ligands, obtained by fluorescence polarization method using bodilisant.[a]

	Kinetic estimation[b]		Competitive displacement assay[c]			[³H]-N^α-methylhistamine displacement assays[d]	
	pK_D	*n*	pK_i	*n*	p$K_{i,ex}$	pK_i	n
Pitolisant	7.89 ± 0.14	6	7.68 ± 0.08	3	7.67 ± 0.06	7.92 ± 0.04	5
Ciproxifan	5.54 ± 0.06	5	5.30 ± 0.12	3	5.35 ± 0.11	6.49 ± 0.12	3
Thioperamide	5.70 ± 0.11	6	5.32 ± 0.09	4	5.33 ± 0.10	7.15 ± 0.04	3
Clobenpropit	6.40 ± 0.13	4	5.87 ± 0.18	3	5.89 ± 0.19	8.73 ± 0.08	3

[a] Data represent means ± S.E.M. from the indicated number of experiments (*n*).
[b] pK_D values were calculated from association and dissociation rate constants of each independent experiment (*n*) and expressed as means ± S.E.M.
[c] Competitive displacement assays were carried out by titration of ten different concentrations of competitors in duplicates against bodilisant at H₃ receptor. Fluorescence polarization was measured after 90 min pK_i values were calculated from IC_{50} values via the correction by Cheng and Prusoff (1973) and compared to p$K_{i,ex}$ values representing the affinities calculated from the exact correction by Munson and Rodbard (1988), using pK_D of bodilisant obtained by kinetic estimation (Eq. (11) and Fig. 2).
[d] Competitive displacement assays using [³H]-N^α-methylhistamine as radiolabeled ligand as described by Kottke et al. (2011).

prominent ligands were determined as representatives (Table 1). Whereas comparable dissociation rate constants were observed among them, showing a tenfold faster dissociation rate than the fluorescently labeled ligand bodilisant, drastic differences were observed between the association rate constants: pitolisant displayed the highest association rate constant comparable with bodilisant, followed by the imidazole-based ligand clobenpropit. Ciproxifan and thioperamide showed a k_{on} being about one twentieth of clobenpropit. The corresponding K_D values were calculated according to Eq. (2), transformed into pK_D and reported in Table 2. Pitolisant showed the highest affinity whereas a tenfold lower affinity was observed for clobenpropit. Thioperamide and ciproxifan appeared even less active, showing only micromolar K_D values. Comparison with data obtained by a recently published method show significantly slower dissociation rate constants for thioperamide and clobenpropit with k_{off} 0.026 ± 0.01 min⁻¹ for both compounds (p < 0.05). Significance was not assessed for pitolisant as two different dissociation rate constants were observed in recently published literature, one being lower (k_{off} = 0.086 ± 0.07 min⁻¹), whereas another revealing a faster dissociation (k_{off} = 1.53 ± 0.94 min⁻¹) (Mocking et al., 2018; Riddy et al., 2019).

3.4. Fluorescence polarization-based displacement assay

As no kinetic rate constants for the chosen competitors were available during the assay development, competitive displacement assays based on fluorescence polarization were conducted to validate the pK_D values derived by the kinetic approach (Fig. 4A). Thereby, pK_i values validated the pK_D values obtained above (p – values being 0.68, 0.12,

0.18, 0.23 for pitolisant, ciproxifan, thioperamide and clobenpropit, respectively), showing a good correlation (r = 0.992, P = 0.008, Fig. 4B). Pitolisant appeared with affinities in nanomolar concentration range, whereas ciproxifan, thioperamide and clobenpropit showed only micromolar K_i values, thereby deviating from values obtained by [³H]-N^α-methylhistamine displacement assays (Table 2). F_{SR} in competition binding assays showed maximum binding below saturation that is important as erroneous K_i values may occur if an exceedingly high amount of labeled ligand is bound. To assess the influence of this termed 'ligand depletion' as possible reason for the observed discrepancies, the exact correction to Cheng and Prusoffs translation of IC_{50} values into K_i values was applied (Munson and Rodbard, 1988), not showing significant differences (Table 2). Thus, errors were not due to large contents of H₃ receptor preparation.

3.5. Characterization of concerted N^α-methylhistamine and bodilisant binding to H₃R

The results obtained above prompted us to investigate the binding behavior of N^α-methylhistamine and bodilisant. Competitiveness of bodilisant and N^α-methylhistamine at H₃ receptors was assessed by saturation binding curves of tritiated ligand in presence of different bodilisant concentrations that should cause a linear shift in affinity (Fig. 5A) (Christopoulos, 2002). Indeed, such a correlation (R² = 0.94) could be found if examining the data in a Schild-type plot using specific binding instead of functional data (Fig. 5B). However, the slope being 0.58 ± 0.06 showed a large deviation from unity.

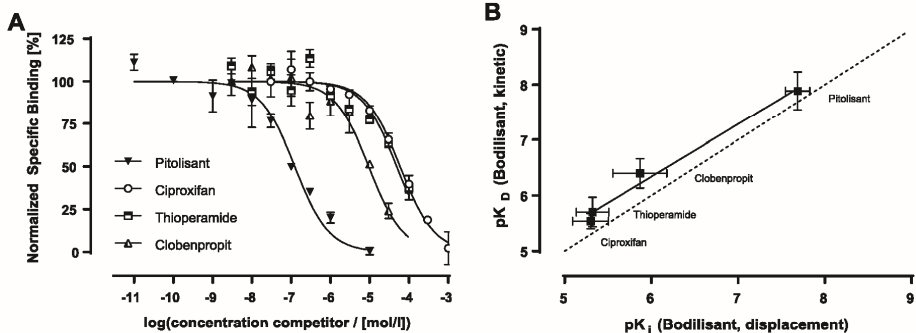Taking advantage of the newly developed method above, we also

Fig. 4. Fluorescence polarization-based bodilisant displacement assays. **(A)** Different competitors were titrated against 12 nM bodilisant. Anisotropy data were transformed to specific binding (F_{SB}) and normalized to fitted maximum for presentation (%-F_{SB} ± S.D.). **(B)** Pearson correlation of kinetically derived affinity measure (pK_D) with that derived by fluorescence polarization-based displacement assay (pK_i) adopted from Table 2, resulting in a correlation of r = 0.992 (P = 0.008). The dashed line indicates a line with unity slope.

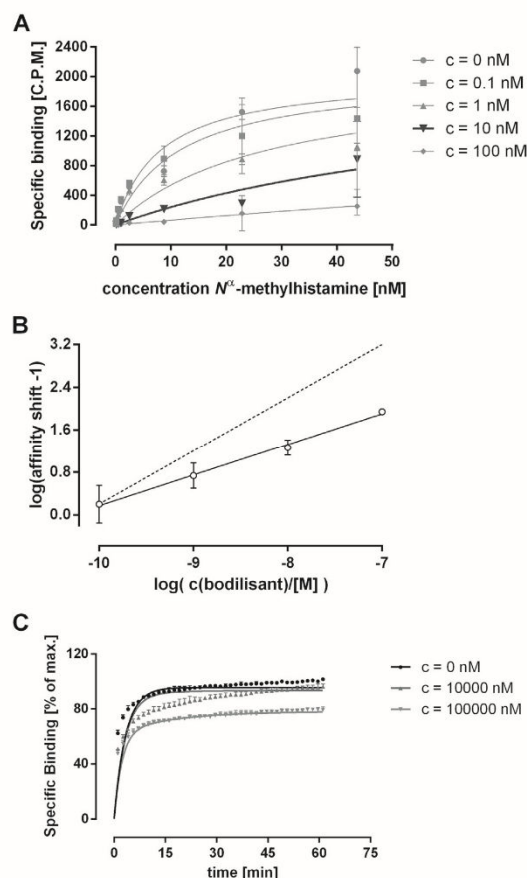**Fig. 5.** Characterization of binding properties of N^α-methylhistamine and bodilisant **(A)** Saturation experiments included eight different concentrations of [^{3}H]-N^α-methylhistamine against 0.1, 1, 10 and 100 nM bodilisant and membrane preparations containing histamine H$_3$ receptor (20 µg/well) within binding buffer (75 mM Tris, 100 mM NaCl, 10 mM MgCl$_2$; 0.01% Pluronic® F-127 pH = 7.4). Incubation was terminated after 90 min according to the procedure described by Khanfar et al. (2018). Data display one representative of two independent experiments performed in duplicate while highlighting the approximate conditions for N^α-methylhistamine within a fluorescence polarization-based kinetic assay in black. **(B)** Apparent K_D values from both independent experiments were converted to logarithmic affinity shifts - 1 (concentration ratio) and plotted against bodilisant concentration in a Schild-type regression. Linear regression yielded a straight line (R^2 = 0.94) with a slope = 0.58 ± 0.06 (mean ± S.D.). The dashed line indicates a straight line with unity slope. **(C)** Fluorescence polarization-based kinetics of N^α-methylhistamine as described previously, revealing high concentrations of competitor needed to inhibit binding of bodilisant. The panel shows data of a representative of three independent experiments as means ± S.D. of triplicates.

determined binding kinetics of unlabeled N^α-methylhistamine and the corresponding affinity (Fig. 5C). With k_{off} = 0.045 ± 0.015 min^{-1} a residence time of 22 ± 8 min was demonstrated. A remarkably slow association rate (with k_{on} = 0.86 ± 0.32 mM^{-1} min^{-1}) emanate from unusually high concentrations of N^α-methylhistamine (> 10 µM) demanding to cause inhibition of bodilisant (c = 12 nM) within the time-curve, consequently resulting in affinity of high micromolar concentration range (pK_D = 4.25 ± 0.06). In contrast, the highlighted

dataset of Fig. 5A shows the radiolabeled congener still binding to the receptor despite remarkably lower concentrations.

4. Discussion

The newly established method permits measurements of binding kinetics at the H$_3$ receptor and thereby allows determination of τ. Taking the considerations of setting up assays using fluorescence polarization into account, the protocol consists of typical elements of competition assays based on this technique and subsequently, it was expanded to established frameworks for studying receptor binding kinetics. Once this pair was found, signal stability was determined as the method should be capable of monitoring long residence times without conflicting with receptor degradation processes. The receptor concentration appeared relatively high when compared to classical radioligand binding experiments due to the need of binding a significant amount of fluorescence ligand for detecting a signal. Occurrence of ligand depletion might cause alterations of basic terms in mathematical derivations used for affinity estimates as the K_i value derived by Cheng and Prusoff (1973) or the previously mentioned mathematical model by Motulsky and Mahan. In this case, measured affinity estimates as K_D and K_i would appear erroneous and may be a reason for the estimates derived by our method. However, $K_{i,ex}$ derived by an exact solution accounting for ligand depletion did not show significantly different values compared to K_i estimations by the first method. K_D values were validated with consistent K_i values observed within the displacement assay using bodilisant as labeled ligand.

We detected medium τ for the fluorescently labeled ligand bodilisant. k_{off} values did not differ among the representative H$_3$ inverse agonists ciproxifan, pitolisant as well as for thioperamide and clobenpropit, that show slower dissociation rate constants in literature (Mocking et al., 2018). We attribute these findings to an apparent high dependency of kinetic off-rates on the respective assay conditions as in recent literature higher k_{off} values can be found for pitolisant as well (Riddy et al., 2019). The association rate constants were distinct, ranging about three orders of magnitude from bodilisant (fastest association) to thioperamide and ciproxifan (slowest association). For interpretation of the results we take the initial considerations of the drug-target residence time concept by Copeland into account, namely k_{off} being an indicator accounting for interactions between ligand and receptor that control the dissociation, whereas k_{on} being influenced by many further processes such as diffusion, desolvation and conformational changes of the ligand as well as of the receptor (Copeland et al., 2006; Schuetz et al., 2017). This would indicate that the observed inverse agonists display comparable interaction modes and the binding event of ciproxifan and thioperamide appear more complex as of clobenpropit or pitolisant.

Due to low association rate constants, the method seems to lack observing affinities within the range of those typically found for the assessed ligands in literature and by radioligand displacement experiments, while no difference was found for pitolisant and bodilisant. From a structural point of view, this observation might be rationalized as with bodilisant, our assay makes use of a non-imidazole H$_3$ receptor ligand, whereas most radioligand binding experiments at H$_3$ receptor are performed using [^{3}H]-N^α-methylhistamine, [^{3}H]-histamine or [^{125}I]-iodoproxyfan. Various research projects revealed the existence of further receptor states for G-protein coupled receptors beyond the active and inactive state (Baker and Hill, 2007). Taking this and the structural differences of pitolisant and bodilisant compared to the imidazole-based H$_3$ receptor ligands into account, we postulate that bodilisant addresses another affinity state if not another binding site at the histamine H$_3$ receptor. The second hypothesis may be supported by the evidence of further binding sites at many other G-protein coupled receptors beyond those for endogenous ligand and G-proteins that is increasingly presumed to exist at many more receptors (Christopoulos, 2002; Wootten et al., 2013; Chan et al., 2018; Goulding et al., 2018).

Despite such evidence being best provided by resolving the structure of the target in question, the results of the present study do not minimize this probability but contribute with indicators thereover as a Schild-type plot using tritiated N^α-methylhistamine and bodilisant (Christopoulos and Kenakin, 2002). This approach revealed a straight line, an indicator for potential competitive behavior within the examined concentration range but a slope smaller than unity, describing a disproportional increase of competitor action at the receptor with increasing concentrations. The latter finding may be attributable to experimental factors such as inactivation or uptake processes of labeled species within the receptor compartment but seems unlikely while working with membrane preparations and the well-established use of N^α-methylhistamine as radiolabeled ligand (Alexander et al., 2015). Alternatively, competition of the investigated ligands for two binding sites may emerge to such extend (Neubig et al., 2003). We could additionally demonstrate binding of [³H]-N^α-methylhistamine while not displacing bodilisant at even higher concentration of untritiated analogue. Despite this unusual approach, it expands our hypothesis of bodilisant binding to a second binding site with higher receptor occupancy but similar affinity that is consistent with bodilisant kinetics indicating a single class of binding sites (Fig. 2B). N^α-methylhistamine would then exhibit different affinities for both sites.

Reflection of the kinetic results in this context deduce the observed differences in affinity of competing ligands from molecular perspective: Measurable dissociation rate constants show a degree of drug-receptor interaction that would have been concealed by simple displacement assays. Slow association rates among the presented imidazole-containing H₃ receptor ligands describe the complexity of the binding event, hindering the association and leading to weaker binding affinities to this binding site. In addition, this method does not preclude different rate constants and affinity to other binding sites. Qualitatively, the results exemplify the possible high probe-dependency of competitive displacement assays that are never direct affinity measurements, but estimations depending on assay conditions as well as of the properties of the labeled ligand in use.

5. Conclusion

Growing demand for hit-to-lead optimization oriented on drug-target residence time prompted us to set up a method to assess receptor binding kinetics. Application of fluorescence polarization instead of binding experiments using radiolabeled ligands appeared as a modern and useful approach as it enables multiparameter analysis through real-time resolution and to further extend the recently burgeoning portfolio of methods to investigate drug-target residence times at the H₃ receptor. Upon this, embedded into an automated pipetting surrounding, this method can be further applied to high throughput screening programs. Residence times of clobenpropit, thioperamide, ciproxifan and pitolisant adjoined around five min. Large differences for association rate constants were observed resulting in a reduced affinity of ciproxifan, thioperamide, clobenpropit and N^α-methylhistamine compared to that observed in radioligand displacement assays. Validation of the kinetically derived affinity estimates was successfully performed using bodilisant as labeled analogue within displacement assays based on fluorescence polarization. However, the results suggest the presumptive existence of a second binding site needing further pharmacological elucidation. Thus, this method would not serve as substitute for radioligand binding experiments, but provides useful kinetic information about a binding event even in case of low-affinity ligands. Moreover, the given protocol provides a roadmap for expanding fluorescence-polarization based investigations using other fluorescently labeled ligands at histamine H₃ receptors.

Acknowledgements

Declarations of interest

None.

Author contributions

DR planned, conducted the experiments, analyzed the data, wrote and approved the manuscript. HS initiated and supervised the project, provided reagents, read and approved the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejphar.2019.01.041.

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<u>**Supplementary Material**</u>

**Ligand binding kinetics
at histamine H3 receptors by fluorescence-polarization
with real-time monitoring**

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S1.   Optimization of Assay conditions
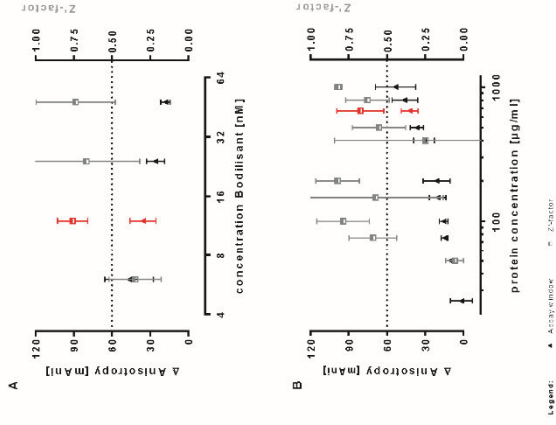
S1.1   Z′-values

Fig. 1 Optimisation of anisotropy measurements of bodilisant at H3 receptor. The panels show assay windows (left ordinate, difference of anisotropy signals of samples with and without 10 µM of pitolisant) and Z′-factors (right ordinate) calculated as means ± S.D. from three independent experiments performed in duplicate. The dashed line indicates Z′ > 0.5, a factor signifying high-throughput screening compatibility. Red highlighted data pairs indicate the chosen assay conditions for later experiments. (**A**) Four concentrations of bodilisant (6 nM, 12 nM, 24 nM and 48 nM, abscissa) were incubated with 800 µg/ml of protein for 60 min.
(**B**) Various protein concentrations were titrated against 12 nM of bodilisant and incubated for 60 min.

3.2. Novel pyrrolidinone derivative lacks claimed histamine H₃ receptor stimulation in receptor binding and functional studies

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Contribution to research: DR designed, prepared and conducted radioligand displacement experiments and fluorescence polarisation-based binding studies and was involved in planning and interpretation of LC-MS based purity analysis. He analysed and interpreted the data from binding and functional assays, wrote the manuscript and processed the revision.

Abstract:

Since the discovery and early characterization of the histamine H₃ receptor (H₃R) in the 1980´s, predominantly imidazole-based agonists were presented to the scientific community such as Nα-methylhistamine (N^{α}-MeHA) or (*R*)- α-methylhistamine ((*R*)α-MeHA). Whereas therapeutic applications have been prompted for H₃R agonists such as treatment of pain, asthma and obesity, several drawbacks associated with imidazole-containing ligands makes the search for new agonists for this receptor demanding. Accordingly, high interest arose after publication of several pyrrolidindione-based, highly affine H₃R agonists within this journal that avoid the imidazole moiety and thus, presenting a novel type of potential pharmacophores (Ghoshal, Anirban et al., 2018). In our present study performed in two independent laboratories, we further evaluated the exposed lead-compound (*EC*₅₀ = 0.1 nM) of the previous research project with regards to pharmacological behavior at H₃R. Thereby, no binding affinity was observed in neither [³H]N^{α}-MeHA nor bodilisant displacement assays that contradicts the previously published activity. Additional functional exploration employing GTPγ[³⁵S], cAMP-accumulation assay and cAMP response element (CRE)-driven reporter gene assays exhibited slight partial agonist properties of such pyrrolidindiones but acting apart from the reported concentration range. We conclude, that the previously reported actions of such pyrrolidindiones result from an overestimation based on the method of measurement and thus, we cast doubt on the new pharmacophores with H₃R agonist activity.

Research paper

Novel pyrrolidinone derivative lacks claimed histamine H₃ receptor stimulation in receptor binding and functional studies

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ABSTRACT

Since the discovery and early characterization of the histamine H₃ receptor (H₃R) in the 1980's, pre-dominantly imidazole-based agonists were presented to the scientific community such as N^α-methyl-histamine (N^α-MeHA) or (R)-α-methylhistamine ((R)α-MeHA). Whereas therapeutic applications have been prompted for H₃R agonists such as treatment of pain, asthma and obesity, several drawbacks associated with imidazole-containing ligands makes the search for new agonists for this receptor demanding. Accordingly, high interest arose after publication of several pyrrolidindione-based, highly affine H₃R agonists within this journal that avoid the imidazole moiety and thus, presenting a novel type of potential pharmacophores (Ghoshal, Anirban et al., 2018). In our present study performed in two independent laboratories, we further evaluated the exposed lead-compound (EC₅₀ = 0.1 nM) of the previous research project with regards to pharmacological behavior at H₃R. Thereby, no binding affinity was observed in neither [³H]N^α-MeHA nor bodilisant displacement assays that contradicts the previously published activity. Additional functional exploration employing GTPγ[³⁵S], cAMP-accumulation assay and cAMP response element (CRE)-driven reporter gene assays exhibited slight partial agonist properties of such pyrrolidindiones but acting apart from the reported concentration range. We conclude, that the previously reported actions of such pyrrolidindiones result from an overestimation based on the method of measurement and thus, we cast doubt on the new pharmacophores with H₃R agonist activity.

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1. Introduction

The discovery of the histamine H₃ receptor (H₃R) back in 1983 consolidated the role of histamine as a neurotransmitter [1–3]. Histamine was former known as tissue hormone to be involved in the etiology of allergies and gastritis that led to the development of successful drugs. After this successful period that relied on thera-peutically influencing histamine H₁ and H₂ receptor mediated ef-fects, new hope to resume this blockbuster period was raised by targeting brain-histamine function through the newly recognized

histamine receptor family member [4,5]. In parallel, the physio-logical characterization revealed constitutive activity and the re-sponsibility of H₃Rs for modulating neuronal histamine release as autoreceptors [6–8] and as heteroreceptors being responsible for the release of further neurotransmitters such as acetylcholine, noradrenaline and dopamine [9–13].

Owing to the pivotal role of histamine as neurotransmitter and the corresponding involvement in pathophysiological processes, numerous H₃R ligands entered the clinical research stage, proving the therapeutic potential in disorders as Alzheimer's disease, Par-kinson's disease, schizophrenia, epilepsy, attention-deficit and hy-peractivity disorder (ADHD) and sleep-disorders [7,9,14,15]. Finally, the intense work was rewarded with a market authorization of pitolisant for the treatment of narcolepsy [16]. Whereas most preclinical and clinical research projects employ H₃R antagonists/inverse agonists, the development of H₃R agonists has been focused marginally. Potential applications were prompted for conditions of cardiac dysfunction, asthma, pain and migraine [17] as well as for

obesity [18,19]. Structural features of well investigated H₃R agonists encompass an obligate 4- or 5-monosubstituted imidazole core, whereas the side chain tolerates some structural modifications. (R)-α-Methylhistamine $((R)α\text{-MeHA})$ as one of the first H₃R agonists used for pharmacological characterization of H₃R showed lacking applicability due to its high metabolism by histamine N-methyl-transferase (HMT) that inactivates the agonist by N^{τ}-methylation, leading to the development of several prodrugs with low HMT affinity [20]. Additionally, their liability to interactions with the cytochrome P-450 enzyme system make 4-substituted imidazoles less attractive for optimization towards promising lead compounds [21].

Concluding such open questions in therapeutic application of H₃R agonists, some efforts for developing non-imidazole-based H₃R agonists have been currently presented to the scientific community [22]. In addition, we have been attracted by a recent publication that claims the identification of H₃R agonists comprising pyrrolidindione cores as option to avoid the imidazole moiety (Fig. 1, compound **6k**) [23]. In remarkable contrast to all previously presented H₃R ligands, the compounds are devoid of basic amine with the property to be protonated at physiological pH values. The comprehensively investigated lead compound was identified in a screening program among 94 compounds employing cAMP accumulation in transiently H₃R-transfected HEK-293T cells based on the GloSensor™ technique [24]. Thereby, it displayed inhibition of cAMP-accumulation corresponding to an agonist in the low nanomolar concentration range as well as selectivity over histamine H₁, H₂ and H₄ receptors [23]. Specificity of H₃R mediated effects was investigated by monitoring the cAMP-response using such biosensors in HEK-293T cells without H₃R transfection, reversal of agonism by a H₃R-selective agonist (GSK334429) at 1 μM of compound and by *in silico* screening excluding the compound belonging to pan assay interference compounds (PAINS) [23,25].

2. Results & discussion

The promising results from pyrrolidindiones as new chemical entity for H₃R pharmacophores prompted us for further elucidation but starting with the pharmacological investigation of binding properties at membrane preparations of HEK-293T cells stably transfected with the human 445-amino acid isoform encoding cDNA of H₃R. Therefore, compound **6k** was synthesized as described by Ghoshal et al. [23,25] (see materials & methods) as a yellow solid.
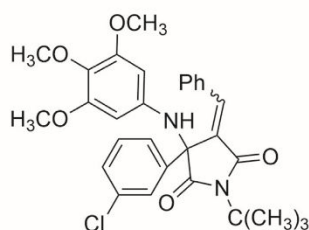
6k

H₃R: $EC_{50} = 0.1$ nM[23]

Fig. 1. Structure of **6k** that was claimed as H₃R selective agonist by Ghoshal et al. [23]. The EC_{50} was derived from HEK-293T cells transiently expressing the human H₃R isoform and biosensors for monitoring cAMP accumulation (GloSensor™, Promega).

Interestingly, the compound did not show entire displacement of the highly affine H₃R agonist $[^{3}H]N^{α}$-methylhistamine ($N^{α}$-MeHA, c = 2.0 nM, K_D = 3.1 nM) in radioligand displacement assays that were performed as described elsewhere [26]. Due to insolubility of the substance in assay buffer at 100 μM (75 mM TRIS, pH = 7.4, 100 mM NaCl, 10 mM MgCl₂), only concentrations up to 10 μM could be monitored, while full inhibition following a hill coefficient equal to unity was not observed (Fig. 2(A)). This effect may be attributable to different affinity states of receptor that two agonists are able to differentiate [27]. We are aware that the same effect may occur from impurities that were detected in the purchased compounds, however significant inhibition in low nanomolar concentration range corresponding to the reported EC_{50} was lacking in our studies. We therefore subjected **6k** for further binding studies based on fluorescence polarization in an orthogonal screening system that makes use of bodilisant, a fluorescently labelled second generation H₃R ligand with binding characteristics directed towards secondary binding sites [28]. Through monitoring the time course of competition between **6k** and bodilisant (bodilisant: c = 12 nM, K_D = 1.9 nM) binding to H₃R, no inhibition was observed at concentrations of 1 μM or 10 μM (Fig. 2(B)), prohibiting the determination of kinetic rate constants. In contrast, pitolisant as reference antagonist markedly displaced bodilisant, leading to an affinity consistent with literature determinations (K_D = 8.6 nM, k_{on} = 1.5 10⁷ mol⁻¹ min⁻¹; k_{off} = 0.13 min⁻¹). Therefore, we conclude that binding of **6k** at H₃R is not the trigger of the effects reported by Ghoshal et al. [23].

We further applied three complementary functional systems, being GTPγ[³⁵S], cAMP-accumulation assay and a cAMP or multiple response element (CRE/MRE)-driven reporter-gene assay, as described below. With regards to effects most proximal within the signal cascade of H₃R mediated signal-transduction, the results of GTPγ[³⁵S] assay using human-isoform H₃R expressing HEK-293 cells failed to demonstrate G-protein activation by **6k**, though that should occur upon receptor activation by an agonist such as (R)
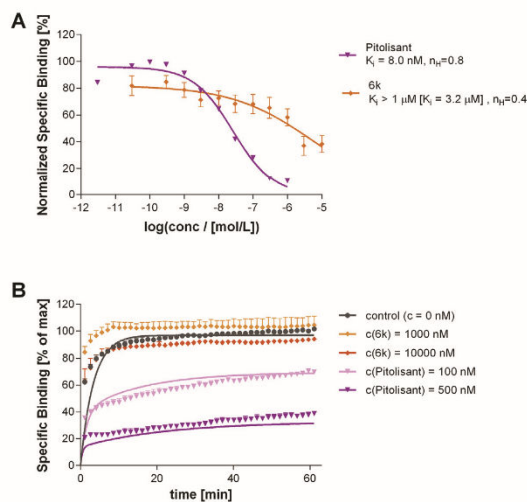

Fig. 2. Displacement curves of compound **6k** employing $[^{3}H]N^{α}$-methylhistamine (**A**) or bodilisant (**B**). (**A**): $[^{3}H]N^{α}$-methylhistamine displacement by pitolisant (K_i = 15.9 [10.1; 24.9] nM) and **6k** (K_i > 1 μM). Values were derived from global fitting normalized specific binding of four independent experiments performed in duplicate as described by Khanfar et al. [26]. (**B**): The time course of bodilisant being displaced by pitolisant and **6k**, determined by fluorescence-polarization as described previously [28].

α-MeHA (EC_{50} = 40 nM; Fig. 3 (A)) [29]. To test for presumable antagonist efficacy, reversion of *(R)*α-MeHA by **6k** was investigated. In contrast to pitolisant (K_B = 3.7 nM), compound **6k** exerted a slight inhibition in micromolar concentration range and thereby, was in consistence with the observations of the binding studies outlined above. The next distal step in the transduction pathway was examined, being the mitigation of cAMP accumulation upon inhibition of adenylyl cyclase by interaction with $G\alpha_i$-proteins. This effect was illustrated for agonism by histamine (EC_{50} = 30 nM) within our studies using CHO cells with stably transfected H₃R,
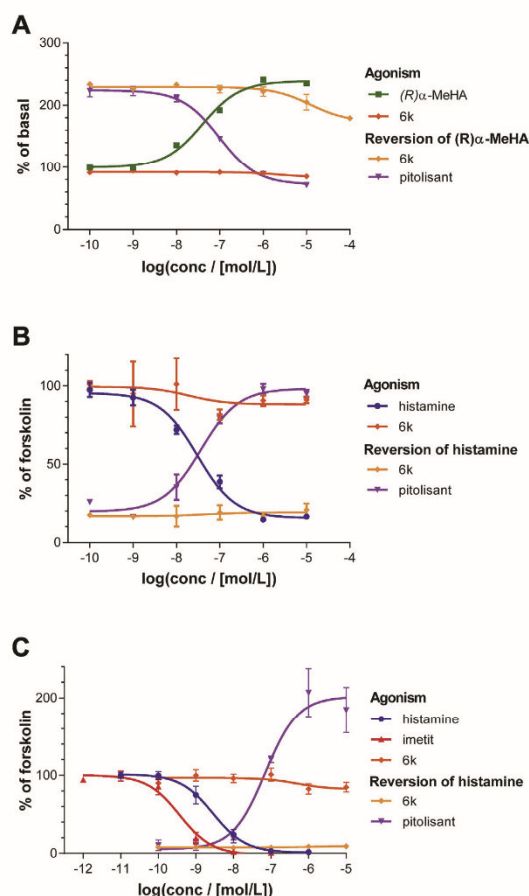

Fig. 3. Efficacy of compound 6k investigated in three orthogonal assays. **(A)**: GTPγ [³⁵S]-binding in membrane preparations of H₃R-expressing HEK-293 cells, showing changes of H₃R activity relative to basal receptor activity that was exerted by agonist *(R)*α-MeHA and compound **6k**. Additionally, reversion of *(R)*α-MeHA agonist (1 μM) response according to antagonism by pitolisant and **6k** is displayed. **(B)**: cAMP accumulation as assayed with a HitHunter® cAMP assay for small molecules (DiscoverX) within CHO-DUKX cells stably expressing H₃R and a CRE/MRE-luciferase reporter gene [36]. The curves illustrate the extend of inhibition of adenylyl cyclase mediated by **6k** and histamine as reference agonist, as well as reversion of histamine (1 μM) activity by **6k** and pitolisant as reference inverse agonist. **(C)**: CRE/MRE-luciferase reporter gene assays conducted in the same cells as described in Panel (B). Data correspond to luciferase expression resulting from changes in cAMP accumulation due to agonism by histamine, imetit or **6k** or reversal of histamine (100 nM) by pitolisant and **6k**.

whereas weak partial agonist properties with a low intrinsic activity of 0.14 (relative to histamine) could be observed for the compound in question (Fig. 3(B)). However, the corresponding partial antagonist properties could not be confirmed in presence of histamine, again in contrast to the well characterized H₃R inverse agonist/antagonist pitolisant (K_B = 1.0 nM). A similar effect could be observed in more distal CRE/MRE-luciferase reporter gene assays relying on the expression of luciferase upon binding of the cAMP response element binding protein (CREB) to the corresponding promotor-region within DNA (Fig. 3(C)). As well, a response corresponding to full agonism was observed for both H₃R agonists, imetit and histamine (EC_{50} = 2.9 and 0.4 nM, respectively), but not for **6k**. Additionally, the latter was not able to reverse the effects of histamine while antagonization of agonist-mediated inhibition of cAMP accumulation with pitolisant resulted in K_B = 2.0 nM. Control measurements using paternal CHO-cells deprived of H₃R but being transfected with CRE/MRE-luciferase reporter gene construct suggest that the observed effects are H₃R mediated (cf. Supporting Information, Fig. S2.1).

The herein presented studies from our characterization being performed in two independent laboratories show contrary results to those obtained by the originators of **6k**. The initial investigation by Ghoshal et al. was comprehensively performed based on cAMP accumulation [23]. Whereas cAMP can evolve from various cellular sources [30], the group did not observe same effects in HEK-293T cells that were not transfected with H₃R being indicative of H₃R mediated effects. Nonetheless, herein described results from [³H]N^α-MeHA and bodilisant displacement studies raise strong concerns about the presented specificity of those effects. Secondly, the group reported reversal of agonism exerted by **6k** (see Ref. [23]) by GSK334429 as H₃R selective inverse agonist [31]. However, thorough inspection of the data shows that **6k** shows only slight abrogation of forskolin induced response. The GloSensor™ application relies on complementation of functional Firefly luciferase enzyme in presence of cAMP [24]. Due to the affiliation of this technique to competition following the law of mass-action, the signal is not linear to the accumulated second messenger [24]. As extensively reviewed by Hill et al. (2010), this provokes false positive results if the dynamic range of the assay is exceeded and/or results being interpreted on luminescence data instead of transforming such to cAMP-levels before further analysis [32]. This, in combination with the slight inhibition of forskolin-induced response displayed in the Supplementary Information to the research paper and seen in our investigation to some extent, is one potential explanation for the observations by Ghoshal et al. (2018) [23].

3. Conclusion

The cAMP accumulation displayed in the presented study by Ghoshal et al. (2018) may originate from unspecific effects or misinterpretation of the measurement data, but we do not support the hypothesis of them being ligands at the H₃R. We conclusively cast doubt on the presented pyrrolidinediones being representatives for a new pharmacophoric entity with affinity and agonist efficacy at H₃R, though such new pharmacophores would be of high interest for the H₃R research community.

4. Materials & methods

4.1. Synthesis & analytics of compound 6k [(E/Z)-4-benzylidene-1-(tert-butyl)-3-((3,4,5-trimethoxyphenyl)amino)-3-(3-chlorophenyl)pyrrolidine-2,5-dione]

Compound **6k** [[(*E/Z*)-4-benzylidene-1-(*tert*-butyl)-3-((3,4,5-trimethoxyphenyl)amino)-3-(3-chlorophenyl)pyrrolidine-2,5–

dione] was resynthesized as a yellow solid according to the procedure described by Ghoshal et al. (2018) [23].

Briefly, a solution of phenylpropiolic acid (500 mg, 3.42 mmol), 3,4,5-trimethoxyaniline (627 mg, 1eq.), *m*-chlorobenzaldehyde (388 µL, 1 eq.) and *tert*-butyl isocyanate (387 µL, 1 eq.) in methanol (5 mL) was stirred for 12 h at room temperature. Solid potassium carbonate (1.42 g, 3 eq.) was then added and the mixture refluxed for 5 h. After cooling to room temperature, the mixture was filtered on a pad of Celite, rinsed with methanol and the filtrate concentrated under reduced pressure. Two consecutive flash chromatographic separations on silica (40 g/50 µm and 25 g/20 µm, elution with ethyl acetate/n-heptane from 4/1 to 2/1) were necessary to afford the sample (120 mg, yellow solid, m.p. = 79 °C).

^{1}H and ^{13}C NMR spectra were recorded on a Varian 400 MHz instrument and matched the reference ones. ^{1}H NMR (400 MHz, CDCl3): δ 7.90 (s, 1H, Ph-CH=C−), 7.69 (s, 1H, ArH), 7.46 (d, J = 7.2 Hz, 1H, ArH), 7.37−7.22 (m, 7H, ArH), 5.58 (s, 2H, ArH), 4.26 (s, 1H, NH), 3.68 (s, 3H, OCH₃), 3.58 (s, 6H, −(OCH₃)₂), 1.54 (s, 9H, −(CH₃)₃); ^{13}C NMR (125 MHz, CDCl₃): δ 176.25 (−C=C−CO), 170.30 (CO), 153.15, 140.37, 140.06, 138.52, 135.28, 133.05, 132.80, 131.04, 130.34, 130.12, 129.29, 128.61, 128.32, 126.64, 124.39, 96.05, 67.61 (C-quaternary), 60.91 (C-(CH₃)₃), 59.17 (OCH₃), 55.72 (OCH₃)₂, 28.34 (−(CH₃)₃) (Full spectra are depicted in supp. inf., Fig. S1.1 and Fig. S1.2.).

UV purity was found to be over 94% by HPLC (Sunfire C18 5 µm 4.6 × 150mm column, eluents: water/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B), linear gradient from 5% (B) to 95% (B) in 10 min) (supp. inf., Fig. S1.3), comparable to the method used by the inventors of the compound [23]. Additional LC-MS analysis was performed using an Intensity Solo 2C18 2.1 × 100mm column (temperature = 50 °C, flow rate = 0.2 ml/min, eluents: water/0.1% formic acid (A) and acetonitrile (B), linear gradient from 5% (B) to 95% (B) in 10 min), revealing a hidden impurity (m/z = 509.1) eluting with the main analyte. Isolation and quantification of the containing impurity (14.9%) could be performed using different eluent composition and thereby, resulted in purity of the main-analyte to be 82% (eluents: water/0.1% formic acid (A) and acetonitrile (B). Isocratic elution: 55% (B) within the first 10 min. A linear gradient (10−12 min) from 55% (B) to 60% (B), followed by isocratic elution with 60% (B) for 2 min) than used within the UV purity method (see supporting information, section S1.2).

4.2. [³H]N^α^-methylhistamine displacement assays

Membrane preparations and cell culture of HEK-293T cells that were stably transfected with the human H₃ receptor encoding cDNA, as well as radioligand ([³H]N^α^-methylhistamine) displacement studies were conducted as described previously [26]. Reference compound pitolisant was synthesized as oxalate-salt according to the procedure described by Meier et al. [33].

4.3. Fluorescence polarization-based bodilisant displacement assays

The same membrane preparations as used for radioligand displacement assays (section 4.2) were used for fluorescence polarization-based displacement studies using bodilisant that originated from the laboratory stocks of Tomasch et al. [34]. Assay conditions and data evaluation were as described previously [28].

4.4. GTPγ[³⁵S] assay

HEK-293 cells stably expressing human H₃ receptor were grown until confluence and centrifuged at 300×g for 15 min at 4 °C. Pellets were resuspended in buffer I (Tris-HCl 50 mM, MgCl₂ 10 mM, NaCl 140 mM, pH = 7,4 supplemented by Phenyl Methyl Sulphonyl Fluoride (PMSF) 1 mM). The obtained suspension was stirred gently and submitted to a 25-26×g mechanic pressure exerted through a syringe. The cell lysate was then centrifuged at 300×g for 5 min at 4 °C in order to eliminate nucleus and cell scraps. The obtained supernatant was then centrifuged at 48,000 g for 30 min at 4 °C. The final pellet was resuspended in buffer I. Aliquots were frozen in liquid nitrogen and stored until use at −80 °C. Protein content was measured by the Bradford method [35].

For conducting GTPγ[³⁵S] binding assays, defreezed membranes were diluted at a final concentration of 2.5 µg/180 µL/well and incubated at room temperature with compounds for 30 min in a binding buffer containing Tris-HCl 50 mM, MgCl₂ 10 mM, NaC 140 mM, GDP 10 µM, pH = 7,4 and distributed in 96-well polystyrene microplate. GTPγ[³⁵S] labelled ligand (0.2−0.3 nM) was added for additional 30 min. After transfer in a Millipore GF/C HTS® microplate, the filtration of the reactional mix followed by a three time 250 µL wash was used to terminate the reaction. The filter-bound radioactivity was measured in a liquid scintillation counter Microbeta TRILUX® with 50 µL of scintillation fluid. GTPγ[³⁵S] dependent binding activity was determined in vitro for histamine, imetit, (R)-α-methylhistamine and the compound of interest.

4.5. cAMP-accumulation assay

Chinese hamster ovary cells (CHO-DUKX, ATCC No. CRL-9010) expressing stable human histamine H₃ receptor cDNA (GenBank accession No. NM_007232) and stable multiple-responsive element/cAMP-responsive-element driving luciferase gene reporter were used [36].

The assay was performed with non-adherent cells in a 96 wells format using 20,000 cells per well in alphaMEM without serum in the presence of IBMX (500 µM). cAMP was measured in whole cells in the presence of forskolin (10 µM) using the HitHunter® cAMP Assay for Small Molecules kit (Art. No. 90-0075SM2, DiscoverX) according to the manufacturer's recommendations. Bioluminescence was measured using a FDSS/µcell apparatus (Hamamatsu). Results were expressed as percentage of forskolin-stimulated bioluminescence.

4.6. CRE/MRE luciferase reporter-gene assay

Employed CHO cells were the same as for the cAMP accumulation assay. Cells were seeded overnight at a density of 25,000 cells per well in a 96 well microplate with black wall and clear flat bottom (Art. No. 3904, Costar). Cells were washed twice with alphaMEM (Art. No. BE02-002F, Lonza) without serum, then treated with forskolin (Art. No. F6686, Sigma) at 0.3 µM or 1 µM final concentration, in the agonist or the antagonist mode, respectively, and with the compounds of interest.

After 4 h placed in a cell incubator (37 °C), the medium was removed and replaced with 50 µl of luciferase reporter gene assay kit reagent (Steady-Glo, Promega) (50/50 Steady-Glo reagent/alphaMEM). The bioluminescence was measured 10−20 min later using a FDSS/µcell apparatus (Hamamatsu). Results have been expressed as percentage of forskolin-stimulated bioluminescence.

5

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.112150.

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**Novel pyrrolidinone derivative lacks claimed histamine H₃ receptor
stimulation
in receptor binding and functional studies**

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Contents:

S1.   Analytics of compound 6k
S1.1.   NMR-Spectrometry (¹H/¹³C)

Fig. S1.1. ¹H NMR spectrum of compound 6k

Fig. S1.2. ¹³C NMR spectrum of compound 6k

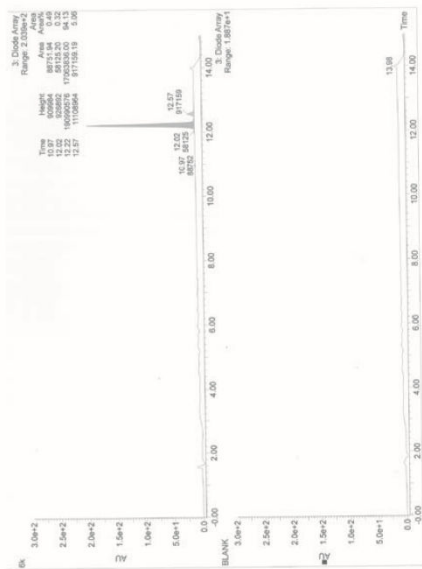**Fig. S1.3.** HPLC chromatogram (UV detection).

S1.2.   Purity determination (HPLC / LC-MS)

HPLC-UV-determined purity was found to be over 94% (instrument data: Sunfire C18 5μm 4.6x150mm

column, eluents: water/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B), linear gradient from

5% (B) to 95% (B) in 10 min) (Fig. S1.3). LC-MS analysis was performed using an Intensity Solo 2 C18

2.1x100mm column (temperature = 50 °C, flow rate = 0.2 ml/min). Eluents: water/0.1% formic acid (A)

and acetonitrile (B) with a linear gradient from 5% (B) to 95% (B) in 10 min, followed by isocratic elution

with 95% (B) for 4 min). The LC-MS based method revealed a hidden impurity eluting with the main-

peak (Fig. S1.4). By modification of the method using initially higher elution strength (Eluents:

water/0.1% formic acid (A) and acetonitrile (B). Isocratic elution: 55% (B) within the first 10 min. A

linear gradient (10-12 min) from 55% (B) to 60% (B), followed by isocratic elution with 60% (B) for

2 min), a faster elution of the main peak could be achieved, whereas full isolation of the impurity and final

quantification of compound 6k could be achieved (Fig. S1.5) at lower elution strength during the elution

of main analyte (60% (B) instead of 95% (B)).

3

4
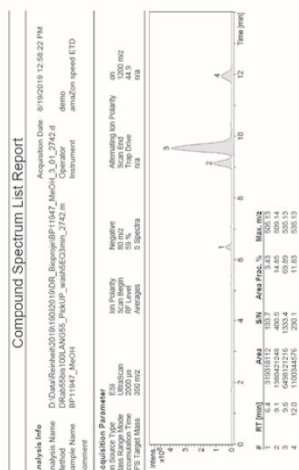
Fig. S1.5. LC-MS chromatogram with isolated impurity (m/z = 509.1) and main-analyte (m/z = 535.1) eluting at 9.1 min.

S2. *In vitro* **Pharmacology**

S2.1 **CRE/MRE luciferase reporter gene assays**
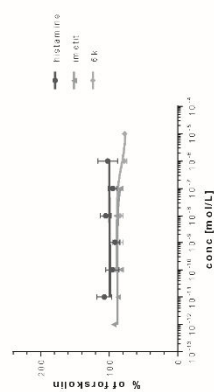
Fig. S2.1. Control measurement of luciferase expression within CHO-DUKX cells, stably transfected with CRE/MRE-luciferase reporter gene but without H₃R cDNA. Neither histamine, imetit, nor compound **6k** exerted remarkable activity.
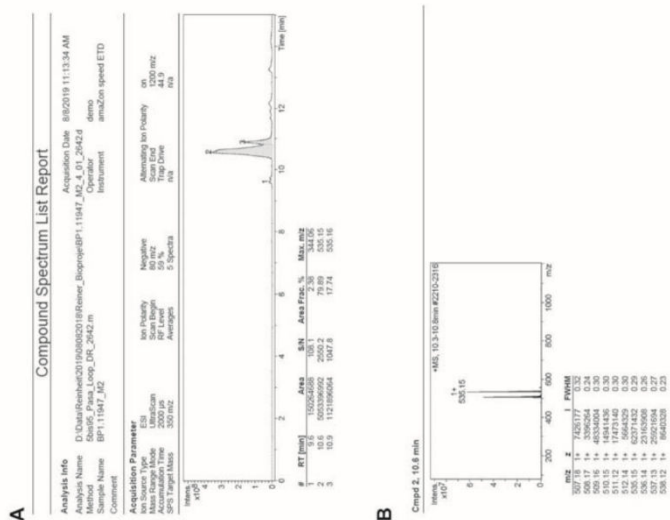
Fig. S1.4. LC-MS chromatogram **(A)** and mass-spectrum corresponding to second peak **(B)**, revealing the hidden impurity (m/z = 509.1) eluting with main analyte (m/z = 535.2) at 18.9 min.

3.3. Design, synthesis, and biological evaluation of novel oxadiazole- and thiazole-based histamine H₃R ligands

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3) College of Pharmacy, Alfaisal University, Al Takhassusi Rd, Riyadh 11533, Saudi Arabia

Contribution to research: DR was in major parts involved in preparation, planning and conduction of radioligand displacement experiments, in maintenance of the corresponding cell-culture and evaluated corresponding data to determine H₃R affinity. DR drafted the supplementary material, wrote the pharmacological parts of the manuscript, and processed the revision.

Abstract:

Histamine H₃ receptor (H₃R) is largely expressed in the CNS and modulation of the H₃R function can affect histamine synthesis and liberation, and modulate the release of many other neurotransmitters. Targeting H₃R with antagonists/inverse agonists may have therapeutic applications in neurodegenerative disorders, gastrointestinal and inflammatory diseases. This prompted us to design and synthesize azole-based H₃R ligands, i.e. having oxadiazole- or thiazole-based core structures. While ligands of oxadiazole scaffold were almost inactive, thiazole-based ligands were very potent and several exhibited binding affinities in a nanomolar concentration range. Ligands combining 4-cyanophenyl moiety as arbitrary region, *para*-xylene or piperidine carbamoyl linkers, and/or pyrrolidine or piperidine basic heads were found to be the most active within this series of thiazole based H₃R ligands. The most active ligands were in silico screened for ADMET properties and drug-likeness. They fulfilled Lipinski's and Veber's rules and exhibited potential activities for oral administration, blood-brain barrier penetration, low hepatotoxicity, combined with an overall good toxicity profile.

Design, synthesis, and biological evaluation of novel oxadiazole- and thiazole-based histamine H₃R ligands

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ARTICLE INFO

ABSTRACT

Histamine H₃ receptor (H₃R) is largely expressed in the CNS and modulation of the H₃R function can affect histamine synthesis and liberation, and modulate the release of many other neurotransmitters. Targeting H₃R with antagonists/inverse agonists may have therapeutic applications in neurodegenerative disorders, gastro-intestinal and inflammatory diseases. This prompted us to design and synthesize azole-based H₃R ligands, i.e. having oxadiazole- or thiazole-based core structures. While ligands of oxadiazole scaffold were almost inactive, thiazole-based ligands were very potent and several exhibited binding affinities in a nanomolar concentration range. Ligands combining 4-cyanophenyl moiety as arbitrary region, *para*-xylene or piperidine carbamoyl linkers, and/or pyrrolidine or piperidine basic heads were found to be the most active within this series of thiazole-based H₃R ligands. The most active ligands were *in silico* screened for ADMET properties and drug-likeness. They fulfilled Lipinski's and Veber's rules and exhibited potential activities for oral administration, blood–brain barrier penetration, low hepatotoxicity, combined with an overall good toxicity profile.

1. Introduction

Histamine belongs to biogenic amines, which influence several intracellular pathways, including its neurotransmission activities.[1–4] Histamine's regulatory character in cellular activities is attributed to its binding to four subtypes of G-protein-coupled receptors (GPCRs); H₁, H₂, H₃, and H₄ that are differentially expressed in several cell types.[5,6]

Histamine H₃ receptor (H₃R) is a member of transmembrane class A of GPCR family.[7,8] H₃R is largely expressed on the histaminergic neurons of the CNS, located pre- and postsynaptically.[9] It plays an essential role in the biosynthesis and release of histamine as well as in the modulation the release of different neurotransmitters (e.g., dopamine, serotonin, acetylcholine, noradrenaline, GABA, and glutamate).[10,11] Peripherally, H₃R is marginally distributed in the peripheral nervous system and regulates the sympathetic effector systems and pain sensation.[12] Consequently, modulation of the H₃R function can potentially affect histaminergic neurotransmission. Therefore, targeting H₃R with antagonists/inverse agonists may have therapeutic applications in neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, as well as in depression, epilepsy, schizophrenia and in sleep disorders.[13–19]

Recently, a number of H₃R antagonists/inverse agonists have entered late clinical trials for the treatment of several CNS disorders.[15,20,21] In March 2016, the European Medicines Agency authorized marketing of pitolisant (Wakix®) as the first H₃R inverse agonist for the treatment of narcolepsy, designated as an orphan drug since narcolepsy belongs to the rare diseases.[22–24] Pitolisant is also currently in phase III clinical trials for the treatment of hypersomnia.[25]

First H₃R antagonists/inverse agonists reported in literature were imidazole-based compounds followed by non-imidazoles in the next generation. They share a general pharmacophore generated from numerous chemical scaffolds (Fig. 1).[15,26,27] Prototype H₃R antagonists/inverse agonists pharmacophore consist of basic moiety (mainly tertiary amine electrostatically interacting with Asp114 within H₃R binding site) separated by a spacer to a central core (mainly electron-rich moiety).[28] The distance between the basic head and the electron rich moiety is approximately 4–5 Å, equivalent to 4 bonds in linear arrangement. The central core is usually connected to an arbitrary region, mainly lipophilic, but polar or basic and even acidic moieties are also tolerable.[29] This arbitrary region modulates potency and pharmacokinetic properties of H₃R antagonists/inverse agonists.[27,30]

Oxadiazole and thiazole nuclei have attracted a wide attention in the search for new therapeutic molecules. They are widely exploited in various applications because of their versatile applicability in multiple therapeutic agents and their blood–brain barrier (BBB)
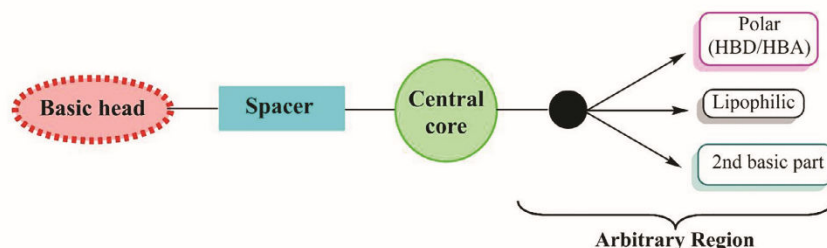
Fig. 1. Schematic presentation of the pharmacophore model of H₃R antagonists/inverse agonists.
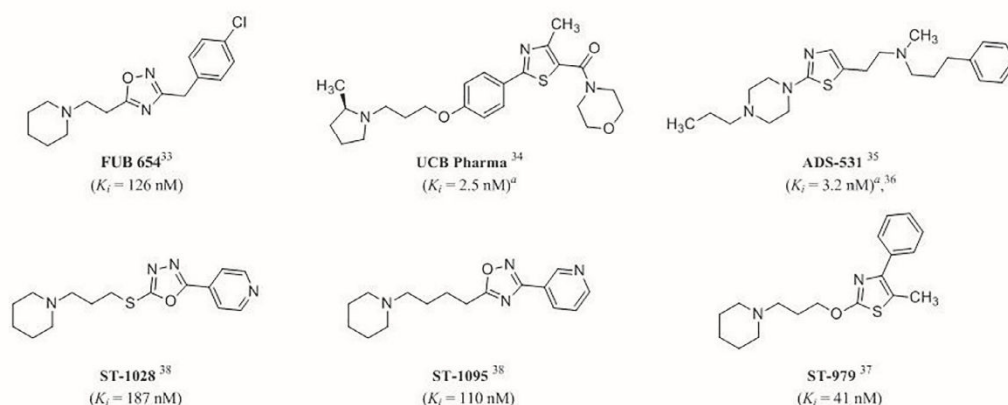

Fig. 2. Selected oxadiazole- and thiazole-based H₃ receptor ligands serving as a basis for design of new H₃R ligands. $^{a}K_i$ values calculated from published pK_i values.

permeability.[31,32] Five-membered heterocycles as core motifs in non-imidazole H₃R ligands with structural resemblance to the presented structures were explored in earlier research projects resulting in affine compounds (Fig. 2).[33,34] Among them ADS–531 that recently underwent *in vivo* examination upon effects on food-intake and neurotransmitter systems.[35,36] The former progress in the design of potent oxadiazole- and thiazole-based H₃R ligands prompted our research group to explore 2-arylthiazole-4-ylethers as analogues to ST-979 but differing in the arrangement of the substituents.[37] Furthermore, *N*-aryl-1,3,4-oxadiazole-2-amines and 3-phenyl-1,2,4-oxadiazole-5-carbox-amides (cf. GSK247246 and ST-1095) were designed to increase structural and linkage diversity in the H₃R central core motif, basically linked to our previous work on variations of polar azole core motifs in H₃R ligands (Fig. 2).[37–40] Our synthetic effort combines various aliphatic and aromatic spacers of variable length, and arbitrary region of electronically and sterically variable substituents. Several H₃R analogues with binding affinities in low nanomolar concentrations were obtained within this project.

2. Results and discussions

### 2.1. Hit-identification and hit-to-lead-optimization

Synthetic design strategy was driven by an initial structural hypothesis for various azole-based H₃R ligands fitting the H₃R-pharmacophoric map (Fig. 1).[37–39] The following structural modification strategies were applied to design potent H₃R ligands: (1) employing oxadiazole (1,2,4 and 1,3,4) or thiazole rings as a polar, electron-rich central core; (2) several cyclic and noncyclic amines were examined as a basic head of the designed H₃R ligands including: pyrrolidine, piperidine, piperazine and noncyclic aliphatic tertiary amines; (3) variable spacers of different chain length connecting the central core and the basic head were examined using aliphatic and aromatic linkers. Linkers of 2 to 5 elements of cyclic or noncyclic structure were tested for optimal length; (4) substituted and unsubstituted aromatic and heteroaromatic rings of variable size and electronic properties were placed on the arbitrary region. Substantial modifications can be conducted on the arbitrary region to modulate potency, solubility and pharmacokinetic properties. Initially, only a few ligands of each compound series without changes in the arbitrary region were synthesized and evaluated for hit identification. Hit-to-lead optimization was conducted for those structures with promising results.

2.2. Chemistry

An efficient one-pot synthesis of substituted 2-anilino-1,3,4-oxadiazoles was applied. A carboxylic acid (1.0 eq.) and 4-phenyl-3-thiosemicarbazide (1.0 eq.) were mixed in DCM at room temperature with three equivalents of *N*-(3-dimethylaminopropyl)-*N*¹-ethylcarbodiimide (EDCI) as coupling reagent, producing the corresponding 2-anilino- 1,3,4-oxadiazoles (**2a-m**) (Scheme 1). The thiosemicarbazides **1a-e** were readily prepared by reacting the corresponding isothiocyanate with hydrazine under reflux in MeOH.

The synthesis of 1,2,4-oxadiazole derivatives started from commercially available nitriles that were reacted with hydroxylamine in EtOH/H₂O in the presence of NaHCO₃ to give the corresponding phenylamidoximes **3a–b** in good yields. The latter were suspended in DCM and then refluxed for 5 h in pyridine with ethyl chlorooxalate to provide 3-aryl-1,2,4-oxadiazole-5-carboxylates **4a–b**. Then, the obtained esters were eadily converted into the desired compounds **5a-c** by reaction
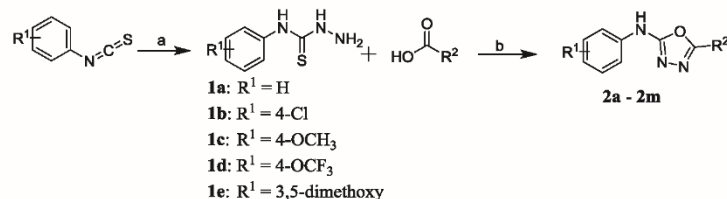
Scheme 1. Synthesis of 2-Anilino-1,3,4-oxadiazoles. Reagents and Conditions: (a) (1) N$_2$H$_4$, MeOH, rt, 1 h. (2) 65–80 °C, 20 min (88–96%); (b) EDCI (3 eq), CH$_2$Cl$_2$, rt, 12 h (50–84%). Structure of compounds **2a–2m** are listed in Table 1.
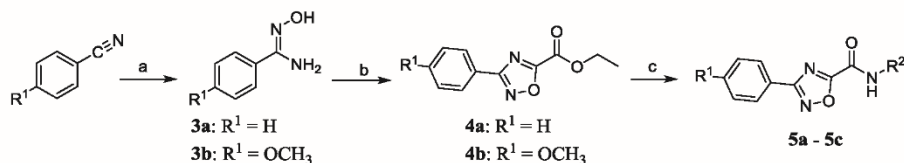
Scheme 2. Synthesis of 3-phenyl-1,2,4-oxadiazole derivatives. Reagents and Conditions: (a) NH$_4$OH, EtOH, NaHCO$_3$, reflux, 3 h (67–77%); (b) CHCl$_3$, ethyl chlorooxalate, rt for 30 min, then refluxed for 5 h (79–82%); (c) EtOH, alkyl amine, reflux for 8 h (73–87%). Structure of compounds **5a–5c** are listed in Table 1.

with 3-substituted amine-1-yl propan-1-amine (Scheme 2).

The synthesis of 5-methyl thiazole analogues (**7–36**) was based on literature procedure where the aryl/heteroaryl nitrile, α-mercapto-carboxylic acid, and pyridine were reacted to form the corresponding hydroxymethylthiazole (**6**).[41] Subsequently, the hydroxymethiazole was O-alkylated following a Williamson-type etherification with K$_2$CO$_3$ as the base to afford the final compounds (**7–36**) (Scheme 3). Analogues **39–42** were synthesized by first refluxing 4-hydroxythiazole with 4 equivalents of α,α′-dibromo-p-xylene in acetone and K$_2$CO$_3$. After column chromatography, the monoether products (**38a-b**) were reacted with either piperidine or pyrrolidine to furnish the corresponding final compounds (**39–42**) (Scheme 4). The 5-ethyl thiazole analogues (**44–46**) were synthesized by first mixing the corresponding phenylthioamide, ethyl 2-bromobutanoate and pyridine under argon and slowly heating up to 100–110 °C for 2 h to form hydroxyethylthiazole (**43**), which was O-alkylated to afford the final compounds (**44–46**) (Scheme 5).[42]

2.3. Biological evaluation

All final synthesized compounds were tested for their *in vitro* H₃R binding affinity in a binding assay by competitive displacement of [³H]-N$^\alpha$-methylhistamine as radioligand. Their corresponding K$_i$ values with 95% confidence intervals are listed in Tables 1 and 2.

2.3.1. Oxadiazole-based ligands

As it was previously demonstrated that oxadiazoles, e.g. linked either via oxy-/thioethers or carboxamides to the basic amine moiety, are accepted as central core in H₃R ligands, a small series of 1,2,4- and 1,3,4-oxadiazole derivatives were designed.[38,43]

Analogues of 1,3,4-oxadiazole scaffold showed low binding affinities at H₃R (K$_i$ > 1000 nM), thus, permitting only limited conclusions for structural variations to improve H₃R affinity. The prototypes (**2a–2d**) without modifications at the anilino substructure resulted in ligand **2d** showing weak affinity. Only a few variations of these ligands were constructed to examine possibilities for improving this binding behavior, resulting in **2e–2m**. Ligands with alicyclic basic head and 2–3 carbon linkers showed comparable activity with **2d** exhibiting low micromolar K$_i$ values (**2c 2 g**, **2j**, **2k**, and **2m**). However, analogues with the same linker length but rigid linker and basic head have negligible binding affinity (**2b**, **2f**, **2i**). This behavior implies the necessity of flexibility to position the basic head in favorable orientation for salt bridge formation with Asp114, described as crucial for H₃R antagonists/inverse agonists binding.[44] Moreover, ligands with noncyclic basic head and/or shorter linker (**2a**, **2h**, **2l**) appeared inactive, except the 4-chlorine derivative showing micromolar affinity (**2e**). These data suggest the need of a defined distance between the central core and the cyclic basic head for these compounds. Nevertheless, the modifications performed here were not able to improve affinity substantially, limiting any SAR interpretations in this series.
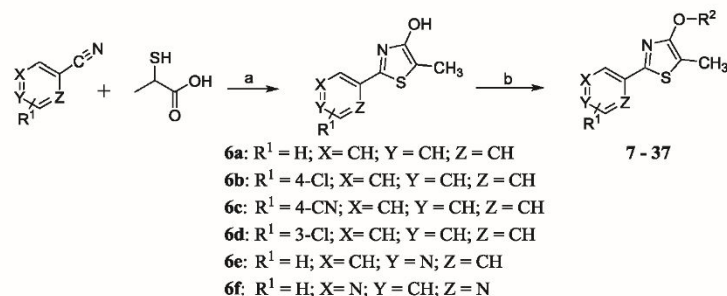
Scheme 3. Synthesis of 5-methyl thiazole derivatives. Reagents and Conditions: (a) pyridine, 100 °C for 2 h (69–96%); (b) DMF (or acetone), Cs$_2$CO$_3$ (or K$_2$CO$_3$) (3.0 eq.), KI (1.0 eq.), alkyl halide (0.9 eq.) (57–95%). Structure of compounds **7–37** are listed in Table 2.
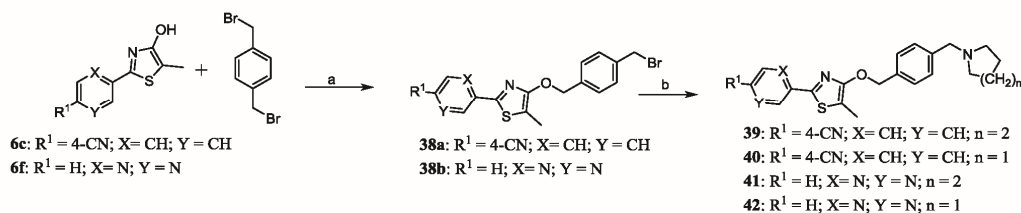
Scheme 4. Synthesis of xylene linker thiazole derivatives. Reagents and conditions: (a) K₂CO₃, acetone, reflux, 12 h (74–86%); (b) alicyclic amine, K₂CO₃, DMF, 60 °C, 12 h (84–90%).

The smaller series of 1,2,4-oxadiazole-5-carboxamides analogues structurally related to previously described compounds FUB 654, ST-1095 or GSK247246 were inactive (**5a**, **5b** and **5c**, Table 1).[33,38,39] Therefore, they are considered as unsuitable fundamental structure for further synthetic efforts, compared to 1,2,4-oxadiazole FUB 654 (Fig. 2), a non-imidazole analogue of the highly affine Glaxo Wellcome compound GR175737 (K_i = 2.5 nM).[33,45] Compared to previously published more potent alkyl-/thioether-linked oxadiazole derivatives (Fig. 2),[38] N-phenyl-1,3,4-oxadiazole-2-amines and 1,2,4-oxadiazole-5-carboxamides seem to bear a less promising or more restricted core motif on substitution and linkage, respectively. Subsequently, the more promising thiazole class has been followed in more detail.

2.3.2. Thiazole-based ligands

The synthetic approach for thiazole-based ligands was supported by the previously published structure ST-979 (Fig. 1).[37] They can be considered as analogues were the substituents were shifted by one carbon atom yielding **11** as initial structure with moderate affinity. The resulting analogues exhibited superior binding affinities with K_i values ranging from low micromolar to nanomolar concentrations. Similar to 1,3,4-oxadiazole analogues, optimum binding activities are achieved with pyrrolidine or piperidine basic heads. For example, compound **21** with pyrrolidine basic head and three-carbon chain linker demonstrated a K_i value of 4 nM. Yet, analogues with *para*-xylene (e.g., **41** and **42** with K_i values 7.0 nM and 72 nM, respectively) or piperidine carbamoyl (e.g., **14**, **20**, **23** with K_i values 5.4 nM, 54 nM and 42 nM, respectively) linkers showed high binding affinities. Interestingly, the methylpiperazine carbamoyl substituted thiazole **13** showed only weak affinity (K_i = 5800 nM) compared to the piperidine carbamoyl linked analogue **14** being more active by more than three orders of magnitude. Different affinities of 2- and 3-carbon linked pyrrolidine or piperidine-analogues were detected between the corresponding structures of (pyrrolidine-1-yl)alkoxy linked 2-phenylthiazoles with K_i of 47 nM and 520 nM for dimethylen **8** and trimethylen analogue **10**, respectively. Comparable binding behavior was detected between the corresponding 4-chlorophenylthiazole analogues **16** and **18** as well as between **17** and **19**, both carrying a (piperidin-1-yl)alkoxy linker instead (Table 2). The arbitrary region (i.e. the phenyl moiety) showed wide electronic and steric tolerability. Replacing the phenyl ring with pyridine was tolerable (or slightly less active) but not with pyrimidine (except when it is coupled with *para*-xylene linker in **41** and **42**). A cyano group in *para*-position generated the most active analogues (e.g., **21**, **22**), which may indicate the favorable positioning of hydrogen bond acceptor at this site presumably interacting with the Threonine residue identified by Roche

et al.[27] Replacing the 5-methyl by an 5-ethyl substituent showed inconsistent pattern; it improves the binding affinity as with **46** (K_i = 200 nM), which is about five fold more active than its methyl counterpart (**19**, K_i = 930 nM), but not with **44** and **45**.

Accordingly, analogues combining optimum moieties, i.e. 4-cyanophenyl moiety as arbitrary region, *para*-xylene or piperidine carbamoyl linkers, and/or pyrrolidine or piperidine basic heads were found to be the most active within this series of thiazole-based H₃R ligands.

2.4. In silico molecular and ADMET properties of designed thiazole ligands

Prediction of how much a ligand would have proper pharmacokinetic (ADME) and pharmacodynamic (e.g., toxicological) properties (drug-likeness) is of great importance during pre-clinical evaluation to assist drug discovery/development process, to guide the optimization from a lead compound to a successful drug candidate, and to reduce attrition rates during clinical trials, hence increasing the chance of reaching the market.[46]

Some important chemical descriptors correlate well with ADMET properties such as polar surface area (PSA) and low molecular weight (MW) for high oral absorption.[47] Likewise, rapid renal clearance is correlated with hydrophilic and small compounds. The hepatic metabolism of most drugs is associated with large and hydrophobic compounds. Higher lipophilicity of compounds leads to increased metabolism and poor absorption, along with an increased probability of binding to undesired hydrophobic macromolecules, thereby increasing the potential for toxicity.[48]

Lipinski's[48] and Veber's[49] rules are one of the most important measures to evaluate the drug-likeness and to predict if a compound of certain chemical properties would be orally bioavailable. In spite of some observed exceptions to Lipinski's and Veber's rules, the vast majority (90%) of the orally bioavailable compounds are within their cut-off limits. Lipinski's rule of five states that, generally, an orally bioavailable compound should not violate the following criteria: ≤ 5 hydrogen bond donors (HBD); ≤ 10 hydrogen bond acceptors (HBA); MW < 500; and logP value of < 5. Alternatively, Veber's findings described the role of PSA and number of rotatable bonds as criteria to estimate the oral bioavailability. Veber's rule stated that for a compound to be orally bioavailable it should have either: a PSA ≤ 140 Å and ≤ 10 rotatable bonds, or ≤ 12 HBD and HBA in total and ≤ 10 rotatable bonds. Clearly from Table 3, the most potent ligands (those with K_i < 100 nM) followed all Lipinski's and Veber's rules without any exception, which highlight their drug-likeness properties and their potential to pass the drug development process.
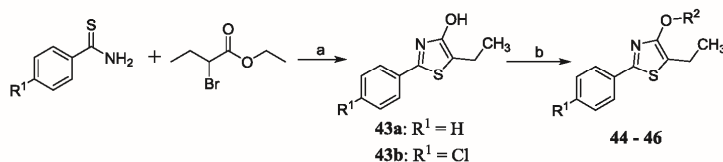
Scheme 5. Synthesis of 5-ethyl thiazole derivatives. Reagents and Conditions: (a) pyridine (1.0 eq.), 100–110 °C, 3 h (76–98%); (b) DMF, K₂CO₃ (3.0 eq.), KI (1.0 eq.), alkyl halide (0.9 eq.) (62–94%). Ligand structures of **44–46** are listed in Table 2.
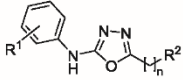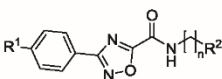
Table 1
Oxadiazole-based ligands along with H_3R binding affinities.

I   II

Compd No.	Scaffold	R^1	n	R^2	H_3R K_i [nM] [95% CI] [a]; (n)[b]
2a	I	H	3		> 5000; (3)
2b	I	H	0		> 5000; (2)
2c	I	H	2		2000 [100; 72,200]; (2)
2d	I	H	3		1300 [700; 2500]; (2)
2e	I	4-Cl	3		5700 [300; 112,800]; (3)
2f	I	4-Cl	0		> 5000; (2)
2g	I	4-Cl	2		1600 [100; 55,200]; (2)
2h	I	4-OCH₃	1		> 5000; (2)
2i	I	4-OCH₃	0		> 5000; (2)
2j	I	4-OCH₃	2		1600 [700; 3800]; (2)
2k	I	4-OCF₃	2		1300 [100; 34,800]; (2)
2l	I	3,5-dimethoxy	1		> 5000 (2)
2m	I	3,5-dimethoxy	2		1100 [1000; 1200]; (2)
5a	II	H	3		> 5000 (2)
5b	II	H	3		> 5000 (4)
5c	II	OCH₃	3		> 5000; (2)

[a] Binding affinity values (K_i) are expressed as mean with 95% confidence intervals.
[b] Number of experiments.

The most active ligands were *in silico* screened for predicted pharmacokinetic properties (BBB penetration, absorption, solubility (Sw), hepatotoxicity, inhibition of CYP2D6, and plasma protein binding (PPB)) being summarized in Table 4. The intestinal absorption and BBB penetration were predicted by developing an ADME model using descriptors 2D PSA and AlogP98 that include 95% and 99% confidence ellipses. These ellipses define regions where well-absorbed compounds are expected to be found. All active ligands showed medium (brain-blood ratio between 0.3:1 and 1:1) to high (brain-blood ratio between 1:1 and 5:1) penetration of BBB and good intestinal absorption (at least 90% absorbed into bloodstream) within 95% confidence ellipses (Table 4 and Fig. 3). These results are therapeutically crucial for H_3R

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Table 2
Thiazole-based ligands along with H₃R binding affinities.

Compd No.	X	Y	Z	R^1	n	R^2	R^3	H₃R K_i [nM] [95% CI][c], (n)[b]
7	CH	CH	CH	H	3		CH₃	4100 [1400; 12,200]; (3)
8	CH	CH	CH	H	2		CH₃	47 [20; 110]; (3)
9	CH	CH	CH	H	2		CH₃	200 [90; 490]; (3)
10	CH	CH	CH	H	3		CH₃	520 [340; 780]; (3)
11	CH	CH	CH	H	3		CH₃	870 [130; 5710]; (5)
12	CH	CH	CH	H	1		CH₃	220 [70; 650]; (8)
13	CH	CH	CH	H	0		CH₃	5800 [1200; 27,000]; (4)
14	CH	CH	CH	H	0		CH₃	5.4 [2.2; 13.2]; (3)
15	CH	CH	CH	4-Cl	3		CH₃	280 [70; 1170]; (4)
16	CH	CH	CH	4-Cl	2		CH₃	22 [5; 98]; (3)
17	CH	CH	CH	4-Cl	2		CH₃	44 [12; 167]; (3)
18	CH	CH	CH	4-Cl	3		CH₃	450 [180; 1150]; (3)
19	CH	CH	CH	4-Cl	3		CH₃	930 [570; 1540]; (4)
20	CH	CH	CH	4-Cl	0		CH₃	54 [22; 131]; (3)
21	CH	CH	CH	4-CN	3		CH₃	4.2 [2.6; 6.6]; (3)
22	CH	CH	CH	4-CN	3		CH₃	21 [2; 201]; (3)
23	CH	CH	CH	4-CN	0		CH₃	42 [15; 122]; (4)
24	CH	CH	CH	3-Cl	2		CH₃	600 [310; 1150]; (3)
25	CH	CH	CH	3-Cl	3		CH₃	430 [120; 1550]; (3)

(continued on next page)

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Table 2 (continued)

Compd No.	X	Y	Z	R^1	n	R^2	R^3	H₃R K_i [nM] [95% CI][a], (n)[b]
26	CH	CH	CH	3-Cl	3	piperidine	CH₃	300 [130; 700]; (4)
27	CH	CH	CH	3-Cl	0	acyl-piperidinyl-piperidine	CH₃	98 [56; 170]; (6)
28	CH	N	CH	H	3	dimethylamino	CH₃	110 [40; 300]; (4)
29	CH	N	CH	H	2	pyrrolidine	CH₃	100 [30; 380]; (4)
30	CH	N	CH	H	2	piperidine	CH₃	57 [42; 78]; (3)
31	CH	N	CH	H	3	pyrrolidine	CH₃	410 [170; 1010]; (4)
32	CH	N	CH	H	3	piperidine	CH₃	130 [20; 820]; (3)
33	N	CH	N	H	3	dimethylamino	CH₃	5900 [2800; 12,600]; (4)
34	N	CH	N	H	2	pyrrolidine	CH₃	2100 [800; 6100]; (4)
35	N	CH	N	H	3	piperidine	CH₃	1100 [500; 2400]; (6)
36	N	CH	N	H	0	acyl-(4-methylpiperazine)	CH₃	> 10,000
37	CH	CH	CH	(oxolanone, 3,4)	3	piperidine	CH₃	87 [16; 462]; (5)
39	CH	CH	CH	4-CN	1	benzyl-piperidine	CH₃	450 [190; 1100]; (4)
40	CH	CH	CH	4-CN	1	benzyl-piperidine	CH₃	190 [130; 280]; (3)
41	N	CH	N	H	1	benzyl-piperidine	CH₃	7.0 [2.6; 18.4]; (4)
42	N	CH	N	H	1	benzyl-piperidine	CH₃	72 [11; 492]; (4)
44	CH	CH	CH	H	3	piperidine	CH₂CH₃	380 [180; 810]; (3)
45	CH	CH	CH	4-Cl	3	pyrrolidine	CH₂CH₃	170 [30; 870]; (4)
46	CH	CH	CH	4-Cl	3	piperidine	CH₂CH₃	200 [100; 370]; (4)

[a] Binding affinity values (K_i) are expressed as average along with 95% confidence intervals.
[b] Number of experiments.

Table 3
Compliance of the most active ligands to Lipinski's and Veber's rules.

Compound	AlogP (< 5)	MW (<5 0 0)	No. HBA[a] (≤ 10)	No. HBD[b] (≤ 5)	No. rotatable bonds (≤ 10)	Molecular PSA[c] (< 140 Å)
14	2.33	386.5	5	1	4	75
20	3.00	421.0	5	1	4	75
21	1.80	314.4	4	1	5	79
23	2.21	411.5	6	1	4	99
27	3.00	421.0	5	1	4	75
41	1.56	367.5	5	1	6	81

[a] Number of Hydrogen-bond acceptors (HBA).
[b] Number of Hydrogen-bond donors (HBD).
[c] Polar Surface Area (PSA).

Table 4
Predicted ADME profiles of the most active ligands.

Compound	BBB[a]	Absorption[b]	Solubility[c]	Hepatotoxicity[c]	CYP2D6[e]	PPB[f]
14	2	0	3	Nontoxic	Inhibitor	2 (> 95%)
20	1	0	2	Nontoxic	Non-inhibitor	0 (90%)
21	2	0	3	Nontoxic	Non-inhibitor	1 (> 90%)
23	2	0	3	Nontoxic	Non-inhibitor	0 (< 90%)
27	1	0	2	Nontoxic	Non-inhibitor	0 (< 90%)
41	2	0	3	Nontoxic	Non-inhibitor	2 (> 95%)

[a] Predicts ability of the compound to penetrate the blood-brain barrier (BBB). Levels 0, 1, 2, 3, or 4 correspond to very high, high, medium, low, or undefined penetration, respectively.
[b] Predicts human intestinal absorption after oral administration. Levels 0, 1, 2, or 3 correspond to good, moderate, poor, or very poor absorption, respectively.
[c] Predicts the solubility of each compound in water at 25 °C. Levels 0, 1, 2, 3, 4, 5, or 6 correspond to extremely low, very low, low, good, optimal, too soluble, or unknown solubility, respectively.
[d] Predicts the occurrence of dose-dependent human hepatotoxicity.
[e] Predicts cytochrome P450 2D6 inhibition.
[f] Predicts the likelihood that a compound will be highly bound to carrier proteins on the blood (PPB, plasma protein binding).

ligands to be effective for targeting neurodegenerative diseases and their potential for oral administration. Moreover, these ligands showed low (−6.0 < logSw < −4.0) to good (−4.0 < logSw < −2.0) solubility, non-hepatotoxicity, and variable properties for inhibition of CYP2D6 and PPB (Table 4). However, the compounds with highest affinity in the series (**14**, **21** and **41**) provide some deficiencies regarding BBB permeability and display high PPB (PPB > 95% as with **14** and **41**).

As ligand efficiency measures emerge as increasingly important parameters in hit-to-lead optimization, we calculated ligand efficiency (*LE*) and lipophilic ligand efficiency (*LLE*) according to literature, based on *in silico* AlogP-values and *in vitro* binding affinities.[50] *LE* values were within the commonly accepted limits with *LE* > 0.3 kcal per mole per non-H-atoms (nHA) (0.42, 0.50 and 0.41 kcal/mole/nHA for compounds **14**, **21** and **41** respectively).[51] *LLE* values conform to the acceptance criteria of *LLE* > 5, being 5.9, 6.6 and 6.6 for **14**, **21** and **41**, respectively.[52]

The United States Food and Drug Administration (US FDA) standard toxicity risk predictor software TOPKAT (Discovery Studio, Accelrys, USA) locates fragments within the compound that indicate a potential
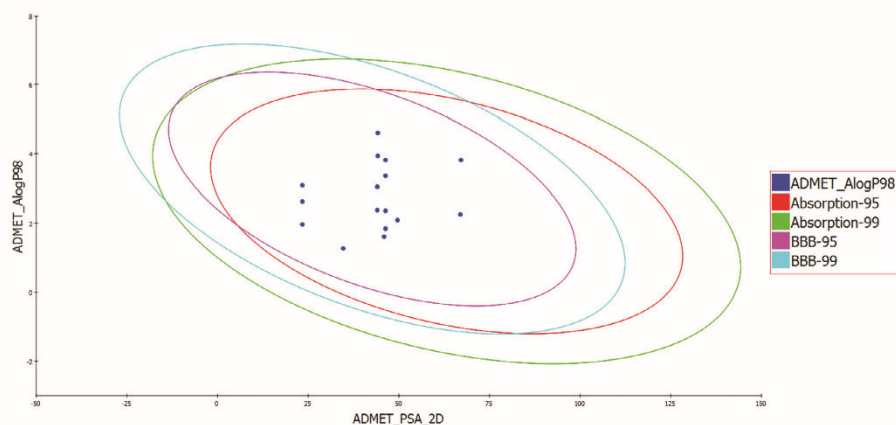

Fig. 3. Plot of PSA *vs* AlogP (calculated via "Calculate Molecular Properties" module of Discovery Studio 2.5.5) for the most active ligands showing 95% and 99% confidence limit ellipses corresponding to penetration of BBB and intestinal absorption.

Table 5
Toxicity profile of the most active ligands using toxicity prediction, extensible protocol of Accelrys Discovery studio 2.5.5.

Compound	Aerobic BioDegradability[a]	AMES Mutagenicity[b]	Ocular Irritancy[c]	Skin Irritancy[c]	Skin Sensitizer[c]	Carcinogenicity_USFDA[d]			
						Female Mouse	Male Mouse	Female rat	Male Rat
14	−	−	+	−	−	−	+	+	−
20	−	−	+	−	−	−	−	−	−
21	−	−	+	−	+ +	−	+	+ +	+ +
23	−	−	+	−	−	−	+	+ +	+ +
27	−	−	+	−	−	−	−	−	−
41	−	−	+	+	+ +	+	+	+	+ +

  [a] Prediction of oxidative degradability-properties of compounds. Indicators −, or + correspond to non-biodegradability, or degradability by bioorganisms, respectively.
  [b] Predicted mutagenicity in AMES mutagenicity test. Indicators −, or + correspond to non-mutagenicity, or mutagenicity, respectively.
  [c] Prediction of organic toxicity. Indicators −, +, or + + correspond to none, mild, or severe/strong toxic properties, respectively.
  [d] Prediction of carcinogenic properties. Indicators −, +, or + + correspond to classification as Non-Carcinogen, Single-Carcinogen, or Multi-Carcinogen, respectively.

toxicity risk. TOPKAT (TOxicity Prediction by Komputer Assisted Technology) employs robust and cross-validated Quantitative Structure Toxicity Relationship (QSTR) models for assessing various measures of toxicity and utilizing the patented Optimal Predictive Space validation method to assist in interpreting the results. Toxicity screening results of TOPKAT for the most active H₃R ligands showed no risk of AMES mutagenicity, and tolerable ocular and skin irritation (Table 5); however, they possess potential US FDA rodent carcinogenicity, except against female mouse. Compound **14** is the only non-skin sensitizing agent among the three most active compounds and appears as single-carcinogen in male mouse and female rat, whereas the cyano-containing ligands **21** and **41** differentiate as skin sensitizing and multi-carcinogenic agents. Moreover, particular attention was paid to the risk of aerobic biodegradability as 2-phenylthiazoles are known to produce hepatotoxic and/or nephrotoxic thioamides and 1,2-dicarbonyl-species upon oxidative metabolism.[53,54] Thus, exclusively in 5-position alkylated thiazole derivatives were synthesized to achieve reduced oxidative ring-scission and biodegradable probability, confirmed by the TOPKAT screening results.[55]

Besides the biological evaluation, this *in silico* screening provided additional information that could be useful for further lead optimization of the presented compounds (cf. additional *in silico* ADMET and toxicity information on compounds **8**, **16**, **17**, **22**, **30**, **37**, and **42** is provided in Supplementary Material).

3. Conclusions

A novel series of oxadiazole and thiazole derivatives as H₃R ligands are explored in the present study. Oxadiazole ligands showed mediocre affinities unlike the thiazole, which showed potent H₃R affinities in low nanomolar concentration range. The highest affinities are observed with ligands of pyrrolidine or piperidine basic heads and 2–3 carbon linkers. However, thiazole ligands of *para*-xylene or piperidine carbamoyl linkers showed high binding affinities, too. The examined compounds show excellent drug-like properties in compliance with Lipinski's and Veber's rules and satisfactory *in silico* ADMET properties. The results from this study will be helpful for further improvements of potent H₃R ligands.

4. Experimental

4.1. Chemistry

Reagents and solvents for synthesis were purchased from Sigma-Aldrich, VWR Chemicals, Fisher Scientific, Alfa Aesar and Chemsolute and were used without further purifications. ¹H NMR and ¹³C NMR were recorded on a Bruker AMX spectrometer (Bruker, Germany) at

300 and 75 MHz respectively, where CDCl₃ or DMSO-d_6 was used as a solvent. Tetramethylsilane was used as standard and chemical shifts are reported in parts per million (ppm). Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quintet) or m (multiplet). Approximate coupling constants (J) are given in Hertz (Hz). Number and assignment of protons (ax, axial; eq, equatorial; ph, phenyl; pip, piperidine; pyra, pyrazine; pyr, pyridine; pyrr, pyrrolidine). Elementary analyses (C, H, N) were measured on a CHN-Rapid (Heraeus, Germany) and all final compounds were within 0.4% of the theoretical values. Electrospray ionization mass spectrometry (ESI-MS) was performed on an amaZon speed (Bruker, Germany) in positive polarity. Data are listed as mass number ([M + H⁺]). High-resolution mass spectra (HRMS) were run in electrospray ionization (ESI) mode. Melting points (m.p., uncorrected) were determined on a M-564 Büchi melting point apparatus (Büchi, Germany). Preparative column chromatography was performed on silica gel 60 M, 0.04–0.063 mm (Macherey-Nagel, Germany) and thin-layer chromatography (TLC) was carried out using pre-coated silica gel 60 with fluorescence indicator at UV 254 nm (Macherey-Nagel, Germany).

4.1.1. General reaction procedures
4.1.2.1. General procedure (A). Preparation of 4-Phenyl-3-thiosemicarbazide. The phenylisothiocyanate (30 mmol) was added, dropwise, over a period of 1 h to a stirred solution of hydrazine (30 mmol) in methanol (8 mL) at 65–80 °C. The reaction mixture was stirred for 20 min more at the same temperature. The solvent was removed by evaporation *in vacuo* and the precipitate was collected by filtration and washed with petroleum ether. The compound was used without further purification.

4.1.2.2. General procedure (B). Preparation of 2-Anilino-5-alkyl-1,3,4-oxadiazole. A carboxylic acid (0.33 mmol), a 4-phenyl-3-thiosemicarbazide (0.33 mmol), and EDCI (182 mg, 1.0 mmol) were mixed in CH₂Cl₂ (15 mL), and the reaction mixture was stirred at room temperature for 12 h. The organic layer was washed three times with saturated NaHCO₃, dried over MgSO₄, filtered, and concentrated. Chromatography (5:95 MeOH/DCM, silica gel) of the residue afforded the title compound.

4.1.2.3. General procedure (C). Preparation of Phenyl amidoximes. To a stirred solution of phenyl nitrile (0.1 mol) in ethanol (100 mL), a solution of hydroxylamine (0,6 mol) in H₂O (150 mL) was slowly added followed by NaHCO₃ (0.3 mol). The resulting mixture was refluxed for 3 h. The solvent was evaporated *in vacuo* and the resulting residue was poured into cold water. The formed precipitate was filtered off, washed with water and dried *in vacuo*. The obtained product was recrystallized from methanol.

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4.1.2.4. General procedure (D). Preparation of 1,2,4-Oxadiazole-5-carboxylates. Pyridine (0.015 mol) was added to a solution of phenyl amidoxime (0.01 mol) dissolved in dry CHCl₃ (100 mL). Ethyl chloroxalate (0.025 mol) was then added to the solution under vigorous stirring. The mixture was stirred at room temperature for 30 min, then refluxed for 5 h, cooled to rt, washed with water and HCl (5%). Organic layer was dried under MgSO₄, concentrated *in vacuo* and the formed residue was purified by flash-chromatography on silica gel.

4.1.2.5. General procedure (E). Preparation of 1,2,4-Oxadiazole-5-carboxamides. A primary amine (0.5 mmol.) was added to the carboxylate (0.5 mmol) dissolved in dry ethanol (5 mL). The reaction mixture was refluxed for 12 h, and then cooled to rt. The organic solvent was dried *in vacuo* and the crude product was purified by flash-chromatography on silica gel to give the target compounds.

4.1.2.6. General procedure (F). Preparation of 4-Hydroxy-5-methylthiazole. Pyridine (0.005 mol) was added to a mixture of thiolactic acid (0.02 mol) and nitrile (0.02 mol) at 23 °C under argon. The reaction mixture was then heated at 100 °C for 2 h. After cooling, the precipitate was collected, washed with absolute ethanol, and recrystallized from methanol to afford the product.

4.1.2.7. General procedure (G). O-Alkylations of Hydroxy thiazoles. To a solution of thiazole (1.0 eq.) dissolved in DMF (4 mL), K₂CO₃ (3.0 eq.) was added, and the reaction mixture was left for 30 min at 60 °C. Then, KI (1.0 eq.) and the alkyl halide (0.9 eq.) was added and the reaction was left for an additional 8 h. The reaction mixture was taken up in DCM, washed with Na₂CO₃ (3x) and dried over MgSO₄ and concentrated *in vacuo*. The concentrate was purified by flash chromatography yielding the desired compound.

4.1.2.8. General procedure (H). Preparation of 2-Phenyl-4-hydroxy-5-ethylthiazole. Pyridine (5.83 mmol) was added to a mixture of ethyl 2-bromobutanoate (6.41 mmol) and thiobenzamide (5.83 mmol) at 23 °C under argon. The reaction mixture was then heated at 100 °C for 2 h. After cooling, the precipitate was collected and washed with absolute ethanol, and the recrystallized from methanol to afford the desired product.

4.1.2.9. General procedure (I). Preparation of Bromoethyl benzyl thiazole. 1,4-Bis(bromomethyl)benzen (4.0 mmol), 4-hydroxythiazole (1.0 mmol) and K₂CO₃ (2.0 mmol) dissolved in 50 mL acetone was refluxed for 12 h. After cooling, acetone was evaporated and the crude product was constituted in DCM. The organic layer was washed with NaHCO₃ solution (3x) and dried over Na₂SO₃. The final compound was purified with flash chromatography.

4.1.2.10. General procedure (J). Nucleophilic Substitution of Bromoethyl benzyl thiazole. The bromoethyl benzyl thiazole (1.0 eq.) was dissolved in DMF (4 mL) and K₂CO₃ (3.0 eq.) was added. The reaction mixture was left for 30 min at 60 °C, then the piperidine or pyrolidine (0.9 eq.) was added and the reaction was left for an additional 8 h. The reaction mixture was taken up in DCM, washed with Na₂CO₃ (3x) and dried over MgSO₄ and concentrated *in vacuo*. The concentrate was purified by flash chromatography yielding the desired compound.

4.1.2.11. 5-Methyl-2-phenyl-4-(2-(pyrrolidin-1-yl)ethoxy)thiazole (8). Compound **8** was prepared according to procedure G from 5-methyl-2-phenylthiazol-4-ol and 1-(2-chloroethyl)pyrrolidine to afford 62 mg of yellow solid (70%). ^{1}H NMR (CDCl₃, 300 MHz) δ 7.77 (dd, J = 8.1, 1.8 Hz, 2H, ph-2,6H), 7.38–7.23 (m, 3H, ph-3,4,5H), 4.45 (t, J = 6.0 Hz, 2H, OCH_2), 2.87 (t, J = 6.0 Hz, 2H, OCH₂CH_2N), 2.73–2.50 (m, 4H, pyrr-2,5H_2), 2.22 (s, 3H, CH_3), 1.77 (p, J = 1.8 Hz, 4H, pyrr-3,4H_2). ^{13}C NMR (151 MHz, CDCl₃) δ 9.40, 23.53, 54.67, 55.32, 69.26, 107.09, 125.34, 128.78, 129.29, 134.00, 159.47, 159.53. HRMS *m/z*

289.1384 [M+H⁺] (calcd for C₁₆H₂₁N₂OS, 289.1375). Anal. Calcd for C₁₆H₂₀N₂OS: C 66.63, H 6.99, N 9.51, S 11.12. Found: C 66.82, H 7.24, N 9.18, S 11.51.

4.1.2.12. 5-Methyl-2-phenylthiazol-4-yl [1,4′-bipiperidine]-1′-carboxylate (14). Compound **14** was prepared according to procedure G from 5-methyl-2-phenylthiazol-4-ol and [1,4′-bipiperidine]-1′-carbonyl chloride to afford 74 mg of yellow solid (80%). ^{1}H NMR (CDCl₃, 300 MHz) δ 7.79–7.74 (dd, J = 2.1, 1.8 Hz, 2H, ph-2,6H), 7.31 (d, J = 1.8 Hz, 1H, ph-4H), 7.29 (d, J = 2.1 Hz, 2H, ph-3,5H), 4.27 (dd, J = 30.2, 12.8 Hz, 2H, pip^1-2,6H_{eq}), 2.85 (m, 2H, pip^1-2,6H_{ax}), 2.55–2.32 (m, 5H, pip^1-4H, pip^2-2,6$H_{2ax/eq}$), 2.22 (s, 3H, CH_3), 1.94–1.72 (m, 2H, pip^1-3,5H_{eq}), 1.67–1.42 (m, 6H, pip^1-3,5H_{ax}, pip^2-3,5$H_{2ax/eq}$), 1.42–1.27 (m, 2H, pip^2-4$H_{2ax/eq}$). ^{13}C NMR (75 MHz, CDCl₃) δ 10.03, 24.63, 26.21, 28.10, 44.40, 50.20, 62.36, 117.88, 125.72, 128.76, 129.81, 133.37, 151.79, 152.31, 161.20. HRMS *m/z* 386.1910 [M+H⁺] (calcd for C₂₁H₂₈N₃O₂S, 386.1902). Anal. Calcd for C₂₁H₂₇N₃O₂S: C 65.43, H 7.06, N 10.90, S 8.32. Found: C 66.18, H 7.12, N 10.84, S 8.38.

4.1.2.13. 2-(4-Chlorophenyl)-5-methyl-4-(2-(pyrrolidin-1-yl)ethoxy) thiazole (16). Compound **16** was prepared according to procedure G from 2-(4-chlorophenyl)-5-methylthiazol-4-ol and 1-(2-chloroethyl) pyrrolidine to afford 79 mg of yellow solid (91%). ^{1}H NMR (CDCl₃, 300 MHz) δ 7.66 (d, J = 8.6 Hz, 2H, ph-2,6H), 7.24 (d, J = 8.6 Hz, 2H, ph-3,5H), 4.40 (t, J = 6.0 Hz, 2H, OCH_2), 2.81 (t, J = 6.0 Hz, 2H, OCH₂CH_2N), 2.63–2.47 (m, 4H, pyrr-2,5H_2), 2.19 (s, 3H, CH_3), 1.72 (p, J = 3.0 Hz, 4H, pyrr-3,4H_2). ^{13}C NMR (75 MHz, DMSO) δ 9.28, 23.42, 54.59, 55.26, 69.42, 107.31, 126.37, 128.82, 132.38, 134.89, 157.85, 159.64. HRMS *m/z* 323.0991 [M+H⁺] (calcd for C₁₆H₁₉ClN₂OS, 323.0985). Anal. Calcd for C₁₆H₂₀ClN₂OS: C 59.52, H 5.93, N 8.68, S 9.93. Found: C 59.88, H 6.07, N 8.41, S 10.37.

4.1.2.14. 2-(4-Chlorophenyl)-5-methyl-4-(2-(piperidin-1-yl)ethoxy) thiazole (17). Compound **17** was prepared according to procedure G from 2-(4-chlorophenyl)-5-methylthiazol-4-ol and 1-(3-chloroethyl) piperidine to afford 43 mg of yellow solid (57%). ^{1}H NMR (CDCl₃, 300 MHz) δ 7.69 (d, J = 8.5 Hz, 2H, ph-2,6H), 7.28 (d, J = 8.5 Hz, 2H, ph-3,5H), 4.44 (t, J = 6.0 Hz, 3H, OCH_2), 2.76 (t, J = 6.0 Hz, 3H, OCH₂CH_2N), 2.62–2.47 (m, 4H, pip-2,6$H_{2ax/eq}$), 2.21 (s, 3H, CH_3), 1.72–1.52 (m, 4H, pip-3,5$H_{2ax/eq}$), 1.45–1.32 (m, 2H, pip-4$H_{2ax/eq}$). ^{13}C NMR (75 MHz, CDCl₃) δ 9.73, 24.63, 26.34, 55.33, 58.66, 68.65, 107.37, 126.48, 128.93, 132.48, 134.99, 158.01, 159.78. HRMS *m/z* 337.1148 [M+H⁺] (calcd for C₁₇H₂₂ClN₂OS, 337.1141). Anal. Calcd for C₁₇H₂₁ClN₂OS: C 60.61, H 6.28, N 8.32, S 9.52. Found: C 61.08, H 6.51, N 8.41, S 9.85.

4.1.2.15. 2-(4-Chlorophenyl)-5-methylthiazol-4-yl [1,4′-bipiperidine]-1′-carboxylate (20). Compound **20** was prepared according to procedure G from 2-(3-chlorophenyl)-5-methylthiazol-4-ol and [1,4′-bipiperidine]-1′-carbonyl chloride to afford 84 mg of yellow solid (89%). ^{1}H NMR (CDCl₃, 300 MHz) δ 7.69 (d, J = 8.6 Hz, 2H, ph-2,6H), 7.27 (d, J = 8.6 Hz, 2H, ph-3,5H), 4.27 (dd, J = 28.1, 13.3 Hz, 2H, pip^1-2,6H_{eq}), 2.86 (m, 2H, pip^2-2,6H_{eq}), 2.62–2.34 (m, 5H, pip^1-4H, pip^2-2,6$H_{2ax/eq}$), 2.22 (s, 3H, CH_3), 1.96–1.74 (m, 2H, pip^1-3,5H_{eq}), 1.68–1.45 (m, 6H, pip^1-3,5H_{ax}, pip^2-3,5$H_{2ax/eq}$), 1.44–1.29 (m, 2H, pip^2-4H_2). ^{13}C NMR (75 MHz, CDCl₃) δ 10.02, 24.46, 25.96, 27.40, 27.97, 44.04, 44.33, 50.16, 62.39, 118.40, 126.90, 128.99, 131.86, 135.69, 151.88, 152.22, 159.79. HRMS *m/z* 420.1514 [M+H⁺] (calcd for C₂₁H₂₆ClN₃O₂S, 420.1513). Anal. Calcd for C₂₁H₂₇ClN₃O₂S: C 60.06, H 6.24, N 10.01, S 7.63. Found: C 60.12, H 6.20, N 10.18, S 7.66.

4.1.2.16. 4-(5-Methyl-4-(2-(pyrrolidin-1-yl)ethoxy)thiazol-2-yl) benzonitrile (21). Compound **21** was prepared according to procedure G from 2-(3-cyanophenyl)-5-methylthiazol-4-ol and 1-(2-chloroethyl) pyrrolidine to afford 67 mg of yellow solid (80%). ^{1}H NMR (CDCl₃,

300 MHz) δ 7.74 (d, J = 8.7 Hz, 2H, ph-2,6H), 7.49 (d, J = 8.7 Hz, 2H, ph-3,5H), 4.38 (t, J = 5.4 Hz, 2H, OCH_2), 2.84 (t, J = 5.5 Hz, 2H, OCH$_2$CH_2N), 2.66–2.55 (m, 4H, pyrr-2,5H_2), 2.16 (s, 3H, CH_3), 1.80–1.64 (m, 4H, pyrr-3,4H_2). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.36, 31.25, 36.37, 54.98, 68.92, 109.48, 111.85, 118.48, 125.35, 132.44, 137.43, 156.31, 160.10. HRMS m/z 314.1332 [M+H$^+$] (calcd for C$_{17}$H$_{20}$N$_3$OS, 314.1327). Anal. Calcd for C$_{17}$H$_{19}$N$_3$OS: C 66.03, H 6.46, N 12.83, S 9.79. Found: C 66.18, H 6.51, N 12.79, S 9.72.

4.1.2.17. 4-(5-Methyl-4-(3-(piperidin-1-yl)propoxy)thiazol-2-yl) benzonitrile (22).

Compound **22** was prepared according to procedure G from 2-(3-cyanophenyl)-5-methylthiazol-4-ol and 1-(3-chloropropyl) piperidine to afford 75 mg of yellow solid (83%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.81 (d, J = 8.7 Hz, 2H, ph-2,6H), 7.55 (d, J = 8.7 Hz, 2H, ph-3-5H), 4.29 (t, J = 6.4 Hz, 2H, OCH_2), 2.45–2.40 (m, 2H, OCH$_2$CH_2), 2.39–2.31 (m, 4H, pip-2,6$H_{2ax/eq}$), 2.21 (s, 3H, CH_3), 2.03–1.82 (m, 2H, OCH$_2$CH$_2$CH_2N), 1.68–1.48 (m, 4H, pip-3,5$H_{2ax/eq}$), 1.45–1.27 (m, 2H, pip-4$H_{2ax/eq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.40, 24.31, 25.80, 26.97, 54.54, 55.91, 69.11, 109.34, 112.00, 118.62, 125.45, 132.53, 137.66, 156.33, 160.53. HRMS m/z 342.1645 [M+H$^+$] (calcd for C$_{19}$H$_{24}$N$_3$OS, 342.1640). Anal. Calcd for C$_{19}$H$_{23}$N$_3$OS: C 66.83, H 6.79, N 12.31, S 9.39. Found: C 66.79, H 6.80, N 12.31, S 9.44.

4.1.2.18. 2-(4-Cyanophenyl)-5-methylthiazol-4-yl [1,4′-bipiperidine]-1′-carboxylate (23).

Compound **23** was prepared according to procedure G from 4-(4-hydroxy-5-methylthiazol-2-yl)benzonitrile and [1,4′-bipiperidine]-1′-carbonyl chloride to afford 91 mg of yellow solid (91%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.85 (d, J = 8.7 Hz, 2H, ph-2,6H), 7.59 (d, J = 8.2 Hz, 2H, ph-3,5H), 4.26 (dd, J = 31.1, 13.0 Hz, 2H, pip^1-2,6H_{eq}), 2.84 (m, 2H, pip^2-2,6H_{eq}), 2.56–2.44 (m, 5H, pip^1-4H, pip^2-2,6$H_{ax/eq}$), 2.26 (s, 3H, CH_3), 2.01–1.76 (m, 2H, pip^1-3,5H_{eq}), 1.66–1.47 (m, 6H, pip^1-3,5H_{ax}, pip^2-3,5$H_{ax/eq}$), 1.45–1.30 (m, 2H, pip^2-4H_2). ^{13}C NMR (75 MHz, CDCl$_3$) δ 10.08, 24.41, 25.93, 27.41, 27.96, 44.03, 44.31, 50.13, 62.24, 112.75, 118.37, 120.43, 125.93, 132.55, 137.00, 152.02, 152.55, 158.24. HRMS m/z 411.1862 [M+H$^+$] (calcd for C$_{22}$H$_{27}$N$_4$O$_2$S, 411.1855). Anal. Calcd for C$_{22}$H$_{26}$N$_4$O$_2$S: C 64.37, H 6.38, N 13.65, S 7.81. Found: C 64.44, H 6.51, N 13.51, S 7.70.

4.1.2.19. 2-(3-Chlorophenyl)-5-methylthiazol-4-yl [1,4′-bipiperidine]-1′-carboxylate (27).

Compound **27** was prepared according to procedure G from 2-(3-chlorophenyl)-5-methylthiazol-4-ol and [1,4′-bipiperidine]-1′-carbonyl chloride to afford 85 mg of yellow solid (89%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.81–7.78 (m, 1H, ph-2H), 7.64–7.58 (m, 1H, ph-5H), 7.35–7.19 (m, 2H, ph-3,4H), 4.27 (dd, J = 29.0, 13.3 Hz, 2H, pip^1-2,6H_{eq}), 2.86 (m, 2H, pip^1-2,6H_{ax}), 2.54–2.34 (m, 5H, pip^1-4H, pip^2-2,6$H_{ax/eq}$), 2.23 (s, 3H, CH_3), 1.72–1.94 (m, 2H, pip^1-3,5H_{eq}), 1.67–1.46 (m, 6H, pip^1-3,5H_{ax}, pip^2-3,5$H_{ax/eq}$), 1.44–1.33 (m, 2H, pip^2-4H_2). ^{13}C NMR (75 MHz, CDCl$_3$) δ 10.05, 24.54, 26.08, 27.47, 28.03, 44.09, 44.38, 50.19, 62.36, 118.83, 123.79, 125.64, 129.70, 130.04, 134.90, 134.95, 151.99, 152.21, 159.37. HRMS m/z 420.1504 [M+H$^+$] (calcd for C$_{21}$H$_{27}$ClN$_3$O$_2$S, 420.1513). Anal. Calcd for C$_{21}$H$_{26}$ClN$_3$O$_2$S: C 60.06, H 6.24, N 10.01, S 7.63. Found: C 60.10, H 6.29, N 10.10, S 7.58.

4.1.2.20. 5-Methyl-4-(2-(piperidin-1-yl)ethoxy)-2-(pyridin-4-yl)thiazole (30).

Compound **30** was prepared according to procedure G from 5-methyl-2-(pyridin-4-yl)thiazol-4-ol and 1-(3-chloroethyl)piperidine to afford 78 mg of yellow solid (82%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.55 (dd, J = 4.6, 1.7 Hz, 2H, pyr-3,5H), 7.61 (dd, J = 4.6, 1.7 Hz, 2H, pyr-2,6H), 4.46 (t, J = 6.0 Hz, 2H, OCH_2), 2.75 (t, J = 6.0 Hz, 2H, OCH$_2$CH_2N), 2.58–2.44 (m, 4H, pip-2,6$H_{2ax/eq}$), 2.25 (s, 3H, CH_3), 1.69–1.52 (m, 4H, pip-3,5$H_{2ax/eq}$), 1.46–1.32 (m, 2H, pip-4$H_{2ax/eq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.51, 24.01, 25.68, 54.87, 58.04, 68.07, 109.80, 119.04, 140.55, 150.42, 155.92, 160.37. HRMS m/z

304.1482 [M+H$^+$] (calcd for C$_{16}$H$_{22}$N$_3$OS, 304.1484). Anal. Calcd for C$_{16}$H$_{21}$N$_3$OS: C 63.33, H 6.98, N 13.85, S 10.57. Found: C 63.67, H 7.22, N 13.92, S 10.67.

4.1.2.21. 5-(5-Methyl-4-(3-(piperidin-1-yl)propoxy)thiazol-2-yl) isobenzofuran-1(3H)-one (37).

Compound **37** was prepared according to procedure G from 5-(4-hydroxy-5-methylthiazol-2-yl)isobenzofuran-1(3H)-one and 1-(3-chloropropyl)piperidine to afford 72 mg of yellow solid (84%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.92–7.85 (m, 2H, isobenzofuran-4,7H), 7.82 (dd, J = 8.1, 0.8 Hz, 1H, isobenzofuran-6H), 5.27 (s, 2H, isobenzofuran-3H_2), 4.33 (t, J = 6.3 Hz, 2H, OCH_2), 2.68–2.39 (m, 6H, OCH$_2$CH$_2$CH_2N, pip-2,6$H_{2ax/eq}$), 2.24 (s, 3H, CH_3), 2.09–1.89 (m, 2H, OCH$_2$CH_2), 1.70–1.52 (m, 4H, pip-3,5$H_{2ax/eq}$), 1.50–1.33 (m, 2H, pip-4$H_{2ax/eq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.45, 23.98, 25.33, 26.59, 54.41, 55.84, 68.92, 69.55, 109.43, 118.37, 125.74, 126.15, 126.19, 139.17, 147.32, 156.86, 160.40, 170.53. HRMS m/z 373.1593 [M+H$^+$] (calcd for C$_{20}$H$_{25}$N$_2$O$_3$S, 373.1586). Anal. Calcd for C$_{20}$H$_{24}$N$_2$O$_3$S: C 64.49, H 6.49, N 7.52, O 12.89, S 8.61. Found: C 64.57, H 6.35, N 7.62, S 8.67.

4.1.2.22. 5-Methyl-4-((4-(piperidin-1-ylmethyl)benzyl)oxy)-2-(pyrazin-2-yl)thiazole (41).

Compound **41** was prepared according to procedure J from 4-((4-(bromomethyl)benzyl)oxy)-5-methyl-2-(pyrazin-2-yl) thiazole and piperidine to afford 91 mg of yellow solid (89%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.22 (d, J = 1.5 Hz, 1H, pyra-6H), 8.60–8.12 (m, 2H, pyra-3,4H), 7.34 (d, J = 8.1 Hz, 2H, ph-2,6H), 7.25 (d, J = 8.1 Hz, 2H, ph-3,5H), 5.33 (s, 2H, phCH_2O), 3.42 (s, 2H, phCH_2N), 2.36–2.27 (m, 4H, pip-2,6$H_{2ax/eq}$), 2.23 (s, 3H, CH_3), 1.56–1.42 (m, 4H, pip-3,5$H_{2ax/eq}$), 1.40–1.26 (m, 2H, pip-4$H_{2ax/eq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.63, 24.23, 25.76, 54.37, 63.39, 72.04, 112.07, 127.90, 129.40, 136.17, 137.86, 140.81, 143.68, 144.16, 146.96, 156.57, 160.42. HRMS m/z 381.1758 [M+H$^+$] (calcd for C$_{21}$H$_{25}$N$_4$OS, 381.1749). Anal. Calcd for C$_{21}$H$_{24}$N$_4$OS: C 66.29, H 6.36, N 14.72, S 8.43. Found: C 66.37, H 6.35, N 14.62, S 8.67.

4.1.2.23. 5-Methyl-2-(pyrazin-2-yl)-4-((4-(pyrrolidin-1-ylmethyl)benzyl) oxy)thiazole (42).

Compound **42** was prepared according to procedure J from 4-((4-(bromomethyl)benzyl)oxy)-5-methyl-2-(pyrazin-2-yl) thiazole and pyrrolidine to afford 87 mg of yellow solid (84%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.21 (d, J = 1.5 Hz, 1H, pyra-4H), 8.42 (d, J = 2.6 Hz, 1H, pyra-6H), 8.38 (dd, J = 2.6, 1.5 Hz, 1H, pyra-3H), 7.37 (d, J = 8.4 Hz, 2H, ph-2,6H), 7.32 (d, J = 8.4 Hz, 2H, ph-3,5H), 5.33 (s, 2H, phCH_2O), 3.66 (s, 2H, phCH_2N), 2.68–2.47 (m, 4H, pyrr-2,5H), 2.24 (s, 3H, CH_3), 1.85–1.58 (m, 4H, pyrr-3,4H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.62, 23.36, 53.88, 59.88, 71.87, 112.08, 128.10, 129.33, 136.77, 137.02, 140.78, 143.68, 144.18, 146.92, 156.60, 160.32. HRMS m/z 367.1587 [M+H$^+$] (calcd for C$_{20}$H$_{23}$N$_4$OS, 367.1593). Anal. Calcd for C$_{20}$H$_{22}$N$_4$OS: C 65.55, H 6.05, N 15.29, S, 8.75. Found: C 65.67, H 6.11, N 15.32, S 8.67.

4.2. Pharmacology

4.2.1. [³H]-N$^{\alpha}$-Methylhistamine hH₃ receptor displacement assay

The procedure was performed as described previously[56] with slight modification: In brief, membrane preparations (20 μg/well) of HEK-293 cells stably expressing the human H₃ receptor (hH3R) were incubated in a mixture of [³H]-N$^{\alpha}$-methylhistamine (2 nM; K$_D$ = 3.08 nM as determined by saturation binding experiments) and appropriate concentrations of the present competitors (seven to eleven concentrations between 0.01 nM and 10 μM) in 96-well microtiter plates with a final assay volume of 200 μl per well. Preparation of competitor-concentrations was carried out by serial dilution of 10 mM and 3 mM stocks using a Freedom EVO pipetting instrument (TECAN®, Männedorf, Switzerland). For sample filtration ice-cold demineralized water was used. Specific binding was analyzed by non-linear squares fit via GraphPad-Prism™ (2012, vers. 6.01, La Jolla, CA, USA). Affinities (K$_i$) were

calculated from IC_{50}-values using the Cheng-Prusoff equation and expressed as means from at least two independent experiments in triplicates within 95% confidence intervals.[57]

4.3. Molecular descriptor analysis

Molecular properties of most active ligands (those with $K_i < 100$ nM) were calculated using "Calculate Molecular Properties" module of Discovery Studio 2.5.5 client package (Accelrys, San Diego, USA). These descriptors include MW, number HBD and HBA, an octanol/water partition coefficient (log P), number of rotatable bonds, and molecular PSA.

4.4. ADME and toxicity studies

In silico ADME profiling for the most active ligands were measured using "ADMET Descriptors" module of Accelrys Discovery studio 2.5.5. The calculated ADME descriptors include BBB, intestinal absorption, solubility, hepatotoxicity, inhibition of CYP2D6, and plasma protein binding.

Toxicity profiling of the active ligands were conducted using TOPKAT toxicity estimation of Discovery Studio 2.5.5. TOPKAT computes a probable value of toxicity for a submitted chemical structure from a quantitative structure–toxicity relationship (QSTR) equation. The product of a structure descriptors and its corresponding coefficient is the descriptors contribution to the probable toxicity. Toxicity profile involves screening for aerobic biodegradability, AMES (activity in the salmonella/mammalian microsome mutagenicity) mutagenicity, ocular and skin irritancy, skin sensitizer, and carcinogenicity.

Acknowledgements

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2018.06.028.

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Supplementary Material

Design, Synthesis, and Biological Evaluation of Novel Oxadiazole- and Thiazole-Based Histamine H3R Ligands

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Table of Contents

S.1) Analytical Data

N-(4-Chlorophenyl)hydrazinecarbothioamide (1b). 1b was prepared according to procedure A from 1-chloro-4-isothiocyanatobenzene to afford 4.87 g of yellow solid (96%). ¹H NMR (300 MHz, DMSO-*d₆*) δ 9.23 (s, 1H), 7.70 (d, *J* = 7.9 Hz, 2H), 7.34 (d, *J* = 8.8 Hz, 2H), 4.85 (bs, 2H), 3.34 (s, 1H).

N-(4-Methoxyphenyl)hydrazinecarbothioamide (1c). 1c was prepared according to procedure A from 1-methoxy-4-isothiocyanatobenzene to afford 5.2 g of yellow solid (88%). ¹H NMR (300 MHz, DMSO-*d₆*) δ 9.23 (s, 1H), 7.32 (d, *J* = 9.0, 2H), 6.79 (d, *J* = 9.0, 2H), 4.85 (bs, 2H), 3.70 (s, 3H), 3.34 (s, 1H).

N-(4-(Trifluoromethoxy)phenyl)hydrazinecarbothioamide (1d). 1d was prepared according to procedure A from 1-isothiocyanato-4-(trifluoromethoxy)benzene to afford 6.78 g of yellow solid (90%). ¹H NMR (300 MHz, DMSO-*d₆*) δ 9.25 (s, 1H), 7.72 (d, *J* = 7.9 Hz, 2H), 7.33 (d, *J* = 8.8 Hz, 2H), 4.89 (bs, 2H), 3.34 (s, 1H).

N-(3,5-Dimethoxyphenyl)hydrazinecarbothioamide (1e). 1e was prepared according to procedure A from 1-isothiocyanato-3,5-dimethoxybenzene to afford 6.13 g of yellow solid (91%). δ 9.25 (s, 1H), 7.15 (d, *J* = 2.6 Hz, 1H), 6.88 (dd, *J* = 8.6, 2.6 Hz, 1H), 6.74 (d, *J* = 8.6 Hz, 1H), 4.89 (bs, 2H), 3.81 (s, 3H), 3.77 (s, 3H), 3.53 (s, 1H).

5-(3-(Dimethylamino)propyl)-N-phenyl-1,3,4-oxadiazol-2-amine (2a). Compound 2a was prepared according to procedure B from *N*-phenylhydrazinecarbothioamide and 4-(dimethylamino)butanoic acid to afford 50 mg of white powder (61%). ¹H NMR (CDCl₃, 300 MHz) δ 9.17 (bs, 1H, N*H*), 7.42 (dd., *J* = 8.7, 1.2 Hz, 2H, ph-2,6*H*), 7.26 (d, *J* = 8.7 Hz, 2H, ph-3,5*H*), 6.99 – 6.88 (m, 1H, ph-4*H*), 2.74 (t, *J* = 7.5 Hz, 2H, 1C*H₂*), 2.36 (t, *J* = 8.4 Hz, 2H, 3C*H₂*), 2.21 (s, 6H, 2 × C*H₃*), 1.88 (m, 2H, 2C*H₂*). ¹³C NMR (75 MHz, CDCl₃)

2

1

δ 23.14, 24.19, 45.20, 58.34, 117.57, 122.58, 129.22, 138.33, 160.40, 160.53. HRMS m/z 247.1556 [M+H⁺] (calcd 247.1559). Anal. Calcd for C$_{13}$H$_{18}$N$_4$O: C 63.39, H 7.37, N 22.75. Found: C 63.77, H 7.54, N 23.04.

5-(1-Methylpiperidin-4-yl)-N-phenyl-1,3,4-oxadiazol-2-amine (2b). Compound **2b** was prepared according to procedure B from *N*-phenylhydrazinecarbothioamide and 1-methylpiperidine-4-carboxylic acid to afford 69 mg of white powder (81%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.46 (bs, 1H, N*H*), 7.38 (d, J = 7.8 Hz, 2H, ph-2,6*H*), 7.26 (dd, , J = 7.8, 7.2 Hz, 2H, ph-3,5*H*), 6.97 (dd, J = 7.2, 1.2 Hz, 1H, ph-4*H*), 3.03 – 2.75 (m, 3H, pip-2,6*H*$_{eq}$, pip-4*H*), 2.34 (s, 3H, C*H$_3$*), 2.26 – 1.82 (m, 6H, pip-2,6*H*$_{ax}$, pip-3,5*H*$_{2axeq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 14.26, 29.19, 32.67, 44.70, 46.28, 54.71, 117.50, 122.75, 129.29, 138.09, 160.27, 162.81. ESI-MS m/z 259.2 [M+H⁺] (calcd 259.2). Anal. Calcd for C$_{14}$H$_{18}$N$_4$O: C 65.09, H 7.02, N 21.69. Found: C 64.49, H 7.17, N 21.01.

N-Phenyl-5-(2-(piperidin-1-yl)ethyl)-1,3,4-oxadiazol-2-amine (2c). Compound **2c** was prepared according to procedure B from *N*-phenylhydrazinecarbothioamide and 3-(piperidin-1-yl)propanoic acid to afford 55 mg of white powder (61%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.45 (bs, 1H, N*H*), 7.41 (d, J = 7.8 Hz, 2H, ph-2,6*H*), 7.26 (dd, J = 7.8, 7.2 Hz, 2H, ph-3,5*H*), 6.98 (dd, J = 7.2, 1.2 Hz, 1H, ph-4*H*), 3.00 (t, J = 6.8 Hz, 2H, 1C*H$_2$*), 2.94 – 2.74 (m, 2H, 2C*H$_2$*), 2.66 – 2.39 (m, 4H, pip-2,6*H*$_{2axeq}$), 1.59 (dt, J = 10.0, 5.4 Hz, 4H, pip-3,5*H*$_{2axeq}$), 1.41 (t, J = 5.8 Hz, 2H, pip-4*H*$_{2axeq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 23.05, 23.82, 25.34, 54.14, 55.08, 117.55, 122.79, 129.30, 138.03, 159.14, 160.43. HRMS m/z 273.1719 [M+H⁺] (calcd 273.1715). Anal. Calcd for C$_{15}$H$_{20}$N$_4$O: C 66.15, H 7.40, N 20.57. Found: C 65.69, H 7.37, N 19.96.

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N-Phenyl-5-(3-(piperidin-1-yl)propyl)-1,3,4-oxadiazol-2-amine (2d). Compound **2d** was prepared according to procedure B from *N*-phenylhydrazinecarbothioamide and 4-(piperidin-1-yl)butanoic acid to afford 50 mg of white powder (53%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.28 (bs, 1H, N*H*), 7.39 (dd, J = 8.7, 1.2 Hz, 2H, ph-2,6*H*), 7.24 (dd, J = 8.7, 7.2 Hz, 2H, ph-3,5*H*), 6.94 (dd, J = 7.2, 1.2 Hz, 1H, ph-4*H*), 2.72 (t, J = 7.4 Hz, 2H, 1C*H$_2$*), 2.45 – 2.26 (m, 6H, 3C*H$_2$*, pip-2,6*H*$_{2axeq}$), 1.99 – 1.82 (m, 2H, 2C*H$_2$*), 1.51 (dt, J = 11.0, 5.2 Hz, 4H, pip-3,5*H*$_{2axeq}$), 1.40 – 1.28 (m, 2H, pip-4*H*$_{2axeq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 23.14, 24.19, 44.69, 45.20, 58.34, 117.57, 122.58, 129.22, 138.33, 160.40, 160.53. HRMS m/z 287.1869 [M+H⁺] (calcd 287.1872). Anal. Calcd for C$_{16}$H$_{22}$N$_4$O: C 67.11, H 7.74, N 19.56. Found: C 67.43, H 7.81, N 18.89.

N-(4-Chlorophenyl)-5-(3-(dimethylamino)propyl)-1,3,4-oxadiazol-2-amine (2e). Compound **2e** was prepared according to procedure B from *N*-(4-chlorophenyl)hydrazinecarbothioamide and 4-(dimethylamino)butanoic acid to afford 45 mg of white powder (49%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.38 (d, J = 9.0 Hz, 2H, ph-2,6*H*), 7.19 (d, J = 9.0 Hz, 2H, ph-3,5*H*), 2.74 (t, J = 7.5 Hz, 2H, 1C*H$_2$*), 2.39 – 2.33 (m, 2H, 3C*H$_2$*), 2.20 (s, 6H, 2 x C*H$_3$*), 1.99 – 1.79 (m, 2H, 2C*H$_2$*). ^{13}C NMR (75 MHz, CDCl$_3$) δ 23.10, 44.75, 45.21, 58.30, 118.80, 127.47, 129.14, 136.95, 160.18, 160.57. ESI-MS m/z 281.1 [M+H⁺] (calcd 281.1). Anal. Calcd for C$_{13}$H$_{17}$ClN$_4$O: C 55.62, H 6.10, N 12.63. Found: C 55.81, H 6.28, N 12.61.

N-(4-Chlorophenyl)-5-(1-methylpiperidin-4-yl)-1,3,4-oxadiazol-2-amine (2f). Compound **2f** was prepared according to procedure B from *N*-(4-chlorophenyl)hydrazinecarbothioamide and 1-methylpiperidine-4-carboxylic acid to

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N-(4-Chlorophenyl)-5-(2-(piperidin-1-yl)ethyl)-1,3,4-oxadiazol-2-amine **(2g)**. Compound **2g** was prepared according to procedure B from *N*-(4-chlorophenyl)hydrazinecarbothioamide and 3-(piperidin-1-yl)propanoic acid to afford 85 mg of white powder (84%). ¹H NMR (CDCl₃, 300 MHz) δ 7.35 (d, J = 9.0 Hz, 2H, ph-2,6*H*), 7.21 (d, J = 9.0 Hz, 2H, ph-3,5*H*), 2.94 (t, J = 7.5 Hz, 2H, 1C*H₂*), 2.77 (t, J = 7.5 Hz, 2H, 2C*H₂*), 2.58 – 2.36 (m, 4H, pip-2,6*H*₂ₐₓₑq), 1.58 (dt, J = 10.8, 5.1 Hz, 4H, pip-3,5*H*₂ₐₓₑq), 1.42 (t, J = 5.8 Hz, 2H, pip-4*H*₂ₐₓₑq). ¹³C NMR (75 MHz, CDCl₃) δ 22.63, 23.64, 25.12, 54.01, 54.90, 118.61, 127.29, 129.03, 136.88, 158.93, 160.23. ESI-MS *m/z* 307.1 [M+H⁺] (calcd for C₁₅H₁₉ClN₄O [M+H⁺], 307.1).

5-((Dimethylamino)methyl)-*N*-(4-methoxyphenyl)-1,3,4-oxadiazol-2-amine **(2h)**. Compound **2h** was prepared according to procedure B from *N*-(4-methoxyphenyl)hydrazinecarbothioamide and dimethylglycine to afford 49 mg of white powder (60%). ¹H NMR (CDCl₃, 300 MHz) δ 8.69 (bs, 1H, N*H*), 7.32 (d, J = 9.0, 2H, ph-2,6*H*), 6.79 (d, J = 9.0, 2H, ph-3,5*H*), 3.70 (s, 3H, OC*H₃*), 3.62 (s, 2H, C*H₂*), 2.30 (s, 6H, 2 x C*H₃*). ¹³C NMR (75 MHz, CDCl₃) δ 44.91, 52.82, 55.53, 114.52, 119.85, 131.15, 155.68, 157.03, 161.44. ESI-MS *m/z* 249.1 [M+H⁺] (calcd 249.1). Anal. Calcd for C₁₂H₁₆N₄O₂: C 58.05, H 6.50, N 22.57. Found: C 58.41, H 6.84, N 22.77.

afford 77 mg of white powder (80%). ¹H NMR (MeOD, 300 MHz) δ 7.35 (d, J = 9.0 Hz, 2H, ph-2,6*H*), 7.21 (d, J = 9.0 Hz, 2H, ph-3,5*H*), 3.03 – 2.75 (m, 2H, pip-2,6*H*ₑq pip-4*H*), 2.34 (s, 3H, C*H₃*), 2.26 – 1.82 (m, 6H, pip-2,6*H*ₐₓ, pip-3,5*H*₂ₐₓₑq). ¹³C NMR (MeOD) δ 28.01, 31.60, 44.00, 54.59, 120.13, 128.61, 130.12, 138.53, 161.73, 162.53. ESI-MS *m/z* 293.1 [M+H⁺] (calcd for C₁₄H₁₈ClN₄O [M+H⁺], 293.1).

N-(4-Methoxyphenyl)-5-(1-methylpiperidin-4-yl)-1,3,4-oxadiazol-2-amine **(2i)**. Compound **2i** was prepared according to procedure B from *N*-(4-methoxyphenyl)hydrazinecarbothioamide and 1-methylpiperidine-4-carboxylic acid to afford 52 mg of white powder (54%). ¹H NMR (CDCl₃, 300 MHz) δ 8.77 (bs, 1H, N*H*), 7.37 (d, J = 9.0 Hz, 2H, ph-2,6*H*), 6.87 (d, J = 9 Hz, 2H, ph-3,5*H*), 3.03 – 2.75 (m, 3H, pip-2,6*H*ₑq, pip-4*H*), 2.34 (s, 3H, C*H₃*), 2.26 – 1.82 (m, 6H, pip-2,6*H*ₐₓ, pip-3,5*H*₂ₐₓₑq). ¹³C NMR (75 MHz, CDCl₃) δ 29.01, 32.51, 46.15, 54.59, 55.54, 114.53, 119.58, 131.45, 155.50, 160.87, 162.40. ESI-MS *m/z* 289.2 [M+H⁺] (calcd 289.2). Anal. Calcd for C₁₅H₂₀N₄O₂: C 62.48, H 6.99, N 19.43. Found: C 62.48, H 6.78, N 18.81.

N-(4-Methoxyphenyl)-5-(2-(piperidin-1-yl)ethyl)-1,3,4-oxadiazol-2-amine **(2j)**. Compound **2j** was prepared according to procedure B from *N*-(4-methoxyphenyl)hydrazinecarbothioamide and 3-(piperidin-1-yl)propanoic acid to afford 72 mg of white powder (72%). ¹H NMR (CDCl₃, 300 MHz) δ 8.43 (bs, 1H, N*H*), 7.32, (d, J = 9.0 Hz, 2H, ph-2,6*H*), 6.79 (d, J = 9.0 Hz, 2H, ph-3,5*H*), 3.72 (s, 3H, C*H₃*), 2.96-3.01 (m, 2H, 1C*H₃*), 2.80-2.85 (m, 2H, 2C*H₃*), 2.50-2.54 (m, 4H, pip-2,6*H*₂ₐₓₑq), 1.55-1.63 (m, 4H, pip-3,5*H*₂ₐₓₑq), 1.36-1.44 (m, 2H, pip-4*H*₂ₐₓₑq). ¹³C NMR (75 MHz, CDCl₃) δ 22.91, 23.72, 25.19, 54.09, 54.98, 55.55, 114.49, 119.68, 131.34, 155.55, 158.72, 160.92. HRMS *m/z* 303.1827 [M+H⁺] (calcd 303.1816). Anal. Calcd for C₁₆H₂₂N₄O₂: C 63.55, H 7.33, N 18.53. Found: C 63.49, H 7.17, N 18.60.

5-(2-(Piperidin-1-yl)ethyl)-*N*-(4-(trifluoromethoxy)phenyl)-1,3,4-oxadiazol-2-amine **(2k)**. Compound **2k** was prepared according to procedure B from *N*-(4-(trifluoromethoxy)phenyl)hydrazinecarbothioamide and 3-(piperidin-1-yl)propanoic to

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afford 75 mg of white powder (64%). ¹H NMR (CDCl₃, 300 MHz) δ 7.43 (d, J = 8.25, 2H, ph-2,6H), 7.10 (d, J = 8.25, 2H, ph-3,5H), 2.91 (t, J = 8.6 Hz, 2H, 1CH_2), 2.73 (t, J = 8.6 Hz, 2CH_2), 2.42 (t, J = 5.4 Hz, 4H, pip-2,6H_2), 1.51 (m, 4H, pip-3,5H_2), 1.37 (m, 2H, pip-4H_2). ¹³C NMR (75 MHz, CDCl₃) δ 22.70, 23.64, 25.10, 48.47, 48.76, 49.04, 49.33, 49.61, 49.90, 50.18, 54.06, 54.90, 118.37, 122.02, 137.01, 143.98, 158.97, 160.25. ESI-MS m/z 357.2 [M+H⁺] (calcd 357.2). Anal. Calcd for $C_{18}H_{19}F_3N_4O_2$: C 53.93, H 5.37, N 15.72. Found: C 53.72, H 5.31, N 15.57.

N-(3,5-Dimethoxyphenyl)-5-((dimethylamino)methyl)-1,3,4-oxadiazol-2-amine (2). Compound **2l** was prepared according to procedure B from N-(3,5-dimethoxyphenyl)hydrazinecarbothioamide and dimethylglycine to afford 51 mg of white powder (55%). ¹H NMR (CDCl₃, 300 MHz) δ 7.15 (d, J = 2.6 Hz, 1H, ph-4H), 6.88 (dd, J = 8.6, 2.6 Hz, 1H, ph-2H), 6.74 (d, J = 8.6 Hz, 1H, ph-6H), 3.81 (s, 3H, CH_3), 3.77 (s, 3H, CH_3), 3.63 (s, 2H, CH_2), 2.31 (s, 6H, 2 x CH_3). ¹³C NMR (75 MHz, CDCl₃) δ 44.87, 52.78, 56.20, 103.29, 109.87, 111.83, 131.57, 145.06, 149.44, 157.01, 161.18. ESI-MS m/z 279.1 [M+H⁺] (calcd 279.1). Anal. Calcd for $C_{13}H_{18}N_4O_3$: C 56.10, H 6.52, N 20.13. Found: C 56.28, H 6.55, N 20.49.

N-(3,5-Dimethoxyphenyl)-5-(2-(piperidin-1-yl)ethyl)-1,3,4-oxadiazol-2-amine (2m). Compound **2m** was prepared according to procedure B from N-(3,5-dimethoxyphenyl)hydrazinecarbothioamide and 3-(piperidin-1-yl)propanoic to afford 57 mg of white powder (52%).¹H NMR (CDCl₃, 300 MHz) δ 8.74 (s, 1H, NH), 7.10 (d, J = 2.5 Hz, 1H, ph-4H), 6.86 (d, J = 2.5 Hz, 1H, ph-2H), 6.72 (d, J = 2.5 Hz, 1H, ph-6H), 3.80 (s, 3H, CH_3), 3.77 (s, 3H, CH_3), 2.91 (t, J = 8.6 Hz, 2H, 1CH_2), 2.73 (t, J = 8.6 Hz, 2H,

2CH_2), 2.42 (t, J = 5.4 Hz, 4H, pip-2,6H_2), 1.51 (m, 4H, pip-3,5H_2), 1.37 (m, 2H, pip-4H_2). ¹³C NMR (75 MHz, CDCl₃) δ 23.24, 24.02, 25.60, 54.18, 55.32, 55.92, 56.20, 103.26, 109.71, 111.83, 131.88, 144.87, 149.41, 159.08, 160.79. HRMS m/z 333.1918 [M+H⁺] (calcd 333.1927). Anal. Calcd for $C_{17}H_{24}N_4O_3$: C 61.43, H 7.28, N 16.86. Found: C 61.80, H 7.37, N 16.17.

(E)-N'-Hydroxybenzimidamide (3a). 3a was prepared according to procedure C from benzonitrile to afford 7.93 of white solid (77%). ¹H NMR (300 MHz, DMSO-d₆) δ 9.66 (s, 1H), 7.77 – 7.62 (m, 2H), 7.46 – 7.30 (m, 3H), 5.81 (s, 2H).

(E)-N'-Hydroxy-4-methoxybenzimidamide (3b). 3b was prepared according to procedure C from 4-methoxybenzonitrile to afford 8.91 of white solid (67%). ¹H NMR (300 MHz, DMSO-d₆) δ 9.46 (s, 1H), 7.61 (d, J = 8.9 Hz, 2H), 6.92 (d, J = 8.9 Hz, 2H), 5.72 (s, 2H), 3.77 (s, 3H).

Ethyl 3-phenyl-1,2,4-oxadiazole-5-carboxylate (4a). 4a was prepared according to procedure D from MAK-A to afford 1.07 of white solid (79%). ¹H NMR (300 MHz, Chloroform-d) δ 8.08 (dd, J = 8.0, 1.7 Hz, 2H), 7.54 – 7.36 (m, 3H), 4.50 (q, J = 7.1 Hz, 2H), 1.41 (t, J = 7.1 Hz, 3H).

Ethyl 3-(4-methoxyphenyl)-1,2,4-oxadiazole-5-carboxylate (4b). 4b was prepared according to procedure D from MAK-C to afford 1.36 of white solid (82%). ¹H NMR (300 MHz, Chloroform-d) δ 8.01 (d, J = 9.0 Hz, 2H), 6.92 (d, J = 9.0 Hz, 2H), 4.49 (q, J = 7.1 Hz, 2H), 3.79 (s, 3H), 1.41 (t, J = 7.1 Hz, 3H).

N-(3-(Diethylamino)propyl)-3-phenyl-1,2,4-oxadiazole-5-carboxamide (5a). Compound 5a was prepared according to procedure E from ethyl 3-phenyl-1,2,4-

oxadiazole-5-carboxylate and N^1,N^1-diethylpropane-1,3-diamine to afford 124 mg of yellow oil (82%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 10.08 (s, 1H, NH), 8.49 – 7.64 (m, 3H, ph-2,4,6H), 7.52 – 6.89 (m, 2H, ph-3,5H), 3.90 – 3.06 (m, 2H, CONHCH_2), 2.69 – 2.27 (m, 6H, CONHCH$_2$CH$_2$CH_2, N(CH_2CH3)$_2$), 1.67 (m, 2H, CONHCH$_2$CH_2), 1.01 (t, J = 7.1 Hz, 6H, N(CH$_2$C$H3$)$_2$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 11.49, 24.11, 41.22, 46.73, 53.29, 126.04, 127.34, 128.88, 131.53, 152.80, 168.65, 169.29. ESI-MS m/z 303.2 [M+H$^+$] (calcd for C$_{16}$H$_{22}$N$_4$O$_2$: C 63.55, H 7.33, N 18.53. Found: C 64.12, H 7.58, N 18.57.

3-Phenyl-N-(3-(piperidin-1-yl)propyl)-1,2,4-oxadiazole-5-carboxamide (5b). Compound 5b was prepared according to procedure E from ethyl 3-phenyl-1,2,4-oxadiazole-5-carboxylate and 3-(piperidin-1-yl)propan-1-amine to afford 115 mg of yellow oil (73%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.99 (s, 1H, NH), 8.00 – 7.99 (m, 2H, ph-2,6H), 7.64 – 7.28 (m, 3H, ph-3,4,5H), 3.52 (t, J = 3.9 Hz, 2H, CONHCH_2), 2.47 (t, J = 2.7 Hz, 2H, CONHCH$_2$CH$_2$CH_2), 2.44 – 2.32 (m, 4H, pip-2,6$H_{2(eq)}$), 1.76 – 1.59 (m, 6H, pip-3,5$H_{2(eq)}$, pip-$H_{2(eq)}$). 1.43 (m, 2H, CONHCH$_2$CH_2CH$_2$). ^{13}C NMR (151 MHz, CDCl$_3$) δ 23.42, 24.40, 25.67, 41.45, 54.80, 59.22, 126.12, 127.41, 128.88, 131.56, 152.95, 168.73, 169.26. HRMS m/z 315.1833 [M+H$^+$] (calcd for C$_{17}$H$_{22}$N$_4$O$_2$, 315.1821).

N-(3-(Diethylamino)propyl)-3-(4-methoxyphenyl)-1,2,4-oxadiazole-5-carboxamide (5c). Compound 5c was prepared according to procedure E from ethyl 3-(4-methoxyphenyl)-1,2,4-oxadiazole-5-carboxylate and N^1,N^1-diethylpropane-1,3-diamine to afford 145 mg of yellow oil (87%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.95 (s, 1H, NH), 7.91 (d, J = 8.8 Hz, 2H, ph-2,6H), 6.88 (d, J = 8.8 Hz, 2H, ph-3,5H), 3.75 (s, 3H, CH_3), 3.63 –

3.38 (m, 2H, CONHCH_2), 2.93 – 2.32 (m, 6H, CONHCH$_2$CH$_2$CH_2, N(CH_2CH$_3$)$_2$), 1.71 (m, 2H, CONHCH$_2$CH_2CH$_2$), 1.04 (t, J = 7.1 Hz, 6H, N(CH$_2$CH_3)$_2$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 11.29, 24.10, 40.92, 46.70, 53.03, 55.34, 114.28, 118.38, 129.00, 152.97, 162.16, 168.38, 168.94. ESI-MS m/z 333.2 [M+H$^+$] (calcd for C$_{17}$H$_{24}$N$_4$O$_3$, 333.2).

5-Methyl-2-phenylthiazol-4-ol (6a) 6a was prepared according to procedure F to afford 1.55 g of yellow solid (75%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.31 (s, 1H), 7.79 (dd, J = 8.1, 1.6 Hz, 2H), 7.50 – 7.39 (m, 3H), 2.22 (s, 3H).

2-(4-Chlorophenyl)-5-methylthiazol-4-ol (6b). 6b was prepared according to procedure F to afford 2.27 g of yellow solid (83%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.36 (s, 1H), 7.77 (d, J = 8.7 Hz, 2H), 7.48 (d, J = 8.8 Hz, 2H), 2.21 (s, 3H). ^{13}C NMR (75 MHz, DMSO) δ 9.10, 103.27, 126.37, 129.10, 132.15, 133.83, 156.78, 158.91.

4-(4-Hydroxy-5-methylthiazol-2-yl)benzonitrile (6c). 6c was prepared according to procedure F to afford 2.05 g of yellow solid (80%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.55 (s, 1H), 7.97 – 7.87 (m, 4H), 2.25 (s, 3H).

2-(3-Chlorophenyl)-5-methylthiazol-4-ol (6d). 6d was prepared according to procedure F to afford 1.89 g of yellow solid (69%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.40 (s, 1H), 7.78 (dt, J = 2.5, 1.0 Hz, 1H), 7.74 – 7.68 (m, 1H), 7.50 – 7.45 (m, 2H), 2.23 (s, 3H).

5-Methyl-2-(pyridin-4-yl)thiazol-4-ol (6e). 6e was prepared according to procedure F to afford 1.93 g of yellow solid (93%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.61 (s, 1H), 8.81 – 8.50 (dd, J = 1.65, 4.47, 2H), 7.81 – 7.58 (dd, J = 1.65, 4.47, 2H), 2.25 (s, 3H). ^{13}C NMR (75 MHz, DMSO) δ 9.25, 105.86, 118.62, 139.77, 150.49, 155.04, 159.63.

5-Methyl-2-(pyrazin-2-yl)thiazol-4-ol (6f). 6f was prepared according to procedure F to afford 2.02 g of yellow solid (96%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.38 (s, 1H), 9.18 (d, J = 1.5 Hz, 1H), 8.40 (d, J = 2.6 Hz, 1H), 8.36 (dd, J = 2.6, 1.5 Hz, 1H), 2.23 (s, 3H).

N,N-Dimethyl-3-((5-methyl-2-phenylthiazol-4-yl)oxy)propan-1-amine (7). Compound 7 was prepared according to procedure G from 5-methyl-2-phenylthiazol-4-ol and 3-chloro-N,N-dimethylpropan-1-amine to afford 51 mg of yellow solid (73%). ^{1}H NMR (CDCl₃, 300 MHz) δ 7.76 (dd, J = 8.1, 1.8 Hz, 2H, ph-2,6H), 7.35 – 7.24 (m, 3H, ph-3,4,5H), 4.31 (t, J = 6.4 Hz, 2H, OCH_2), 2.44 (t, J = 7.4 Hz, 2H, OCH₂CH₂CH_2), 2.22 (s, 6H, 2 x CH_3), 2.21 (s, 3H, CH_3), 1.90 (dt, J = 8.8, 7.4, 6.4 Hz, 2H, OCH₂CH_2CH₂). ^{13}C NMR (75 MHz, CDCl₃) δ 9.33, 27.72, 45.32, 56.38, 68.81, 107.00, 125.34, 128.76, 129.26, 134.00, 159.47, 159.67. HREIMS m/z 277.1389 [M H]⁺ (calcd 277.1375). Anal. Calcd for C₁₅H₂₀N₂OS: C 65.18, H 7.29, N 10.14, S 11.60. Found: C 65.22, H 7.57, N 10.27, S 12.07.

5-Methyl-2-phenyl-4-(2-(piperidin-1-yl)ethoxy)thiazole (9). Compound 9 was prepared according to procedure G from 5-methyl-2-phenylthiazol-4-ol and 1-(3-chloroethyl)piperidine to afford 59 mg of yellow solid (61%). ^{1}H NMR (CDCl₃, 300 MHz) δ 7.75 (dd, J = 8.1, 18 Hz, 2H, ph-2,6H), 7.33 – 7.22 (m, 3H, ph-3,4,5H), 4.40 (t, J = 6.1 Hz, 2H, OCH_2), 2.69 (t, J = 6.1 Hz, 2H, OCH₂CH_2), 2.45 (t, J = 5.6 Hz, 4H, pip-2,6H'2xeq), 2.19 (s, 3H, CH_3), 1.63 – 1.42 (m, 4H, pip-3,5H2xeq), 1.36 (dt, J = 6.3, 3.3 Hz, 2H, pip-4H2xeq). ^{13}C NMR (126 MHz, CDCl₃) δ 9.73, 24.63, 26.34, 55.33, 58.66, 68.65, 107.48, 125.73, 129.14, 129.62, 134.45, 159.81, 160.06. HRMS m/z 303.1533 [M+H]⁺ (calcd 303.1531). Anal. Calcd for C₁₇H₂₂N₂OS: C 67.51, H 7.33, N 9.26, S 10.60. Found: C 67.89, H 7.48, N 9.18, S 10.63.

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5-Methyl-2-phenyl-4-(3-(pyrrolidin-1-yl)propoxy)thiazole (10). Compound 10 was prepared according to procedure G from 5-methyl-2-phenylthiazol-4-ol and 1-(3-chloropropyl)pyrrolidine to afford 78 mg of yellow solid (85%). ^{1}H NMR (CDCl₃, 300 MHz) δ 7.74 (dd, J = 8.0, 1.6 Hz, 2H, ph-2,6H), 7.31 – 7.19 (m, 3H, ph-3,4,5H), 4.30 (t, J = 6.4Hz, 2H, OCH_2), 2.61 – 2.53 (m, 2H, OCH₂CH₂CH_2), 2.50 – 2.41 (m, 4H, pyrr-2,5H_2), 2.18 (s, 3H, CH_3), 2.01 – 1.83 (m, 2H, OCH₂CH_2), 1.78 – 1.64 (m, 4H, pyrr-3,4H_2). ^{13}C NMR (75 MHz, CDCl₃) δ 9.32, 23.46, 29.14, 53.20, 54.23, 68.96, 106.82, 125.30, 128.73, 129.21, 134.01, 159.37, 159.72. ESI-MS m/z 303.1 [M+H⁺] (calcd 303.1). Anal. Calcd for C₁₇H₂₂N₂OS: C 67.51, H 7.33, N 9.26, S 10.60. Found: C 67.55, H 7.20, N 9.28, S 10.63.

5-Methyl-2-phenyl-4-(3-(piperidin-1-yl)propoxy)thiazole (11). Compound 11 was prepared according to procedure G from 5-methyl-2-phenylthiazol-4-ol and 1-(3-chloropropyl)piperidine to afford 64 mg of yellow solid (68%). ^{1}H NMR (CDCl₃, 300 MHz) δ 7.76 (dd, J = 8.1, 1.8 Hz, 2H, ph-2,6H), 7.34 – 7.24 (m, 3H, ph-3,4,5H), 4.30 (t, J – 6.4 Hz, 2H, OCH_2), 2.48 – 2.41 (m, 2H, OCH₂CH_2), 2.40 – 2.33 (m, 4H, pip-2,6H2xeq), 2.20 (s, 3H, CH_3), 1.99 – 1.83 (m, 2H, OCH₂CH₂CH_2), 1.61 – 1.46 (m, 4H, pip-3,5H2xeq), 1.43 – 1.29 (m, 2H, pip-4H_2). ^{13}C NMR (75 MHz, CDCl₃) δ 9.34, 24.35, 25.81, 27.04, 54.57, 56.05, 69.14, 106.98, 125.33, 128.75, 129.23, 134.02, 159.43, 159.71. HREIMS m/z 317.1672 [M H]⁺ (calcd 317.1688). Anal. Calcd for C₁₈H₂₄N₂OS: C 68.32, H 7.64, N 8.85, S 10.13. Found: C 68.40, H 7.69, N 8.57, S 10.51.

5-Methyl-4-((1-methylpiperidin-4-yl)methoxy)-2-phenylthiazole (12). Compound 12 was prepared according to procedure G from 5-methyl-2-phenylthiazol-4-ol and 4-(chloromethyl)-1-methylpiperidine to afford 67 mg of yellow solid (77%). ^{1}H NMR

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$(CDCl_3$, 300 MHz) δ 7.76 (dd, J = 7.9, 1.7 Hz, 2H, ph-2,6H), 7.37 – 7.26 (m, 3H, ph-3,4,5H), 4.18 (d, J = 5.6 Hz, 2H, OCH_2), 3.39 – 3.14 (m, 2H, pip-2,6H_{eq}), 2.53 (s, 3H, CH_3), 2.48 – 2.40 (m, 2H, pip-2,6H_{ax}), 2.21 (s, 3H, CH_3), 2.03 – 1.66 (m, 5H, pip-3,5$H_{2ax/eq}$, pip-4H). HRMS m/z 303.1530 [M+H]⁺ (calcd 303.1531). Anal. Calcd for $C_{17}H_{22}N_2OS$: C 67.51, H 7.33, N 9.26, S 10.60. Found: C 67.59, H 7.23, N 9.37, S 10.77.

5-Methyl-2-phenylthiazol-4-yl 4-methylpiperazine-1-carboxylate (13). Compound 13 was prepared according to procedure G from 5-methyl-2-phenylthiazol-4-ol and 4-methylpiperazine-1-carbonyl chloride to afford 88 mg of yellow solid (95%). 1H NMR $(CDCl_3$, 300 MHz) δ 7.80 – 7.70 (m, 2H, ph-2,6H), 7.35 – 7.24 (m, 3H, ph-3,4,5H), 3.64 (dd, J = 6.6, 3.9 Hz, 2H, piperazine-2,6H_{eq}), 3.51 (dd, J = 6.6, 3.9 Hz, 2H, piperazine-2,6H_{ax}), 2.44 – 2.32 (m, 4H, piperazine-3,5H_{2xeq}), 2.24 (s, 3H, CH_3), 2.21 (s, 3H, CH_3). 13C NMR (75 MHz, $CDCl_3$) δ 9.99, 43.94, 44.48, 46.06, 54.43, 54.62, 117.94, 125.67, 128.76, 129.83, 133.29, 151.60, 152.35, 161.21. ESI-MS m/z 318.1 [M+H]⁺ (calcd for $C_{16}H_{19}N_3O_2S$, 318.1).

3-((2-(4-Chlorophenyl)-5-methylthiazol-4-yl)oxy)-N,N-dimethylpropan-1-amine (15). Compound 15 was prepared according to procedure G from 2-(4-chlorophenyl)-5-methylthiazol-4-ol and 3-chloro-N,N-dimethylpropan-1-amine to afford 56 mg of yellow solid (79%). 1H NMR $(CDCl_3$, 300 MHz) δ 7.68 (dd, J = 8.5, 1.5 Hz, 2H, ph-2,6H), 7.26 (dd, J = 8.5, 1.6 Hz, 2H, ph-3,5H), 4.29 (td, J = 6.4, 1.1 Hz, 2H, OCH_2), 2.53 – 2.36 (m, 2H, OCH_2CH_2), 2.23 (s, 6H, N(CH_3)₂), 2.20 (s, 3H, CH_3), 2.00 – 1.77 (m, 2H, O$CH_2CH_2CH_2$). 13C NMR (75 MHz, $CDCl_3$) δ 9.31, 27.64, 45.27, 56.33, 68.77, 107.37, 126.48, 128.93, 132.48, 134.99, 158.01, 159.78. HRMS m/z 311.0990 [M+H]⁺ (calcd

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311.0985). Anal. Calcd for $C_{15}H_{19}ClN_2OS$: C 57.96, H 6.16, N 9.01, S 10.31. Found: C 57.19, H 6.18, N 8.65, S 9.50.

2-(4-Chlorophenyl)-5-methyl-4-(3-(pyrrolidin-1-yl)propoxy)thiazole (18). Compound 18 was prepared according to procedure G from 2-(4-chlorophenyl)-5-methylthiazol-4-ol and 1-(3-chloropropyl)pyrrolidine to afford 59 mg of yellow solid (74%). 1H NMR $(CDCl_3$, 300 MHz) δ 7.67 (d, J = 8.6 Hz, 2H, ph-2,6H), 7.25 (d, J = 8.6 Hz, 2H, ph-3,5H), 4.30 (t, J = 6.3 Hz, 2H, OCH_2), 2.69 – 2.62 (m, 2H, O$CH_2CH_2CH_2$), 2.61 – 2.54 (m, 4H, pyr-2,5H_2), 2.19 (s, 3H, CH_3), 2.03 – 1.91 (m, 2H, OCH_2CH_2), 1.84 – 1.71 (m, 4H, pyr-3,4H_2). 13C NMR (75 MHz, $CDCl_3$) δ 9.33, 23.44, 28.73, 53.13, 54.10, 68.82, 107.35, 126.48, 128.93, 132.48, 135.00, 158.00, 159.75. HRMS m/z 337.1137 [M+H]⁺ (calcd 337.1141). Anal. Calcd for $C_{17}H_{21}ClN_2OS$: C 60.61, H 6.28, N 8.32, S 9.52. Found: C 60.86, H 6.34, N 8.27, S 9.62.

2-(4-Chlorophenyl)-5-methyl-4-(3-(piperidin-1-yl)propoxy)thiazole (19). Compound 19 was prepared according to procedure G from 2-(4-chlorophenyl)-5-methylthiazol-4-ol and 1-(3-chloropropyl)piperidine to afford 72 mg of yellow solid (88%). 1H NMR $(CDCl_3$, 300 MHz) δ 7.69 (d, J = 8.7 Hz, 2H, ph-2,6H), 7.27 (d, J = 8.7 Hz, 2H, ph-3,5H), 4.30 (t, J = 6.3 Hz, 2H, OCH_2), 2.53 – 2.47 (m, 2H, pip-2,6H_{eq}), 2.43 (t, J = 5.7 Hz, 6H, O$CH_2CH_2CH_2$), 2.20 (s, 3H, CH_3), 2.03 – 1.88 (m, 2H, pip-2,6H_{ax}), 1.68 – 1.51 (m, 4H, pip-3,5H_{2xeq}), 1.48 – 1.31 (m, 2H, pip-4H_{2xeq}). 13C NMR (75 MHz, $CDCl_3$) δ 9.34, 24.16, 25.54, 26.79, 54.48, 55.95, 69.03, 107.45, 126.51, 128.96, 132.50, 135.04, 158.06, 159.78. ESI-MS m/z 351.1 [M+H]⁺ (calcd 351.1). Anal. Calcd for $C_{18}H_{23}ClN_2OS$: C 61.61, H 6.61, N 7.98, S 9.14. Found: C 61.85, H 6.60, N 7.73, S 9.57.

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2-(3-Chlorophenyl)-5-methyl-4-(2-(piperidin-1-yl)ethoxy)thiazole (24). Compound 24 was prepared according to procedure G from 2-(3-chlorophenyl)-5-methylthiazol-4-ol and 1-(2-chloroethyl)piperidine to afford 73 mg of yellow solid (87%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.74 (m, 1H, ph-2*H*), 7.55 (ddd, *J* = 4.9, 3.4, 1.7 Hz, 1H, ph-6*H*), 7.22 – 7.15 (m, 2H, ph-4,5*H*), 4.38 (t, *J* = 6.1 Hz, 2H, OC*H*$_2$), 2.67 (t, *J* = 6.1 Hz, 2H, OCH$_2$C*H*$_2$), 2.44 (m, 4H, pip-2,6*H*$_{2axeq}$), 2.17 (s, 3H, C*H*$_3$), 1.60 – 1.45 (m, 4H, pip-3,5*H*$_{2axeq}$), 1.42 1.28 (m, 2H, pip-4*H*$_{2axeq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.36, 24.20, 25.92, 54.92, 58.20, 68.21, 107.81, 123.32, 125.17, 129.00, 129.95, 134.81, 135.59, 157.44, 159.81. ESI-MS *m/z* 337.1 [M+H$^+$] (calcd 337.1). Anal. Calcd for C$_{17}$H$_{21}$ClN$_2$OS: C 60.61, H 6.28, N 8.32, S 9.61. Found: C 60.82, H 6.38, N 8.43, S 9.61.

2-(3-Chlorophenyl)-5-methyl-4-(3-(pyrrolidin-1-yl)propoxy)thiazole (25). Compound 25 was prepared according to procedure G from 2-(3-chlorophenyl)-5-methylthiazol-4-ol and 1-(3-chloropropyl)pyrrolidine to afford 73 mg of yellow solid (87%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.78 – 7.71 (m, 1H, ph-2*H*), 7.55 (ddd, *J* = 5.0, 3.6, 1.7 Hz, 1H, ph-6*H*), 7.22 – 7.16 (m, 2H, ph-4,5*H*), 4.30 (t, *J* = 6.4 Hz, 2H, OC*H*$_2$), 2.65 – 2.40 (m, 6H, OCH$_2$CH$_2$C*H*$_2$, pyrr-2,5*H*$_2$), 2.18 (s, 3H, C*H*$_3$), 2.01 – 1.84 (m, 2H, OCH$_2$C*H*$_2$CH$_2$), 1.77 – 1.64 (m, 4H, pyrr-3,4*H*$_2$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.31, 23.44, 29.00, 53.13, 54.19, 68.91, 107.61, 123.33, 125.14, 128.98, 129.95, 134.79, 135.60, 157.43, 159.88. ESI-MS *m/z* 337.1 [M+H$^+$] (calcd 337.1). Anal. Calcd for C$_{17}$H$_{21}$ClN$_2$OS: C 60.61, H 6.28, N 8.32, S 9.52. Found: C 60.80, H 6.34, N 8.44, S 9.67.

2-(3-Chlorophenyl)-5-methyl-4-(3-(piperidin-1-yl)propoxy)thiazole (26). Compound 26 was prepared according to procedure G from 2-(3-chlorophenyl)-5-methylthiazol-4-ol

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and 1-(3-chloropropyl)piperidine to afford 70 mg of yellow solid (76%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.76 – 7.73 (m, 1H, ph-2*H*), 7.56 (ddd, *J* = 5.0, 3.6, 1.7 Hz, 1H, ph-6*H*), 7.21 – 7.16 (m, 2H, ph-4,5*H*), 4.28 (t, *J* = 6.4 Hz, 2H, OC*H*$_2$), 2.51 – 2.28 (m, 6H, OCH$_2$CH$_2$C*H*$_2$, pip-2,6*H*$_{2axeq}$), 2.18 (s, 3H, C*H*$_3$), 1.99 – 1.77 (m, 2H, OCH$_2$C*H*$_2$), 1.64 – 1.43 (m, 4H, pip-3,5*H*$_{2axeq}$), 1.41 – 1.25 (m, 2H, pip-4*H*$_{2axeq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.31, 24.31, 25.78, 26.98, 54.54, 55.97, 69.07, 107.65, 123.34, 125.15, 128.99, 129.95, 134.80, 135.61, 157.45, 159.90. HRMS *m/z* 351.1288 [M+H$^+$] (calcd 351.1298). Anal. Calcd for C$_{18}$H$_{23}$ClN$_2$OS: C 61.61, H 6.61, N 7.98, S 9.14. Found: C 61.73, H 6.81, N 8.11, S 9.26.

N,N-Dimethyl-3-((5-methyl-2-(pyridin-4-yl)thiazol-4-yl)oxy)propan-1-amine (28). Compound 28 was prepared according to procedure G from 5-methyl-2-(pyridin-4-yl)thiazol-4-ol and 3-chloro-*N,N*-dimethylpropan-1-amine to afford 125 mg of yellow solid (79%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.6 (dd, *J* = 3.7, 1.7 Hz, 2H, pyr-3,5*H*), 7.6 (dd, *J* = 3.7, 1.7 Hz, 2H, pyr-2,6*H*), 4.34 (t, *J* = 6.4 Hz, 2H, OC*H*$_3$), 2.55 – 2.40 (m, 2H, OCH$_2$C*H*$_2$), 2.25 (s, 3H, C*H*$_3$), 2.24 (s, 6H, N(C*H*$_3$)$_2$), 1.93 1.99 – 1.84 (m, 2H, OCH$_2$C*H*$_2$). ^{13}C NMR (126 MHz, CDCl$_3$) δ 9.82, 28.01, 45.66, 56.72, 69.24, 109.96, 119.46, 141.00, 150.83, 156.33, 160.97. HRMS *m/z* 278.1326 [M+H$^+$] (calcd 278.1327). Anal. Calcd for C$_{14}$H$_{19}$N$_3$OS: C 60.62, H 6.90, N 15.15, S 11.56. Found: C 60.85, H 7.24, N 15.27, S 11.84.

5-Methyl-2-(pyridin-4-yl)-4-(2-(pyrrolidin-1-yl)ethoxy)thiazole (29). Compound 29 was prepared according to procedure G from 5-methyl-2-(pyridin-4-yl)thiazol-4-ol and 1-(2-chloroethyl)pyrrolidine to afford 103 mg of yellow solid (86%). ^{1}H NMR (CDCl$_3$, 300

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MHz) δ 8.55 (d, J = 6.3 Hz, 2H, pyr-3,5H), 7.60 (d, J = 6.3 Hz, 1H, pyr-2,6H), 4.49 (t, J = 5.9 Hz, 2H, OCH_2), 2.92 (t, J = 5.9 Hz, 2H, OCH$_2$CH_2), 2.73 – 2.59 (m, 4H, pyrr-2,5H_2), 2.25 (s, 3H, CH_3), 1.85 – 1.68 (m, 4H, pyrr-3,4H_2). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.52, 23.47, 54.65, 55.13, 69.01, 109.76, 119.02, 140.49, 150.41, 155.96, 160.24. HRMS m/z 290.1327 [M+H$^+$] (calcd 290.1327). Anal. Calcd for C$_{15}$H$_{19}$N$_3$OS: C 62.26, H 6.62, N 14.52, S 11.08. Found: C 62.49, H 6.72, N 14.58, S 11.17.

5-Methyl-2-(pyridin-4-yl)-4-(3-(pyrrolidin-1-yl)propoxy)thiazole (31). Compound **31** was prepared according to procedure G from 5-methyl-2-(pyridin-4-yl)thiazol-4-ol and 1-(3-chloropropyl)pyrrolidine to afford 72 mg of yellow solid (85%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.55 (dd, J = 4.5, 1.7 Hz, 2H, pyr-3,5H), 7.60 (dd, J = 4.5, 1.7 Hz, 2H, pyr-2,6H), 4.34 (t, J = 6.3 Hz, 2H, OCH_2), 2.77 – 2.50 (m, 6H, OCH$_2$CH$_2$CH_2, pyrr-2,5H_2), 2.23 (s, 3H, CH_3), 2.11 – 1.92 (m, 2H, OCH$_2$CH_2), 1.86 – 1.70 (m, 4H, pyrr-3,4H_2). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.45, 23.42, 28.68, 53.11, 54.15, 68.81, 109.53, 119.01, 140.52, 150.40, 155.86, 160.45. ESI-MS m/z 304.12 [M+H$^+$] (calcd 304.2). Anal. Calcd for C$_{16}$H$_{21}$N$_3$OS: C 63.33, H 6.98, N 13.85, S 10.57. Found: C 63.41, H 7.08, N 13.89, S 10.59.

5-Methyl-4-(3-(piperidin-1-yl)propoxy)-2-(pyridin-4-yl)thiazole (32). Compound **32** was prepared according to procedure G from 5-methyl-2-(pyridin-4-yl)thiazol-4-ol and 1-(3-chloropropyl)piperidine to afford 68 mg of yellow solid (84%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.6 (dd, J = 3.9, 1.8 Hz, 2H, pyr-3,5H), 7.6 (dd, J = 3.9, 1.8 Hz, 2H, pyr-2,6H), 4.33 (t, J = 6.4 Hz, 2H, OCH_2), 2.53 – 2.45 (m, 2H, OCH$_2$CH$_2$CH_2N), 2.44 – 2.37 (m, 4H, pip-2,6$H_{2ac/eq}$), 2.24 (s, 3H, CH_3), 2.02 – 1.90 (m, 2H, OCH$_2$CH_2), 1.57 (p, J = 5.6 Hz, 4H, pip-3,5$H_{2ac/eq}$), 1.45 – 1.36 (m, 2H, pip-4$H_{2ac/eq}$). ^{13}C NMR (126 MHz, CDCl$_3$) δ 9.76,

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24.58, 26.02, 27.23, 54.85, 56.22, 69.42, 109.83, 119.35, 140.91, 150.73, 156.18, 160.93. HRMS m/z 318.1632 [M+H$^+$] (calcd 318.1640). Anal. Calcd for C$_{17}$H$_{23}$N$_3$OS: C 64.32, H 7.30, N 13.24, S 10.10. Found: C 64.57, H 7.72, N 13.35, S 10.28.

N,N-Dimethyl-3-((5-methyl-2-(pyrazin-2-yl)thiazol-4-yl)oxy)propan-1-amine (33). Compound **33** was prepared according to procedure G from 5-methyl-2-(pyrazin-2-yl)thiazol-4-ol and 3-chloro-N,N-dimethylpropan-1-amine to afford 84 mg of yellow solid (94%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.19 (d, J = 1.5 Hz, 1H, pyra-6H), 8.42 (d, J = 2.6 Hz, 1H, pyra-4H), 8.38 (dd, J = 2.6, 1.5 Hz, 1H, pyra-3H), 4.33 (t, J = 6.5 Hz, 2H, OCH_2), 2.41 (t, J = 7.7 Hz, 2H, OCH$_2$CH$_2$CH_2N), 2.25 (s, 3H, CH_3), 2.20 (s, 6H, N(CH_3)$_2$), 2.03 – 1.79 (m, 2H, OCH$_2$CH_2). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.56, 27.78, 45.46, 56.35, 68.84, 111.49, 140.75, 143.62, 144.09, 146.94, 156.50, 160.69. ESI-MS m/z 279.1 [M+H$^+$] (calcd 279.1). Anal. Calcd for C$_{13}$H$_{18}$N$_4$OS: C 56.09, H 6.52, N 20.13, S 11.52. Found: C 56.20, H 6.49, N 19.99, S 10.92.

5-Methyl-2-(pyrazin-2-yl)-4-(2-(pyrrolidin-1-yl)ethoxy)thiazole (34). Compound **34** was prepared according to procedure G from 5-methyl-2-(pyrazin-2-yl)thiazol-4-ol and 1-(2-chloroethyl)pyrrolidine to afford 92 mg of yellow solid (71%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.18 (d, J = 1.5 Hz, 1H, pyra-6H), 8.40 (d, J = 2.6 Hz, 1H, pyra-4H), 8.36 (dd, J = 2.6, 1.5 Hz, 1H, pyra-3H), 4.43 (t, J = 6.1 Hz, 2H, OCH_2), 2.82 (t, J = 6.1 Hz, 2H, OCH$_2$CH_2N), 2.64 – 2.50 (m, 4H, pyrr-2,5H_2), 2.24 (s, 3H, CH_3), 1.79 – 1.60 (m, 4H, pyrr-3,4H_2). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.60, 23.49, 54.68, 55.26, 69.51, 111.55, 140.72, 143.58, 144.06, 146.87, 156.48, 160.57. ESI-MS m/z 291.1 [M+H$^+$] (calcd 291.1). Anal.

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Calcd for C$_{14}$H$_{17}$N$_5$O$_2$S: C 52.65, H 5.37, N 21.93, S 10.04. Found: C 52.62, H 5.48, N 22.51, S 10.16.

4-(4-((Bromomethyl)benzyl)oxy)-5-methylthiazol-2-yl)benzonitrile (38a). 38a was prepared according to procedure I to afford 0.87 g of yellow solid (86%). ^{1}H NMR (300 MHz, Chloroform-d) δ 7.86 (d, J = 8.8 Hz, 2H), 7.60 (d, J = 8.7 Hz, 2H), 7.37-7.33 (m, 4H), 5.34 (s, 2H), 4.43 (s, 2H), 2.24 (s, 3H).

4-(4-((Bromomethyl)benzyl)oxy)-5-methyl-2-(pyrazin-2-yl)thiazole (38b). 38b was prepared according to procedure I to afford 0.72 g of yellow solid (74%). ^{1}H NMR (600 MHz, Chloroform-d) δ 9.21 (d, J = 1.1 Hz, 1H), 8.54 – 8.36 (m, 2H), 7.38 (d, J = 8.1 Hz, 2H), 7.33 (d, J = 8.1 Hz, 2H), 5.35 (s, 2H), 4.43 (s, 2H), 2.26 (s, 3H).

4-(5-Methyl-4-((4-(piperidin-1-yl)methyl)benzyl)oxy)thiazol-2-yl)benzonitrile (39). Compound **39** was prepared according to procedure J from 4-(4-((4-(bromomethyl)benzyl)oxy)-5-methylthiazol-2-yl)benzonitrile and piperidine to afford 92 mg of yellow solid (90%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.85 (d, J = 8.7 Hz, 2H, ph^1-2,6H), 7.58 (d, J = 8.7 Hz, 2H, ph^1-3,5H), 7.32 (d, J = 8.2 Hz, 2H, ph^2-2,6H), 7.24 (d, J = 8.2 Hz, 2H, ph^2-3,5H), 5.31 (s, 2H, OCH_2), 3.39 (s, 2H, phCH_2N), 2.33 – 2.26 (m, 4H, pip-2,6$H_{2×eq}$), 2.22 (s, 3H, CH_3), 1.58 – 1.44 (m, 4H, pip-3,5$H_{2×eq}$), 1.39 – 1.25 (m, 2H, pip-4$H_{2×eq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.49, 24.35, 25.94, 54.50, 63.56, 72.16, 109.95, 112.15, 118.69, 125.55, 127.86, 129.31, 132.63, 135.99, 137.72, 138.44, 156.47, 160.32. HRMS m/z 404.1790 [M+H$^+$] (calcd for C$_{24}$H$_{25}$N$_3$OS. 404.1797).

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Calcd for C$_{14}$H$_{18}$N$_4$OS: C 57.91, H 6.25, N 19.29, S 11.04. Found: C 57.95, H 6.29, N 19.00, S 10.76.

5-Methyl-4-(3-(piperidin-1-yl)propoxy)-2-(pyrazin-2-yl)thiazole (35). Compound **35** was prepared according to procedure G from 5-methyl-2-(pyrazin-2-yl)thiazol-4-ol and 1-(3-chloropropyl)piperidine to afford 91 mg of yellow solid (75%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.18 (d, J = 1.5 Hz, 1H, pyra-6H), 8.41 (d, J = 2.6 Hz, 1H, pyra-4H), 8.38 (dd, J = 2.6, 1.5 Hz, 1H, pyra-3H), 4.32 (t, J = 6.4 Hz, 2H, OCH_2), 2.51 – 2.45 (m, 4H, pip-2,6$H_{2×eq}$), 2.40 (t, J = 5.61 Hz, 2H, OCH$_2$CH_2CH$_2$), 2.24 (s, 3H, CH_3), 2.03 – 1.87 (m, 2H, OCH$_2$CH_2), 1.62 – 1.50 (m, 4H, pip-3,5$H_{2×eq}$), 1.43 – 1.31 (m, 2H, pip-4$H_{2×eq}$). ^{13}C NMR (75 MHz, CDCl$_3$) δ 9.55, 24.19, 25.63, 26.81, 54.48, 55.89, 68.98, 111.50, 140.71, 143.61, 144.08, 146.89, 156.50, 160.63. ESI-MS m/z 319.2 [M+H$^+$] (calcd 319.2). Anal. Calcd for C$_{16}$H$_{22}$N$_4$OS: C 60.35, H 6.96, N 17.59, S 10.07. Found: C 59.45, H 6.75, N 17.82, S 9.62.

5-Methyl-2-(pyrazin-2-yl)thiazol-4-yl 4-methylpiperazine-1-carboxylate (36). Compound **36** was prepared according to procedure G from 5-methyl-2-(pyrazin-2-yl)thiazol-4-ol and 4-methylpiperazine-1-carbonyl chloride to afford 66 mg of yellow solid (75%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 9.23 (d, J = 1.5 Hz, 1H, pyra-6H), 8.47 (d, J = 2.6 Hz, 1H, pyra-4H), 8.42 (dd, J = 2.6, 1.5 Hz, 1H, pyra-3H), 3.69 (dd, J = 6.6, 3.9 Hz, 2H, piperazine-2,6$H_{2×eq}$), 3.58 – 3.52 (t dd, J = 6.6, 3.9 Hz, 2H, piperazine-2,6$H_{2×}$), 2.49 – 2.35 (m, 4H, piperazine-3,5$H_{2×eq}$), 2.29 (s, 2H, CH_3), 2.28 (s, 3H, CH_3). ^{13}C NMR (75 MHz, CDCl$_3$) δ 10.24, 43.99, 44.53, 46.06, 54.41, 54.62, 122.42, 140.93, 143.65, 144.84, 146.24, 152.19, 152.41, 158.69. ESI-MS m/z 320.1 [M+H$^+$] (calcd 320.1). Anal. Calcd for

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5-Ethyl-2-phenylthiazol-4-ol (43a). 43a was prepared according to procedure H to afford 0.71 g of yellow solid (89%) ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.39 (s, 1H), 7.76 (dd, J = 8.1, 1.6 Hz, 2H), 7.34 – 7.23 (m, 3H), 2.66 (q, J = 7.5 Hz, 2H), 1.18 (t, J = 7.5 Hz, 3H).

2-(4-Chlorophenyl)-5-ethylthiazol-4-ol (43b). 43b was prepared according to procedure H to afford 0.73 g of yellow solid (76%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.38 (s, 1H), 7.80 (d, J = 8.8 Hz, 2H), 7.51 (d, J = 8.8 Hz, 2H), 2.66 (q, J = 7.5 Hz, 2H), 1.18 (t, J = 7.5 Hz, 3H).

5-Ethyl-2-phenyl-4-(3-(piperidin-1-yl)propoxy)thiazole (44). Compound **44** was prepared according to procedure G from 5-ethyl-2-phenylthiazol-4-ol and 1-(3-chloropropyl)piperidine to afford 66 mg of yellow solid (71%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.76 (dd, J = 8.1, 1.6 Hz, 2H, ph-2,6H), 7.34 – 7.23 (m, 3H, ph-3,4,5H), 4.30 (t, J = 6.4 Hz, 2H, OCH_2), 2.64 (q, J = 7.5 Hz, 2H, ethyl-CH_2), 2.49 – 2.29 (m, 6H, pip-2,6H_{2axeq}, O$CH_2CH_2CH_2$N), 2.00 – 1.79 (m, 2H, OCH$_2CH_2$), 1.59 – 1.47 (m, 4H, pip-3,5H_{2axeq}), 1.46 – 1.28 (m, 2H, pip-4H_{2axeq}), 1.16 (t, J = 7.5 Hz, 3H, ethyl-CH_3). ^{13}C NMR (75 MHz, CDCl$_3$) δ 15.83, 18.00, 24.35, 25.82, 27.03, 54.56, 56.06, 69.03, 114.65, 125.32, 128.73, 129.18, 134.13, 158.93, 159.35. ESI-MS m/z 331.2 [M+H$^+$] (calcd for C$_{19}$H$_{26}$N$_2$OS, 331.2).

2-(4-chlorophenyl)-5-ethyl-4-(2-(pyrrolidin-1-yl)ethoxy)thiazole (45). Compound **45** was prepared according to procedure G from 4-chloro-2-(4-chlorophenyl)-5-ethylthiazole and 1-(3-chloropropyl)pyrrolidine to afford 49 mg of yellow solid (62%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.69 (d, J = 8.6 Hz, 2H, ph-2,6H), 7.27 (d, J = 8.6 Hz, 2H, ph-3,5H), 4.44 (t, J = 6.0 Hz, 2H, OCH_2), 2.86 (t, J = 6.0 Hz, 2H, OCH$_2CH_2$), 2.75 – 2.54 (m, 6H, pyrr-2,5H_2,

ethyl-CH_2), 1.88 – 1.66 (m, 4H, pyrr-3,4H_2), 1.17 (t, J = 7.5 Hz, 3H, ethyl-CH_3). ^{13}C NMR (75 MHz, CDCl$_3$) δ 15.81, 18.01, 23.51, 54.65, 55.23, 69.20, 115.24, 126.48, 128.94, 132.58, 135.00, 157.98, 158.81. ESI-MS m/z 351.1 [M+H$^+$] (calcd 351.1). Anal. Calcd for C$_{18}$H$_{23}$ClN$_2$OS: C 61.61, H 6.61, N 7.98, S 9.14. Found: C 61.70, H 6.57, N 8.04, S 9.20.

2-(4-Chlorophenyl)-5-ethyl-4-(3-(piperidin-1-yl)propoxy)thiazole (46). Compound **46** was prepared according to procedure G from 4-chloro-2-(4-chlorophenyl)-5-ethylthiazole and 1-(3-chloropropyl)piperidine to afford 142 mg of yellow solid (94%). ^{1}H NMR (CDCl$_3$, 300 MHz) δ ^{1}H NMR (300 MHz, Chloroform-d) δ 7.70 (d, J = 8.8 Hz, 2H, ph-2,6H), 7.27 (d, J = 8.8 Hz, 2H, ph-3,5H), 4.30 (t, J = 6.3 Hz, 2H, OCH_2), 2.64 (q, J = 7.5 Hz, 2H, ethyl-CH_2), 2.55 – 2.33 (m, 6H, pip-2,6H_{2axeq}, OCH_2CH_2), 2.04 – 1.85 (m, 2H, OCH$_2CH_2CH_2$N), 1.66 – 1.52 (m, 4H, pip-3,5H_{2axeq}), 1.47 – 1.33 (m, 2H, pip-4H_{2axeq}), 1.17 (t, J = 6.3 Hz, 3H, ethyl-CH_3). ^{13}C NMR (75 MHz, CDCl$_3$) δ 15.79, 17.99, 24.16, 25.55, 26.79, 54.47, 55.97, 68.93, 115.13, 126.49, 128.94, 132.61, 134.98, 157.97, 158.99. HRMS m/z 365.1445 [M+H$^+$] (calcd 365.1454). Anal. Calcd for C$_{19}$H$_{25}$ClN$_2$OS: C 62.53, H 6.91, N 7.68, S 8.79. Found: C 62.42, H 6.98, N 7.74, S 8.70.

Table S.2) Compliance of the most active ligands to Lipinski's and Veber's rules.

Compound	AlogP[b] (≤5)	MW (<500)	No. HBA[a] (≤10)	No. HBD[b] (≤5)	No. rotatable bonds (≤10)	Molecular PSA[c] (<140 Å)
8	1.92	289.4	3	1	5	54
16	2.58	323.9	3	1	5	55
17	3.04	337.9	3	1	5	55
22	2.32	342.5	4	1	6	79
30	1.22	304.4	4	1	5	68
37	2.05	373.5	5	1	6	81
42	1.56	367.5	5	1	6	81

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[a]Number of Hydrogen-bond acceptors (HBA); [b]Number of Hydrogen-bond donors
(HBD); [c]Polar Surface Area (PSA).

Table S.3) Predicted ADME profiles of further active ligands.

Compound	BBB[a]	Absorption[b]	Solubility[c]	Hepatotoxicity[d]	CYP2D6[e]	PPB[f]
8	1	0	3	nontoxic	inhibitor	1 (> 90%)
16	1	0	3	nontoxic	inhibitor	2 (> 95%)
17	1	0	2	nontoxic	inhibitor	2 (> 95%)
22	2	0	3	nontoxic	inhibitor	1 (> 90%)
30	2	0	3	nontoxic	non-inhibitor	1 (> 90%)
37	2	0	3	nontoxic	inhibitor	0 (< 90%)
42	2	0	3	nontoxic	non-inhibitor	2 (> 95%)

[a]Predicts ability of the compound to penetrate the blood brain barrier (BBB). Levels 0, 1, 2, 3, or 4 correspond
to very high, high, medium, low, or undefined penetration, respectively; [b]Predicts human intestinal absorption
after oral administration. Levels 0, 1, 2, or 3 correspond to good, moderate, poor, or very poor absorption,
respectively; [c]Predicts the solubility of each compound in water at 25°C. Levels 0, 1, 2, 3, 4, 5, or 6 correspond
to extremely low, very low, low, good, optimal, too soluble, or unknown solubility, respectively; [d]Predicts
the occurrence of dose-dependent human hepatotoxicity. Levels 0 or 1 correspond to nontoxic or toxic effect,
respectively; [e]Predicts cytochrome P450, 2D6 inhibition. Levels 0 or 1 correspond to non-inhibitor or
inhibitor, respectively; [f]Predicts the likelihood that a compound will be highly bound to carrier proteins on
the blood (PPB, plasma protein binding). Levels 0, 1, or 2 correspond to binding < 90%, binding > 90%, or
binding > 95%, respectively.

Table S.4) Toxicity profile of further active ligands using toxicity prediction, extensible protocol of Accelrys Discovery studio 2.5.5.
[a]Prediction of oxidative degradability-properties of compounds. Indicators -, or + correspond to non-biodegradability, or degradability by bioorganisms,

Compound	Aerobic Biodegradability[a]	AMES Mutagenicity[b]	Ocular Irritancy[b]	Skin Irritancy[c]	Skin Sensitizer[c]	Carcinogenicity USEDA[c]			
						Female Mouse	Male Mouse	Female rat	Male Rat
8	-	-	-	-	++	-	+	++	+
16	-	-	+	-	++	-	-	-	+
17	-	-	+	-	++	-	-	++	++
22	-	-	+	-	++	-	-	++	-
30	-	-	++	-	++	+	+	+	+
37	-	-	+	+	++	-	+	+	+
42	-	-	+	+	++	++	+	+	++

respectively; [b]Predicted mutagenicity in AMES mutagenicity test. Indicators -, or + correspond to non-mutagenicity, or mutagenicity, respectively; [c]Prediction of
organic toxicity. Indicators -, +, or ++ correspond to none, mild, or severe / strong toxic properties, respectively; [d]Prediction of carcinogenic properties. Indicators
-, +, or ++ correspond to classification as Non-Carcinogen, Single-Carcinogen, or Multi-Carcinogen, respectively.

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4. Multitarget-directed histamine H₃ receptor ligands in neurogenetic disorders

4.1. Profiling of LINS01 compounds at human dopamine D_2 and D_3 receptors

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Contribution to research: DR was involved in cell-culture, prepared and conducted radioligand displacement experiments at dopamine D_2 and D_3 receptors, evaluated inhibition and affinity data, wrote pharmacological parts of the manuscript draft and reviewed the manuscript.

Abstract:

Histamine and dopamine neuronal pathways display interesting overlapping in the CNS, especially in the limbic areas, making them very attractive to designing drugs with synergistic and/or additive effects. The roles of these systems to treat schizophrenia, drug addiction, Parkinson's and Alzheimer's diseases, among others are widely known. The LINS01 compounds were previously reported as histamine H_3 receptor (H_3R) antagonists and some of them are under evaluation in rodent memory models. Considering their pharmacological potential and similarities to literature dopamine D_2 receptor (D_2R) and dopamine D_3 receptor (D_3R) ligands, this work aimed to evaluate these compounds as ligands these receptors by using [^{3}H]spiperone displacement assays. A set of 11 compounds containing the dihydrobenzofuranyl-piperazine core with substituents at 5-position of dihydrobenzofuran ring and at the piperazine nitrogen was examined. The compounds showed low to moderate affinities at both, D_2R and D_3R. N-Phenyl compounds LINS01005 (1d), LINS01011 (1h), LINS01012 (1i) and LINS01016 (1k) showed the highest affinities in the set to D_3R (K_i 0.3–1.5 μM), indicating that N-phenylpiperazine moiety increases the affinity to this receptor subtype with some selectivity, since they showed lower affinities to D_2R (K_i 1.3-5.5 μM). With the LINS01 compounds showing moderate binding affinity, new lead structures for optimisation with regards to combined H_3R and D_2R/D_3R-ligands are provided.

REGULAR ARTICLE

Profiling of LINS01 compounds at human dopamine D_2 and D_3 receptors

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Abstract. Histamine and dopamine neuronal pathways display interesting overlapping in the CNS, especially in the limbic areas, making them very attractive to designing drugs with synergistic and/or additive effects. The roles of these systems to treat schizophrenia, drug addiction, Parkinson's and Alzheimer's diseases, among others are widely known. The LINS01 compounds were previously reported as histamine H_3 receptor (H_3R) antagonists and some of them are under evaluation in rodent memory models. Considering their pharmacological potential and similarities to literature dopamine D_2 receptor (D_2R) and dopamine D_3 receptor (D_3R) ligands, this work aimed to evaluate these compounds as ligands these receptors by using [^{3}H]spiperone displacement assays. A set of 11 compounds containing the dihydrobenzofuranyl-piperazine core with substituents at 5-position of dihydrobenzofuran ring and at the piperazine nitrogen was examined. The compounds showed low to moderate affinities at both, D_2R and D_3R. *N*-Phenyl compounds LINS01005 (**1d**), LINS01011 (**1h**), LINS01012 (**1i**) and LINS01016 (**1k**) showed the highest affinities in the set to D_3R (K_i 0.3–1.5 μM), indicating that *N*-phenylpiperazine moiety increases the affinity to this receptor subtype with some selectivity, since they showed lower affinities to D_2R (K_i 1.3–5.5 μM). With the LINS01 compounds showing moderate binding affinity, new lead structures for optimization with regards to combined H_3R and D_2R/D_3R-ligands are provided.

Keywords. Antihistamine; dopamine receptor ligand; D2 receptor; D3 receptor.

1. Introduction

Classically, the drug discovery process focuses on the "one drug, one target" paradigm, which means that a drug must interact specifically with a defined biological target in the organism, to assure the maximum efficacy (potency) and fewer side effects (selectivity). However, this philosophy has changed in the last years to a more comprehensive view of the diseases, coining a "one drug, multiple targets" paradigm, also known as polypharmacology.[1,2] Several diseases have been treated using a polypharmacological approach with multitarget drugs that were not designed by purpose.

For example, schizophrenia is an affective disorder that has been treated with typical and atypical antipsychotics, targeting the dopamine receptors but far from being defined as "selective drugs". The efficacy of the classical antipsychotics such as haloperidol is attributed to the dopamine receptor antagonism, leading to the desired therapeutic outcome, but also causing extrapyramidal side effects (EPS) and worsening the cognitive and negative symptoms.[3] On the other hand, atypical antipsychotics display a better therapeutic profile (especially against the negative and cognitive symptoms of schizophrenia) possibly due to their additional actions on

serotonin and histamine systems in the brain.[4] Considering that most of these effects are attributed to the GPCR targeting, and taking advantage of the anatomy and physiology of the neural network of the synapses, CNS diseases have been explored for designing multi target GPCR ligands.[1]

The histamine receptors are class-A GPCRs that are divided into 4 subtypes, H_1R to H_4R.[5] The H_1R and H_2R are widely expressed in the brain and are related to the control of sleep, food intake, body temperature and cognition. The H_4R is expressed in glial cells and may play a role in the inflammatory processes in the CNS.[6] The H_3R is mainly distributed in the CNS as an auto and/or heteroreceptor that regulates the production and releasing of histamine and other neurotransmitters, such as dopamine, and so it is a potential target for several CNS disorders.[7,8]

The dopamine receptors are also class-A GPCRs which are expressed in 5 subtypes, D_1R-D_5R. Considering their signalling profile, they are grouped into D_1-like (coupled to G_s) and D_2-like (coupled to $G_{i/o}$) families.[3] Classically, D_2-like receptors (D_2R, D_3R and possibly D_4R) have been widely explored to the treatment of schizophrenia. However, the distribution profile of the subtypes in CNS may lead to different applications. For instance, the high density of D_2R in the movement-related and cortical areas and the high density of both D_2R and D_3R in brain areas such as the limbic system may explain why non-selective D_2R antagonists lead to the motor-related EPS and cognitive decline as caused by the classical antipsychotics, but also the efficacy in the psychotic effects.[9,10] D_3R blockade also increases the acetylcholine release in the cortex, related to the improvement in the cognitive processes.[11] In spite of this, selective D_3R antagonists would be effective drugs against conditions such as schizophrenia, drug addiction, AD, PD and depression.[9]

The histamine and dopamine systems in the brain are noteworthy due to very interesting common characteristics. Both systems are originated in the tuberomammillary nucleus (TMN), with projections to the cortex, (hypo)thalamus, hippocampus, striatum and amygdala. In particular, H_3R and D_3R present a considerable density distribution overlapping in the limbic areas, such as hippocampus, stratum and amygdala.[12] It is interesting to note that 95% of the neurons expressing dopamine D_1-like receptors and 89% of those expressing D_2-like receptors in the striatum also express H_3R leading to complex interactions between both neurotransmitter systems.[13] Several results from pharmacological studies suggest that antagonists of both H_3R and D_3R can present additive and/or

synergistic effects, making them attractive multi-targeting tools for the treatment of schizophrenia, drug addiction, PD, AD, dementias and certain types of epilepsy.[8,14,15]

The LINS01 compounds (e.g., **1c** and **1g**, Figure 1) were previously described as selective H_3R antagonists.[16,17] However, these compounds present some similarity to dopamine D_2R/D_3R ligands, since the overlap between the H_3R and D_2R/D_3R pharmacophores can be noted.[8,18] The *N*-phenylpiperazine motif present in some LINS01 compounds is found in several D_2R/D_3R ligands such as compounds **2**, **3** and **4** in Figure 1.[19,20] Moreover, the similarity of LINS01 compounds to compound **4** and its analogue **5** is clearly evident, which are potent ligands of D_2R and D_3R.[11] Considering the potential of these compounds and the similarities, the present report intended to assess the affinity of the LINS01 compounds to the D_2R/D_3R.

2. Experimental

2.1 *Preparation of the compounds LINS01*

All chemicals were purchased with adequate purity from Sigma-Aldrich Co. (Brazil) and LabSynth (Brazil) and used as supplied. The compounds were prepared and characterized as described in previous reports from our group (Figure 2).[16,17,21,22] The analytical characterization of the newly synthesized compounds **1j** and **1k** is stated below. All
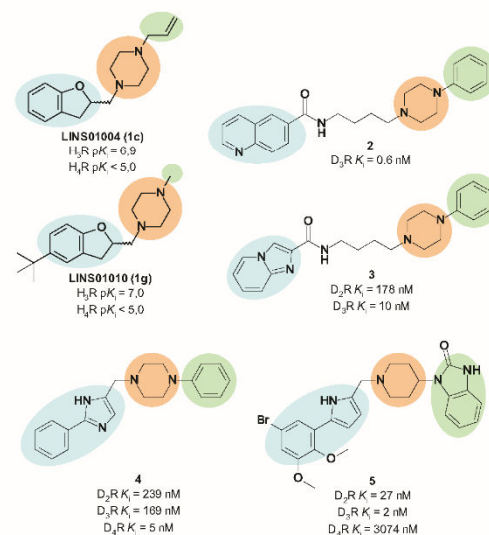
Figure 1. LINS01 compounds and literature ligands of D_2R/D_3R.
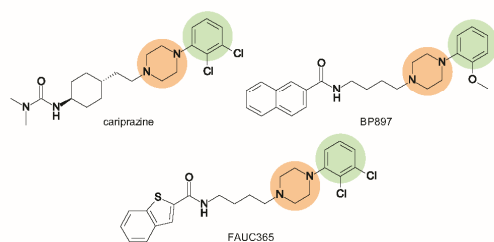
Figure 2. Ligands of D₂R and D₃R reported in the literature.

compounds were checked for purity through chromatography and considered adequate when purity was >95%.

1-Methyl-4-[(5-phenyl-2,3-dihydro-1-benzofuran-2-yl)methyl]piperazine (**1j**). The reaction between 1-methylpiperazine and prepared 2-(iodomethyl)-5-phenyl-2,3-dihydrobenzofuran yielded 45% of **1j**. ^{1}H NMR (300 MHz, CDCl₃): δ 2.31 (*s*, 3H), 2.41–2.74 (*m*, 9H), 2.82 (*dd*, 1H, *J* = 13.3, 7.7 Hz), 3.0 (*dd*, 1H, *J* = 15.7, 8.0 Hz), 3.32 (*dd*, 1H, *J* = 15.6, 9.1 Hz), 5.01 (*dq*, 1H, *J* = 8.2, 4.1 Hz), 6.85 (*d*, 1H, *J* = 8.3 Hz), 7.27–7.46 (*m*, 5H), 7.52 (*d*, 2H, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, CDCl₃): δ 34.2, 46.1, 53.8, 55.0, 63.2, 81.3, 109.8, 123.8, 126.5, 126.8, 127.1, 127.2, 128.9, 133.9, 141.3, 159.2.

1-Phenyl-4-[(5-phenyl-2,3-dihydro-1-benzofuran-2-yl)methyl]piperazine (**1k**). The reaction between 1-phenylpiperazine and prepared 2-(iodomethyl)-5-phenyl-2,3-dihydrobenzofuran yielded 78% of **1k**. ^{1}H NMR (300 MHz, CDCl₃): δ 2.67 (*dd*, 1H, *J* = 13.3, 4.1 Hz), 2.72–2.81 (*m*, 4H), 2.88 (*dd*, 1H, *J* = 13.4, 7.8 Hz), 2.98–3.10 (*m*, 2H), 3.26 (*t*, 4H, *J* = 4.9 Hz), 3.36 (*dd*, 1H, *J* = 15.8, 9.2 Hz), 5.00–5.13 (*m*, 1H), 6.83–6.91 (*m*, 2H), 6.95 (*d*, 2H, *J* = 8.2 Hz), 7.28–7.45 (*m*, 6H), 7.52 (*d*, 2H, *J* = 7.2 Hz). ^{13}C NMR (75 MHz, CDCl₃): δ 34.2, 49.1, 53.9, 63.2, 77.3, 81.3, 109.8, 116.1, 119.7, 123.8, 126.5, 126.8, 127.2, 128.7, 129.1, 134.1, 141.3, 151.3, 159.2.

2.2 *Binding assays on D₂R and D₃R*

Membranes from CHO cells stably expressing either the short transcript of D₂ receptors or D₃ receptors were prepared as described previously.[23] For inhibition screening, freshly thawed membrane preparations (containing 25 and 20 μg/well of D₂R and D₃R, respectively) were incubated on microtiter plates with the indicated concentration of compounds and 0.2 nM [^{3}H]spiperone in binding buffer [50 mM TRIS (pH = 7.4) 120 mM NaCl, 5 mM KCl, 1 mM CaCl₂ and 1 mM MgCl₂]. After an incubation period of 120 min, the mixture was harvested on glass-fiber mats, presoaked with 3% polyethylene-imine solution, followed by three wash-steps using cold demineralized water (approx. 1 mL/sample). The workup for scintillation-counting followed the standard procedure described before.[24] Non-specific binding was determined by an excess of unlabeled

haloperidol (10 μM). Inhibition was calculated as residual of specific binding of [^{3}H]spiperone in presence of compound relative to specific binding in the absence of inhibitor. For affinity measurements, the same procedure as above was used but including a titration pattern of the investigated ligands (0.01–100,000 nM, final concentration). The finally determined specific binding was analyzed by non-linear least-square fitting to a four-parameter logistic equation. Conversion of the determined IC₅₀ to K_i values was performed as described elsewhere.[25]
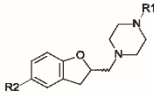
3. Results and Discussion

The LINS01 compounds (**1a–1k**, Scheme 1) were prepared as reported previously by our group.[16,17] The derivatives **1a–1d** were prepared from 2-allylphenol through iodine-promoted cyclization, using water as a solvent.[21] Finally, the iodinated heterocycle was used to alkylate the *N*-substituted piperazine in aprotic solvent (THF), with potassium carbonate as base, with moderate yield (∼50%). The 5-phenyl derivatives **1j** and **1k** were prepared from the corresponding 4-phenyl-phenol following the same procedure, with moderate to good yields (45–78%). A novel microwave-assisted methodology[17] was employed to avoid the considerable excess of 1-phenylpiperazine to obtain compounds **1h**, **1i** and **1k**, leading to good yields (>60%). Although this method gave comparable yields to the conventional methodology, it also saved reaction time and required less 1-phenylpiperazine (1.1 eq.) indeed, therefore comprising the green chemistry principles.[22] The spectroscopic data for the final compounds and intermediates are in accordance with the literature reports.[16,17,21, 26]

In an initial screening as shown in Table 1, the LINS01 compounds showed variable inhibition profiles at D₂R and D₃R, depending on the presence of certain groups. In general, a slight preference for
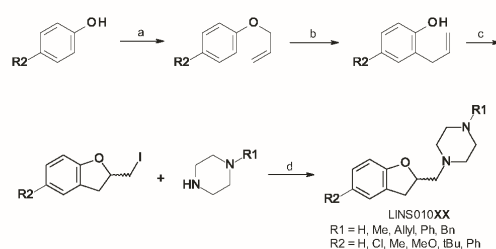
Scheme 1. Reagents and conditions. (a) Allyl bromide (2 eq.), K₂CO₃ (2 eq.), acetone, 60 °C, 2–4 h; (b) DMF, MW 200 °C (300 psi, 300 W), 1,5 h; (c) I₂ (1.1 eq.), water or EtOH/water, *r.t.*, 4–6 h; (d) K₂CO₃ (1.2 eq.), THF, reflux, 12–24 h or MW 120 °C (300 psi, 300 W), 1.5 h.

Table 1. Screening of the compounds LINS01 towards inhibition and affinity at the human dopamine D_2R and D_3R.

Compounds	R 1	R 2	% inhibition (10 μM) ± SD (n)		K_i (μM) [95% CI]	
			D_2R	D_3R	D_2R	D_3R
1a (LINS01001)	H	H	0.0 ± 0.0 (12)	9.0 ± 6.4 (12)	n.d. (>10 μM)	n.d. (>10 μM)
1b (LINS01003)	Me	H	1.5 ± 13.9 (15)	23.5 ± 11.7 (15)	n.d. (>10 μM)	n.d. (>10 μM)
1c (LINS01004)	Allyl	H	33.6 ± 9.7 (15)	62.3 ± 14.4 (15)	5.5 [5.0–6.0]	1.5 [0.8–3.0]
1d (LINS01005)	Ph	H	34.1 ± 10.5 (15)	55.1 ± 10.3 (15)	2.4 [2.0–2.9]	0.89 [0.45–1.75]
1e (LINS01007)	Me	Cl	10.3 ± 10.9 (15)	31.6 ± 10.1 (15)	n.d. (>10 μM)	n.d. (>10 μM)
1f (LINS01008)	Me	Me	11.1 ± 11.7 (15)	29.9 ± 9.6 (15)	n.d. (>10 μM)	n.d. (>10 μM)
1g (LINS01010)	Me	*t*Bu	12.9 ± 12.7 (15)	31.7 ± 8.5 (15)	n.d. (>10 μM)	n.d. (>10 μM)
1h (LINS01011)	Ph	Cl	44.0 ± 3.6 (6)	64.0 ± 7.1 (6)	2.6 [1.2–6.1]	0.50 [0.10–2.66]
1i (LINS01012)	Ph	Me	45.1 ± 4.2 (6)	60.9 ± 10.7 (6)	2.4 [1.1–5.3]	1.5 [0.4–4.8]
1j (LINS01016)	Me	Ph	3.3 ± 12.3 (15)	27.2 ± 7.1 (15)	n.d. (>10 μM)	n.d. (>10 μM)
1k (LINS01017)	Ph	Ph	39.5 ± 9.6 (6)	62.0 ± 5.7 (6)	1.3 [0.6–3.0]	0.39 [0.04–3.40]
Haloperidol			100.0 ± 2.5 (15)	99.3 ± 2.6 (15)	n.d.	n.d.

inhibiting [³H]spiperone binding to D_3R was observed. Compounds **1a** to **1d** bear the 1-(2,3-dihydrobenzofuranyl)methylpiperazine core with different substituents attached to the nitrogen. Whereas **1a** did not show important inhibition at both D_2R and D_3R, compounds presenting bigger and/or aromatic substituents attached to the piperazine displayed increased inhibition at the dopamine receptors. The *N*-methylpiperazine analogues (**1e–1g**, **1j**) displayed inhibition below 50% at both receptors leading to affinities above the 10 μM concentration range. There instead, substitution of *N*-allylpiperazine by *N*-phenylpiperazine (compounds **1c** and **1d**, respectively) showed comparable inhibition profiles at both receptors but a slight increase in affinity at D_2R. Furthermore, the results suggest that the presence of these groups drive the preference toward D_3R.

These results motivated us to further explore the affinities of the *N*-phenylpiperazine derivatives. The *N*-arylpiperazine group can be considered a privileged group to design ligands of D_2-like receptors as embodied in the LINS01 series.[10,11] The compounds **1d**, **1h**, **1i** and **1k** presented the highest inhibition at the investigated targets with affinities in the low micromolar concentration range at D_2R. Though only **1d** showing significant D_3R preference among them, even submicromolar K_i values were determined at the D_3R.

The influence of the groups attached in 5-position of dihydrobenzofuran (R2) on the affinity of the compounds seems minor. When comparing compounds with different substitution profiles in this part of the molecule, it can be noted that the inhibition did not significantly change among the *N*-methylpiperazine derivatives **1e**, **1f** and **1g**, as well as comparable affinities were observed among the *N*-phenylpiperazine compounds **1d**, **1h**, **1i** and **1k**.

The role of the 2,3-dihydrobenzofuran group in the affinity of the compounds remains unclear as this element originates from the initial H₃R design

strategy. A search in literature reveals that several compounds containing the aromatic benzofuran were already reported as dopamine receptor ligands, however, only few compounds containing the dihydro analogue were tested so far. The aromatic analogues of LINS01 compounds have been demonstrating activity as sigma- and serotonin receptor ligands,[27,28] but usually with poor affinity to dopamine receptors,[26,29] Despite a closely related aromatic *N*-benzylpiperazine analogue being reported in literature,[29] no compounds containing the unsubstituted *N*-allyl-, *N*-methyl- or *N*–phenylpiperazine moieties were found. On the other hand, homologues containing a longer linker between the benzofuran and the piperazine showed increased affinity to dopamine receptors,[30] suggesting that longer homologues of LINS01 compounds would shed light on the role of the dihydrobenzofuran in the D₂R and D₃R affinities, and should be considered in future evaluations. Secondly, derivatization of *N*-phenylpiperazine may be used for enhancing affinity at D₂R and D₃R. For instance, the substitution with a 2-methoxy or 2,3-dichloro groups in the phenyl ring usually leads to increased D₃R selectivity, as can be seen in cariprazine (a subnanomolar affinity D₃R ligand and nanomolar affinity at D₂R) and other compounds such as BP897 (bearing a 2-methoxyphenyl piperazine) and FAUC365 (bearing a 2,3-dichlorophenyl piperazine), indicating potential substitutions in this direction for designing improved ligands[10] (Figure 2).

4. Conclusions

This is the first report exploring the histamine H₃R-targeting LINS01 compounds being ligands at dopamine receptors as well. Although these molecules showed low affinity to D₂R and D₃R, some of them show slight D₃R preference. The *N*-phenylpiperazine and *N*-benzylpiperazine fragments increased the binding of these compounds to D₂R and D₃R, although the latter reduces the selectivity. Additionally, the substitution in the dihydrobenzofuran seems not to influence the affinity of these compounds to D₂R or D₃R. The presented characterization of the LINS01 series lays the foundation for further profiling of the detected hits, profiling them towards attractive lead-compounds with combined dopaminergic and histaminergic activity.

Supplementary Information (SI)

Supplementary information is available at www.ias.ac.in/chemsci.

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Figure S1. ^{1}H-NMR of compound 1j

SUPPLEMENTARY INFORMATION

Profiling of LINS01 compounds at human dopamine D₂ and D₃ receptors

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Figure S2. ¹H-NMR of compound 1k

4.2. Dual target ligands with 4-*tert*-butylphenoxy scaffold as histamine H₃ receptor antagonists and monoamine oxidase B inhibitors

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Contribution to research: DR was involved in preparation, planning and conduction of cell-culture and radioligand displacement experiments, and evaluated corresponding data to determine H₃R affinity. DR wrote the pharmacological parts of and reviewed the manuscript.

Abstract:

Dual target ligands are a promising concept for the treatment of Parkinson's disease (PD). A combination of monoamine oxidase B (MAO B) inhibition with histamine H₃ receptor (H₃R) antagonism could have positive effects on dopamine regulation. Thus, a series of twenty-seven 4-*tert*-butylphenoxyalkoxyamines were designed as potential dual-target ligands for PD based on the structure of 1-(3-(4-tert-butylphenoxy)propyl)piperidine (DL76). Probed modifications included the introduction of different cyclic amines and elongation of the alkyl chain. Synthesized compounds were investigated or human H₃R (hH₃R) affinity and human MAO B (hMAO B) inhibitory activity. Most compounds showed good hH₃R affinities with K_i values below 400 nM, and some of them showed potent inhibitory activity for hMAO B with IC_{50} values below 50 nM. However, the most balanced activity against both biological targets showed DL76 (hH₃R: K_i = 38 nM and hMAO B: IC_{50} = 48 nM). Thus, DL76 was chosen for further studies, revealing the nontoxic nature of DL76 in HEK293 and neuroblastoma SH-SY5Ycells. However, no neuroprotective effect was observed for DL76 in hydrogen peroxide-treated neuroblastoma SH-SY5Y cells. Furthermore, *in vivo* studies showed antiparkinsonian activity of DL76 in haloperidol-induced catalepsy (Cross Leg Position Test) at a dose of 50 mg/kg body weight.

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Dual Target Ligands with 4-*tert*-Butylphenoxy Scaffold as Histamine H$_3$ Receptor Antagonists and Monoamine Oxidase B Inhibitors

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Abstract: Dual target ligands are a promising concept for the treatment of Parkinson's disease (PD). A combination of monoamine oxidase B (MAO B) inhibition with histamine H$_3$ receptor (H$_3$R) antagonism could have positive effects on dopamine regulation. Thus, a series of twenty-seven 4-*tert*-butylphenoxyalkoxyamines were designed as potential dual-target ligands for PD based on the structure of 1-(3-(4-*tert*-butylphenoxy)propyl)piperidine (**DL76**). Probed modifications included the introduction of different cyclic amines and elongation of the alkyl chain. Synthesized compounds were investigated for human H$_3$R (hH$_3$R) affinity and human MAO B (hMAO B) inhibitory activity. Most compounds showed good hH$_3$R affinities with K$_i$ values below 400 nM, and some of them showed potent inhibitory activity for hMAO B with IC$_{50}$ values below 50 nM. However, the most balanced activity against both biological targets showed **DL76** (hH$_3$R: K$_i$ = 38 nM and hMAO B: IC$_{50}$ = 48 nM). Thus, **DL76** was chosen for further studies, revealing the nontoxic nature of **DL76** in HEK293 and neuroblastoma SH-SY5Ycells. However, no neuroprotective effect was observed for **DL76** in hydrogen peroxide-treated neuroblastoma SH-SY5Y cells. Furthermore, in vivo studies showed antiparkinsonian activity of **DL76** in haloperidol-induced catalepsy (Cross Leg Position Test) at a dose of 50 mg/kg body weight.

Keywords: dual-target ligands; histamine H$_3$ receptor; monoamine oxidase B; 4-*tert*-butylphenyl derivatives; antagonists; inhibitors; neurodegenerative disease; Parkinson's disease

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by motor problems. Although the entire pathology of PD is still unknown, several factors have been proposed to contribute to PD development, such as environmental toxins, neuroinflammation, genetic mutations, oxidative stress, or mitochondrial dysfunction [1]. Generally, PD is characterized by a severe lack of dopamine (DA) (80–90%) in *striatum* due to a progressive loss of dopaminergic neurons in the *substantia nigra* [1]. Current therapy for PD can only mitigate symptoms and slow the progress. However, there is no cure for the disease to date. Commonly administered drugs include levodopa

**Molecular pharmacology of histamine H₃ receptor ligands
and their implications in neurogenetic disorders**
David Reiner-Link (2020)

(as a DA precursor), DA agonists (e.g., pramipexole and rotigotine), and monoamine oxidase (MAO) B inhibitors (e.g., selegiline, rasagiline, or safinamide; Figure 1).
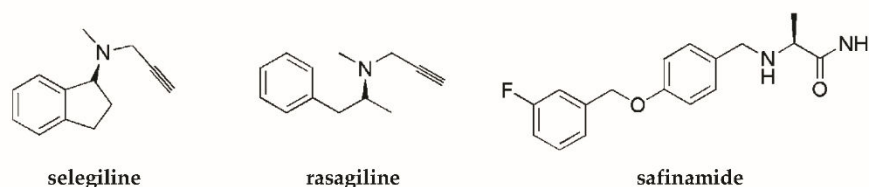

Figure 1. Structures of antiparkinsonian drugs—monoamine oxidase (MAO) B inhibitors.

MAO B plays a crucial role in the pathogenesis of PD. This enzyme belongs to the family of MAOs that catalyze the deamination of neurotransmitters (e.g., DA) and release reactive oxygen species as by-products. MAO B dominates in the human brain and deaminates β-phenylethylamine (PEA). PEA increases the synaptic levels of DA and blocks its reuptake into neurons. An increase in the activity of MAO B with age and some diseases as PD was observed in humans. Inhibitors of MAO B stop the activity of this enzyme and block the breakdown of DA. Moreover, MAO B inhibitors show neuroprotection and reduce oxidative stress [2]. Thus, MAO B inhibition is an important factor in the search for effective drugs in the treatment of PD. However, due to the multifactorial etiology of PD, it is thought that ligands acting on several targets at the same time (so-called Multi-Target-Directed Ligands (MTDL)) will be more effective in treatment than a one-target compound [3]. Thus, for improving the pharmacotherapy of PD, it is important to find MAO B inhibitors with combined activity at other targets.

Histamine H₃ receptors (H₃Rs) are widely distributed in the human brain and dominantly in areas connected with cognition (such as the striatum, cortex, or hippocampus). H₃Rs influence the release not only of histamine itself but also of other neurotransmitters, such as DA or acetylcholine [4], and increase the level of mentioned neurotransmitters in the synaptic cleft. Numerous pharmacological studies show that blocking H₃Rs provides beneficial effects in the treatment of various neurological diseases such as narcolepsy, neurodegenerative diseases (e.g., Alzheimer's disease and PD), attention deficit hyperactivity disorder, epilepsy, obesity, or neuropathic pain [5]. For years, many scientific centers and pharmaceutical companies have been involved in the search for active ligands of these receptors. Intensive synthetic work has led to a large number of structurally diverse compounds. Some of them have reached clinical trials, but so far, only one (pitolisant (Wakix®); Figure 2) has entered into the market as an orphan drug for narcolepsy [6].

One strategy to obtain MTDL is to combine two or more pharmacophores into a single molecule. Pharmacophores can be connected by linkers, attached directly (fused), or merged [7]. A propargylamine moiety is known to be important for MAO B inhibition [8], and it is present in the marketed drugs selegiline and rasagiline. The piperidinepropoxy motif is a part of many potent H₃R ligands, e.g., pitolisant (Figure 2). The idea to combine MAO B inhibition with H₃R antagonism is quite new. In 2017, the first of such compounds, contilisant (Figure 2), was described by Bautista-Aguilera et al. [9]. Contilisant not only proved to be a H₃R antagonist (K_i = 11 nM) and human MAO B (hMAO B) inhibitor (IC_{50} = 78 nM) but also showed moderate inhibition of cholinesterases (AChE IC_{50} = 530 nM; BuChE IC_{50} = 1690 nM). Further, this idea was continued by Lutsenko et al. [10] with the fused rasagiline derivative **1** as a dual-target ligand (DTL) with high hH₃R affinity (K_i = 6.7 nM) and good hMAO B inhibitory activity (IC_{50} = 256 nM) (Figure 2). Moreover, Affini et al. [11] described indanone derivatives as DTL for PD (compound **2**; hH₃R K_i = 6.5 nM; hMAO B IC_{50} = 276 nM; Figure 2). Recently, we have described a new group of DTL hMAO B inhibitors, *tert*-amylphenoxy derivatives [12]. These compounds showed also affinity for hH₃R (e.g., compound **3**; Figure 2). In contrast to the previously described DTL (contilisant, **1** and **2**), some of them showed an inhibitory activity for hMAO B that was higher than their affinity for hH₃R (**3**: hH₃R K_i = 63 nM; hMAO B IC_{50} = 4.5 nM).
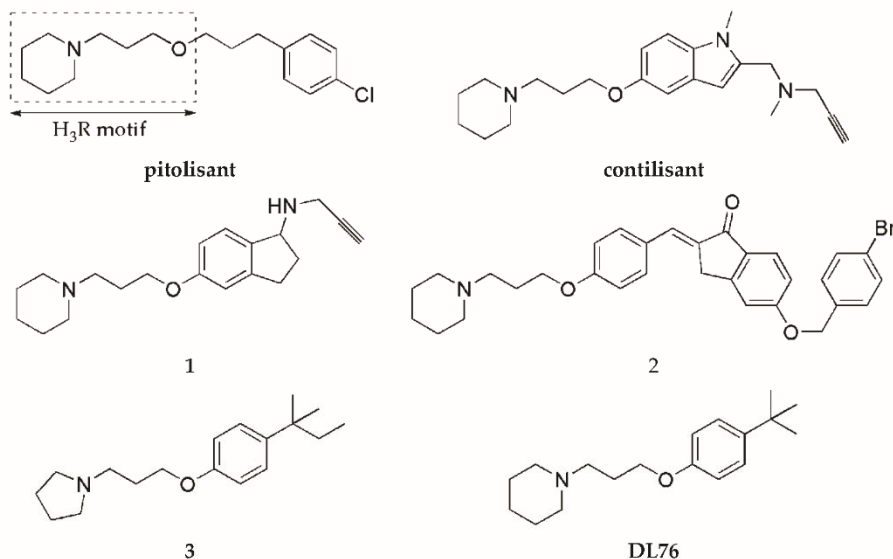
Figure 2. Structures of pitolisant and histamine H₃ receptor ligands with MAO B inhibitory activity.

To continue this work, we synthesized a series of 4-*tert*-butylphenoxy derivatives as analogues of histamine H₃R ligand **DL76** (hH₃R K_i = 22 nM in CHO K1 cells [13]; Figure 2) which showed also good inhibitory activity for hMAO B with an IC_{50} of 48 nM. Encouraged by these results, we designed a series of novel 4-*tert*-butylphenoxy derivatives. Designed structural modifications included the following:

- exchange of piperidine moiety for other cyclic amines (pyrrolidine, substituted piperidine, or azepane)
- elongation of alkyl chain from three up to six atoms.

All compounds were evaluated for their affinity towards hH₃R and inhibition of hMAO B. Furthermore, we selected one of compounds for antiparkinsonian activity tests in vivo (in haloperidol-induced catalepsy) and neuroprotection studies in vitro (in neuroblastoma SH-SY5Y cells). Moreover, the toxicity of this compound in HEK293 cells and neuroblastoma SH-SY5Y cells was evaluated.

2. Results and Discussion

2.1. Synthesis of Compounds

Compounds were synthesized as shown in Scheme 1. Briefly, proper 4-*tert*-butylphenoxyalkyl bromides (**4a–4d**) were obtained by nucleophilic substitution of 4-*tert*-butylphenol with α,ω-dibromoalkanes in freshly prepared sodium propanolate as described previously [14]. Then, the bromides **4a–4d** were refluxed with corresponding amines in the mixture of ethanol–water (21:4) with powdered potassium carbonate and a catalytic amount of potassium iodide. The purified free bases were converted into hydrogen oxalates. Structures and purity of novel compounds **5–31** were confirmed by spectral analyses (¹H and ¹³C NMR spectra; see Supplementary Materials S1), mass spectrometry (MS), and elemental analysis.
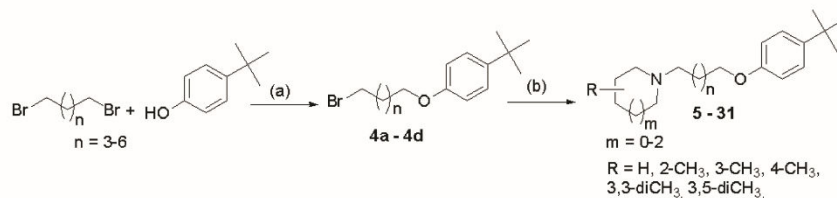
Scheme 1. General synthetic pathway of synthesized compounds **5–31**. Reagents and conditions: (**a**) sodium propanolate (0.05 mol Na in 50 mL), room temperature, 15′; 60 °C 3 h; reflux 3 h; 69–78%; (**b**) (i) amine, K_2CO_3, KI, $EtOH:H_2O$ (21:4), and reflux 20 h and (ii) oxalic acid, EtOH, room temperature, 1 h; $(Et)_2O$; crystallization from EtOH, 5–53%.

2.2. Human Histamine H₃ Receptor Affinity

The affinity of compounds (**5–31**) for H₃R was evaluated in a radioligand-binding assay in HEK293 cells stably expressing hH₃R [14]. $[^3H]N^\alpha$-methylhistamine was used as a radioligand. Results are presented as K_i values in Table 1. The compounds showed variable affinities for hH₃R ranging from good ($K_i < 100$ nM) to weak ($K_i > 1500$ nM), depending on the kind of the introduced cyclic amine moiety and alkyl chain length. Analyzing the influence of a cyclic amine moiety on the affinity for hH₃R, it was noticed that derivatives of piperidine (**DL76, 6–8**), 3-methylpiperidine (**12–15**), and azepane (**28–31**) were the most active. On the other hand, 3,3-dimethylpiperidine derivatives (**16–19**) showed weaker affinities ($K_i > 1600$ nM). Unfortunately, none of the synthesized compounds (**5–31**) showed higher hH₃R affinity than the lead structure **DL76** ($K_i = 38$ nM). The most potent was compound **9** with a K_i of 69 nM.

Table 1. Human histamine H₃ receptor affinity and human MAO B inhibition of tested compounds **5–31** and **DL76**.

Compounds	R	n	H₃R [a] K_i (nM) (95%CI)	MAO B [b] IC_{50} (nM) (%Inh.) [c]	MAO A [b] IC_{50} (nM) (%Inh.) [d]
5		1	371 (136, 1009)	2.7 ± 0.4	nt [e]
DL76		1	38 [f] (8, 181)	48 ± 15	>10,000 (9%)
6		2	309 (166, 574)	290 ± 7	>10,000 (10%)
7		3	252 (64, 990)	(28%)	nt [e]
8		4	225 (98, 519)	(13%)	nt [e]
9		1	69 (49, 96)	11 ± 1	>10,000 (5%)
10		2	153 (46, 505)	475 ± 38	>10,000 (2%)
11		3	1556 (349, 6941)	(36%)	nt [e]

Table 1. *Cont.*

Compounds	R	n	H_3R [a] K_i (nM) (95%CI)	MAO B [b] IC_{50} (nM) (%Inh.) [c]	MAO A [b] IC_{50} (nM) (%Inh.) [d]
12		1	98 (43, 226)	117 ±12	>10,000 (10%)
13		2	102 (18, 571)	1405 ± 494	nt [e]
14		3	114 (33, 397)	(33%)	nt [e]
15		4	351 (223, 552)	(13%)	nt
16		1	1624 (1075, 2453)	476 ± 38	>10,000 (6%)
17		2	3437 (2701, 4374)	(38%)	nt [e]
18		3	3535 (2528, 4942)	2777 ± 66	>10,000 (19%)
19		4	2575 (542, 12227)	1953 ± 45	>10,000 (24%)
20		1	341 (49, 2388)	(37%)	nt [e]
21		2	1381 (923, 2066)	(35%)	nt [e]
22		3	2235 (1136, 4397)	(41%)	nt [e]
23		4	2083 (936, 4637)	(34%)	nt [e]
24		1	316 (123, 808)	(37%)	nt [e]
25		2	400 (152, 1050)	(33%)	nt [e]
26		3	531 (344, 822)	(39%)	nt [e]
27		4	1350 (651, 2798)	(10%)	nt [e]
28		1	111 (68, 180)	45 ± 4	>10,000 (10%)
29		2	299 (105, 855)	1627 ± 78	>10,000 (23%)
30		3	324 (121, 870)	(18%)	nt [e]
31		4	829 [g] (313, 2194)	(23%)	nt [e]
rasagiline			nt [e]	15 ± 1	nt [e]
pargiline			nt [e]	360 ± 138	nt [e]
safinamide			nt [e]	7.7 ± 1.2	nt [e]
clorgiline			nt [e]	nt [e]	1.76 ± 0.5 nM

[a] [³H]Nᵅ-Methylhistamine-binding assay in HEK293 cells stably expressing the human H₃R; mean value within the 95% confidence interval (CI); [b] fluorometricAmplex™ Red MAO assay [15]; mean ± SEM of 2–5 independent experiments; [c] the percent of inhibition at 1 μM, mean values of two independent experiments; [d] the percent of inhibition at 10 μM, mean values of two independent experiments; [e] nt = not tested; [f] K_i (±SEM) = 22 ± 3 nM in [¹²⁵I]Iodoproxyfan binding assay in CHO K1 cells, data from Reference [13]; [g] data from Reference [16].

2.3. Human Monoamine Oxidase B Inhibitory Activity

2.3.1. Screening and Determination of IC_{50}

The inhibitory activity of the compounds against hMAO B was evaluated using Amplex Red® Monoamine Oxidase kits [15]. Rasagiline, pargyline, and safinamide were used as reference inhibitors. Following the initial screening of compounds at 1 µM concentration, IC_{50} values were determined for those which exhibited inhibitory activity greater than 50% (Table 1). Sixteen compounds out of the thirty-one showed a percentage of inhibition between 10% and 41%. Results of IC_{50} determinations indicated the influence of both an amine moiety and a length of alkyl chain on hMAO B inhibition. Except the series of 3,5-dimethylpiperidine and 4-methylpiperidine derivatives, the inhibitory activity of the compounds was more pronounced with a shorter (three or four) alkyl linker. Thus, an elongation of alkyl chain caused a drop of activity. The highest activity was observed for the compounds with the propylene linker (**DL76, 9, 12, 16, 20, 24,** and **28**). This observation is simillar to our previous finding [12] concerning 4-*tert*-amylphenoxy derivatives as DTL ligands. Exchange of a cyclic amine group (piperidine in **DL76**) for other moieties (pyrrolidine, substituted piperidine, or azepane) caused variable influences: increased (pyrrolidine-**5** or 2-methylpiperidine-**9**), maintained (azepane-**28**), or decreased activity (3-methylpiperidine-**12**, 3,3-dimethylpiperidine-**16**, 3,5-dimethylpiepridine-**20**, or 4-methylpiperidine-**24**). The most potent inhibitor **5** (IC_{50} = 2.7 nM) showed higher activity than the reference compounds rasagiline (IC_{50} = 15 nM) and safinamide (IC_{50} = 7.7 nM).

2.3.2. Reversibility Studies

To investigate the type of inhibition (reversible or irreversible) of hMAO B by 4-*tert*-butylphenoxy derivatives, experiments were performed with selected compounds (**DL76, 5,** and **9**) and conducted as described previously [12]. Rasagiline (irreversible inhibitor) and safinamide (reversible inhibitor) were used as standards. Results from the experiment are shown in Figure 3A–C. All tested compounds presented a signal similar to the reversible reference inhibitor safinamide so they were considered as reversible. However, the signal for rasagiline was slightly higher than expected for the IC_{80} concentration. High signal for rasagiline can be explained by the fact that irreversible inhibitors need more time to create the covalent bond with the enzyme. The used protocol for the reversibility testing did not contain the preincubation of enzyme with inhibitors. Lack of preincubation could also change the observed reversibility curves for reversible inhibitors because they could present different affinity towards the free enzyme and the enzyme that was bound to the substrate [17]. Thus, the experiment was modified and the reversibiblity of investigated inhibitors with and without the preincubation with the enzyme was performed (see Materials and Methods). Results from the experiments after the modification are shown in Figure 3D,E. Safinamide, **5**, and **9** did not show differences between experiments with and without the preincubation, suggesting that the inhibitors did not bind covalently to the enzyme. On the other hand, rasagiline (irreversible inhibitor) showed higher inhibition when preincubated with hMAOB.
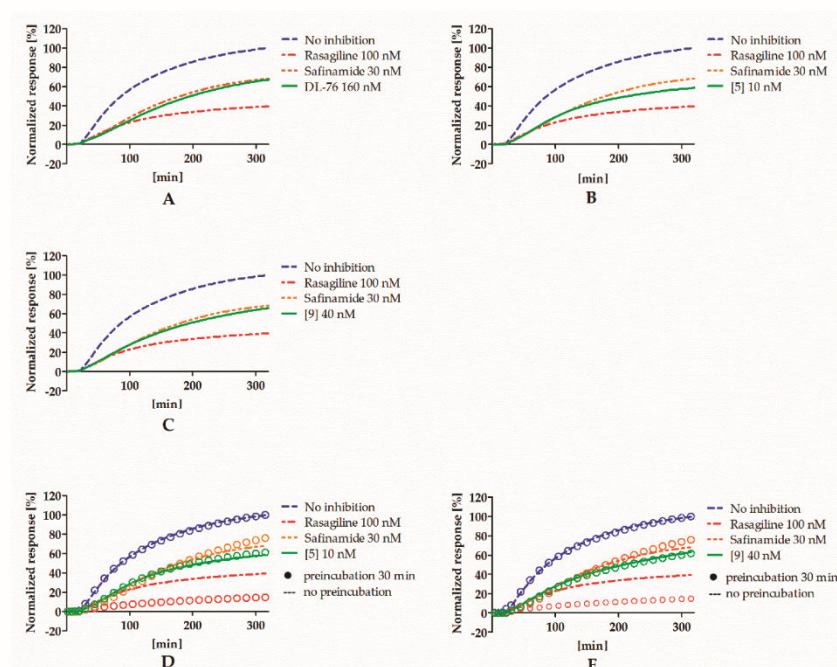
Figure 3. Reversibility of **DL76** (**A**), **5** (**B**), **9** (**C**), and reference inhibitors without the preincubation and comparison of reversibility of **5** (**D**), **9** (**E**), and reference inhibitors that were (rings) or were not (lines) preincubated with hMAOB. Concentrations of compounds correspond to their IC_{80} values.

2.3.3. Kinetic Studies

In order to determine the mode of hMAO B inhibition, compounds **9** and **DL76** were chosen and used in three concentrations corresponding to their IC_{20}, IC_{50}, and IC_{80} values (0.2 nM, 10 nM, and 40 nM for **9** and 7 nM, 48 nM, and 164 nM for **DL76**). Results from the kinetic experiment were used for determination of Michaelis–Menten curves (Figure 4A,C). Later, data were transformed using the Lineweaver–Burk equation to double reciprocal plot (lines from different concentrations of both compound **9** and **DL76** converged to the left of *y*-axis and above *x*-axis; Figure 4B,D). From Michaelis–Menten curves, V_{max} and K_M values were fitted (Table 2). For both compounds, V_{max} values decreased curvilinearly and K_M values increased curvilinearly with the increased inhibitor concentrations. The behavior of these parameters suggested the mixed mode inhibition of **9** and **DL76** [17,18]. This observation was further confirmed by a value of calculated constant α ($\alpha = 1.6$ for **9** and $\alpha = 4.6$ for **DL76**) by GraphPad Prism software from the nonlinear regression (see Supplementary Materials S2). According to Copeland [18,19], $\alpha > 1$ is characteristic for the mixed mode of inhibition where an inhibitor is able to bind to both the free enzyme and the enzyme–substrate complex unequally and its affinity for binding to the free enzyme is higher (see Table S1, Supplementary Materials S2).
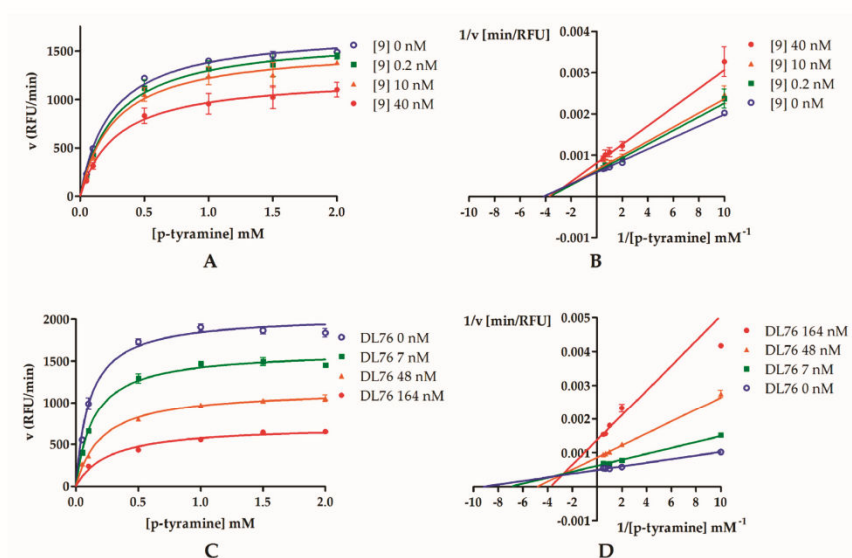
Figure 4. Michaelis–Menten curves for compound **9** (**A**) and **DL76** (**C**): Inhibitors were tested in three concentrations (0.2 nM, 10 nM, and 40 nM for **9** and 7 nM, 48 nM, and 164 nM for **DL76**) representing their IC_{20}, IC_{50}, and IC_{80}, respectively. Substrate (p-tyramine) was used in six concentrations: 0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 1.5 mM, and 2 mM. (**B,D**) Double-reciprocal (Lineweaver–Burk) plots from enzyme kinetic studies.

Table 2. V_{max} and K_m calculated from Michaelis–Menten curves of compound **9** and **DL76**.

Parameters	Compound 9				DL76			
Concentration (nM)	0	0.2	10	40	0	7	48	164
V_{max} (RFU/min)	1713	1652	1544	1238	2046	1628	1165	726.3
K_M (mM)	0.24	0.27	0.27	0.28	0.11	0.14	0.21	0.27

2.4. Human Monoamine Oxidase A Inhibitory Activity

Selected compounds (**DL76, 6, 9, 10, 12, 16**, and **28**) were screened for hMAO A inhibition using Amplex Red® Monoamine Oxidase kits [15]. Tested compounds showed a percentage of inihibition <25% at a concentration of 10 μM. Results indicated very weak or no inhibition of hMAO A by these compounds.

2.5. Toxicity and Neuroprotection Studies In Vitro

For toxicity and neuroprotection studies, **DL76** was chosen as the most promising DTL. The human embryonic kidney (HEK-293) cell line was used for the estimation of safety of **DL76**. As shown in Figure 5, the statistically significant decrease of HEK-293 cell viability in the presence of **DL76** was observed only at the highest doses of 125 μM and 250 μM. The used reference cytostatic drug doxorubicin (DX) decreased cell viability <50% under the same conditions at very low concentrations of 0.2 and 0.05 μM. The neuroprotection effect of **DL76** was investigated in vitro in the model of neuronal damage. Oxidative damage was induced by very high toxic levels of hydrogen peroxide (H_2O_2; 300 μM), the popular cell model for PD research, in neuroblastoma SH-SY5Y cell line. Compound **DL76** was tested at two doses, 10 μM and 50 μM, within two independent experiments. Salsolinol (SAL) was used at a concentration of 50 μM as the reference compound with the proven neuroprotection

activity [20]. **DL76** displayed no increase of cell survival either at 10 μM (Figure 6A) or 50 μM (Figure 6B), whereas SAL statistically significant increased cells viability in both experiments. However, the safety of **DL76** was confirmed here, as no toxic effect against SH-SY5Y cells was observed at both used concentrations 10 and 50 μM (Figure 6A,B).
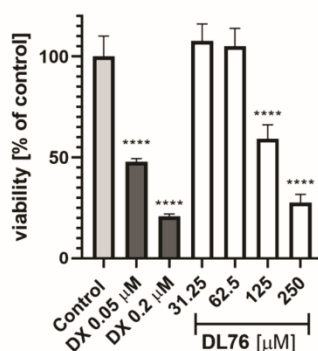
Figure 5. The effect of **DL76** and doxorubicin (DX) on HEK-293 viability: Statistical significance (**** $p < 0.0001$) was analyzed by GraphPadPrism™ 8 software using one-way ANOVA and Bonferroni's multiple comparison posttest in comparison with control (1% DMSO in cell culture medium).
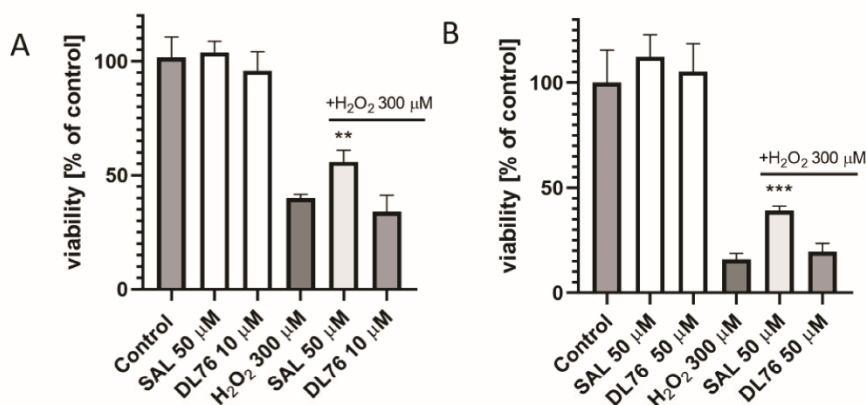
Figure 6. The effect of salsolinol (SAL) at 50 μM and **DL76** at 10 μM (**A**) or at 50 μM (**B**) on SH-SY5Y neuroblastoma cells viability damaged by 300 μM of H_2O_2 after 24 h of incubation: Statistical significance was set at *** $p < 0.001$ and ** $p < 0.01$ by GraphPadPrism™ 8 software using one-way ANOVA and Bonferroni's multiple comparison posttest in comparison with the positive control H_2O_2 (300 μM).

2.6. Antiparkinsonian Activity in Haloperidol-Induced Catalepsy in Wistar Rats

Haloperidol (dopamine D_2 antagonist)-induced catalepsy in rodents is a popular model for screening of antiparkinsonian activity of compounds [21]. Caused symptoms in animals are similar to the inability of PD patients to initiate movements. We used this test to preliminary evaluate antiparkinsonian activity of **DL76**. Haloperidol (0.63 mg/kg; s.c.) was administered to induce catalepsy in Wistar rats; 5 min later, **DL76** was added at doses of 25 or 50 mg/kg (i.p.). The reversal of catalepsy was tested for 6 min (3 times in 3 min intervals) after 1 h of haloperidol administration. As the reference compound was used **MSX-3**, the adenosine A_{2A} receptor antagonist with confirmed ability to reverse

catalepsy mediated by D_1 and D_2 receptor antagonists [22]. To characterize the antiparkinsonian activity of **DL76**, two tests were performed: bar test and crossed-leg position test.

2.6.1. Bar Test

Haloperidol-induced catalepsy was not reduced by **DL-76** administrated at a dose of 25 mg/kg body weight (Table 3). After administration of **DL-76** at a dose of 50 mg/kg body weight, low antiparkinsonian activity in the bar test was observed. The compound reduced the duration of catalepsy by 36.4% compared to the control group, which was obtained after administration of haloperidol (16.4 versus 25.8). This effect was weaker than the anti-Parkisonian activity of **MSX-3**.

Table 3. Antiparkinsonian activity of **DL76** in the bar test.

Compound	Dose (mg/kg; i.p.)	Times of Observation of Catalepsy (s) [1]		
		0 min	3 min	6 min
control	-	17.5 ± 5.3	30.0 ± 0	30.0 ± 0
DL-76	25	19.5 ± 4.2	26.5 ± 3.5	30.0 ± 0
	50	13.8 ± 5.9	13.5 ± 4.2 *	22.0 ± 5.0
MSX-3	25	0 ± 0 ***	0 ± 0 ***	0 ± 0 ***
	50	0 ± 0 ***	0 ± 0 ***	0 ± 0 ***

[1] mean $\pm$ SEM ($n = 6$); * $p < 0.05$; *** $p < 0.001$.

2.6.2. Crossed-Leg Position Test

The strongest, statistically significant antiparkinsonian activity was demonstrated by **DL-76** at a dose of 50 mg/kg body weight, which, compared to the control group, reduced the duration of haloperidol-induced catalepsy by 99.7% (0.053 versus 19.25) (Table 4). This effect was comparable to the antiparkinsonian activity of **MSX-3**.

The weaker, antiparkinsonian activity was demonstrated by **DL-76** administered at a dose of 25 mg/kg body weight. **DL76**, in comparison to the control group, reduced the duration of haloperidol-induced catalepsy by 54.5% (8.75 versus 19.25) (Table 4), but the effect was not statistically significant.

Table 4. Antiparkisonian activity of **DL76** in the crossed-leg position test.

Compound	Dose (mg/kg)	Times of Observation of Catalepsy (s) [1]		
		0 min	3 min	6 min
control	-	4.2 ± 1.6	23.5 ± 6.5	30.0 ± 0
DL-76	25	0.66 ± 0.68	10.6 ± 6.1	15.0 ± 6.7
	50	0 ± 0 ***	0 ± 0 ***	0.16 ± 0.4 ***
MSX-3	25	0 ± 0 ***	0 ± 0 ***	0 ± 0 ***
	50	0 ± 0 ***	0 ± 0 ***	0 ± 0 ***

[1] mean $\pm$ SEM ($n = 6$); *** $p < 0.001$.

3. Materials and Methods

3.1. Chemistry

All reagents were purchased from commercial suppliers Alfa Aesar (Karlsruhe, Germany) or Sigma Aldrich (Darmstadt, Germany) and were used without further purification. TLC data were obtained with Merck silica gel 60F₂₅₄ aluminum sheets with UV light detection and evaluation with Dragendorff's reagent (solvent systems: methylene chloride: methanol 9:1 or methylene chloride). Melting points (m.p.) were determined on a MEL-TEMP II (LD Inc., Long Beach, CA, USA) melting

point apparatus and are uncorrected. Mass spectra (LC/MS) were performed on Waters TQ Detector (Water Corporation., Milford, CT, USA) mass spectrometer. Retention times (t_R) are given in minutes. The UPLC/MS purity of all final compounds was determined (%). All compounds (except **14**, **19**, and **26**) showed purity above 96%. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Mercury 300 MHz PFG spectrometer (Varian, Palo Alto, CA, USA), Avance III HD 400 MHz spectrometer (Bruker, Billerica, MA, USA), or FT-NMR 500 MHz spectrometer (Jeol Ltd., Akishima, Tokyo, Japan). Chemical shifts were expressed in parts per million (ppm) using the solvent signal as an internal standard. Data are reported in the following order: multiplicity (br, broad; d, dublet; m, multiplet; quin, quintet; s, singlet; and t, triplet), approximate coupling constants J expressed in Hertz (Hz), and number of protons. Elemental analyses were measured on analyzer Vario EL III 2 (Elementar, Langenselbold, Germany) and are within ±0.5% of the theoretical values (except carbon for **7** (±0.6%) and **28** (±0.7%).

4-*tert*-Butylphenoxyalkyl bromides (**4a–4d**) were synthesized according to the method described previously [14]. All of them are known and reported in Chemical Abstract Database:

1-(3-bromopropoxy)-4-*tert*-butylbenzene(**4a**):CAS3245-63-4; 1-(4-bromobutoxy)-4-*tert*-butylbenzene (**4b**): CAS53669-73-1;
1-(5-bromopentyloxy)-4-tert-butylbenzene (**4c**): CAS53669-74-2;
1-(6-bromohexyloxy)-4-tert-butylbenzene (**4d**): CAS53669-73-3.

Designed compounds **5–31** were synthesized according to the procedure described previously [12]. Briefly, to a proper 4-*tert*-butylphenylalkoxy bromide (2.5 mmol) in the mixture of C_2H_5OH (52.5 mL) and H_2O (10 mL) and in the presence of K_2CO_3 (7.5 mmol) with the catalytic amount of KI was added a proper amine (5 mmol), and the solution was refluxed from 10 to 48 h. Then, C_2H_5OH was evaporated, and to the residue were added 50 mL of CH_2Cl_2 and 40 mL of water. The organic solution was washed with 50 mL of 1% HCl, evaporated to dryness, dissolved in 3% HCl followed by washing with $(C_2H_5)_2O$, and neutralized with 5% NaOH. The final product was extracted with CH_2Cl_2, dried over Na_2SO_4, and evaporated. The oily residue was transformed into oxalic acid salt in absolute C_2H_5OH and precipitated $(C_2H_5)_2O$. The solid was crystallized from C_2H_5OH.

1-(3-(4-tert-Butylphenoxy)propyl)pyrrolidine hydrogen oxalate (**5**). White solid, yield 27%, m.p. 147–149 °C, $C_{17}H_{27}NO$ x $C_2H_2O_4$ (MW = 351.43). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.29 (d, J = 8.61 Hz, 2H), 6.86 (d, J = 8.61 Hz, 2H), 4.01 (t, J = 6.06 Hz, 2H), 3.20–3.31 (m, 6H), 2.05–2.14 (m, 2H), 1.93 (br s, 4H), 1.25 (s, 9H). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 165.3, 156.5, 143.4, 126.5, 114.4, 65.3, 53.5, 51.9, 34.2, 31.8, 25.9, 23.2.LC-MS: purity 100% t_R = 5.27, (ESI) m/z [M + H]$^+$ 262.24. Anal. calcd. for $C_{19}H_{29}NO_5$: C, 64.93; H, 8.32; N, 3.99%. Found: C, 64.90; H, 8.35; N, 3.93%.

1-(4-(4-tert-Butylphenoxy)butyl)piperidine hydrogen oxalate (**6**). White solid, yield 52%, m.p. 150–152 °C, $C_{19}H_{31}NO$ x $C_2H_2O_4$ (MW = 379.48). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, J = 9.00 Hz, 2H), 6.85 (d, J = 8.61 Hz, 2H), 3.95 (t, J = 5.87 Hz, 2H), 2.95–3.31 (m, 6H), 1.64–1.88 (m, 8H), 1.52 (br. s., 2H), 1.25 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.2, 156.7, 143.2, 126.5, 114.4, 67.2, 56.1, 52.4, 40.9, 34.2, 31.8, 26.5, 23.0, 22.0, 20.8.LC-MS: purity 100% t_R = 5.69, (ESI) m/z [M + H]$^+$ 290.22. Anal. calcd. for $C_{21}H_{33}NO_5$: C, 66.46; H, 8.76; N, 3.69%. Found: C, 66.45; H, 8.48; N, 3.62%.

1-(5-(4-tert-Butylphenoxy)pentyl)piperidine hydrogen oxalate (**7**). White solid, yield 42%, m.p. 170–172 °C, $C_{20}H_{33}NO$ x $C_2H_2O_4$ (MW = 393.51). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, J = 8.61 Hz, 2H), 6.84 (d, J = 8.61 Hz, 2H), 3.93 (t, J = 6.06 Hz, 1H), 2.89–3.39 (m, 6H), 1.72 (br. s., 8H), 1.52 (br. s., 2H), 1.37–1.48 (m, 2H), 1.25 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.1, 156.8, 143.0, 126.5, 114.4, 67.5, 56.3, 52.4, 40.9, 34.2, 31.8, 28.7, 23.4, 23.3, 23.0, 22.0. LC-MS: purity 98.98% t_R = 5.98, (ESI) m/z [M + H]$^+$ 304.18. Anal. calcd. for $C_{22}H_{35}NO_5$: C, 67.14; H, 8.96; N, 3.56%. Found: C, 66.50; H, 8.71; N, 3.42%.

1-(6-(4-tert-Butylphenoxy)hexyl)piperidine hydrogen oxalate (**8**). White solid, yield 16%, m.p. 156–158 °C, $C_{21}H_{35}NO$ x $C_2H_2O_4$ (MW = 407.54). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.27 (d, J = 9.00 Hz, 2H), 6.83 (d, J = 8.61 Hz, 2H), 3.92 (t, J = 6.26 Hz, 2H), 2.86–3.21 (m, 6H), 1.59–1.87 (m, 8H), 1.38–1.56 (m, 4H),

1.34 (d, J = 7.04 Hz, 2H), 1.25 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.3, 156.9, 143.0, 126.5, 114.3, 67.6, 56.3, 52.4, 40.9, 34.2, 31.8, 29.0, 26.4, 25.6, 23.6, 22.9, 22.0.LC-MS: purity 96.38% t_R = 6.39, (ESI) *m/z* [M + H]$^+$ 318.20. Anal. calcd. for C$_{23}$H$_{37}$NO$_5$: C, 67.78; H, 9.15; N, 3.44%. Found: C, 67.26; H, 9.13; N, 3.42%.

1-(3-(4-tert-Butylphenoxy)propyl)-2-methylpiperidine hydrogen oxalate (**9**). White solid, yield 19%, m.p. 114–116 °C, C$_{19}$H$_{31}$NO x C$_2$H$_2$O$_4$ (MW = 379.48). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.30 (d, J = 8.61 Hz, 2H), 6.86 (d, J = 9.00 Hz, 2H), 3.93–4.12 (m, 2H), 3.18–3.44 (m, 3H), 3.06–3.17 (m, 1H), 2.92–3.05 (m, 1H), 2.01–2.20 (m, 2H), 1.78–1.91 (m, 1H), 1.53–1.76 (m, 4H), 1.38–1.52 (m, 1H), 1.28 (d, J = 6.65 Hz, 3H), 1.25 (s, 9H).^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.2, 156.5, 143.4, 126.5, 114.5, 65.4, 57.3, 49.3, 40.9, 34.2, 31.8, 23.4, 22.8. LC-MS: purity 100% t_R = 5.68, (ESI) *m/z* [M + H]$^+$ 290.22. Anal. calcd. for C$_{21}$H$_{33}$NO$_5$: C, 66.46; H, 8.76; N, 3.69%. Found: C, 66.29; H, 8.80; N, 3.64%.

1-(4-(4-tert-Butylphenoxy)butyl)-2-methylpiperidine hydrogen oxalate (**10**). White solid, yield 34%, m.p. 107–110 °C, C$_{20}$H$_{33}$NO x C$_2$H$_2$O$_4$ (MW = 393.51). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.29 (d, J = 9.00 Hz, 2H), 6.85 (d, J = 9.00 Hz, 2H), 3.95 (m, 2H), 3.27 (br.s., 2H), 3.08–3.19 (m, 1H), 2.91–3.08 (m, 2H), 1.68–1.87 (m, 7H), 1.39–1.67 (m, 3H), 1.20 – 1.33 (m, 12H).^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.2, 156.7, 143.2, 126.5, 114.4, 67.3, 51.6, 34.2, 31.8, 26.5, 22.7, 20.2.LC-MS: purity 100% t_R = 5.94, (ESI) *m/z* [M + H]$^+$ 304.24. Anal. calcd. for C$_{22}$H$_{35}$NO$_5$: C, 67.14; H, 8.96; N, 3.56%. Found: C, 67.00; H, 8.95; N, 3.52%.

1-(5-(4-tert-Butylphenoxy)pentyl)-2-methylpiperidine hydrogen oxalate (**11**). White solid, yield 11%, m.p. 96–99 °C, C$_{21}$H$_{35}$NO x C$_2$H$_2$O$_4$ (MW = 407.54). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, J = 6.81 Hz, 2H), 6.84 (d, J = 9.00 Hz, 2H), 3.94 (t, J = 6.26 Hz, 2H), 3.26 (br.s., 2H), 3.03–3.13 (m, 1H), 2.91–3.02 (m, 2H), 1.56–1.86 (m, 9H), 1.40–1.50 (m, 3H), 1.23–1.30 (m, 12H).^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.2, 156.8, 143.0, 126.5, 114.4, 67.5, 51.7, 34.2, 31.8, 28.7, 23.4, 22.9.LC-MS: purity 96.49% t_R = 6.23, (ESI) *m/z* [M + H]$^+$318.27. Anal. calcd. for C$_{23}$H$_{37}$NO$_5$: C, 67.78; H, 9.15; N, 3.44%. Found: C, 67.50; H, 8.97; N, 3.41%.

1-(3-(4-tert-Butylphenoxy)propyl)-3-methylpiperidine hydrogen oxalate (**12**). White solid, yield 29%, m.p. 141–143 °C, C$_{19}$H$_{31}$NO x C$_2$H$_2$O$_4$ (MW = 379.48). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.24 (d, J = 8.88 Hz, 2H), 6.80 (d, J = 8.59 Hz, 2H), 3.95 (t, J = 5.87 Hz, 2H), 3.24–3.43 (m, 2H), 3.07 (t, J = 7.59 Hz, 2H), 2.63–2.77 (m, 1H), 2.37–2.45 (m, 1H), 2.00–2.16 (m, 2H), 1.78–1.90 (m, 1H), 1.61–1.77 (m, 3H), 1.20 (s, 9H), 0.94–1.10 (m, 1H), 0.85 (d, J = 6.59 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 165.4, 156.6, 143.4, 126.6, 114.5, 65.5, 58.0, 54.0, 52.1, 34.3, 31.9, 30.6, 29.0, 24.1, 22.8, 19.1. LC-MS: purity 98.04% t_R = 5.75, (ESI) *m/z* [M + H]$^+$ 290.22. Anal. calcd. for C$_{21}$H$_{33}$NO$_5$: C, 66.46; H, 8.76; N, 3.69%. Found: C, 66.44; H, 8.42; N, 3.65%.

1-(4-(4-tert-Butylphenoxy)butyl)-3-methylpiperidine hydrogen oxalate (**13**). White solid, yield 53%, m.p. 148–150 °C, C$_{20}$H$_{33}$NO x C$_2$H$_2$O$_4$ (MW = 393.51). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, J = 8.61 Hz, 2H), 6.85 (d, J = 8.61 Hz, 2H), 3.95 (t, J = 6.06 Hz, 2H), 3.24–3.45 (m, 2H), 2.94–3.11 (m, 2H), 2.67–2.78 (m, 2H), 1.62–1.97 (m, 8H), 1.25 (s, 9H), 1.06 (m, 1H), 0.89 (d, J = 6.65 Hz, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.2, 156.7, 143.1, 126.5, 114.4, 67.2, 57.9, 56.1, 51.9, 40.9, 34.2, 31.8, 30.6, 28.9, 26.5, 22.6, 20.8, 19.1.LC-MS: purity 99.46% t_R = 6.13, (ESI) *m/z* [M + H]$^+$ 304.24. Anal. calcd. for C$_{22}$H$_{35}$NO$_5$: C, 67.14; H, 8.96; N, 3.56%. Found: C, 66.86; H, 8.62; N, 3.51%.

1-(5-(4-tert-Butylphenoxy)pentyl)-3-methylpiperidine hydrogen oxalate (**14**). White solid, yield 8%, m.p. 179–181 °C, C$_{21}$H$_{35}$NO x C$_2$H$_2$O$_4$ (MW = 407.54). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, J = 8.22 Hz, 2H), 6.84 (d, J = 7.83 Hz, 2H), 3.94 (br. s., 2H), 3.19–3.48 (m, 2H), 2.97 (br. s., 4H), 1.57–1.99 (m, 8H), 1.35–1.54 (m, 2H), 1.26 (br. s., 9H), 1.06 (d, J = 9.39 Hz, 1H), 0.69–0.97 (m, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.0, 156.8, 143.1, 126.5, 114.4, 67.5, 58.0, 56.4, 52.0, 41.0, 34.2, 31.8, 30.6, 28.7, 23.5, 23.3, 19.1.LC-MS: purity 93.40% t_R = 6.40, (ESI) *m/z* [M + H]$^+$ 318.20. Anal. calcd. for C$_{23}$H$_{37}$NO$_5$: C, 67.78; H, 9.15; N, 3.44%. Found: C, 67.44; H, 8.90; N, 3.43%.

1-(6-(4-tert-Butylphenoxy)hexyl)-3-methylpiperidine hydrogen oxalate (**15**). White solid, yield 7%, m.p. 140–142 °C, $C_{22}H_{37}NO$ x $C_2H_2O_4$ (MW = 421.56). ^1H NMR (400 MHz, DMSO-d_6) δ: 7.27 (d, J = 8.61 Hz, 2H), 6.83 (d, J = 9.00 Hz, 2H), 3.92 (t, J = 6.46 Hz, 2H), 3.23–3.43 (m, 2H), 2.88–3.04 (m, 2H), 2.68–2.76 (m, 2H), 1.57–1.95 (m, 8H), 1.43 (quin, J = 7.34 Hz, 2H), 1.33 (quin, J = 7.04 Hz, 2H), 1.25 (s, 9H), 0.97–1.14 (m, 1H), 0.89 (d, J = 6.65 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.2, 156.9, 143.0, 126.5, 114.3, 67.6, 57.8, 56.3, 51.9, 40.9, 34.2, 31.8, 30.6, 29.0, 28.9, 26.4, 25.6, 23.6, 22.6, 19.1. LC-MS: purity 100% t_R = 6.75, (ESI) m/z [M + H]$^+$ 332.22. Anal. calcd. for $C_{24}H_{39}NO_5$: C, 68.37; H, 9.32; N, 3.32%. Found: C, 68.14; H, 9.20; N, 3.28%.

1-(3-(4-tert-Butylphenoxy)propyl)-3,3-dimethylpiperidine hydrogen oxalate (**16**). White solid, yield 44%, m.p. 183–186 °C, $C_{20}H_{33}NO$ x $C_2H_2O_4$ (MW = 393.51). ^1H NMR (400 MHz, DMSO-d_6) δ: 7.30 (d, J = 8.61 Hz, 2H), 6.85 (d, J = 8.61 Hz, 2H), 3.99 (t, J = 5.67 Hz, 2H), 2.79–3.30 (m, 6H), 2.10 (br. s., 2H), 1.74 (br. s., 2H), 1.36 (br. s., 2H), 1.25 (s, 9H), 1.00 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.7, 156.5, 143.4, 126.5, 114.4, 65.6, 62.1, 55.0, 52.8, 40.9, 35.3, 34.2, 31.8, 30.9, 24.2, 20.2. LC-MS: purity 100% t_R = 6.02, (ESI) m/z [M + H]$^+$ 304.24. Anal. calcd. for $C_{22}H_{35}NO_5$: C, 67.14; H, 8.96; N, 3.56%. Found: C, 67.19; H, 8.50; N, 3.51%.

1-(4-(4-tert-Butylphenoxy)butyl)-3,3-dimethylpiperidine hydrogen oxalate (**17**). White solid, yield 23%, m.p. 140–142 °C, $C_{21}H_{35}NO$ x $C_2H_2O_4$ (MW = 407.54). ^1H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, J = 8.61 Hz, 2H), 6.85 (d, J = 8.61 Hz, 2H), 3.95 (t, J = 5.87 Hz, 2H), 2.91–3.20 (m, 4H), 2.80 (br. s., 2H), 1.63–1.90 (m, 6H), 1.37 (d, J = 4.70 Hz, 2H), 1.25 (s, 9H), 0.99 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.0, 156.7, 143.1, 126.5, 114.4, 67.2, 61.8, 57.1, 52.5, 40.9, 35.2, 34.2, 31.8, 30.9, 26.6, 20.8, 20.1. LC-MS: purity 100% t_R = 6.29, (ESI) m/z [M + H]$^+$ 318.27. Anal. calcd. for $C_{23}H_{37}NO_5$: C, 67.78; H, 9.15; N, 3.44%. Found: C, 67.57; H, 9.03; N, 3.39%.

1-(5-(4-tert-Butylphenoxy)pentyl)-3,3-dimethylpiperidine hydrogen oxalate (**18**). White solid, yield 40%, m.p. 150–153 °C, $C_{22}H_{37}NO$ x $C_2H_2O_4$ (MW = 421.56). ^1H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, J = 8.61 Hz, 2H), 6.84 (d, J = 9.00 Hz, 2H), 3.93 (t, J = 6.26 Hz, 2H), 2.88–3.24 (m, 4H), 2.81 (br. s., 2H), 1.61–1.83 (m, 6H), 1.30–1.45 (m, 4H), 1.25 (s, 9H), 0.99 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.0, 156.8, 143.0, 126.5, 114.4, 67.4, 61.8, 57.3, 52.5, 40.9, 35.2, 34.2, 31.8, 30.9, 28.7, 23.4, 23.3, 20.0. LC-MS: purity 96.27% t_R = 6.58, (ESI) m/z [M + H]$^+$ 332.29. Anal. calcd. for $C_{24}H_{39}NO_5$: C, 68.37; H, 9.32; N, 3.32%. Found: C, 67.94; H, 8.99; N, 3.24%.

1-(6-(4-tert-Butylphenoxy)hexyl)-3,3-dimethylpiperidine hydrogen oxalate (**19**). White solid, yield 21%, m.p. 122–125 °C, $C_{23}H_{39}NO$ x $C_2H_2O_4$ (MW = 435.59). ^1H NMR (400 MHz, DMSO-d_6) δ: 7.27 (d, J = 8.61 Hz, 2H), 6.83 (d, J = 8.61 Hz, 2H), 3.83–4.01 (m, J = 6.46, 6.46 Hz, 2H), 2.75–3.13 (m, 6H), 1.57–1.83 (m, 6H), 1.29–1.54 (m, 6H), 1.25 (s, 9H), 0.99 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.1, 156.9, 143.0, 126.5, 114.3, 67.6, 61.7, 57.3, 52.4, 40.9, 35.2, 34.2, 31.8, 30.9, 29.0, 26.4, 25.6, 23.5, 20.0. LC-MS: purity 94.18% t_R = 6.97, (ESI) m/z [M + H]$^+$ 346.25. Anal. calcd. for $C_{25}H_{41}NO_5$: C, 68.93; H, 9.46; N, 3.22%. Found: C, 68.80; H, 9.26; N, 3.22%.

1-(3-(4-tert-Butylphenoxy)propyl)-3,5-dimethylpiperidine hydrogen oxalate (**20**). White solid, yield 43%, m.p. 143–145 °C, $C_{20}H_{33}NO$ x $C_2H_2O_4$ (MW = 393.51). ^1H NMR (400 MHz, DMSO-d_6) δ: 7.29 (d, J = 9.00 Hz, 2H), 6.85 (d, J = 8.61 Hz, 2H), 4.00 (t, J = 5.67 Hz, 2H), 3.34 (d, J = 10.17 Hz, 2H), 2.97–3.23 (m, 2H), 2.40 (t, J = 11.93 Hz, 2H), 2.02–2.23 (m, 2H), 1.91 (d, J = 3.52 Hz, 2H), 1.73 (d, J = 12.91 Hz, 1H), 1.33–1.49 (m, 1H), 1.25 (s, 9H), 0.99 (d, J = 6.65 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.0, 156.5, 143.4, 126.5, 114.4, 65.5, 57.6, 53.9, 34.2, 31.8, 28.8, 24.1, 18.9. LC-MS: purity 15.62% t_R = 6.08, (ESI) m/z [M + H]$^+$ 304.24 and purity 84.38% t_R = 6.15, (ESI) m/z [M + H]$^+$ 304.24. Anal. calcd. for $C_{22}H_{35}NO_5$: C, 67.14; H, 8.96; N, 3.56%. Found: C, 67.03; H, 9.14; N, 3.52%.

1-(4-(4-tert-Butylphenoxy)butyl)-3,5-dimethylpiperidine hydrogen oxalate (**21**). White solid, yield 39%, m.p. 170–172 °C, $C_{21}H_{35}NO$ x $C_2H_2O_4$ (MW = 407.54). ^1H NMR (400 MHz, DMSO-d_6) δ: 7.29 (d, J = 8.61 Hz, 2H), 6.85 (d, J = 8.61 Hz, 2H), 3.96 (t, J = 6.06 Hz, 2H), 3.31 (d, J = 9.78 Hz, 2H), 2.90–3.09 (m, 2H), 2.37 (t, J = 11.93 Hz, 2H), 1.61–2.02 (m, 7H), 1.13–1.44 (s, 10H), 0.87–1.04 (m, 6H). ^{13}C NMR (101 MHz,

DMSO-d_6) δ:165.0, 156.7, 143.2, 126.5, 114.4, 67.2, 57.4, 56.1, 34.2, 31.8, 28.7, 26.5, 20.8, 18.9. LC-MS: purity 11.21% t_R = 6.31, (ESI) *m/z* [M + H]$^+$ 318.46 and purity 88.79% t_R = 6.37, (ESI) *m/z* [M + H]$^+$ 318.46. Anal. calcd. for $C_{23}H_{37}NO_5$: C, 67.78; H, 9.15; N, 3.44%. Found: C, 68.14; H, 8.87; N, 3.40%.

1-(5-(4-tert-Butylphenoxy)pentyl)-3,5- dimethylpiperidine hydrogen oxalate (**22**). White solid, yield 31%, m.p. 175–178 °C, $C_{22}H_{37}NO \times C_2H_2O_4$ (MW = 421.56). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, *J* = 8.61 Hz, 2H), 6.84 (d, *J* = 8.61 Hz, 2H), 3.94 (t, *J* = 6.06 Hz, 2H), 3.31 (d, *J* = 10.56 Hz, 2H), 2.80–3.06 (m, 2H), 2.36 (t, *J* = 11.74 Hz, 2H), 1.59–2.12 (m, 7H), 1.15–1.52 (m, 12H), 0.83–0.99 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.9, 156.8, 143.1, 126.5, 114.4, 67.4, 57.5, 34.2, 31.8, 28.7, 23.5, 23.3, 18.9.LC-MS: purity 6.64% t_R = 6.73, (ESI) *m/z* [M + H]$^+$ 332.29 and purity 93.36% t_R = 6.78, (ESI) *m/z* [M + H]$^+$ 332.29. Anal. calcd. for $C_{24}H_{39}NO_5$: C, 68.37; H, 9.32; N, 3.32%. Found: C, 68.25; H, 9.06; N, 3.30%.

1-(6-(4-tert-Butylphenoxy)hexyl)-3,5- dimethylpiperidine hydrogen oxalate (**23**). White solid, yield 43%, m.p. 143–145 °C, $C_{23}H_{39}NO \times C_2H_2O_4$ (MW = 345.56). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, *J* = 8.61 Hz, 2H), 6.84 (d, *J* = 8.61 Hz, 2H), 3.93 (t, *J* = 6.26 Hz, 2H), 3.30 (d, *J* = 10.17 Hz, 2H), 2.81–3.04 (m, 2H), 2.36 (t, *J* = 11.93 Hz, 2H), 1.88 (br. s., 2H), 1.60–1.78 (m, 5H), 1.20–1.53 (m, 14H), 0.89 (d, *J* = 6.65 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.9, 156.9, 143.0, 126.5, 114.4, 67.6, 57.5, 34.2, 31.8, 28.9, 26.4, 25.6, 23.7, 18.9.LC-MS: purity 5.30% t_R = 6.96, (ESI) *m/z* [M + H]$^+$ 346.31 and purity 94.70% t_R = 7.00, (ESI) *m/z* [M + H]$^+$ 346.31. Anal. calcd. for $C_{25}H_{41}NO_5$: C, 68.93; H, 9.49; N, 3.22%. Found: C, 68.74; H, 9.64; N, 3.19%.

1-(3-(4-tert-Butylphenoxy)propyl)-4-methylpiperidine hydrogen oxalate (**24**). White solid, yield 29%, m.p. 158–160 °C, $C_{19}H_{31}NO \times C_2H_2O_4$ (MW = 379.48). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.25 (d, *J* = 8.88 Hz, 2H), 6.81 (d, *J* = 8.59 Hz, 2H), 3.95 (t, *J* = 6.01 Hz, 8H), 3.35 (d, *J* = 11.74 Hz, 2H), 3.02–3.15 (m, 2H), 2.82 (t, *J* = 11.60 Hz, 2H), 2.00–2.11 (m, 2H), 1.72 (d, *J* = 13.17 Hz, 2H), 1.57 (d, *J* = 6.01 Hz, 1H), 1.29–1.43 (m, 2H), 1.20 (s, 9H), 0.88 (d, *J* = 6.59 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 165.2, 156.5, 143.4, 126.6, 114.5, 65.5, 53.8, 53.7, 52.2, 52.1, 52.1, 52.1, 52.1, 34.3, 31.9, 31.2, 31.2, 31.1, 31.1, 31.1, 31.1, 31.0, 28.5, 24.2, 21.4, 21.4.LC-MS: purity 98.17% t_R = 5.84, (ESI) *m/z* [M + H]$^+$ 290.22. Anal. calcd. for $C_{21}H_{33}NO_5$: C, 66.46; H, 8.76; N, 3.69%. Found: C, 65.97; H, 8.46; N, 3.63%.

1-(4-(4-tert-Butylphenoxy)butyl)-4-methylpiperidine hydrogen oxalate (**25**). White solid, yield 47%, m.p. 155–157 °C, $C_{20}H_{33}NO \times C_2H_2O_4$ (MW = 393.51). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, *J* = 9.00 Hz, 2H), 6.85 (d, *J* = 9.00 Hz, 2H), 3.95 (t, *J* = 5.87 Hz, 2H), 3.37 (d, *J* = 11.74 Hz, 2H), 2.97–3.13 (m, 2H), 2.84 (t, *J* = 11.54 Hz, 2H), 1.67–1.90 (m, 6H), 1.61 (d, *J* = 5.87 Hz, 1H), 1.33–1.50 (m, 2H), 1.25 (s, 9H), 0.91 (d, *J* = 6.65 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.2, 156.7, 143.1, 126.5, 114.4, 67.2, 55.8, 51.8, 40.9, 34.2, 31.8, 30.9, 28.5, 26.5, 21.3, 20.9. LC-MS: purity 99.02% t_R = 6.07, (ESI) *m/z* [M + H]$^+$ 304.24. Anal. calcd. for $C_{22}H_{35}NO_5$: C, 67.14; H, 8.96; N, 3.56%. Found: C, 67.41; H, 8.48; N, 3.56%.

1-(5-(4-tert-Butylphenoxy)pentyl)-4-methylpiperidine hydrogen oxalate (**26**). White solid, yield 8%, m.p. 167–170 °C, $C_{21}H_{35}NO \times C_2H_2O_4$ (MW = 407.54). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, *J* = 8.61 Hz, 2H), 6.84 (d, *J* = 8.61 Hz, 2H), 3.94 (t, *J* = 6.26 Hz, 2H), 3.31–3.41 (m, 2H), 2.93–3.03 (m, 2H), 2.77–2.87 (m, 2H), 1.64–1.82 (m, 6H), 1.54–1.64 (m, 1H), 1.32–1.48 (m, 4H), 1.20–1.31 (m, 9H), 0.92 (d, *J* = 6.26 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.0, 156.8, 143.0, 126.5, 114.4, 67.4, 40.9, 34.2, 31.8, 28.7, 23.6, 23.3.LC-MS: purity 92.34% t_R = 6.34, (ESI) *m/z* [M + H]$^+$ 318.20. Anal. calcd. for $C_{23}H_{37}NO_5$: C, 67.78; H, 9.15; N, 3.44%. Found: C, 67.65; H, 8.81; N, 3.42.

1-(6-(4-tert-Butylphenoxy)hexyl)-4-methylpiperidine hydrogen oxalate (**27**). White solid, yield 12%, m.p. 136–139 °C, $C_{22}H_{37}NO \times C_2H_2O_4$ (MW = 421.56). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.27 (d, *J* = 8.61 Hz, 2H), 6.83 (d, *J* = 9.00 Hz, 2H), 3.92 (t, *J* = 6.46 Hz, 2H), 3.36 (d, *J* = 11.74 Hz, 2H), 2.89–3.03 (m, 2H), 2.76–2.87 (m, 2H), 1.54–1.82 (m, 7H), 1.29–1.48 (m, 6H), 1.25 (s, 9H), 0.91 (d, *J* = 6.26 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.3, 156.9, 143.0, 126.5, 114.3, 67.6, 56.0, 51.8, 40.9, 34.2, 31.8, 30.9, 29.0, 28.5, 26.4, 25.6, 23.7, 21.3. LC-MS: purity 98.49% t_R = 6.77, (ESI) *m/z* [M + H]$^+$ 332.22. Anal. calcd. for $C_{24}H_{39}NO_5$: C, 68.37; H, 9.32; N, 3.32%. Found: C, 68.51; H, 9.40; N, 3.30%.

1-(3-(4-tert-Butylphenoxy)propyl)-azepane hydrogen oxalate (**28**). White solid, yield 32%, m.p. 138–140 °C, $C_{19}H_{31}NO$ x $C_2H_2O_4$ (MW = 379.48). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.24 (d, J = 3.15 Hz, 2H), 6.81 (d, J = 2.86 Hz, 2H), 3.95 (br. s., 2H), 2.88–3.47 (m, 6H), 2.08 (br. s., 2H), 1.76 (br. s., 4H), 1.56 (br. s., 4H), 1.20 (br. s., 9H). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 165.5, 156.6, 143.4, 126.6, 114.5, 65.5, 54.3, 54.1, 34.3, 31.9, 26.6, 24.4, 23.6. LC-MS: purity 99.17% t_R = 5.82, (ESI) m/z [M + H]$^+$ 290.22. Anal. calcd. for $C_{21}H_{33}NO_5$: C, 66.46; H, 8.76; N, 3.69%. Found: C, 65.74; H, 8.41; N, 3.65%.

1-(4-(4-tert-Butylphenoxy)butyl)-azepane hydrogen oxalate (**29**). White solid, yield 49%, m.p. 149–151 °C, $C_{20}H_{33}NO$ x $C_2H_2O_4$ (MW = 393.51). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, J = 8.22 Hz, 2H), 6.85 (d, J = 7.82 Hz, 2H), 3.95 (t, J = 5.67 Hz, 2H), 3.16–3.32 (m, 4H), 3.11 (d, J = 7.04 Hz, 2H), 1.68–1.89 (m, 8H), 1.60 (br. s., 4H), 1.25 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.3, 156.7, 143.1, 126.5, 114.4, 67.2, 56.4, 53.9, 40.9, 34.2, 31.8, 26.6, 26.5, 23.5, 21.2. LC-MS: purity 99.24% t_R = 6.01, (ESI) m/z [M + H]$^+$ 304.24. Anal. calcd. for $C_{22}H_{35}NO_5$: C, 67.14; H, 8.96; N, 3.56%. Found: C, 67.40; H, 8.51; N, 3.55%.

1-(5-(4-tert-Butylphenoxy)pentyl)-azepane hydrogen oxalate (**30**). White solid, yield 38%, m.p. 151–153 °C, $C_{21}H_{35}NO$ x $C_2H_2O_4$ (MW = 407.53). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.28 (d, J = 8.61 Hz, 2H), 6.84 (d, J = 9.00 Hz, 2H), 3.93 (t, J = 6.26 Hz, 2H), 3.13–3.32 (m, 4H), 2.94–3.08 (m, 2H), 1.79 (br. s., 4H), 1.66–1.75 (m, 4H), 1.54–1.64 (m, 4H), 1.43 (d, J = 7.43 Hz, 2H), 1.25 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.3, 156.8, 143.0, 126.5, 114.4, 67.5, 56.6, 53.9, 40.9, 34.2, 31.8, 28.7, 26.6, 23.8, 23.4, 23.3. LC-MS: purity 100% t_R = 6.27, (ESI) m/z [M + H]$^+$ 366.25. Anal. calcd. for $C_{23}H_{37}NO_5$: C, 67.78; H, 9.15; N, 3.44%. Found: C, 67.47; H, 8.75; N, 3.39%.

1-(6-(4-tert-Butylphenoxy)hexyl)-azepane hydrogen oxalate (**31**). White solid, yield 5%, m.p. 130–132 °C, $C_{22}H_{37}NO$ x $C_2H_2O_4$ (MW = 421.56). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.27 (d, J = 8.61 Hz, 2H), 6.83 (d, J = 8.61 Hz, 2H), 3.92 (t, J = 6.46 Hz, 2H), 3.16–3.23 (m, 4H), 2.98–3.04 (m, 2H), 1.58–1.82 (m, 12H), 1.28–1.47 (m, 4H), 1.25 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.3, 156.8, 143.0, 126.5, 114.3, 67.6, 56.7, 53.9, 40.9, 34.2, 31.8, 29.0, 26.6, 26.4, 25.6, 24.0, 23.4. LC-MS: purity 97.93% t_R = 6.79, (ESI) m/z [M + H]$^+$ 332.22. Anal. calcd. for $C_{24}H_{39}NO_5$: C, 68.37; H, 9.32; N, 3.32%. Found: C, 68.46; H, 8.98; N, 3.32%.

3.2. Biological Studies In Vitro

3.2.1. Affinity for Human Histamine H₃ Receptor

The radioligand displacement binding assay was carried out in HEK-293 cells stably expressing the recombinant hH₃R as described by Kottke et al. [23] with slight modifications [14]. [^{3}H]N^α-methylhistamine was used as radiolabeled tracer (2 nM, K_D = 3.08). Obtained data from at least three experiments (in at least duplicates) were analyzed with GraphPad Prism 6.1 (San Diego, CA, USA) using nonlinear regression (one-site competition on logarithmic scale), and K_i values were transformed from IC_{50} according to Cheng-Prusoff [24]. Statistical analysis was performed on $-\log K_i$ values. Mean values and 95% confidence intervals were converted to nanomolar concentrations.

3.2.2. Human Monoamine Oxidase Inhibitory Activity

General Method for Determining Activity Against MAO Isoforms

Inhibitory potency of compounds on MAO isoenzymes were carried out by a fluorometric method with a commercial Amplex™ Red Monoamine Oxidase Assay Kit (ThermoFisher Scientific A12214, Waltham, MA, USA) as described previously [12,25]. For all tests, recombinant human MAO B and MAO A (from Sigma AldrichM7441 and M7316, Darmstadt, Germany) were used. Inhibitors' activity was measured in the presence of p-tyramine (200 μM). In all experiments, reference inhibitors in the concentrations that fully inhibited the MAO isoform were included. Reference inhibitors for MAO-B were pargyline 10 μM, rasagiline 1 μM, and safinamide 1 μM and, for MAO-A, was clorgyline 1 μM.

Screening and Determination of IC_{50}

Firstly, inhibitors' activity was measured in a concentration of 1 μM. The results were normalized (no inhibition = 0% and fully inhibited enzyme 100%). For compounds that inhibited the enzyme by more than 50%, further studies were conducted to obtain IC_{50} from concentration–response curves. All calculations were made in Microsoft Excel and GraphPadPrism software. All experiments were performed in duplicate, and data are expressed as mean ± SEM of 2–5 independent experiments.

Reversibility Studies

Reversibility of MAO B inhibitors was tested as described previously [12] with slight modification. Two experiments were performed simultaneously. In the first experiment, inhibitors (in concentrations corresponding to their IC_{80} values) were incubated with the enzyme and p-tyramine (10 μM) for 22 min (measuring fluorescence every two minutes). Next, the concentration of the substrate was increased to 1 mM and the fluorescence was measured every 5 min for 5 h. In the second experiment, inhibitors and enzyme were preincubated for 30 min in room temperature before addition of 10 μM p-tyramine; then, continuation of the experiment was carried out identically as the first.

Kinetic Studies

The mode of the inhibition was tested according to the standard procedure described in Reversibility Studies using different concentrations of the substrate [12,26]. Inhibitors (compound **9** and **DL76**) were used in three concentrations corresponding to their IC_{20}, IC_{50}, and IC_{80} values. Substrate was used in six concentrations: 0.05 nM, 0.1 mM, 0.5 mM, 1 mM, 1.5 mM, and 2 mM. After the experiment, velocities were calculated and put on the graph (y-axis) against the concentration of the substrate (x-axis). From the Michaelis–Menten plot V_{max}, K_M, and α values were calculated for different concentrations of the inhibitor. Double-reciprocal plot (Lineweaver–Burk plot) were prepared to display the data.

3.2.3. Toxicity and Neuroprotection Evaluation In Vitro

Cell Lines

SH-SY5Y CRL-2266™ neuroblastoma cell line was purchased directly from American Type Culture Collection (Manassas, VG, USA) and were cultured as described previously [20]. Human embryonic kidney HEK-293 cell line (ATCC CRL-1573) was kindly donated by Prof. Dr Christa Müller (Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Germany). The cells were cultured as described previously [27].

Toxicity Studies

The HEK-293 cells were seeded at a concentration of 1.5×10^4 cells/well in 200 μL culture medium and incubated for 24 h at 37 °C and 5% CO_2. Next, the cytostatic reference doxorubicin (DX) or **DL76** dissolved in DMSO were added at various concentrations into microplate (total concentration of DMSO in media was 1%) and incubated for 48 h at 37 °C and 5% CO_2. Then, 25 μL EZ4U labelling mixture (Biomedica, Vienna, Austria) was added and the cells were incubated for 2 h. The spectrophotometric absorbance of the samples was measured using a microplate reader (EnSpire, PerkinElmer, Waltham, MA, USA) at 492 nm. All measurements were performed in triplicate, and results are shown as mean ± SD.

Neuroprotection Studies

SH-SY5Y cells were seeded in microplate at a concentration of 2.5×10^4 cells/well in 100 μL culture medium and cultured for 24 h at 37 C and 5% CO_2 to reach 70% confluence. The cells were preincubated first for 1 h with **DL76** (10 and 50 μM) or with the reference neuroprotectant salsolinol.

(R,S)-salsolinol (purity ≥ 99%) was obtained from Cayman Chemical (Ann Arbor, MI, USA). Next, H_2O_2 was added at final concentration 300 µM and the cells were placed into the incubator. After 24 h of compounds co-incubation with H_2O_2, the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) labeling mixture was added to each well, and the microplates were incubated under the same conditions for 5 h. The absorbance was measured using a microplate reader EnSpire (PerkinElmer, Waltham, MA USA) at 490 nm. All measurements were performed in triplicate, and results are shown as mean ± SD.

3.3. Antiparkinsonian Activity in Haloperidol-Induced Catalepsy In Vivo

3.3.1. Animals

The experiments were carried out on male rats Wistar (180–220 g). Animals were housed in plastic cages in room at a constant temperature of 20 ± 2 °C with natural light–dark cycles. The animals had free access to standard pellet diet and water and were used after a minimum of 3 days of acclimatization to the housing conditions. Control and experimental groups consisted of 6 animals each. All experimental procedures were performed according to the European Union Directive of 22 September 2010 (2010/63/EU) and Polish legislation concerning animal care and use and was approved by the Local Ethics Committee for Experiments on Animals in Kraków, Poland (Resolution No. 70/2014, approval date: 20 May 2014). The examined compound was administered as the suspension in 0.5% methylcellulose in constant volume of 10 mL/kg.

3.3.2. Drugs

Haloperidol was purchased from Sigma Aldrich (Darmstadt, Germany). MSX-3 ((E)-phosphoric acid mono-[3-[8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydro-purin-3-yl]propyl] ester disodium salt) was synthesized in the laboratory of Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Germany and donated by Prof. Dr Christa Müller.

3.3.3. Statistical Analysis

The data are expressed as the means ± SEM. All statistical calculations were carried out with the GraphPad Prism 5 program. The data were evaluated by one-way analysis of variance (ANOVA) followed by Duncan test; $p < 0.05$ was considered significant.

3.3.4. Determination of Antiparkinsonian Activity in Catalepsy Tests

Antiparkinsonian activity in catalepsy tests was performed as described in [28]. Haloperidol was administered s.c. at a dose of 0.63 mg/kg, which in 100% of control animals caused catalepsy. Tested compounds were administered i.p. at the doses 50 and 25 mg/kg body weight, 5 min after haloperidol injection. After 60 min from the injection of haloperidol, to assess the occurrence of catalepsy, the animals were placed in a forced position and the time they remained in this position was measured. Two tests were performed to determine the antiparkinsonian activity: crossed leg position test and bar test. In the first test, the animals were put on the hind paws behind the front (forced position); in the second, the animals were supported on a wooden block (the front paws were placed on a block suspended 10 cm above the ground). Then, the time that the animals remain is measured in a forced position (time was measured to a maximum of 60 s). Observation was carried out 3 times in 3-min intervals. The shortening of the time of catalepsy to the control group was adopted for the antiparkinsonian activity.

4. Conclusions

In summary, in this study, new potential DTL for PD have been designed and synthesized. As the lead structure, **DL76** was chosen, the compound with proven high hH₃R affinity (K_i = 22 nM [13] in CHO K1 cells and K_i = 38 nM in HEK 293 cells) and hMAO B inhibitory activity in vitro (IC_{50} = 48 nM).

The introduced modifications were aimed at assessing the influence of cyclic amines and the length of the alkyl chain on hH₃R affinity and hMAO B inhibition. Most compounds showed nanomolar range affinities for hH₃R. The significant increase in the inhibitory effect for hMAO B occurred for pyrrolidine (**5**) and 2-methylpiperidine (**9**) derivatives. These results confirmed our previous observations concerning 4-*tert*-amylphenoxy derivatives [12] where such compounds were among the most potent hMAO B inhibitors.

In vitro toxicity studies with **DL76** in the HEK293 cells and neuroblastoma SH-SY5Y cells did not show risk of toxicity at the dose of 50 μM of this compound. However, no neuroprotection effect of **DL76** against very high toxic levels of hydrogen peroxide (300 μM) in neuroblastoma SH-SY5Y cells was observed.

Conducted in vivo studies showed that tested **DL76** caused just statistically significant antiparkinsonian activity in the crossed-leg position test. The tested compound, at the dose of 50 mg/kg body weight, practically completely reduced the duration of catalepsy, whereas in the bar test at this dose, a low positive effect was observed. Moreover, **DL76** did not show any cataleptic effects.

Considering the dual functional profile, the most valuable compound proved to be **DL76** with balanced activity against both biological targets (hH₃R K_i = 38 nM and hMAO B IC_{50} = 48 nM). Structural modification in DTL is not easy as it requires optimization towards both targets. By direct exchange of a cyclic amine, it was possible to obtain a very potent hMAO B inhibitor, compound **5** with the IC_{50} of 2.7 nM. However, this compound proved to be only a moderate hH₃R ligand (K_i = 371 nM). Generally, very potent hMAO B inhibitors (**5, 9,** and **28**) showed higher strength to inhibit hMAO B than to block hH₃R. Thus, it seems that, in this class of compounds, a cyclic amine moiety in the western part of a molecule plays the very important role in the interaction with hMAO B, but further studies should be performed to confirm it. However, the presented compounds are promising starting materials in further search for new active DTL for PD.

Abbreviations

AChE	Acetylcholinesterase
BuChE	Butyrylcholinesterase
DA	Dopamine
DMSO	Dimethyl sulfoxide
DTL	Dual Target Ligands
DX	Doxorubicin
H₃R	Histamine H₃ receptor
HEK293	Human embryonic kidney
i.p.	Intraperitoneal
MAO B	Monoamine oxidase B
MTDL	Multi-Target-Directed Ligands
PD	Parkinson's disease
PEA	β-phenylethylamine

SAL	Salsolinol
s.c.	Subcutaneous
TLC	Thin-layer Chromatography

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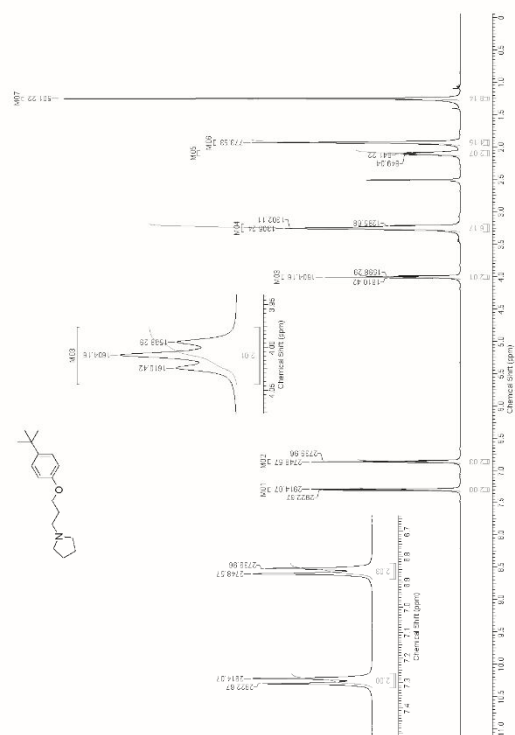
Supplementary Materials S1

Dual target ligands with 4-*tert*-butylphenoxy scaffold as histamine H₃ receptor antagonists and monoamine oxidase B inhibitors

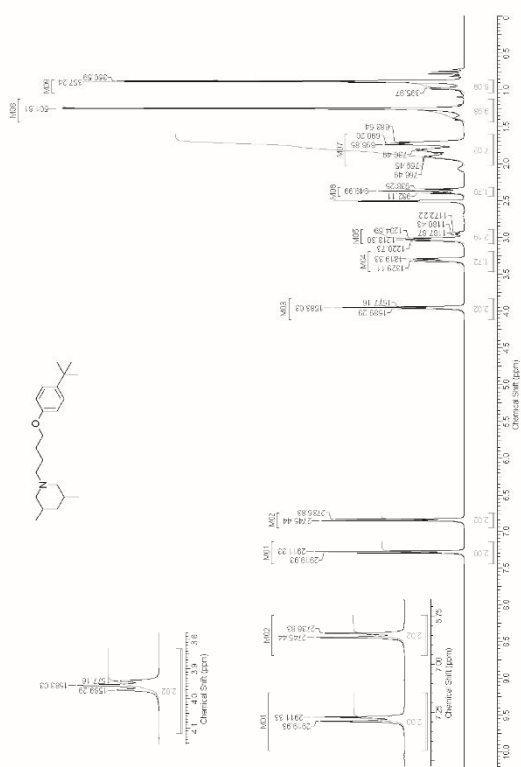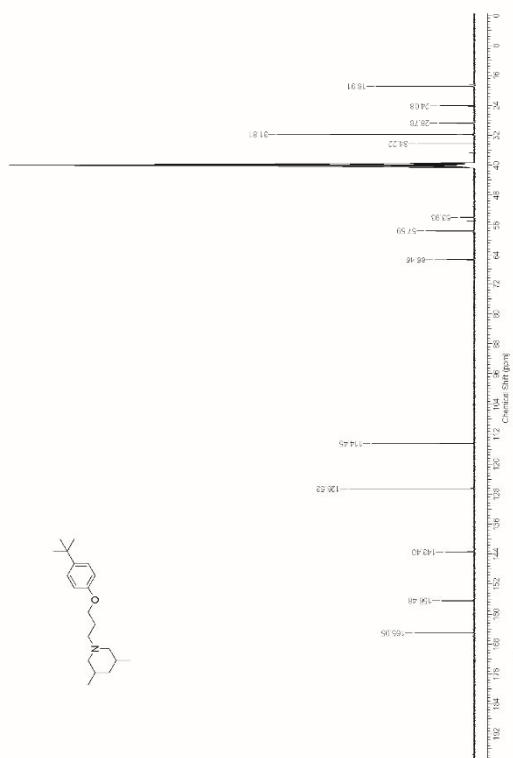Dorota Łażewska[*], Agnieszka Olejarz-Maciej[1], David Reiner[2], Maria Kaleta[1], Gniewomir Latacz[1], Małgorzata Zygmunt[3], Agata Doroz-Płonka[1], Tadeusz Karcz[1], Annika Frank[2], Holger Stark[2] and Katarzyna Kieć-Kononowicz[1*]

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¹H NMR and ¹³C NMR data of synthesized compounds

Fig S1. ¹H NMR spectrum of **5**

Fig S3. ¹H NMR spectrum of **6**

Fig S2. ¹³C NMR spectrum of **5**

Fig S5. ¹H NMR spectrum of **7**

Fig S4. ¹³C NMR spectrum of **6**

Fig S7. ¹H NMR spectrum of **8**
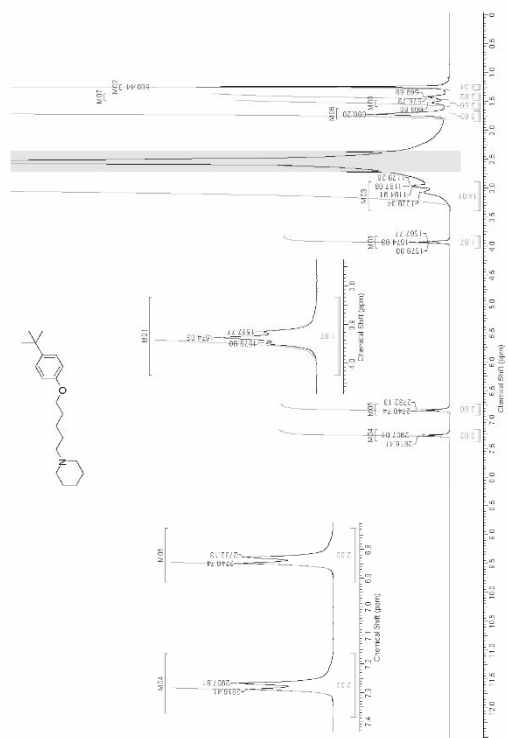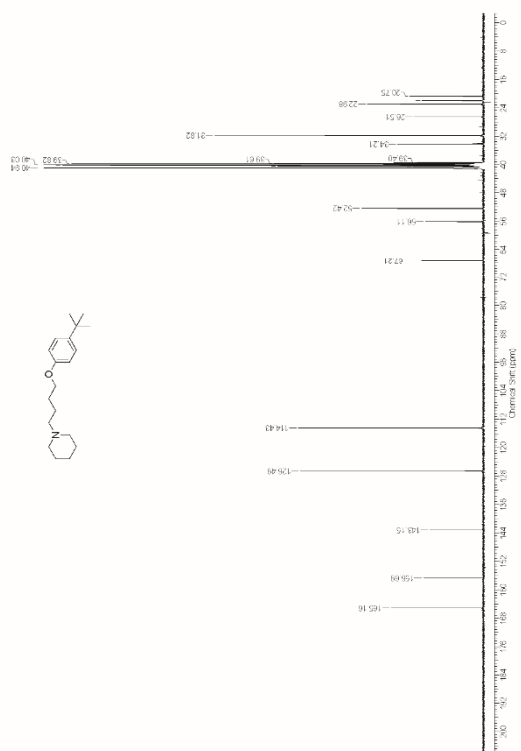
Fig S6. ¹³C NMR spectrum of **7**

Fig S9. ¹H NMR spectrum of **9**

Fig S8. ¹³C NMR spectrum of **8**
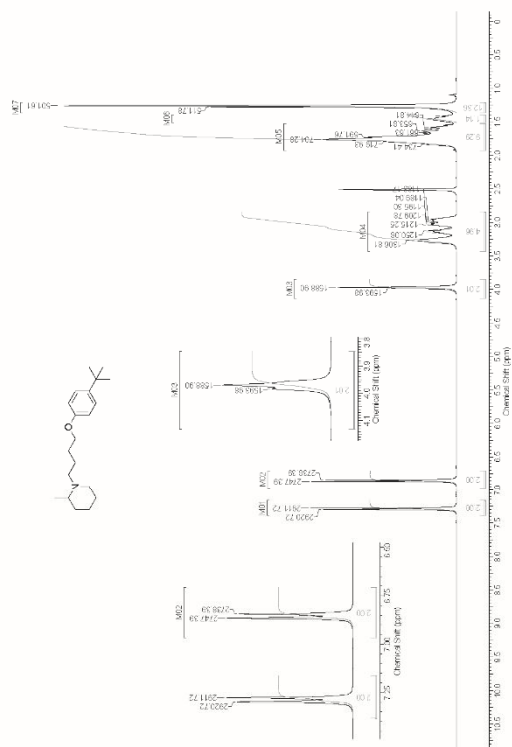
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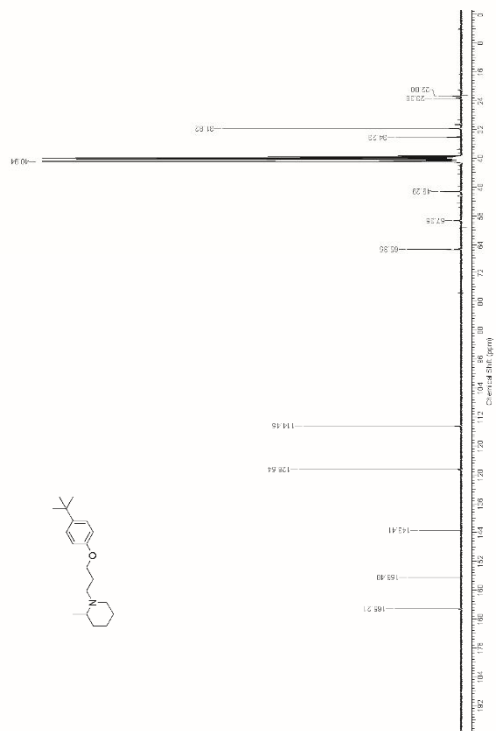
Fig S10. ¹³C NMR spectrum of **9**
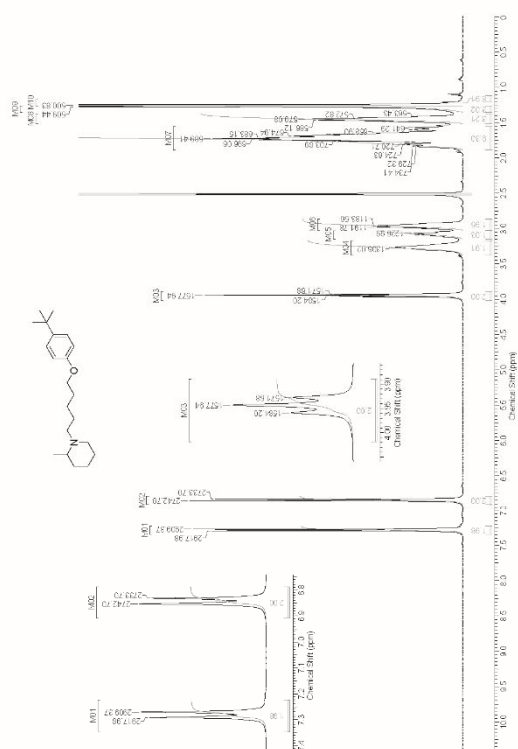
Fig S13. ¹H NMR spectrum of **11**

Fig S12. ¹³C NMR spectrum of **10**
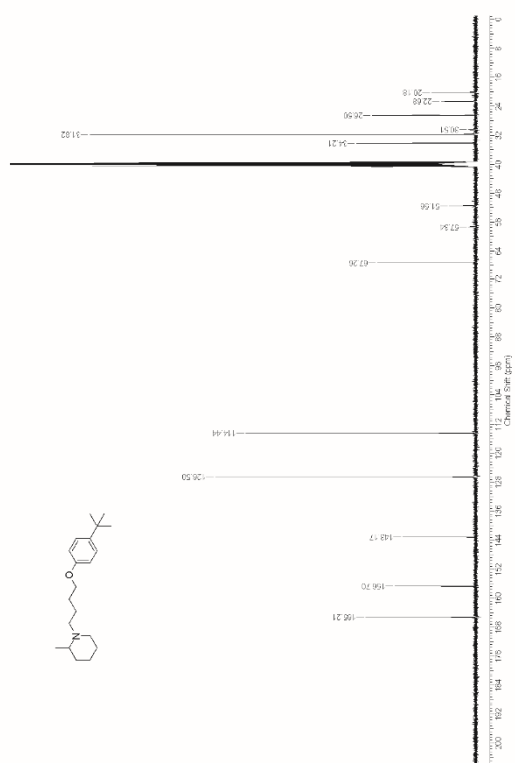
Fig S15. ^{1}H NMR spectrum of **12**
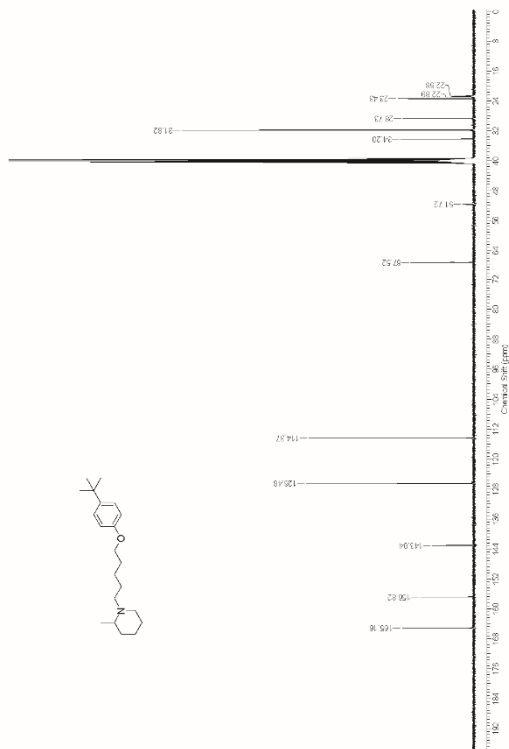
Fig S14. ^{13}C NMR spectrum of **11**
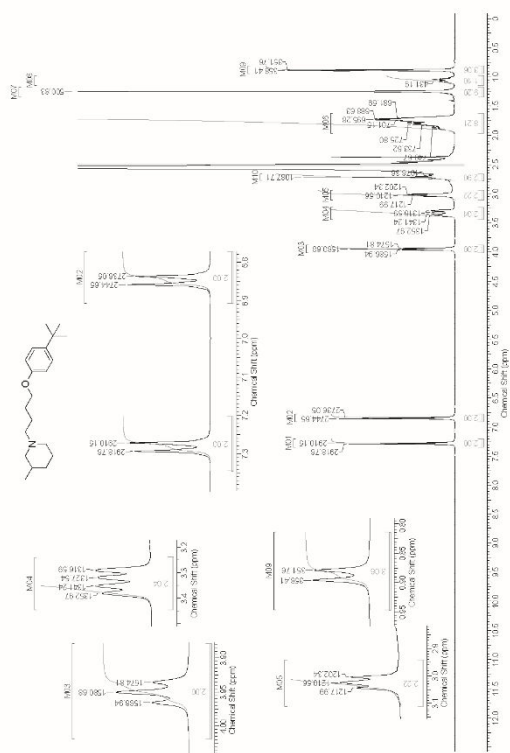
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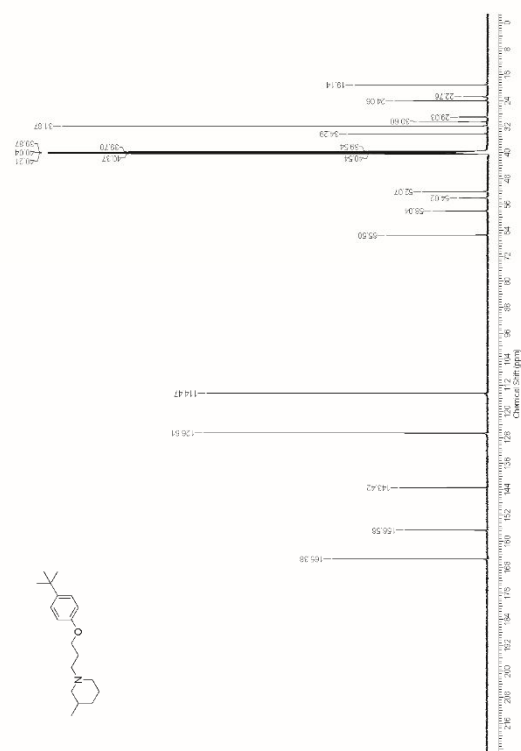
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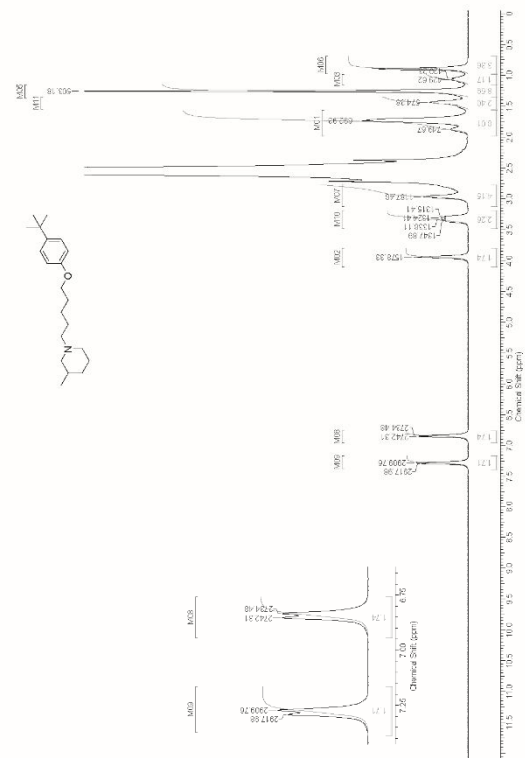
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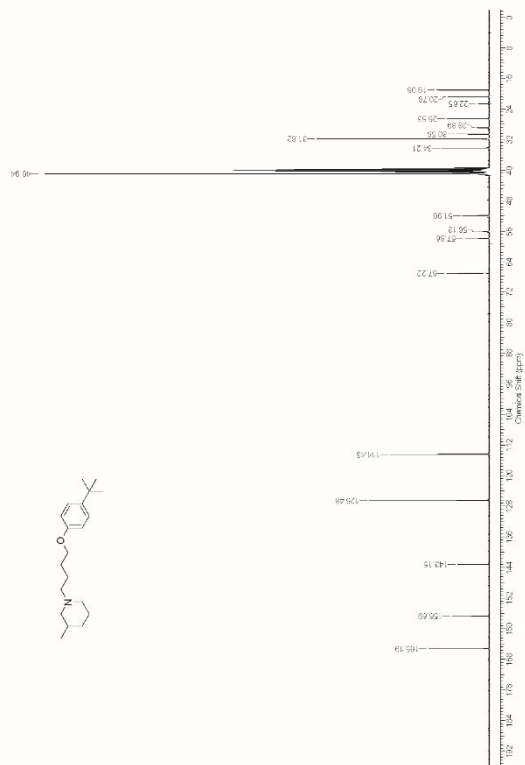
Fig S18. ¹³C NMR spectrum of **13**
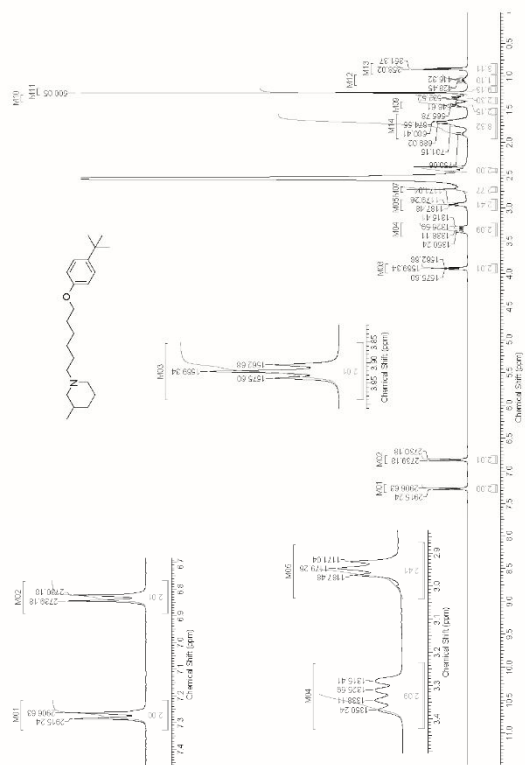
Fig S21. ¹H NMR spectrum of **15**

Fig S20. ¹³C NMR spectrum of **14**
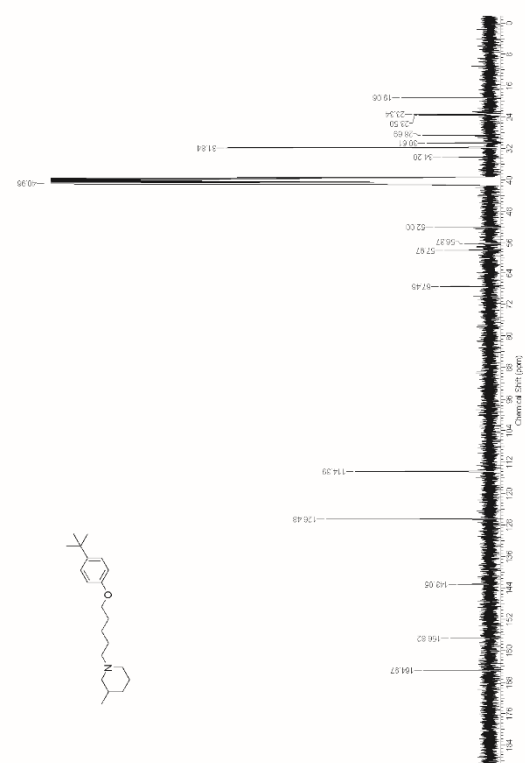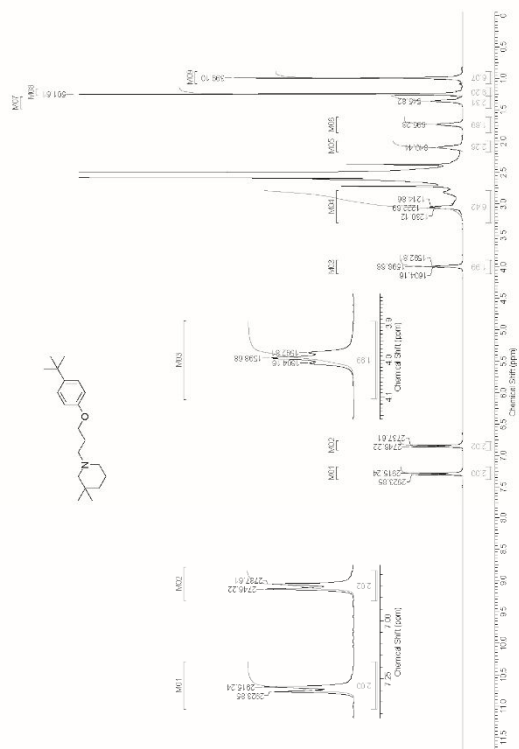
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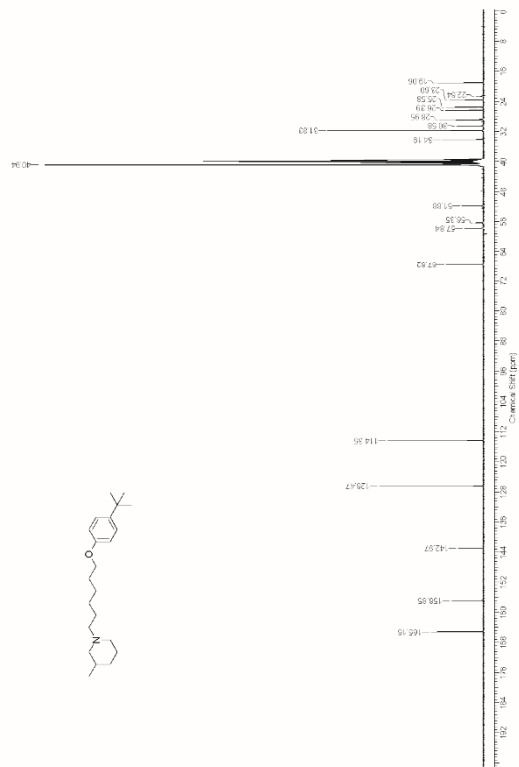
Fig S22. ¹³C NMR spectrum of **15**

Fig S25. ¹H NMR spectrum of **17**

Fig S24. ¹³C NMR spectrum of **16**
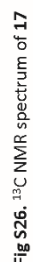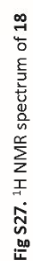
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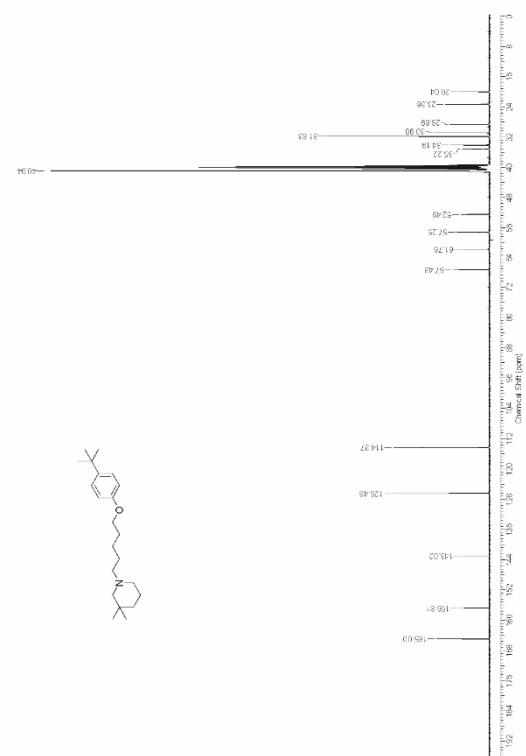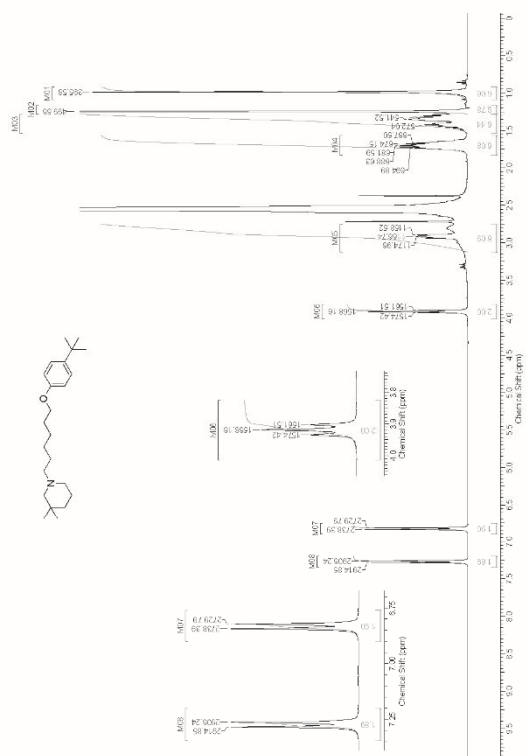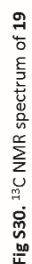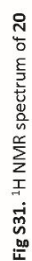
Fig S26. ¹³C NMR spectrum of **17**

Fig S29. ¹H NMR spectrum of **19**

Fig S28. ¹³C NMR spectrum of **18**

Fig S31. ¹H NMR spectrum of **20**

Fig S30. ¹³C NMR spectrum of **19**

Fig S33. ¹H NMR spectrum of **21**

Fig S32. ¹³C NMR spectrum of **20**
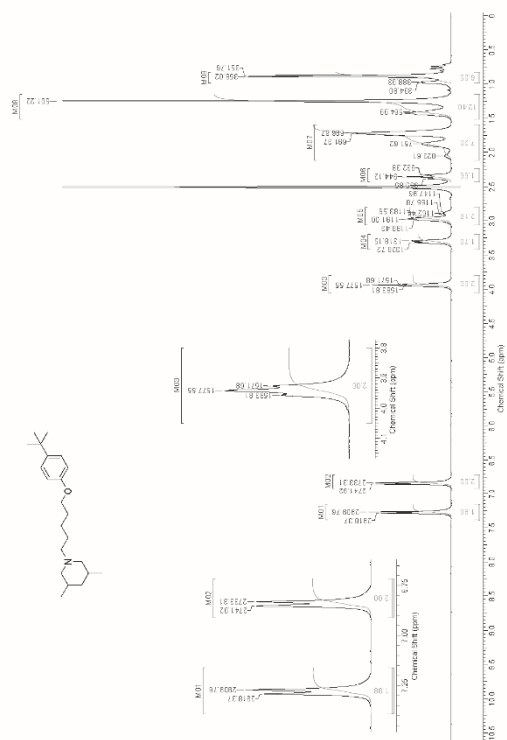
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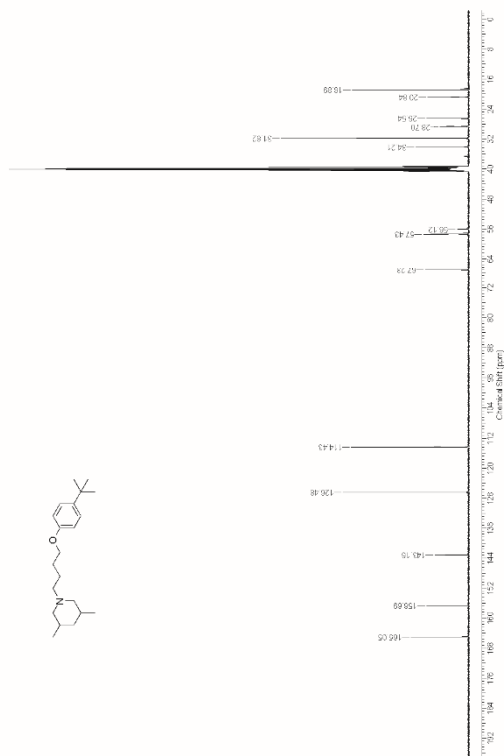
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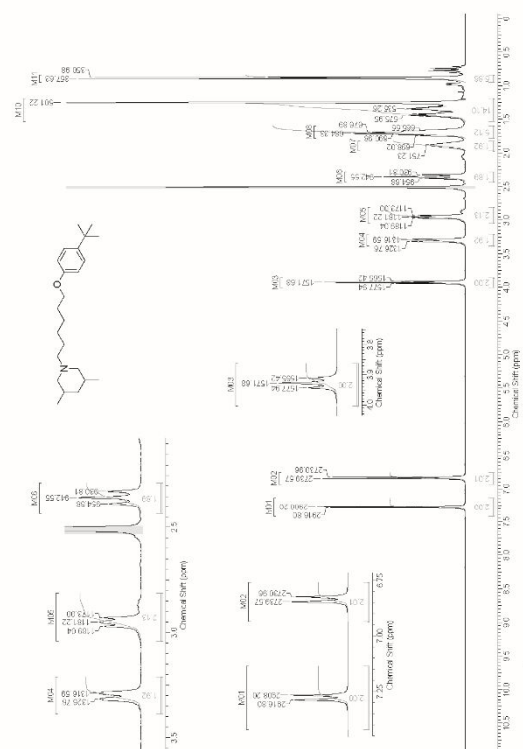
Fig S37. ¹H NMR spectrum of **23**
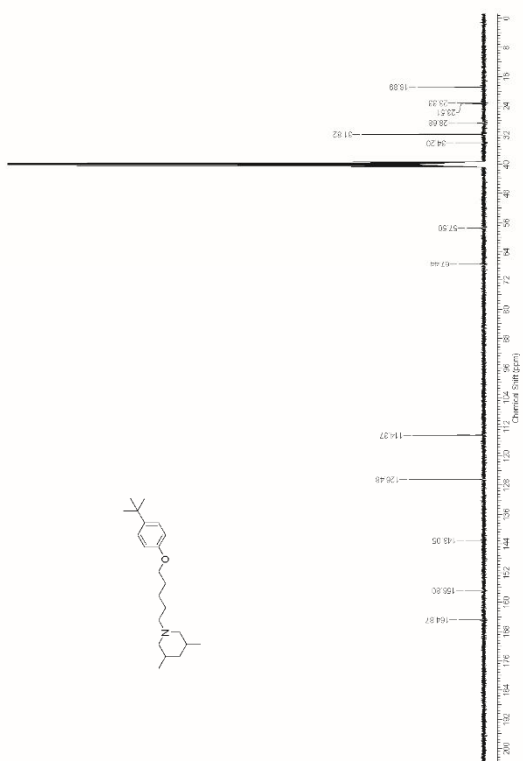
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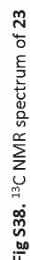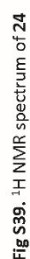
Fig S39. ¹H NMR spectrum of **24**

Fig S38. ¹³C NMR spectrum of **23**
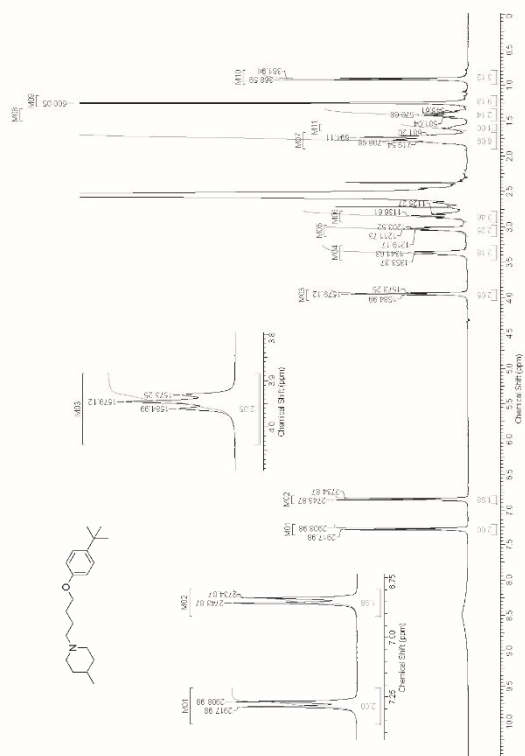
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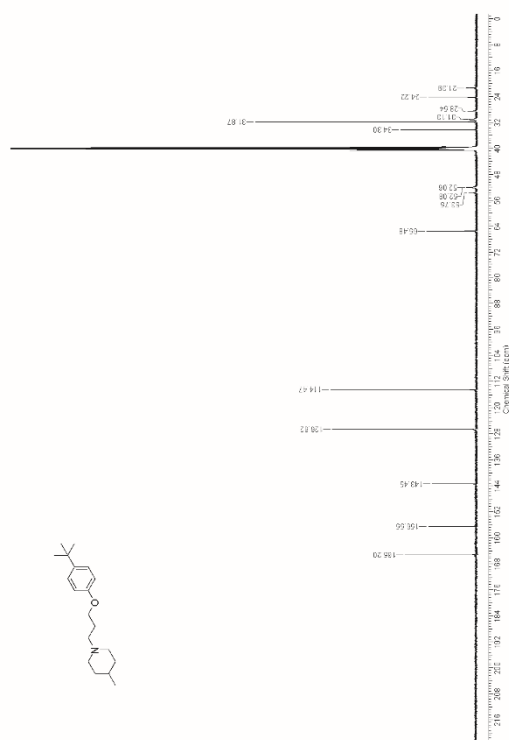
Fig S40. ¹³C NMR spectrum of **24**

Fig S43. ¹H NMR spectrum of **26**
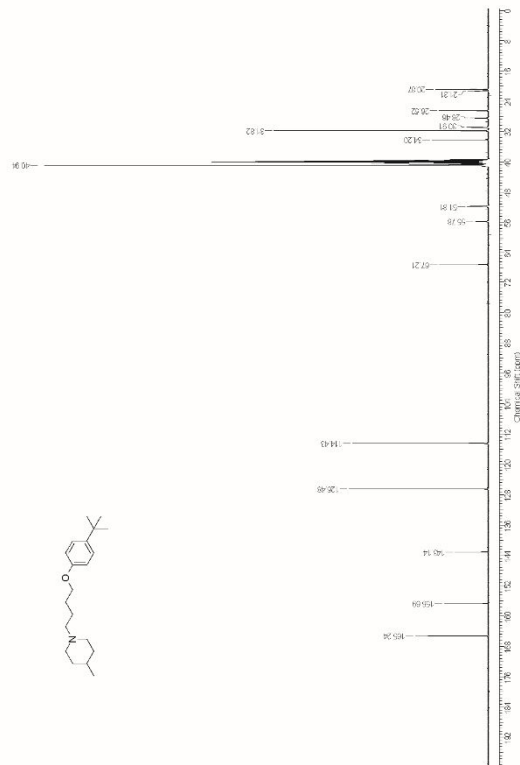
Fig S42. ¹³C NMR spectrum of **25**
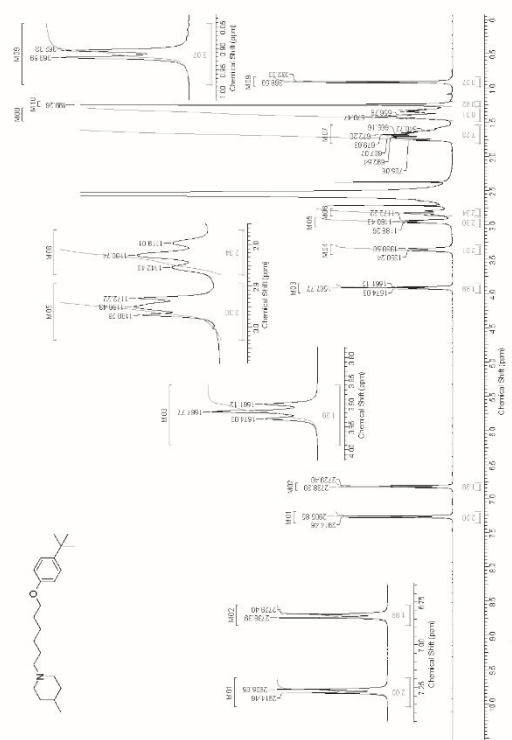
Fig S45. ¹H NMR spectrum of **27**
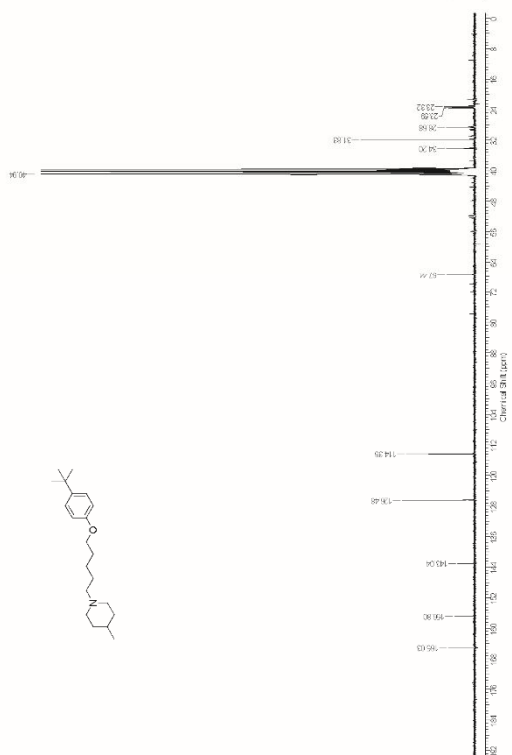
Fig S44. ¹³C NMR spectrum of **26**

Fig S47. ¹H NMR spectrum of **28**
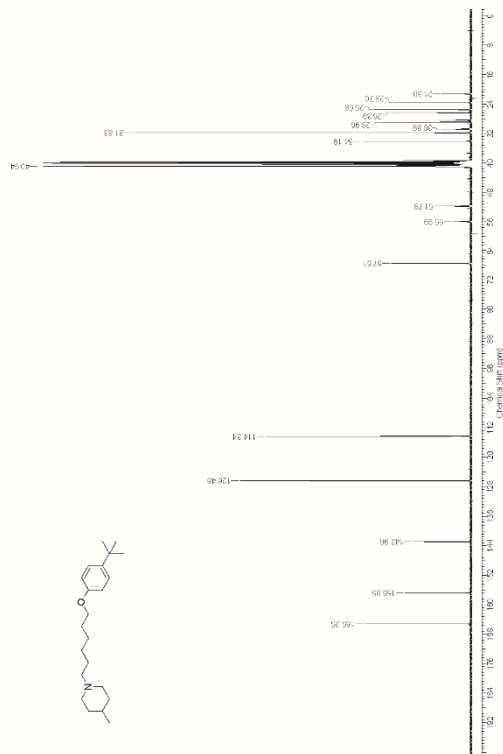
Fig S46. ¹³C NMR spectrum of **27**

Fig S49. ¹H NMR spectrum of **29**
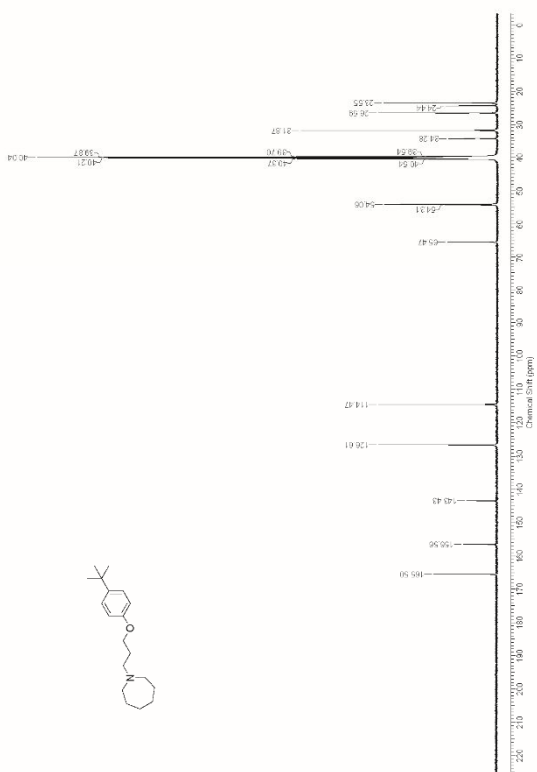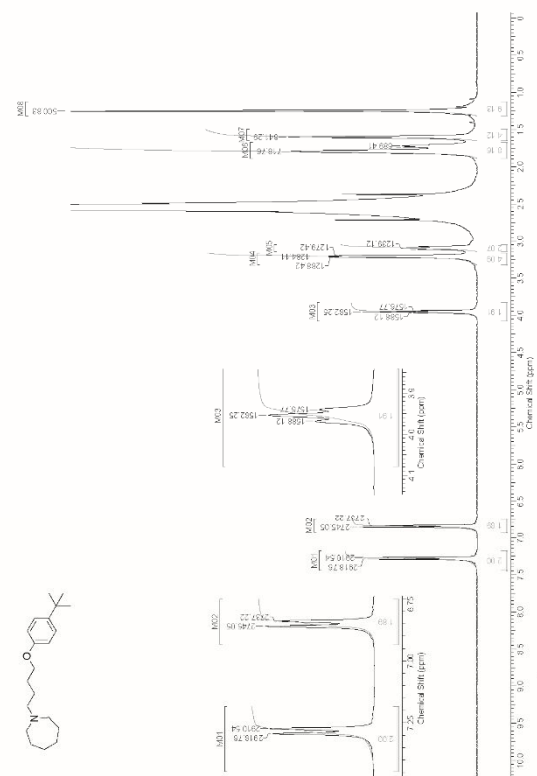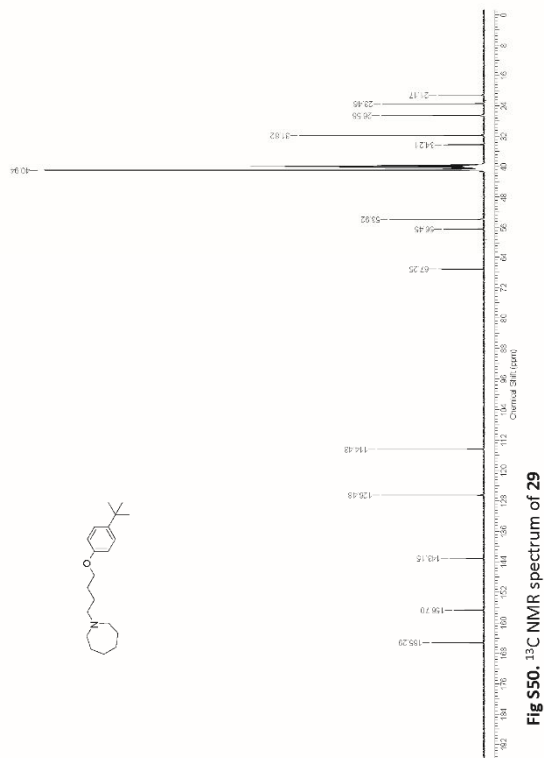
Fig S48. ¹³C NMR spectrum of **28**

Fig S51. ¹H NMR spectrum of **30**

Fig S50. ¹³C NMR spectrum of **29**

Fig S51. ¹H NMR spectrum of **31**

Fig S52. ¹³C NMR spectrum of **30**

Fig S52. ¹³C NMR spectrum of **31**

Supplementary Materials S2

Dual target ligands with 4-*tert*-butylphenoxy scaffold as histamine H₃ receptor antagonists and monoamine oxidase B inhibitors
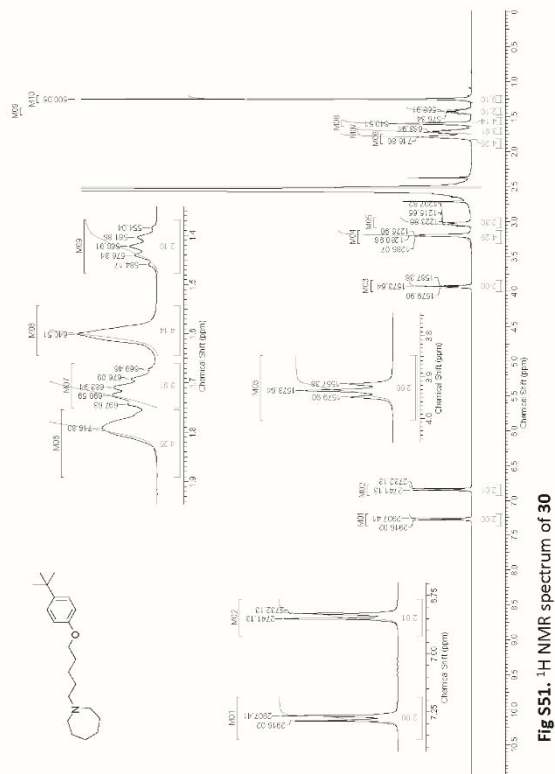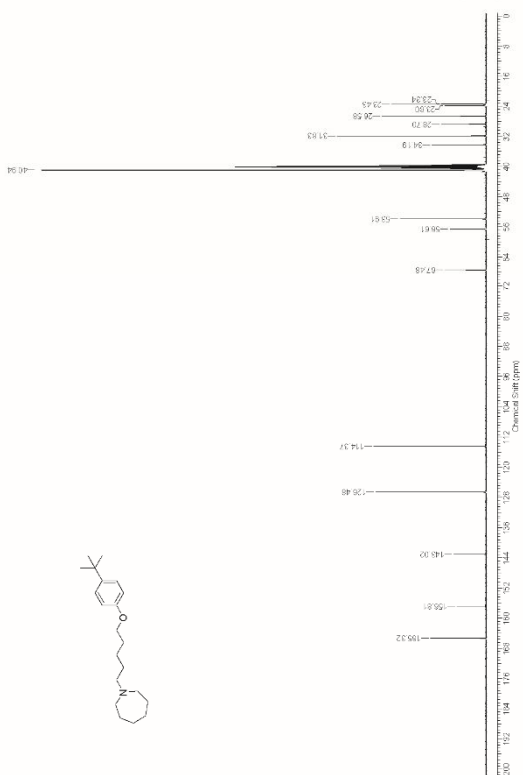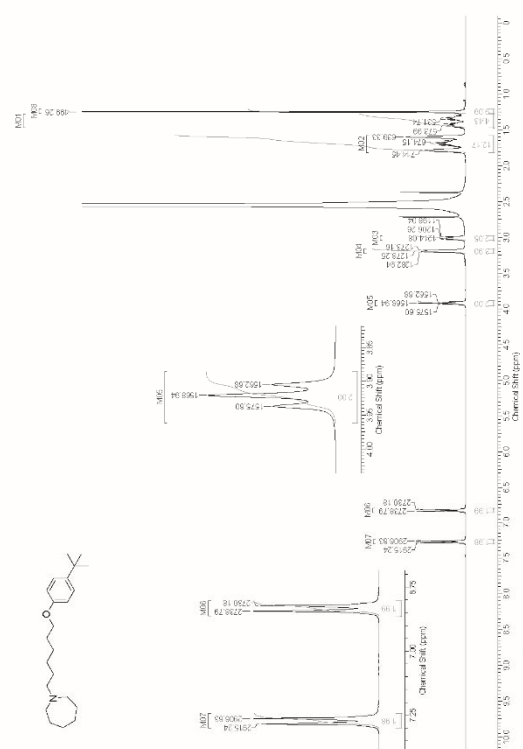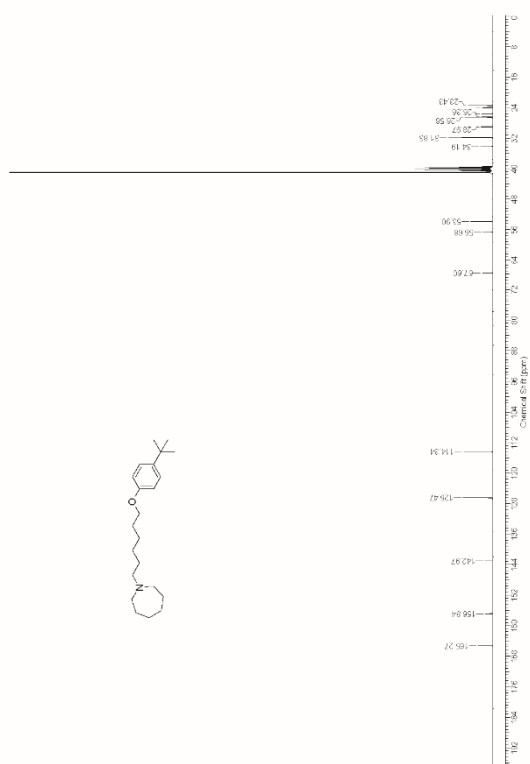
Dorota Łażewska¹, Agnieszka Olejarz-Maciej¹, David Reiner², Maria Kaleta¹, Gniewomir Latacz¹, Małgorzata Zygmunt³, Agata Doroz-Płonka¹, Tadeusz Karcz¹, Annika Frank², Holger Stark² and Katarzyna Kieć-Kononowicz¹*

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hMAO B Kinetic studies

The α values for different modes of reversible inhibition as well as their diagnostic signature on double-reciprocal plot are shown in Table S1. The α value for compound **9** was calculated by GraphPad Prism software from the nonlinear regression curves using following equation for mixed-model of inhibition:

$$v = V_{max}*[S] / [S]*(1+ [I]/ \alpha K_i) + K_m*(1+ [I]/K_i) \qquad (1)$$

v – velocity of enzyme reaction; V_{max} – maximum velocity (at infinite substrate concentration); K_i – dissociation constant (for Enzyme–Inhibitor complex (in the enzymology literature other symbols can be used such as: K_i, K_{is}, K_{ES} etc.); αK_i – dissociation constant for Enzyme–Substrate–Inhibitor complex (in the literature also under the symbols: K_i', K_{ii} and K_{ESI}); K_M – Michaelis-Menten constant; [I] – concentration of the inhibitor; [S] – concentration of the substrate [Ref S1].

Table S1. Relation between inhibition modality, α value, and diagnostic signature on the double-reciprocal plots (i.e Lineweaver–Burk plot) [S2].

α	Inhibition modality	Diagnostic signature on double-reciprocal plot
α = 1	**noncompetitive** (inhibitor binds to free enzyme and enzyme-substrate complex with equal affinity)	lines converge at the x-axis
α > 1	**mixed mode inhibitor** (inhibitor can bind to free enzyme and enzyme-substrate complex, unequally), inhibitor's affinity is higher for free enzyme	lines converge to the left of the y-axis and above the x-axis
α < 1	**mixed mode inhibitor** (inhibitor can bind to free enzyme and enzyme-substrate complex, unequally), inhibitor's affinity is higher for the enzyme-substrate complex)	lines converge to the left of the y-axis and below the x-axis
α → ∞	**competitive** (inhibitor and substrate compete for the same site of binding)	lines converge at the y-axis
α → 0 and α > 0	**uncompetitive** (inhibitor binds only to the enzyme–substrate complex)	parallel lines

References:

S1. Copeland, R.A. Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis.; 2nd ed.; Willey-VCH: New York / Chichester / Weinheim / Brisbane / Singapore /Toronto, 2000; ISBN 0-471-22063-9.

S2. Copeland, R.A. Evaluation of Enzyme Inhibitors in Drug Discovery. A Guide for Medicinal Chemists and Pharmacologists, John Wiley & Sons, Inc., Hoboken, New Jersey, 2005.

4.3. Rasagiline derivatives combined with histamine H3 receptor properties

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Contribution to research: DR was involved in conduction of and data-evaluation from radioligand displacement experiments at histamine H3 receptors, and reviewed the manuscript.

Abstract:

The irreversible monoamine oxidase B (MAO B) inhibitor rasagiline has been described with multiple disease modifying effects in vitro on models of Parkinson's disease. The combination of this established drug to recently developed histamine H3 receptor (H3R) antagonist elements gives new impetus to the design of multitargeting ligands. Surprisingly, the 5-substituted 3-piperidinopropyloxy rasagiline derivative 1 was more potent on both targets than its 6-substituted isomer. It showed nanomolar affinities at the desired targets (MAO B IC_{50} = 256 nM; hH3R K_i = 2.6 nM) with a high preference over monoamine oxidase A (MAO A) and negligible affinity at histamine H_1, H_4, dopamine D_2, D_3 receptors or acetyl-/butyrylcholinesterases.

Rasagiline derivatives combined with histamine H₃ receptor properties

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ABSTRACT

The irreversible monoamine oxidase B (MAO B) inhibitor rasagiline has been described with multiple disease modifying effects in vitro on models of Parkinson's disease. The combination of this established drug to recently developed histamine H₃ receptor (H₃R) antagonist elements gives new impetus to the design of multitargeting ligands. Surprisingly, the 5-substituted 3-piperidinopropyloxy rasagiline derivative **1** was more potent on both targets than its 6-substituted isomer. It showed nanomolar affinities at the desired targets (MAO B $IC_{50} = 256$ nM; hH_3R $K_i = 2.6$ nM) with a high preference over monoamine oxidase A (MAO A) and negligible affinity at histamine H_1, H_4, dopamine D_2, D_3 receptors or acetyl-/butyrylcholinesterases.

Ligands simultaneously acting on a rational combination of target molecules, so called multitargeting ligands (MTLs), may be superior to target-selective drugs by triggering a number of positive synergistic effects. A meaningful target combination should address different pathophysiological pathways or different levels in one pathway. Still, optimizing structure-activity relationships (SARs) for combination of multiple targets of even different target families as well as assuring a balance in target affinities and maintaining drug-likeness at the same time is a high hanging fruit for medicinal chemists.[1–3] This MTL approach is especially valuable when multiple targets have been described for a disease, e.g. for neurodegenerative diseases such as Parkinson's disease (PD).

In PD, patients suffer from progressive loss of dopaminergic neurons in the substantia nigra initiated by cellular oxidative stress and leading to the typical PD motor disorders but also associated non-motor symptoms.[4] Monoamine oxidase B (MAO B) inhibitors are suitable medications in early PD or as add-on therapy, elevating striatal dopamine levels.[5] Some representatives like rasagiline (Fig. 1) show efficacy in treatment of non-motor symptoms, like cognitive impairments and fatigue. Rasagiline is an irreversible propargyl amine-containing MAO B inhibitor, which covalently binds to the flavin adenine dinucleotide (FAD), acting as a cofactor of MAOs.[6] It was shown to have neuroprotective and antiapoptotic effects in multiple studies, not least because it prevents the formation of reactive oxygen species (ROS), which are generated as second product during enzyme turnover.[7] The combined cholinesterase (ChE)/MAO inhibitor ladostigil (Fig. 1),[8] obtained by attaching a ChE active structural element at the 6-position of rasagiline, is currently under investigation for its therapeutic potential in

Alzheimer's disease.[9] Also, its neuroprotective properties were tested in context of dementia associated with extrapyramidal disorders i.e. Parkinsonism and depression,[10] which suggests the potential of MTLs in treatment of neurodegenerative diseases.

Based on previous designed indanone derivatives showing reversible MAO B inhibition,[11] we aimed to develop novel multitargeting rasagiline derivatives by introducing an accepted histamine H₃ receptor (H₃R) antagonist pharmacophore. The H₃R is a member of the GPCR receptor family, contributing to central neurotransmission of histamine and other neurotransmitters such as dopamine or acetylcholine. H₃R antagonists are currently investigated as potential drugs in sleep disorders having pitolisant with EMA market approval against narcolepsy, Alzheimer's disease (AD), PD or attention deficit hyperactivity disorder (ADHD).[12] Considering the previously described MAO inhibition properties of ciproxifan,[13] one of the most prominent H₃R antagonists/inverse agonist, and its non-imidazole analogues UCL2190[11] as well as DL77[14] the 3-piperidinopropyloxy moiety was chosen to be merged with rasagiline (Fig. 1).

The first series of compounds, **1a/2a**[11] were obtained from commercially available 5- and 6-methoxy-1-indanone via demethylation of phenol ethers[15] following the Williamson ether reaction[16] with 1-(3-chloropropyl)piperidine[17] to alkylate 5- or 6-hydroxy-1-indanone.[18,19] with a yield ranging from 81 to 85%. Compound **1** was synthesized via reductive amination using ammonium carbonate as amine source to give the intermediate **1b** with 47% yield[20] followed by nucleophilic substitution with propargyl bromide resulting in compound **1** in 55% yield (Scheme 1).[21] For isomer **2** another reaction sequence was used starting from 6-hydroxy-1-indanone by performing Williamson ether
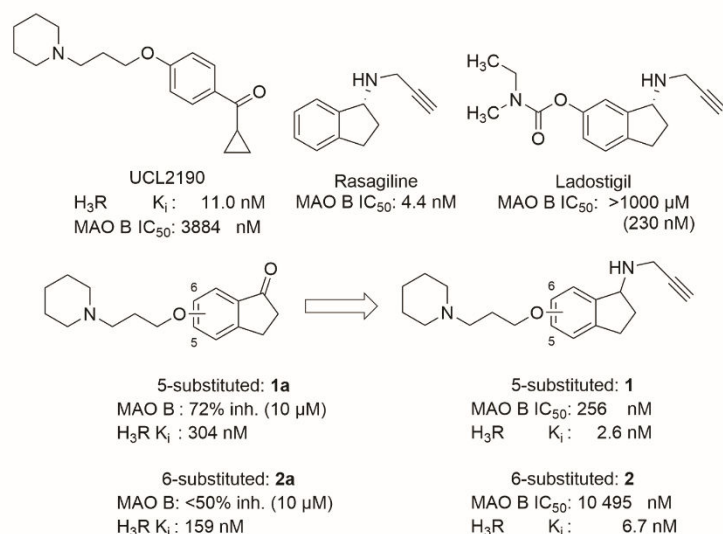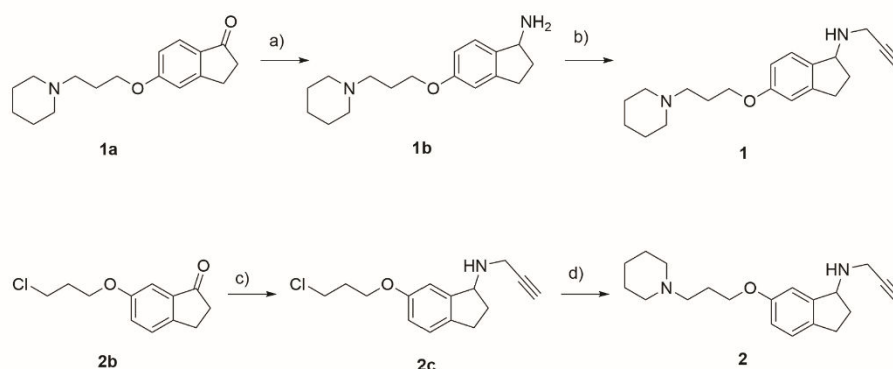
Fig. 1. MTL design of new irreversible MAO B/H₃R MTLs inspired by rasagline,[6] ladostigil (values for active metabolite are given in brackets),[8] UCL2190[11] and previous described indanone lead compounds.[11]

Scheme 1. Synthetic route for the design of indanamine MTLs **1** and **2**: (a) NaCNBH₃, NH₄OAc, EtOH, MW, 130 °C; (b) Propargyl bromide, K₂CO₃, DMF, 30 °C; (c) Propargyl amine, NaCNBH₃, AcOH, DCE, MW, 110 °C; (d) Piperidine, reflux.

reaction with 1-bromo-3-chloropropane to give the intermediate **2b** which was used without further purification for reductive amination with propargyl amine (yield 66% over two steps).[22] The obtained intermediate **2c** was reacted with piperidine to give the final compound **2** in quantitative yield.[20] H₃R affinities of final compounds were determined in [³H]-Nᵅ-methylhistamine radioligand displacement studies using crude membrane extracts from hH₃R-HEK293 cells.[23,24] Monoamine oxidase A/B inhibition assays were performed using the discontinuous fluorimetric method with kynuramine as substrate for both isoforms (human recombinant MAO A/B).[11]

Both indanamines (**1** and **2**) showed high affinities at H₃R (pK$_i$ > 8) in a low nanomolar concentration range (Table 1), whereas at MAO B only compound **1** possessed inhibition in a nanomolar concentration range. However, our previously described indanone derivatives **1a** and **2a**, which are synthetic precursor molecules of MTLs **1** and **2** showed an inhibition < 80% at 10 μM for MAO B, respectively, and moderate affinities at H3R (Ki > 100 nM).[11] Thus, concluding from SAR in these classes the introduction of the benzylic amine led to improvement in H₃R affinity whereas the propargyl amine moiety increased MAO B inhibitory properties.

Interestingly, compound **1** showed an IC₅₀ shift to lower nanomolar concentrations with longer incubation times as expected for irreversible inhibitors. This suggests slow kinetics for the inactivation of MAO B by compound **1**, a time-dependency, which could also be verified by dilution method with excess of substrate (Fig. 2). The subtle but important variations are shown with compound **2** bearing the H₃R pharmacophore in C6 position failing to demonstrate irreversible mode of inhibition under conditions used despite the propargyl amino moiety. This lack of irreversibility together with its about 40-fold lower MAO B inhibition potency compared to **1** led to our assumption, that compound **2** might not or just barely reach the FAD in the active site of MAO B. A linear structure like in compound **1** might be favored to fit inside the MAO B cavity, while the non-linear and more rigid structure of compound **2** hampers binding to the active site.[25] Similar findings in styrylisatin compounds were published by Manley-King and colleagues, where the difference between C5 and C6 position lead to 68-fold

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Table 1
Target affinities (expressed as K_i and IC_{50} values) of novel MAO B/H₃R MTLs, UCL2190 and L-deprenyl (selegiline).

Compound	K_i [nM] [a] [CI 95%] (n) hH₃R	Pre-incubation at 37°	IC_{50} [nM][a] [CI 95%] (n) hMAO B	IC_{50} [nM][a] [CI 95%] (n) hMAO A	MAO SI [b]
1	2.6 [1.3; 5.2] (3)	0 min	991 [461; 2130] (4)	> 100 000 (3)	> 100
		30 min	1052 [469; 2359] (3)	> 100 000 (1)	> 95
		60 min	256 [132; 497] (4)	> 10 000 (2)	> 39
2	6.7 [2.3; 20] (3)	0 min	7920 [5521; 11363] (4)	> 100 000 (2)	> 13
		60 min	10 495 [8372; 13157] (3)	> 50 000 (3)	> 4.8
UCL2190 [c]	11 [3.5; 33] (3)	0 min	3884 [1816; 8311] (3)	> 50 000 (3)	> 12
L-**Deprenyl** [c]	nd	0 min	42 [23; 74] (4)	> 30 000 (4)	> 710
		30 min	3.9 [2.2; 6.8] (5)	nd	

[a] Affinity values (expressed as K_i or IC_{50}) are given as mean values within the 95% confidence interval (CI) of n independent experiments each performed in duplicate.
[b] Selectivity index (SI) = IC_{50} (MAO A)/IC_{50} (MAO B).
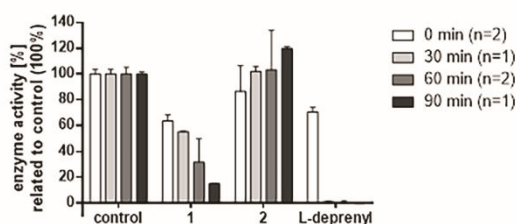[c] Data from Ref. 11, nd = not determined.

Fig. 2. Remained enzyme activity (%) of MAO B after pre-incubation with compound **1**, **2** and the irreversible inhibitor L-deprenyl (10xIC_{50}). Data represent mean ± standard deviation of n independent experiments each performed in duplicates.

decrease of MAO B inhibition.[26] In case of the ChE/MAO inhibitor ladostigil, the ChE active structural motif was also attached at the unfavorable C6 position probably explaining its low binding to MAO B[8]. However, the active metabolite of ladostigil after ester cleavage show comparable MAO B inhibition to that of compound **1** (IC_{50} = 230 nM vs. 256 nM for compound **1**).[13] Compared to contilisant, a combined H₃R/MAO/ChE MTL with similar irreversible MAO inhibition capacities,[27] compound **1** showed an improved preference for MAO B (MAO SI > 100 vs. SI = 0.5 for contilisant) as well as an improved selectivity for histamine H₃ over related histamine H₁, H₄, and dopamine D₂, D₃ receptor subtypes (K_i values > 10 μM; see Supporting information). Both compounds showed low cytotoxicity in a screening with neuroblastoma cells and compound **1** favorable ligand efficiency parameters (see Supporting information). This together with the effective structural pharmacophore overlap leading to a low molecular mass suggest high drug-like properties for compound **1**. Additionally, introducing a second amino moiety improved the H₃R affinity for both indanamines compared to **1a** and **2a**. Nevertheless, enantiomeric separation of compound **1** was not taken into account because of higher nanomolar

MAO B affinity.

In conclusion, the substitution of the approved irreversible MAO B inhibitor rasagiline by a generally accepted H₃R antagonist pharmacophore led to design of two novel multitargeting H₃R/MAO B ligands. Whereas both compounds showed high H₃R affinities, interestingly they vary significantly in their inhibitory properties at MAO B. Only compound **1** bearing the H₃R pharmacophore in 5-position showed promising multitargeting properties in nanomolar concentration ranges with irreversible binding to MAO B for slow kinetics and for binding H₃R. Having this small molecule entity with its well-designed profile and suitable drug-likeness properties, it may serve as lead structure in prospective MTL drug design for the treatment of neurodegenerative diseases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.08.016.

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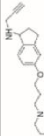
Supplementary Data

Rasagiline derivatives combined with histamine H₃ receptor properties

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Table 1. Off-target affinities for MTL 1 and 2 at histamine/dopamine receptor subtypes and acetyl-/butyrylcholinesterase.

Compound	K_i [μM] (n)				% inhibition (global fit) at 10 μM mean ± SD (n)	
	hH₁R	hH₃R	hD₂R	hD₃R	eeAChE	eqBuChE
1	>10 (2)	>100 (3)	>10 (2)	>10 (2)	57±1.4 (2)	32±11 (2)
2	nd	nd	nd	nd	47±4.4 (2)	47±2.5 (2)

nd – not determined, ee – electric eel, eq – equine/horse serum

Table 2. Evaluation of Lipinski's rule and metric parameters for compound 1

Comp.	Lipinski's rules					H₁R			MAO B		
	HB donors	HB acceptors	M [g/mol]	clogP[a]	PSA[b] (Å²)	LE[c]	LELP[d]	LE[c]	LELP[d]		
1	1	3	312.2	4.8	23.5	0.5	9.3	0.3	13.9		

a Calculation with Marvin Sketch, b Calculation with Molsoft, c hH3R: LE = pKi/HA (heavy atoms), c MAO B: LE = pIC50/HA, d LELP = clogP/LE, HB – hydrogen bond, PSA – polar surface areas, LE – ligand efficacy, LELP – ligand efficacy dependent lipophilicity

Table 3. Cell viability of SH-SY5Y after 24h treatment with test ligands (1 and 10 μM). Data are given as mean ± SD of % inhibition (related to 1% DMSO control; n=1, performed in triplicates).

Compound	% cell viability ± SD	
	10 μM	1 μM
1	95.6 ± 9.7	89.7 ± 12.3
2	100.5 ± 8.4	84.0 ± 13.9
MPP⁺	1.8 ± 0.6 (10 mM)	

Demethylation of the 5- or 6-hydroxy-1-indanone was achieved as described previously.[2] The corresponding 3-(piperidin-1-yl)propylchloride[3] derivatives were synthesized according to already described methods.[4-6] Synthesis of the indanone precursors **1a** and **2a** is described by Affini et al.[7]

5-(3-(Piperidin-1-yl)propoxy)-2,3-dihydro-1H-inden-1-amine (1b)

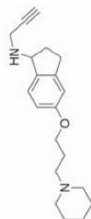Ammonium acetate (2.5 g, 32.8 mmol) and sodium cyano borohydride (0.2 g, 2.6 mmol) were added to a solution of 3a (0.6 g, 2.2 mmol) in absolute ethanol in microwave reactor. The mixture was stirred and heated at 130 °C for 2 min in a microwave reactor. The reaction mixture was concentrated to remove the solvent, treated with 2N NaOH until pH > 10, and extracted with dichloromethane. The organic phase was dried over magnesium sulfate, filtered, and concentrated under reduced pressure to give the crude product, which was purified with column chromatography (methylene chloride: methanol (NH₃ saturated) = 97:3).

Yield: 47%; ¹H NMR (300 MHz, DMSO-d6) δ 7.23–7.15 (m, 1H, phind-7H), 6.75–6.66 (m, 2H, phind-4,6H), 4.18–4.06 (t, 1H, J = 4.1, NH₂CHCH₂), 3.99–3.87 (t, 2H, J = 5.6, prop-1H), 2.87–2.74 (m, 1H, NH₂CHCH₂), 2.72–2.55 (m, 1H, NH₂CHCH₂), 2.40–2.17 (m, 7H, NH₂CHCH₂CH₂, pip-2,6H, prop-3H), 1.89–1.74 (q, 2H, J = 6.4, prop-2H), 1.64–1.43 (m, 5H, pip-3,5H, NH₂CHCH₂CH₂), 1.42–1.30 (m, 2H, pip-4H) ¹³C NMR (75 MHz, DMSO-d6) δ 158.01 (phind-5C), 143.97 (NH₂CHCH₂CH₂C), 140.29 (NH₂CHC), 124.10 (phind-7C), 112.67 (phind-4C), 109.93 (phind-6C), 65.91 (prop-1C), 56.12 (prop-3C), 55.02 (NH₂CH), 54.10 (pip-2,6C), 37.27 (NH₂CHCH₂), 29.78 (NH₂CHCH₂CH₂), 26.39 (prop-2C), 25.61 (pip-3,5C), 24.15 (pip-4C); ESI-HRMS m/z: calc. for C₁₇H₂₆N₂O (MH⁺), 275.2123, found 275.2120.

6-(3-Chloropropoxy)-2,3-dihydro-1H-inden-1-one (2b)

Compound 2b (0.5 g, 3 mmol), 1-bromo-3-chloropropane (0.3 ml, 3.3 mmol) and potassium carbonate (1.0 g, 6.6 mmol) were heated in acetone in a microwave for 2 h. Reaction mixture was taken up in methylene chloride and the unreacted phenolic compound was washed out with 2N NaOH and the crude product was used for the next reaction without further purification.

6-(3-Chloropropoxy)-N-(prop-2-yn-1-yl)-2,3-dihydro-1H-inden-1-amine (2c)

(0.4 ml, 9.2 mmol) propargyl amine and (0.4 g, 6.9 mmol) sodium cyano borohydride were added to a solution of (0.4 ml, 4.6 mmol) glacial acetic acid and (1.1 g, 4.6 mmol) 4b in absolute dichloro ethane in microwave vial. The mixture was stirred and heated at 110 °C for 10 min in a microwave reactor. The reaction mixture was concentrated to remove the solvent, treated with 2N NaOH until pH > 10, and extracted with dichloromethane. The organic phase was dried over magnesium sulfate, filtered, and concentrated under reduced pressure to give the crude product, which was purified with column chromatography (methylene chloride: methanol (NH3 saturated) = 97:3).

Yield: 68%; ¹H NMR (600 MHz, CDCl₃) δ 7.12 (d, J = 8.3 Hz, 1H), 6.91 (d, J = 2.6 Hz, 1H), 6.78 (dd, J = 8.2, 2.5 Hz, 1H), 4.36 (t, J = 6.2 Hz, 1H), 4.10 (td, J = 6.1, 1.1 Hz, 2H), 3.74 (t, J = 6.4 Hz, 2H), 3.61 – 3.45 (m, 2H), 2.95 (ddd, J = 16.2, 8.4, 5.1 Hz, 1H), 2.83 – 2.67 (m, 1H), 2.42 (dddd, J = 12.4, 8.3, 6.9, 5.3 Hz, 1H), 2.27 – 2.26 (m, 1H), 2.22 (p, J = 6.2 Hz, 2H), 1.85 (dddd, J = 12.4, 8.4, 6.5, 5.6 Hz, 1H).
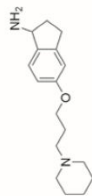
5-(3-(Piperidin-1-yl)propoxy)-N-(prop-2-yn-1-yl)-2,3-dihydro-1H-inden-1-amine hydrochloride (1)

Compound 4a (0.3 g, 1.1 mmol), a propargyl bromide solution 80% wt. in toluene (0.1 ml, 1.1 mmol) and potassium carbonate (0.2 g, 1.1 mmol) were heated in DMF for 4 h. The crude product was purified by column chromatography (methylene chloride: methanol (NH₃ saturated) = 97:3) and crystallized as hydrochloride from 1,4-dioxane (0.2 g, 55%)

Yield: 55%; m.p. 162 °C ¹H NMR (600 MHz, D₂O) δ 10.4 (br s, 1H, NH₄), 7.44–7.36 (m, 1H, phind-7H), 6.96–6.90 (m, 1H, phind-4H), 6.89–6.82 (m, 1H, phind-6H), 4.87–4.80 (dd, 1H, J = 4.8, NHCH), 4.15–4.04 (t, 2H, J = 5.8, prop-1H), 3.88–3.82 (t, 2H, J = 3.9, NHCH-C≡CH), 3.57–3.44 (m, 2H, prop-3H), 3.31–3.18 (m, 2H, NHCHCH₂), 3.14–2.99 (m, 1H, NHCHCH-CH₂), 2.99–2.79 (m, 4H, pip-2,6H), 2.61–2.42 (m, 1H, NHCHCH₂CH₂), 2.30–2.08 (m, 3H, prop-2H, CH₂C≡CH), 1.96–1.32 (m, 6H, pip-3,5H, pip-4H) ¹³C NMR (150 MHz, D₂O) δ 157.02 (phind-5C), 144.95 (NHCHCH₂CH₂C), 125.89 (NHCHC), 123.95 (phind-7C), 111.21 (phind-4C), 108.42 (phind-6C), 75.20 (CH₂C≡CH), 70.54 (CH₂C≡CH), 62.74 (prop-1C), 58.82 (prop-3C), 51.77 (NHCH), 50.63 (pip-2,6C), 31.35 (NHCH₂C≡CH), 27.07 (NHCHCH₂), 26.11 (NHCHCH₂CH₂), 20.71 (prop-2C), 20.13 (pip-3,5C), 18.38 (pip-4C); ESI-HRMS m/z: calc. for C₂₀H₂₈N₂O (MH⁺), 313.2274, found 313.2277. Anal. calc.: C, 62.33; H, 7.85; N, 7.27. Found: C, 62.02; H, 8.07; N, 7.17.
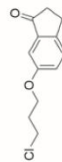
6-(3-(Piperidin-1-yl)propoxy)-N-(prop-2-yn-1-yl)-2,3-dihydro-1H-inden-1-amine dihydro oxalate (2)

Chloride precursor (4c) was refluxed in a large excess absolute piperidine. After 24 h, the reaction was cooled down. The solvent was concentrated under vacuum taken up in methylene chloride and washed with saturated NaHCO₃-solution. The organic phase was then washed with saturated NaCl-solution and water, dried over sodium sulfate, and concentrated under vacuum. The resulting oil was precipitated using oxalic acid and recrystallized from acetone.

Yield: 99% ¹H NMR (600 MHz, CDCl₃) δ 7.20 (d, J = 8.4 Hz, 1H), 7.13 (s, 1H), 6.91 (d, J = 8.5 Hz, 1H), 4.06 (q, J = 5.4 Hz, 2H), 3.97 (d, J = 2.7 Hz, 2H), 3.57 (dq, J = 21.3, 14.2, 11.0 Hz, 2H), 3.28 −3.25 (m, 2H), 3.24 (t, J = 7.9 Hz, 2H), 3.16 (d, J = 2.8 Hz, 1H), 3.07 (dt, J = 15.2, 7.5 Hz, 1H), 2.98 − 2.80 (m, 2H), 2.53 (dq, J = 14.5, 8.0 Hz, 1H), 2.27 (ddt, J = 13.3, 8.7, 4.6 Hz, 1H), 2.19 (dt, J = 10.2, 5.8 Hz, 2H), 1.81 (s, 5H), 1.62 − 1.44 (m, 1H). ¹H NMR (151 MHz, CDCl₃) δ 166.59, 159.32, 139.13, 138.32, 127.26, 118.56, 111.88, 79.01, 75.00, 66.34, 63.42, 55.64, 54.42, 35.45, 30.24, 30.02, 25.03, 24.22, 22.77. ESI-HRMS m/z: calc. for C₂₀H₂₈N₂O (MH⁺), 313.2274, found 313.2274. LC-MS: 96.56% m/z 258,10.

Pharmacological testing

Radioligand displacement assay

Radioligand displacement studies for H₃R affinities were performed as described previously.[1,4] Briefly, crude membrane extracts (20 mg/well) were incubated with [³H]-N-α-methylhistamine (2 nM, 78.3 Ci mmol⁻¹) and various concentrations of test compounds (10⁻¹¹ and 10⁻⁵ M) in a total volume of 0.2 mL binding buffer. The assay was partly automated by using a Tecan Freedom EVO pipetting robot. Data were analyzed using GraphPad PRISM 6 using the implemented non-linear regression fit "one-site competition", where Ki values were calculated according to Cheng-Prusoff equation. Statistical analysis was performed on –log Ki values. Mean values and confidence intervals (95%) were converted to nanomolar concentrations.

Similar methods were used for determination of H₁, H₄, dopamine D₂ and D₃ receptor affinities using [³H]-Pyrilamine (1 nM), [³H]-Histamine (10 nM) and [³H]-Spiperone (0.2 nM), respectively.[30]

Inhibition studies with human recombinant monoamine oxidases A and B

Monoamine oxidase A/B inhibition assays were performed as described previously using the discontinuous fluorimetric method.[7] Briefly, for IC₅₀ determinations (without pre-incubation; 0 min) compounds (ten suitable concentrations between 10⁻¹¹ and 10⁻⁵ M) were incubated with kynuramine as substrate (2×K₍ₘ₎; K₍ₘ₎ of 20 μM and 30 μM for MAO A and B, respectively) and MAO A (1.25 ng mL⁻¹, 900 units mL⁻¹) or MAO B (1.67 ng mL⁻¹, 375 units mL⁻¹) in a final assay volume of 100 μL. In case of pre-incubation experiments, IC₅₀ values were obtained after incubating inhibitors with enzyme for 30 or 60 min at 37 °C prior to addition of substrate. Reactions were conducted in pre-warmed potassium phosphate buffer (50 mM, pH 7.4). All data were analyzed with GraphPad PRISM 6. The IC₅₀ curves were fitted by non-linear regression fit (bottom set to zero).

Reversibility of inhibition was confirmed via pre-incubation of inhibitor (10xIC₅₀) with MAO B for 30, 60 and 90 min (37°C), followed by 50x dilution in potassium phosphate buffer and assayed with an excess of substrate (10xK₍ₘ₎).[7] Data were calculated as percentage of control (DMSO) for each time point.

Inhibition studies with acetyl- and butyrylcholinesterases

Cholinesterase inhibition assays was performed according to a modified Ellman's assay.[11] Compounds (10⁻⁶ and 10⁻⁵ M) were preincubated with electric eel acetylcholine esterase (eeAChE Type VI-S; 0.0025 U mL⁻¹) or equine serum butyrylcholine esterase (eqBuChE; 0.002 U mL⁻¹) at 37°C for 30 min in a total assay volume of 200 μL (0.1 M potassium phosphate buffer pH 8). The enzyme reaction was started by adding a mixture of dithiobis-nitrobenzoic acid (DTNB; 0.5 mM) and acetylthiocholine iodide (ATCl; 1 mM). Absorbance was monitored at 412 nm at 37°C over a period of 30 min in 40 sec intervals. Percentage values were calculated relative to control (no inhibitor) and physostigmine was used as positive control.

Cell viability assay

Cell viability of SH-SY5Y cells was assessed using resazurin fluorescent dye.[12] Resazurin sodium salt was dissolved in NaCl/P₁ buffer (1.06 mM KH₂PO₄, 154 mM NaCl, 3.77 mM Na₂HPO₄, pH = 7.4) to obtain a final concentration of 440 μM (Storage at 4°C, protected from light). Cells (8000 cells/well) were seeded in 96 wells plates (total volume 100 μL/well) and grown at 37°C in a 5% CO₂ atmosphere. After 24 h, media was removed and replaced by culture media containing test compounds in different concentrations (maximum of 1% DMSO per well). Cells were incubated with compounds and 1-methyl-4-phenylpyridinium iodide (MPP⁺, 10 mM) as positive control for 24h. For determination of remained cell viability, cells were incubated with resazurin working solution (44 μM in RPMI medium, 100 μL) for 2h. Fluorescence intensity (Excitation: 535 nm, Emission: 590 nm, bandwidth 5 nm) was measured using an infinite M1000 Pro multimode reader (Tecan, Switzerland).

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7

4.4. Histamine H₃ receptor ligands by hybrid virtual screening, docking, molecular dynamics simulations, and investigation of their biological effects

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<u>Contribution to research:</u> DR planned, conducted and supervised cell-culture. DR co-organised test-ligands, prepared, planned and conducted radioligand displacement experiments, and evaluated corresponding data to determine H₃R affinity. DR wrote pharmacological parts of and reviewed the manuscript.

<u>Abstract:</u>

Histamine H₃ receptors (H₃R), belonging to G protein-coupled receptors (GPCR) class-A superfamily, are responsible for modulating the release of histamine as well as of other neurotransmitters by a negative feedback mechanism mainly in the central nervous system (CNS). These receptors have gained increased attention as therapeutic target for several CNS related neurological diseases.

In the current study, we aimed to identify novel H₃R ligands using in silico virtual screening methods. To this end, a combination of ligand-and structure-based approaches was utilized for screening of ZINC database on the homology model of human H₃R. Structural similarity-and pharmacophore-based approaches were employed to generate compound libraries. Various molecular modelling methodologies such as molecular docking and dynamics simulation along with different drug-likeness filtering criteria were applied to select anti-H₃R ligands as promising candidate molecules based on different known parent lead compounds. In vitro binding assays of the selected molecules demonstrated three of them being active within the micromolar and submicromolar K_i range. The current integrated computational and experimental methods used in this work can provide new general insights for systematic hit identification for novel anti-H₃R agents from large compound libraries.

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Histamine H₃ receptor ligands by hybrid virtual screening, docking, molecular dynamics simulations, and investigation of their biological effects

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Abstract

Histamine H₃ receptors (H₃R), belonging to G-protein coupled receptors (GPCR) class A superfamily, are responsible for modulating the release of histamine as well as of other neurotransmitters by a negative feedback mechanism mainly in the central nervous system (CNS). These receptors have gained increased attention as therapeutic target for several CNS related neurological diseases. In the current study, we aimed to identify novel H₃R ligands using in silico virtual screening methods. To this end, a combination of ligand- and structure-based approaches was utilized for screening of ZINC database on the homology model of human H₃R. Structural similarity- and pharmacophore-based approaches were employed to generate compound libraries. Various molecular modeling methodologies such as molecular docking and dynamics simulation along with different drug likeness filtering criteria were applied to select anti-H₃R ligands as promising candidate molecules based on different known parent lead compounds. In vitro binding assays of the selected molecules demonstrated three of them being active within the micromolar and submicromolar K_i range. The current integrated computational and experimental methods used in this work can provide new general insights for systematic hit identification for novel anti-H₃R agents from large compound libraries.

KEYWORDS

anti-H₃R agents, histamine H₃ receptor, molecular docking, molecular dynamics simulation, virtual screening

1 | INTRODUCTION

Histamine as a biogenic amine exerts its physiological effects via four different subtypes of G-protein coupled histamine receptors: H₁, H₂, H₃, and H₄ receptors (H₁R, H₂R, H₃R, and H₄R, respectively). Although blockade of H₁R and H₂R has led to development of successful marketed drugs for treatment of allergy and gastric acid-related diseases, respectively, the H₃R- and H₄R-related therapeutics are under investigation or in early phases of clinical trials. The discovery of H₃R dates back to 1983 with the observation of regulated histamine release in depolarized slices of rat cerebral cortex by Arrang,

Garbarg, & Schwartz (1983). As presynaptic autoreceptors, they not only control the release of histamine, but also regulate the release of other neurotransmitters such as dopamine, norepinephrine, serotonin, and acetylcholine (Berlin, Boyce, & Ruiz, 2011). From sequence similarity point of view, H_3R shows the highest sequence homology to the H_4R (about ~37%) while less than 20% to H_1 and H_2 receptors (Mocking et al., 2016; Tiligada, Zampeli, Sander, & Stark, 2009). H_3R are mainly found in central nervous system (CNS) of human and animals. Induced signal transduction pathways upon H_3R activation are mediated through $G_{i/o}$ proteins of GPCR, leading to the decrease of intracellular Ca^{+2} and cyclic adenosine monophosphate formation by inhibition of adenylyl cyclase and hence the release of histamine and related neurotransmitters are suppressed (Nieto-Alamilla, Marquez-Gomez, Garcia-Galvez, Morales-Figueroa, & Arias-Montano, 2016).

Since many CNS-related disorders are affected by intracellular level of histamine and other neurotransmitters, in this context, H_3R have gained considerable attention to be targeted. On the basis of several recent investigations, H_3R antagonists and inverse agonists would be effective in CNS-related diseases and neuronal abnormalities such as attention deficit hyperactivity disorder (ADHD), Alzheimer's disease, schizophrenia, learning and memory disorders, sleep disorders epilepsy, and obesity (Gemkow et al., 2009). Recently, pitolisant (also known as tiprolisant and BF2.649) marketed under brand name of Wakix® was approved by European Medical Agency as an inverse agonist of H_3R used for treatment of narcolepsy with or without cataplexy in adults (Syed, 2016).

Accelerating rational drug design and development from bench to bed necessitates involvement of multidisciplinary efforts that are often costly and time-consuming. From this perspective, computer-aided drug design has emerged as invaluable tool significantly contributing in modern drug design pipeline with the major goal to introduce potential bioactive compounds, hit to lead optimization, and eliminating unnecessary experiments in early drug discovery research (Macalino, Gosu, Hong, & Choi, 2015).

Virtual screening is one of the attractive fields of computer-aided drug design which is subdivided into two categories: structure- and ligand-based virtual screening strategies. In structure-based virtual screening, structural information about the target protein (either obtained experimentally from X-ray crystallography and NMR or determined based on molecular modeling techniques) is utilized for screening the compounds through molecular docking. Given a defined pharmacophore model of the target binding site, candidate molecules from large libraries of commercially available compounds are ranked based on predicted interaction energies between molecules and complementary binding sites. In the case of ligand-based approach, the screening is performed according to the information of ligands known

to interact with a target molecule using chemical similarity, substructure searches, and three-dimensional pharmacophore shape matching. This approach is used in case of limited structural information regarding the target of interest (Chen et al., 2012; Scior et al., 2012; Sliwoski, Kothiwale, Meiler, & Lowe, 2014). Virtual screening can serve as an alternative to biological high-throughput screening owing to its potential for finding effective molecules in a timely and cost-effective manner. It provides a very limited set of commercially available candidate molecules for further biological evaluation and optimization achieved by purchasing ready-to-use compounds or synthesizing the compounds (Bielska et al., 2011; Lionta, Spyrou, Vassilatis, & Cournia, 2014).

In the current study, we tried to identify novel H_3R ligands using in silico methods. A combination of ligand-based and structure-based approaches was utilized for screening of databases oriented on the homology model of human H_3R. Structural similarity and pharmacophoric-based approaches were employed to generate compound libraries. Various molecular modeling methodologies such as molecular docking and dynamics simulation along with different filtering criteria such as in silico pharmacokinetic properties were applied to select candidate molecules for further preliminary biological evaluation. In vitro binding assays conducted on the selected compounds examined their capability for binding to H_3R. The current synergistically applied computational and experimental methods used in this work provide a workflow for systematic evaluation of large compound libraries while identifying new hit structures for the development of novel anti-H_3R agents.

2 | METHODS AND MATERIALS

2.1 | Molecular docking and pharmacophore model generation

A homology-based model for histamine H_3 receptor was generated using M_3 muscarinic acetylcholine receptor (PDB code: 4DAJ, Supporting information Appendix S1). In the next step, flexible docking of pitolisant into binding site of the modeled H_3R was performed using GOLD program (version 5.0; CCDC Inc., Cambridge, UK) (Jones, Willett, & Glen, 1995; Jones, Willett, Glen, Leach, & Taylor, 1997) running under LINUX operating system. To define the binding site atoms of the receptor, a point was assigned based on the known residues involved in binding. Geometric center of the residues Asp^{114}, Thr^{119}, Tyr^{189}, Phe^{198}, Glu^{206}, Trp^{371}, and Tyr^{374} (identified to be part of the binding site) was calculated and set as the center of the binding site. All atoms within a radius 10 Å were selected, and flexible docking was performed. Docking was carried out by applying two distance constraints (1.5–3.5 Å). The first constraint was between nitrogen atom of piperidine ring from pitolisant and oxygen atom of Glu^{206}

side chain from protein. The second one imposed on the system was between phenyl ring of pitolisant and phenyl ring of Tyr[189] side chain of protein. The best solution was selected based on score fitness and used for generation of pharmacophore model.

For 3D pharmacophore model generation, LIGANDSCOUT program (version 4.1, InteLigand, GmbH, Vienna, Austria. http://www.inteligand.com/ligandscout) was used (Wolber & Langer, 2005). In this study, the complex of docked pitolisant and H₃R was introduced to LIGANDSCOUT for 3D pharmacophore generation using predefined features, such as hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), hydrophobic (HY), negative ionizable (NI), positive ionizable (PI), aromatic ring (AR). The pharmacophore model was inspected for containing crucial chemical features and used as 3D query for virtual screening procedure.

2.2 | Ligand library preparation and virtual screening

Three approaches including structure- and ligand-based virtual screening or combination of them were used to find new ligands capable of binding to human H₃ receptor including: (a) In the first method, structure-based virtual screening was used through integration of LIGANDSCOUT (version 4.1) derived pharmacophore and ZINCPHARMER database (Kocs & Camacho, 2012). Briefly, the obtained pharmacophore was subjected to ZINCPHARMER web server to find hit compound(s) among the purchasable chemical space available in ZINC database. For filtering the compounds, some stringent criteria were applied: maximum 1 hit per conformation, maximum 1 hit per molecule, a maximum root-mean-square deviation (RMSD) of 0.2, a maximum of 10 rotatable bonds, and maximum molecular weight of 300 Da. (b) The second approach was based on ligand-based virtual screening through 2D/3D similarity-based search. For this purpose, pitolisant was used for similarity search in SWISSSIMILARITY database (Zoete, Daina, Bovigny, & Michielin, 2016) using FP2 fingerprint as 2D and Electroshape and Spectrophores methods as 3D search methods. (c) In this approach, a hybrid of ligand- and structure-based methods was utilized for virtual screening. Results from a ligand-based search on ZINC15 library (Sterling & Irwin, 2015) using pitolisant structure were used for generation of a compound library using internal library preparation features implemented in LIGANDSCOUT. The prepared library was used as input for structure-based virtual screening based on pharmacophore of pitolisant in complex with model structure of H₃R in LIGANDSCOUT program. The hits were ranked according to the pharmacophore-fit score values.

Then, the identified hits from all of three above-mentioned methods were subjected to SWISSTARGETPREDICTION web server for predicting the potential target candidates in a chemical-pharmacological space (Zoete et al., 2016). Accordingly, the molecules with the most probable affinity to the H₃R were selected for further analyses.

2.3 | Drug-likeness and ADME/Tox properties of the identified virtual hits

For further filtering the obtained hit compounds, they were sorted based on drug-likeness properties. A comprehensive set of criteria implemented in SWISSADME web server was considered (Daina, Michielin, & Zoete, 2017) including the following: Lipinski rule of five (Lipinski, Lombardo, Dominy, & Feeney, 2001), bioavailability criteria (Martin, 2005), Ghose filter (Ghose, Viswanadhan, & Wendoloski, 1999), Egan filter (Egan, Merz, & Baldwin, 2000) Muegge filter (Muegge, Heald, & Brittelli, 2001), Veber filter (Veber et al., 2002), and a lead-likeness criteria (Teague Simon, Davis Andrew, Leeson Paul, & Oprea, 1999). Ligands that successfully passed drug-likeness filters were kept for further evaluations. Moreover, SWISSADME web server was used for predicting the pharmacokinetic profile of the selected compounds such as absorption, distribution, metabolism, and excretion (ADME) properties. For toxicity evaluation, OPENVIRTUALTOXLAB (version 5.8) was used for anticipating toxic potential (TP) by providing estimation of binding affinity profile of the candidate molecules to off-target proteins including nuclear receptors (AR, ERα, ERβ, GR, MR, PR, LXR, PPARγ, TRα, and TRβ), cytochrome P450 enzyme family (1A2, 2C9, 2D6, 3A4), a cytosolic transcription factor (AhR) and a potassium ion channel (hERG) (Vedani, Dobler, Hu, & Smiesko, 2015; Vedani, Dobler, & Smiesko, 2012). Then, the selected candidate molecules were introduced to the next step of analyses.

2.4 | Molecular docking and binding free energy calculation

Ligands retrieved following rigorous and different filters in terms of drug-likeness, ADME and toxicity properties were subjected to molecular docking studies. GOLD software was used for flexible docking of the molecules on the modeled H₃R based on the same procedure conducted for pitolisant as parent lead compound. Similarly, two different sets of distance constraints including Tyr[189] and Glu[206] residues were applied to the equivalent atoms in pitolisant structure. This is to simulate the presence of π–π stacking and ionic interactions reported for pitolisant structure. In case of π–π stacking corresponding phenyl groups available in Tyr[189] and pitolisant are involved, whereas ionic interaction is formed between oppositely charged oxygen atom from Glu[206] and nitrogen atom of piperidine ring from pitolisant. Subsequently, the best pose among the docking solutions for each ligand was selected according to the used scoring function.

Following the molecular docking procedure, the molecular dynamics (MD) simulation was conducted for the

complex of candidate molecules and H_3R. Binding free energy was calculated for the complex of candidate ligands and H_3R for the simulation length of 10 ns using MM-Poisson-Boltzmann Surface Area (PBSA)/Generalized-Born Surface Area (GBSA) methods implemented in the AMBER package (Case et al., 2005; Kollman et al., 2000) as described in Supporting information (Appendix S4).

2.5 | Histamine H_3 receptor binding assay

Experimental H_3R affinity estimates were derived as described by Khanfar, Reiner, Hagenow, & Stark (2018). Therefore, a titration pattern of the purchased ligands (ranging from 1 nM to 100 μM) and pitolisant (0.01–1,000 nM) in duplicates was incubated with $[^3H]$-N^α-methylhistamine (2 nM, $K_D = 3.08$ nM) and membrane preparations of HEK-293 cells stably expressing the human H_3 receptor (20 μg/well) on 96-well microplates, including a sample of pitolisant (10 μM) for estimation of non-specific binding. After 90 min, incubation was terminated upon filtration on glass-fiber mats

(presoaked with 3% (m/m) polyethylene-imine solution) using a cell-harvester (Inotech, Dottikon, Switzerland) and removing unbound radioligand by three washing steps with cold water. Subsequently dried filter mats were soaked with scintillation liquid, sealed and subjected to read out using a MicroBeta® TriLuxscintillation counter (Wallac, Turku, Finland). Specific binding data were analyzed using non-linear least squares fit module of GRAPHPAD PRISM® (2012, vers. 6.01, La Jolla, CA, USA) to yield IC_{50} values, being converted to K_i constants (Cheng & Prusoff, 1973).

3 | RESULTS

3.1 | Molecular docking study and pharmacophore modeling

Following the modeling of H_3 receptor, pitolisant was docked into the model using GOLD program and Piecewise Linear Potential (PLP) fitness function (Korb, Stutzle, & Exner, 2009) to rank the docking solutions. The population size, number of
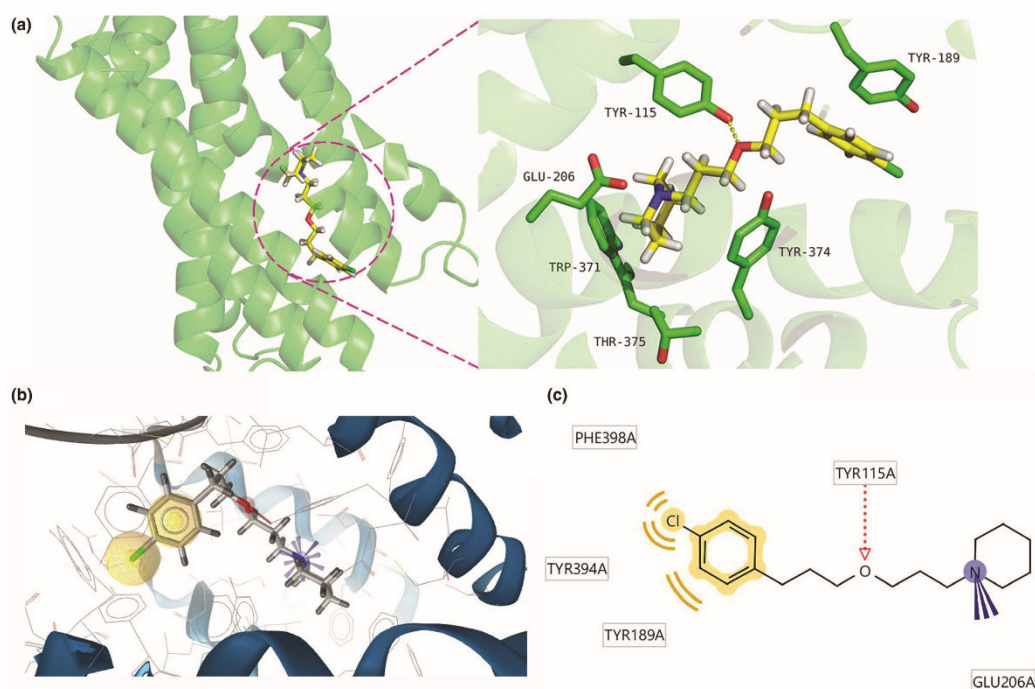
FIGURE 1 (a) 3D representation of the docked pitolisant into binding site of modeled H_3R generated by PYMOL program (version 1.7.x). The ligands and the main interacting residues are illustrated as sticks. Only the side chains of the interacting residues from receptor are shown for further clarity. (b) The structure-based pharmacophore model of pitolisant-H_3R generated by LIGANDSCOUT program (version 4.1). (c) Chemical structures of pharmacophoric elements of pitolisant-H_3R complex used for structure-based virtual screening procedure. The yellow color indicates hydrophobic (HY) interaction, the red dashed arrow demonstrates hydrogen bond acceptor (HBA), and the purple color refers to positive ionizable (PI) area [Colour figure can be viewed at wileyonlinelibrary.com]

operations, and the number of islands were set to 100, 100,000, and 5, respectively. The best solution based on greater value of scoring function was selected and used for pharmacophore generation. Figure 1 illustrates the docked pitolisant in complex with histamine H$_3$ receptor. As shown, phenyl ring of pitolisant is sandwiched between Tyr[189] and Phe[398]. Piperidine nitrogen establishes an ionic bond with Glu[206], and a hydrogen bond is observed between linker oxygen atom of pitolisant and Tyr[115]. The construction of the pharmacophore for structure-based virtual screening was based on the common features of already known active compounds utilizing LIGAND-SCOUT 4.1. Four chemical features including two hydrophobic regions, a positively ionizable moiety, and a hydrogen bond acceptor constituted the pharmacophore model (Figure 1). A π–π stacking interaction pointed to phenyl ring of Tyr[189], an ionic interaction oriented to Glu[206], hydrophobic interactions through Phe[398] and Tyr[394] residues, and a hydrogen bond acceptor toward Tyr[115] were considered as pharmacophoric elements and used as query for structure-based virtual screening procedure.

3.2 | Virtual screening of commercial libraries

Different approaches were employed for virtual screening of the ZINC database presented in Figure 2. In the structure-based virtual screening, 3D pharmacophoric features

generated for H$_3$R-pitolisant complex was used as input in the ZINCPHARMER web server to search purchasable chemical space available in ZINC database containing ~22 million commercially available compounds. Considering primary filtering criteria such as described above resulted to 56 chemical compounds. The remaining molecules were screened by applying additional lead-likeness filter (i.e. MW < 350 Da) leading to the selection of 16 hit compounds. The obtained compounds were subjected to SWISSTARGETPREDICTION web server for predicting the corresponding potential targets. The results showed that only one compound with drug-likeness properties is predicted to bind H$_3$R with the highest probability.

The structural similarity search based on pitolisant (FP2 fingerprint, Electroshape, and Spectrophores methods as 2D and 3D search methods) was employed for screening process among the ~10.6 million drug-like molecules of ZINC database. Similar to the previous method, the identified hits were inspected according to the possible binding affinity toward H$_3$R using SWISSTARGETPREDICTION web server. The findings resulted to introducing 18, 20, and 26 compounds for FP2 fingerprint, Electroshape, and Spectrophores search methods, respectively.

A combined ligand- and structure-based virtual screening protocol (hybrid method) was adopted as third approach in the current study. First, a library was prepared by applying
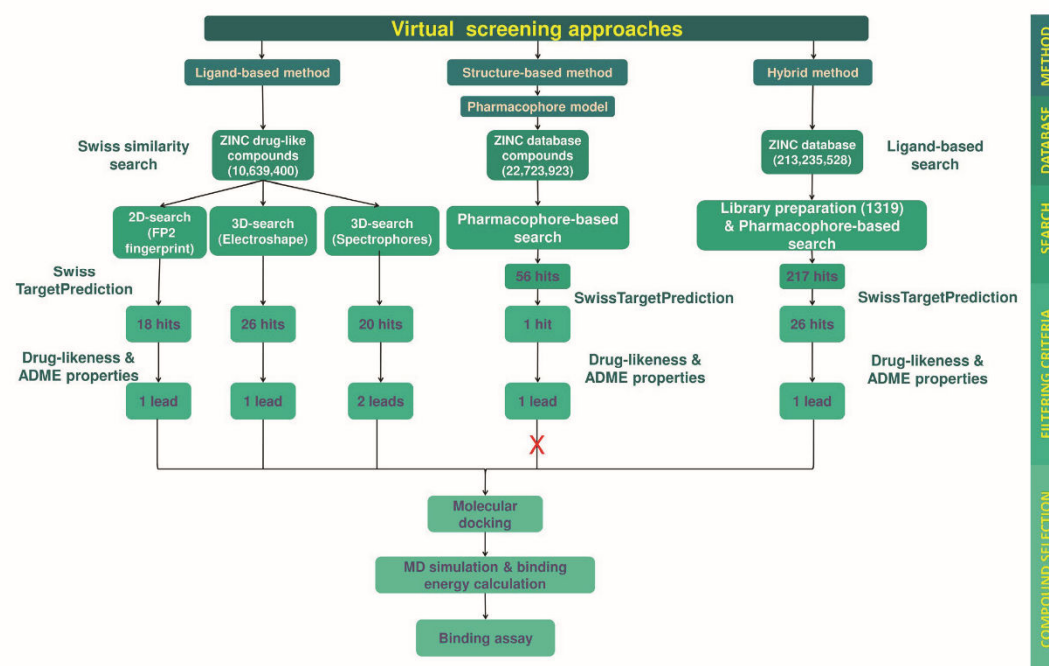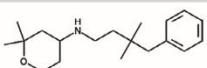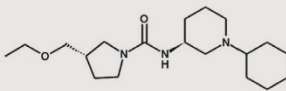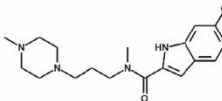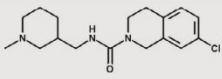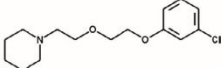

FIGURE 2 Overview of workflow used for virtual screening procedure [Colour figure can be viewed at wileyonlinelibrary.com]

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TABLE 1 List of candidate molecules selected by virtual screening scheme

Compound	ZINC code	Structure	Search method
1	Zinc32124090		Ligand-based approach (FP2 fingerprint search method)
2	Zinc81494423		Ligand-based approach (Electroshape search method)
3	Zinc69700808		Ligand-based approach (Spectrophores search method)
4	Zinc90563066		Ligand-based approach (Spectrophores search method)
5	Zinc2895674		Hybrid approach

ligand-based virtual screening as performed with pitolisant as template structure within 50% Tanimoto similarity index. Following the creation of library composed of 1,319 compounds, structure-based virtual screening was performed by introducing the library to LIGANDSCOUT program. The number of compounds was narrowed down to 217 hits using screening of library against pharmacophore model of pitolisant-H₃R and ranked based on pharmacophore fit value. The included chemical features of the pharmacophore are as follows: two hydrophobic regions, a positive ionizable moiety, and a hydrogen bond acceptor (see Figure 1). These compounds were scrutinized using SWISSTARGETPREDICTION web server and 26 molecules with the highest probability of binding to H₃R were kept for further analyses.

All the selected hits obtained from different approaches were subjected to the additional assessments for selection of final drug-like compounds.

3.3 | Drug-likeness, ADME profiling, and toxicity risk assessment

To investigate the drug-likeness and pharmacokinetic properties of the retrieved hit compounds (shown in Table 1), different criteria were applied using SWISSADME database. The pioneer of drug-likeness criteria corresponds to the study by Christopher A. Lipinski and his coworkers known as Lipinski's "rule-of-five." In this rule, an orally bioactive compound should pass the following criteria: MW of ≤500 Da, logP ≤ 5, hydrogen bond donor ≤5, and hydrogen bond acceptor (N and O atoms) ≤10. The other proposed criteria for drug-likeness property such as Ghose,

Egan, Muegge, and Veber are indicated in Supporting information Table S2.1. Furthermore, a variety of pharmacokinetic parameters such as blood–brain barrier (BBB) permeability, intestinal absorption, being substrate for P-glycoprotein (P-gp) or substrates, inhibitors or inducers of Cytochrome P-450 (CYP450) isoforms are also predicted (See Supporting information Table S2.1 for details). The analysis of the compounds obtained via the identification scheme presented in Figure 2 leads to identification of five drug candidates of which successfully passed those mentioned drug-likeness criteria as defined in Supporting information Table S2.1. One additional compound was identified from structure-based approach but excluded from further investigations due to contained imidazole moiety, since the latter showing detrimental biological properties. Compounds **1–4** originated from ligand-based search, with **1** and **2** detected by FP2 fingerprint and Electroshape methods of SWISSSIMILARITY, respectively, while compounds **3** and **4** were introduced as a result of the Spectrophores method of SWISSSIMILARITY. Compound **5** was obtained from hybrid method of virtual screening.

All the identified molecules have lead-likeness properties except compound **1** containing one violation (i.e. XLOGP3 > 3.5). Also, all the selected candidate molecules are predicted to be of desirable blood–brain barrier (BBB) permeability and of oral bioavailability, inferred from Abbott bioavailability score with potential synthetic accessibilities (Supporting information Table S2.1; Martin, 2005).

For toxicological risk assessment, OPENVIRTUALTOXLAB program was applied as a combination of automated and flexible molecular docking integrated with multi-dimensional

Compound	ZINC ID	$\Delta G_{\text{Binding (GB)}}$	$\Delta G_{\text{Binding (PB)}}$
Pitolisant	Zinc34045468	−38.85 (±2.38)	−32.36 (±3.23)
1	Zinc32124090	−35.56 (±2.35)	−31.91 (±3.08)
2	Zinc81494423	−35.32 (±4.34)	−32.85 (±3.77)
3	Zinc69700808	−36.88 (±2.95)	−31.72 (±3.69)
4	Zinc90563066	−28.72 (±2.98)	−23.60 (±3.89)
5	Zinc2895674	−23.62 (±2.81)	−16.60 (±3.37)

TABLE 2 Calculated mean values of binding free energies for the complex of docked candidate molecules and H₃R using Generalized-Born, $\Delta G_{\text{Binding (GB)}}$ and Poisson–Boltzmann, $\Delta G_{\text{Binding (PB)}}$ method for 10 ns MD simulation

Note. Standard deviations are shown in parentheses.

quantitative structure–activity relationship (QSAR). Endocrine and metabolic disruption, some aspects of carcinogenicity and cardiotoxicity are evaluated in this approach, and the results are presented as an estimation of binding affinity (i.e. TP) toward 16 target proteins most likely to trigger adverse effect. Five classes (i.e. 0, I, II. III, and IV) are considered for assessing the toxic potential of the given compounds ranging from none to extreme in the scale of toxicity alert. The result of toxicity risk assessment for the selected candidate molecules is provided in Supporting information Table S2.2 with no toxic potential predicted for compounds **1** and **2**, moderate classification is found for compounds **4** and **5**, whereas **3** falls into class I (elevated toxic potential).

3.4 | Molecular docking and calculation of binding free energy for ligand–receptor complex

In an attempt for determining the binding mode of the selected candidate molecules with homology-based model of H₃R, the flexible docking was executed by GOLD program based on the procedure outlined for pitolisant structure. Supporting information Figure S3.1 illustrates the interactions between docked ligands and H₃R. For more comprehensive information on molecular docking analysis, see Supporting information S3. The best docked pose in complex with H₃R was subjected for MD simulation for 10 ns using AMBER package. Analysis of the RMSD trajectories and potential energy plot revealed that all systems in the simulation were well-equilibrated and remained stable throughout 10 ns simulation run (Supporting information Figure S4.1). During the simulation, the snapshots were extracted every 10 ps from the trajectory and used for estimation of binding free energy by applying MM-PBSA/GBSA methods implemented in AMBER package. Table 2 shows the binding free energy for the complexes with respect to pitolisant structure (as reference compound).

3.5 | Histamine H₃ receptor binding assay

In vitro human H₃R affinity determination using tritiated N^{α}-methylhistamine revealed three of the preselected

TABLE 3 Affinity at human histamine H₃ receptor (H₃R) determined by [³H]-N$^{\alpha}$-methylhistamine displacement assay

Compound	H₃R K_i (μM) [95% CI][a]	n[b]
Pitolisant	0.0071 [0.0047;0.0106]	8
1	> 10	3
2	> 10	3
3	0.49 [0.12; 1.97]	4
4	0.54 [0.26; 1.16]	3
5	1.2 [0.7; 2.0]	3

[a]Values represent affinity estimates at human H₃R as means and corresponding confidence interval (CI 95%).
[b]Number of independent experiments performed in duplicates.

structures being active within the screened concentration range (Table 3). Purity of purchased compounds was about 89.0%, determined by LC-MS as described in Supporting information (S5). No affinity estimates could be derived for compound **1** consisting of a secondary amine instead of tertiary amine as shown in Table 1. Compound **2**, lacking aromatic moiety appeared inactive as well. Thereinstead, micromolar and submicromolar K_i were detected for the remaining compounds without significant differences within this group. Each of the latter consists of chlorine substituted aromatic core in the eastern part of the molecule and a tertiary alicyclic amine on the facing part.

4 | DISCUSSION

Presynaptic histamine H₃ receptors (H₃R) act as auto- or heteroreceptors, modulating the release of histamine and other neuroactive substances in the central nervous system (CNS). The extracellular levels of several neurotransmitters are enriched by H₃R antagonists, and there is a great interest for brain-penetrating H₃ receptor antagonists/inverse agonists to compensate for the neurotransmitter deficiency existing in various neurological disorders. As a result of relevant investigations in animal models of CNS diseases, administration of anti-H₃R ligands enhances the synaptic neurotransmission and improves the

corresponding neurophysiological processes (Hancock & Fox, 2004). There are lines of evidence suggesting the usefulness of H_3-antagonists/inverse agonists in the treatment of neurological disorders (Haig et al., 2014; Herring et al., 2012; Jarskog et al., 2015; Passani, Cangioli, Bacciottini, Mannaioni, & Blandina, 2000; Schwartzbach et al., 2017). Due to the important physiological role of H_3R in controlling neurotransmitters, special attention has been dedicated to the development of novel H_3 antagonists for their potential therapeutic applicability in neurological diseases.

In spite of tremendous ongoing projects for identification of novel H_3 ligands, there is no crystallography or NMR derived solved 3D structure for H_3R due to obstacles and challenges dealing with solubilization, purification, and crystallization of GPCRs (Carpenter, Beis, Cameron, & Iwata, 2008; Loll, 2003; Zhao & Wu, 2012). By solving the 3D structure of bovine rhodopsin using crystallography technique in 2000, this protein was used for modeling of GPCRs as the only available template for several years as well as for H_3R. Following the introduction of crystal structure for histamine H_1 receptor in complex with doxepin by Shimamura et al. (2011), it was used for developing more realistic homology models for H_3R in comparison with bovine rhodopsin (Harusawa et al., 2013; Tang et al., 2013; Wen, Liu, Hu, Wang, & Wu, 2017). However, the structural features can be considered in addition to sequence similarity for alignment of target and template sequence for modeling purposes. In the current study, we modeled the H_3R using M_3 muscarinic acetylcholine receptor (PDB code: 4DAJ) as the template due to its high sequence similarity to H_3R. Although the similarity of M_3 muscarinic receptor is important for modeling of H_3R, the alignment was further improved according to the structure-based sequence alignment using proposed GPCR Sequence-Structure (GRoSS) alignment of transmembrane (TM) regions for all experimentally solved structures for human GPCR (Cvicek, Goddard, & Abrol, 2016; Kim, Fristrup, Abrol, & Goddard, 2011).

Molecular docking of pitolisant as reference molecule was performed based on the identified key residues in the binding site resulting in docking comparable to those reported elsewhere (Kuder et al., 2016; Łażewska et al., 2016; Levoin et al., 2008; Morini et al., 2006). These amino acids might be the key residues of the binding site of H_3R important for ligand binding as evidenced in several previously published reports (Axe, Bembenek, & Szalma, 2006; Kuder et al., 2016; Łażewska et al., 2016; Levoin et al., 2013; Morini et al., 2006; Schwartz, 2011). As structure-based virtual screening method, the pharmacophore model of the receptor complex outlined above was generated by LIGANDSCOUT and applied to online ZINCPHARMER web server (Figure 1). Based on drug/lead-likeness filtering criteria and target prediction, only one imidazole-based compound was identified. This compound was excluded from further analyses due to its undesirable

properties such as binding to heme iron of cytochrome P450 enzymes (CYP450) as well as lack of selectivity toward histamine H_3/H_4 receptors (Lazewska et al., 2006; Sander, Kottke, & Stark, 2008).

The secondly applied ligand-based similarity search was carried out aiming to reach compounds with more than 50% similarity to pitolisant using ZINC drug-like of SWISSSIMILARITY database employing FP2 fingerprint, Electroshape, and Spectrophores search methods. As two-dimensional search method, descriptors originated from two-dimensional chemical structures are generated followed by computing similarities between descriptors of the molecular fingerprint (FP) and of chemical properties encoded in a vector, quantified using Tanimoto coefficient (Zoete et al., 2016). Thereinstead, as three-dimensional approaches, the 3D geometry of the molecules is considered such as Electroshape and Spectrophores search methods. The latter consist of multi-dimensional vectors considering the shape and molecular features while in Electroshape search methods, properties like atomic charge and partition coefficient are used for defining fourth and fifth coordinates of each atom. The retrieved compounds from each search methods were subjected to a second filtering in terms of drug/lead-likeness criteria and target prediction. Totally four candidate molecules were selected: one from FP2 fingerprint method (compound **1**), one from Electroshape (compound **2**), and two from Spectrophores (compounds **3** and **4**) search methods (Table 1). The last virtual screening method utilized in the current study was a hybrid of ligand- and structure-based methods. Structural similarity- and pharmacophore-based approaches were employed to generate compound libraries to be screened according to the given pharmacophore. This approach led to identification of the fifth molecule (compound **5**) having lead-likeness properties with probable H_3R affinity predicted. The embedded database in SWISSTARGETPREDICTION web server works based on a knowledge-based algorithm using 2D and 3D similarity measures in terms of chemical structure and molecular shape (Gfeller et al., 2014). To satisfy selectivity, H_3R was listed as first rank of target prediction with high probability for selected candidate molecules.

High attrition risk on late stage drug development necessitates early evaluation of pharmacokinetic profile, drug-likeness criteria, and toxicity risk. ADME properties such as absorption, distribution, metabolism, and excretion of drugs are studied on different clinical stages of development. In silico prediction of ADME properties has dramatically reduced high costs associated with labor-extensive experiments. The predicted ADME properties using SWISSADME web server revealed all of the selected candidate molecules having high BBB penetration which is important for H_3R ligands. Moreover, high intestinal absorption was observed for all the compounds. From metabolic point of view, none of them inhibit the CYP3A4 and CYP2C9 as important

FIGURE 3 Schematic presentation of the pharmacophore model proposed for the selected candidate molecules as anti-H₃R ligands [Colour figure can be viewed at wileyonlinelibrary.com]

drug metabolizing isoforms of cytochrome P450 (CYP450) enzymes. Further, all the five selected compounds successfully passed the different drug-likeness criteria, and no violation was observed within the corresponding defined limits (Supporting information Table S2.1; Zanger & Schwab, 2013).

Preliminary potential "off-target" activity evaluation of the drug-like candidates such as endocrine and/or metabolic disruption, carcinogenicity, and cardiotoxicity is of great importance for toxicity assessment. The toxicity profile analysis using OpenVirtualToxLab program suggests the possible safety of the candidate molecules judged from low statistical probability (i.e. TP values assigned to class 0 and I; Supporting information Table S2.2).

Interestingly, the current selected molecules share a set of structural commonalities on the basis of given pharmacophore. Figure 3 shows the schematic representation of the main scaffold proposed for the selected candidate molecules comprised of a basic amine moiety connected to a hydrophobic/aromatic moiety through a linker. To investigate the mode of interactions of the compounds with H₃R and map pharmacophore features with key binding site residues, molecular docking was carried out. The results of docking indicate that the critical amino acids interacting with different parts of selected molecules are as follows: Glu^{206} (TM5) in the interaction with basic moiety, Tyr^{115} (TM3) and Tyr^{374} (TM6) residues interact with linker, and Tyr^{189} (TM5) and Phe^{398} (TM7) forms the hydrophobic pocket with hydrophobic/aromatic moiety (Figure S3.1). These findings are in close agreement with the findings published previously (Axe et al., 2006; Bajda et al., 2012; Harusawa et al., 2013; Kuder et al., 2016; Łażewska et al., 2016; Lepailleur et al., 2014; Levoin et al., 2013; Morini et al., 2006; Sheng et al., 2015; Wen et al., 2017).

Affinity measurements revealed compounds **3–5** being active at H₃R. This is in contrast to the results obtained by MD simulations. Whereas the latter showing similar binding free energies between pitolisant and compounds **1–3** while presenting weaker ones for compounds **4** and **5**, affinity estimates in N^{α}-methylhistamine displacement indicate no affinity for 1 and 2 and similar binding affinities for the remaining compounds. To address these discrepancies, the use of H₃R agonist N^{α}-methylhistamine as radiolabeled species within the binding studies should be considered as the MD simulations were carried out using the receptor conformation while interacting with the H₃R inverse agonist pitolisant.

Structural differences presumably answering for the observations of the radioligand displacement studies comprise of alternating substitution degree of basic amine in compound **1** (secondary instead of tertiary) and lacking aromatic cores in compound **2**. However, these are common structural features of H₃R ligands but no prerequisite to show activity. Namely the endogenous ligand histamine lacks tertiary amine and the sterically restricted steroid alkaloid conessine being active at H₃R without consisting of any aromatic core (Panula et al., 2015). Compound **3** shows structural resemblance to H₄R antagonist JNJ-7777120 with H₃R affinity ranging within the same order of magnitude whereas compound **5** appears as a pitolisant counterpart. In case of the latter pair, the decreased lipophilicity due to the bis-ethyloxy linker and the shortage by one methylene group, thereby changing the spatial orientation of the aromatic core to the tertiary amine, may account for the reduced affinity.

5 | CONCLUSION

In the present investigation, novel anti-H₃R agents were introduced using ligand- and structure-based virtual screening approaches followed by in vitro binding assays. Biological evaluations revealed micromolar and submicromolar K_i values (i.e., 0.49–1.2 μM) for three of the identified molecules. The mode of interactions for the selected compounds was predicted using molecular modeling techniques. Taken together, the presented lead candidates may serve as starting points for further medicinal chemistry optimization for development of novel CNS selective H₃R antagonists to be used in neurodegenerative diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Material

Histamine H₃ Receptor Ligands By Hybrid Virtual Screening, Docking, Molecular Dynamics Simulations, and Investigation of Their Biological Effects

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S1. Homology modeling of human H₃ receptor

Comparative molecular modeling was used for modeling of human histamine H₃ receptor (Uniprot ID: Q9Y5N1). The BLAST search engine, publicly available at National Center for Biotechnology Information (NCBI), was used to find the homologous proteins with known structures as the templates for human H₃ receptor. Based on the obtained results, M₃ muscarinic acetylcholine receptor (PDB code:4DAJ) (1) was selected as the template due to its highest similarity to human H₃ receptor. Clustal Omega from its website at European Bioinformatics Institute (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for sequence alignment of H₃ receptor and M₃ muscarinic acetylcholine receptor accepting default values for the alignment parameters. The alignment was manually adjusted guided by structure-based sequence alignment of all human GPCRs (2, 3). Initial model was built using Swiss PDB viewer (SPDBV, version 4.1, Swiss Institute of Bioinformatics, Lausanne, Switzerland) based on the obtained alignment and submitted to Swiss-Model server to generate energy minimized model structure (https://swissmodel.expasy.org/) (4). The quality of the generated model was evaluated using PROCHECK (5), Molprobity (6), and Verify 3D (7, 8), from their web servers.

The pharmacophore model required for structure-based virtual screening procedure consisted of a homology model of the histamine H3 receptor based on the structure of M₃ muscarinic acetylcholine receptor (PDB ID: 4DAJ) as the template. The initial amino acid sequence alignment between H₃R and M₃ receptor by Clustal Omega resulted to 26.43% sequence identity. This alignment was further manually improved on the basis of structure-based sequence alignment of all human GPCRs. Figure S1.1 shows the sequence alignment and highlights the key conserved residues (red capitals) among GPCR superfamily. Analysis of the optimized final energy minimized model obtained from Swiss-Model server by Procheck program showed that backbone φ and ψ dihedral angles for more than 99% of residues are in the allowed region of the Ramachandran plot (Figure S1.2). The similar quality measure was estimated to be 97.5% using the MolProbity method (Figure 2B). The results are indicative of high quality of the generated model from geometrical perspective. Furthermore, to assess the folding properties of the model in terms of compatibility of proposed model structure (3D) with its sequence (1D), Verify-3D methodology was applied. The result revealed 71.65% of the residues in the 3D/1D profile showing an average score of > 0.2 (Figure S1.3) and those with 3D-1D scores lower than this value not being close to the binding site of the receptor.

1

2

Figure S1.3. Sequence-structure compatibility assessment for H₃R model based on profiles-3D method calculated with verify-3D software from its website. The positive scores suggest that the residues are placed in a proper environment and the protein is folded appropriately.

S2. Drug-likeness, ADME profiling and toxicity risk assessment

Physicochemical, pharmacokinetic, drug-likeness, and medicinal chemistry properties of lead compounds is shown in Table S2.1. Table S2.2 provides the estimated toxic potential of candidate molecules.

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Figure S1.1. Sequence alignment used for building homology model of human H₃ receptor based on M3 muscarinic acetylcholine receptor (PDB ID: 4DAJ).The alignment was manually adjusted guided by structure-based sequence alignment of all human GPCRs (2, 3). The symbols ":*", ":", and ":" represent identical, conserved and semi-conserved substitutions, respectively. The TM regions are shown in different colored boxes (TM1 in violet, TM2 in blue, TM3 in cyan, TM4 in green, TM5 in yellow, TM6 in orange, TM7 in red). Highly conserved amino acids are displayed in red in TM1-6 and white in TM7.

Figure S1.2. The Ramachandran plots for modeled H₃R obtained from (A) PROCHECK and (B)Molprobity model evaluation web servers. Both calculations showed that more than 97% of amino acids are in the allowed regions.

3

Table S2.1 Physicochemical, pharmacokinetic, drug-likeness, and medicinal chemistry properties of lead compounds

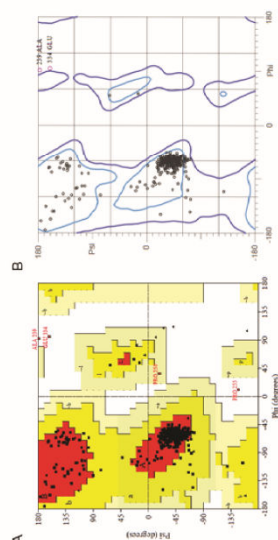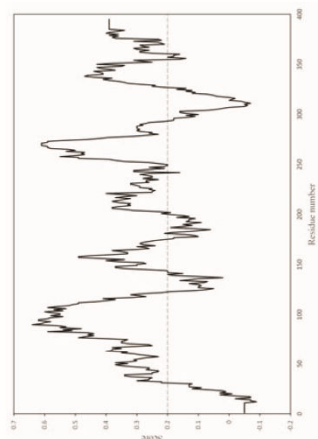| Molecular Descriptor	1	2	3	4	5
Physicochemical Properties					
Formula	C$_{17}$H$_{19}$NO	C$_{19}$H$_{23}$N$_3$O$_2$	C$_{20}$H$_{24}$N$_2$O	C$_{17}$H$_{19}$FN$_2$O	C$_{16}$H$_{21}$ClN$_2$O$_2$
Molecular weight	289.46 g/mol	337.50 g/mol	348.87 g/mol	321.84 g/mol	283.79 g/mol
Num. rotatable bonds	6	7	6	4	7
Num. H-bond acceptors	2	3	3	2	3
Num. H-bond donors	1	1	1	1	0
Molar Refractivity	90.64	105.20	106.36	97.43	82.18
TPSA	21.26 Å^2	44.81 Å^2	42.58 Å^2	35.58 Å^2	21.70 Å^2
Pharmacokinetic properties					
GI absorption	High	High	High	High	High
BBB permeant	Yes	Yes	Yes	Yes	Yes
P-gp substrate	No	No	Yes	Yes	No
CYP1A2 inhibitor	No	No	No	No	Yes
CYP2C19 inhibitor	No	No	Yes	No	Yes
CYP2C9 inhibitor	No	No	No	No	No
CYP2D6 inhibitor	Yes	Yes	Yes	Yes	Yes
CYP3A4 inhibitor	No	No	No	No	No
Log K_p (skin permeation)	-5.00 cm/s	-6.54 cm/s	-6.53 cm/s	-6.50 cm/s	-5.72 cm/s
Druglikeness					
Lipinski	Yes	Yes	Yes	Yes	Yes
Ghose[a]	Yes	Yes	Yes	Yes	Yes
Veber[b]	Yes	Yes	Yes	Yes	Yes
Egan[c]	Yes	Yes	Yes	Yes	Yes
Muegge[d]	Yes	Yes	Yes	Yes	Yes
Bioavailability Score	0.55	0.55	0.55	0.55	0.55
Medicinal Chemistry properties					
Lead-likeness[e]	No; 1 violation: XLOGP3>3.5				
Synthetic accessibility	2.84	4.20	2.47	3.08	2.43

[a] Ghose filter: 160 ≤ MW ≤ 480, -0.4 ≤ WLOGP ≤ 5.6, 40 ≤ MR ≤ 130, 20 ≤ atoms ≤ 70.
[b] Veber filter: Rotatable bonds ≤ 10; TPSA ≤ 140; [c] Egan filter: WLOGP ≤ 5.88; TPSA ≤ 131.6.
[d] Muegge: 200 ≤ MW ≤ 600, -2 ≤ XLOGP ≤5, TPSA ≤ 150; Num. rings ≤ 7; Num. of carbons > 4; Num. heteroatoms > 1; Num. rotatable bonds ≤ 15; Num. H-bond acceptors ≤ 10; and Num. H-bond donors ≤ 5.
[e] Lead-likeness: 250 ≤ MW ≤ 350, XLOGP ≤ 3.5, and Rotatable bonds ≤ 7.

5

Table S2.2 In silico toxicity evaluation of candidate molecules. The calculated binding affinities (IC$_{50}$, Molar) for selected candidate molecules against all 16 target proteins as well as toxic potential using VirtualToxLab are shown.

Molecule	TP	AR	AhR	CYP1A2	CYP2C9	CYP2D6	CYP3A4	ERα	ERβ	GR	hERG	LXR	MR	PPARγ	PR	TRα	TRβ
1	0.269	1.82E-05	NB	NB	NB	NB	NB	9.77E-05	NB	NB	1.41E-05	4.04E-05	NB	NB	NB	NB	1.96E-05
2	0.266	NB	NB	NB	NB	NB	NB	NB	NB	1.62E-05	1.30E-05	5.03E-07	3.79E-05	2.90E-07	6.6CE-05	7.22E-07	2.99E-05
3	0.53	1.91E-05	NB	NB	6.01E-05	3.43E-07	NB	2.31E-07	3.3CE-08	5.93E-05	3.92E-05	2.58E-05	NB	NB	NB	NB	NB
4	0.472	NB	NB	NB	NB	NB	NB	5.93E-05	3.97E-05	2.54E-05	1.03E-07	NB	NB	NB	NB	NB	NB
5	0.463	NB	4.58E-05	NB	NB	NB	NB	6.65E-07	9.77E-07	6.83E-05	2.78E-07	NB	2.07E-05	1.21E-05	NB	5.02E-05	NB

AR, Androgen receptor; AhR, Aryl hydrocarbon receptor; ERα, Estrogen receptor α; ERβ, Estrogen receptor β; GR, Glucocorticoid receptor; hERG, LXR, Liver X receptor; MR, Mineralocorticoid receptor; PR, Progesterone receptor; TRα, Thyroid receptor α; TRβ, Thyroid receptor β; NB no binding. TP = toxic potential derived from calculated binding affinities in respect to series of proteins with known relevant effects. Toxic classes are defined as:
Class I: TP ≤ 0 (above); Class II: 0 < TP ≤ 0.4 (low);
Class I: 0.5 < TP ≤ 0.6 (elevated);
Class II: 0.6 < TP ≤ 0.9 (high);
Class III: 0.7 < TP < 0.9 (Very high);
Class IV: > 0.9 (Extreme).

6

S3. Molecular docking of selected candidate molecules

Analysis of molecular docking results demonstrates the key residues involved in the ligand-receptor interactions (see Table S3 and Figure S3.1). The important amino acids in the binding site of H₃R interacting with selected molecules are: Tyr^{115}, Tyr^{189}, Phe^{193}, Leu^{199}, Glu^{206}, Trp^{371}, Tyr^{374}, Met^{378}, Tyr^{394}, Phe^{398}. For all the selected molecules., π–π stacking and ionic interactions formed by Tyr^{189} and Glu^{206}, respectively, are the two important interactions observed. The main interactions observed for compound 1 bound to the H₃R are: a π–π stacking, two hydrophobic pockets, and an ionic interaction. The π–π stacking is established between Tyr^{189} and phenyl group at the eastern part of molecule. One of hydrophobic pockets is composed of Phe^{193}, Leu^{199}, Tyr^{394}, and Phe^{398} residues accommodating the phenyl and linker dimethyl moiety while the residues Tyr^{115}, Trp^{371}, Tyr^{374}, and Met^{378} consisting the other hydrophobic pocket to interact with tetrahydropyran ring in molecule 1. Additionally, the ionic interaction is established between Glu^{206} and basic amine of linker. In the case of molecule 2, the identified interactions include: an ionic interaction between Glu^{206} and nitrogen of pyrrolidine, a three centered hydrogen bond between NH in the proximity of piperidine ring and Tyr^{115} and Tyr^{374}, and two hydrophobic pockets surrounding the endmost parts of molecule at both ends. Cyclohexyl and piperidine rings of molecule 2 interact hydrophobically with residues Tyr^{189}, Phe^{193}, Leu^{199}, Tyr^{394}, and Phe^{398} from H₃R. It should be noted that Tyr^{189} makes an interaction with cyclohexyl ring like π–π stacking observed for all compounds. Moreover, the other hydrophobic interactions are formed between Trp^{371}, Thr^{375}, Met^{378}, and Leu^{101} of the H₃R and substituent of pyrrolidine at the distal end of molecule. The identified interactions observed for compound 3 are similar to those discussed above. Again a π–π stacking is formed between Tyr^{189} and phenyl group in the indole ring. In addition, indole ring makes hydrophobic interaction with Tyr^{394}. A hydrogen bond is also seen between Tyr^{374} and oxygen of ketone group. Similar to other compounds, molecule 3 contains the ionic interaction between its piperazine nitrogen and Glu^{206}. Furthermore, amino acids Tyr^{115}, Phe^{193}, Leu^{199}, Tyr^{371}, and Met^{378} from H₃R hydrophobically interact with piperazine and alkyl part of linker in compound 3. The main interactions of molecule 4 resemble to those observed for other compounds. Similarly, a hydrophobic pocket formed by Tyr^{189}, Tyr^{394}, and Phe^{398} and surrounds the tetrahydroisoquinoline moiety of the molecule and establishes hydrophobic interactions. The observed π–π stacking in this pocket is also related between Tyr^{189} and phenyl ring of tetrahydroisoquinoline part. In addition, the other hydrophobic interactions are formed between piperidine ring and Tyr^{115}, Phe^{193}, Leu^{199} and Met^{378} from the receptor. Besides, Tyr^{374} of the H₃R is engaged in the hydrogen bond with linker amide NH of the compound 4. Moreover, the ionic interaction is also observed between Glu^{206} and nitrogen of piperidine at the end part of molecule. In the case of molecule 5 which is structurally similar to pitolisant, the observed interactions are as follows: ionic interaction between Glu^{206} and nitrogen of piperdine ring, hydrogen bond between Tyr^{374} and the central ether oxygen, as well as two hydrophobic contacts. The phenyl group is sandwiched in a hydrophobic pocket comprised of Tyr^{189}, Tyr^{394} and Phe^{398} residues from the receptor whereas piperidine ring and linker alkyl located in the hydrophobic pocket formed by Tyr^{115}, Leu^{199}, and Met^{378}.
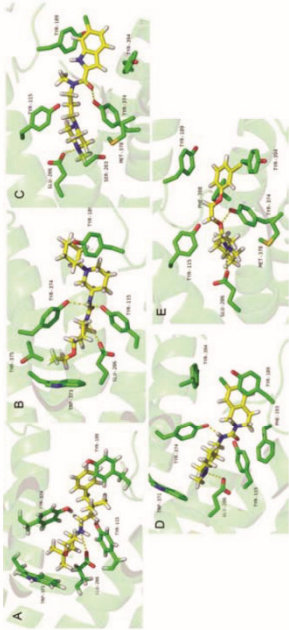

Figure S3.1. 3D representation of selected compounds 1-5 (Panels A to D) docked into the binding site of H₃R generated by PyMol program (version 1.7.x). The ligands and the main interacting residues are shown as sticks. Only the side chains of the interacting residues from receptor are shown for further clarity.

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Table S3.1. Amino acids of H3R involved in interactions with candidates.

Compound	Amino acids of H3R
1	Tyr^{115}, Tyr^{189}, Phe^{198}, Leu^{199}, Glu^{206}, Trp^{371}, Tyr^{374}, Thr^{375}, Met^{378} Tyr^{394}, Phe^{398}, Leu^{401}
2	Tyr^{115}, Tyr^{189}, Ala^{190}, Phe^{193}, Leu^{199} Glu^{206} Trp^{371}, Tyr^{374}, Thr^{375}, Met^{378} Tyr^{394}, Phe^{398}, Leu^{401}
3	Tyr^{115}, Tyr^{189}, Phe^{198}, Leu^{199}, Ser^{203}, Glu^{206}, Tyr^{374}, Met^{378}, Asp^{390}, Tyr^{394}
4	Tyr^{115}, Tyr^{189}, Ala^{190}, Phe^{198}, Leu^{199}, Glu^{206}, Tyr^{374}, Met^{378}, Tyr^{394}, Phe^{398}
5	Tyr^{115}, Tyr^{189}, Leu^{199}, Glu^{206}, Tyr^{374}, Met^{378} Tyr^{394}, Phe^{398}

S4. MD simulation and calculation of binding free energy

All the MD calculations were performed using the Assisted Model Building with Energy Refinement (AMBER) suite of programs (version 14) (9, 10) operating on a Linux-based (Centus6.8) GPU work station consisting of four NvidiaK40M (each has 12 GB RAM and 2880 cuda cores), 2X Intel Xeon E5-2697 v2, 2.7 GHz (total of 48 cores), total RAM = 128 GB. The usable coordinate files for AMBER (i.e. *prmtop and *.inpcrd) were created by *leap* module followed by adding a correct number of counter ions for neutralizing the total charge of the system. The system was solvated with TIP3P water molecules in a rectangular box by the buffering distances set to 12 Å in all directions. Then the solvated system was subjected to an initial energy minimization process by applying *Sander* module (500 steps of steepest descent followed by 500 steps of conjugate gradient) followed by a 50 ps heating step where the temperature was gradually increased from 0 to 300 K. 50 ps of density equilibration was followed by 500 ps of constant pressure equilibration at 300 K with a time step of 2 fs. Only bond lengths involving hydrogen atoms were constrained using the SHAKE algorithm. A final 10 ns MD simulation was performed by applying the Particle Mesh Ewald (PME) method to calculate long-range electrostatic interactions. All calculations were carried out under periodic boundary conditions where no constraint was applied to either the protein or the ligand molecules. The trajectory of the simulation was obtained by writing out the coordinates every 10 ps. After MD simulation on receptor-ligand complex, snapshots were extracted from the 10 ns molecular dynamic trajectory with an interval of 10 ps. The dielectric constant values were set to 1.0 and 80 for the interior of solute and the surrounding solvent, respectively. Binding free energy was calculated for the complex of candidate ligands and H3R using MM-Poisson-Boltzmann Surface Area (PBSA)/Generalized-Born Surface Area (GBSA) methods implemented in the AMBER package (9, 11). Ligand-receptor binding free energy is obtained by summing up the molecular mechanics energies, solvation-free energies, and entropic terms, averaged over a series of equilibrated snapshots from MD simulation trajectory. The interaction energies for the snapshots were calculated by excluding water molecules and counter ions and presented as the average value.

Binding free energy (ΔG_{bind}) is calculated using the following equation:

$$\Delta G_{bind} = G_{water(complex)} - G_{water(protein)} - G_{water(ligand)}$$

In this equation, G_{water}(complex), G_{water}(protein) and G_{water}(ligand) are the free energies of the complex, protein, and ligand, respectively. Free energy, ΔG, for each species is calculated as follows:

$$G = E_{gas} + \Delta G_{solvation} - TS$$

$$\Delta G_{solvation} = \Delta G_{polar} + \Delta G_{non-polar}$$

$$E_{gas} = E_{int} + E_{vdw} + E_{elec}$$

$$E_{int} = E_{bond} + E_{angle} + E_{tors}$$

G is the calculated average free energy, and E_{gas} is the standard force-field energy, including internal energy (E_{int}) in the gas phase as well as non-covalent van der Waals (E_{vdw}) and electrostatic (E_{elec}) energies. E_{bond}, E_{angle} and E_{tors} are the contributions to the internal energy caused by the strain from the deviation of the bonds, angle, and torsion angle from their equilibrium values. ΔG_{polar} and $\Delta G_{non-polar}$ the polar and non-polar contributions to the solvation free energy. The polar contribution is achieved using Poisson-Boltzmann or Generalized Born model while the non-polar contributions are calculated by the solvent accessible surface area (SASA) using the LCPO method (12). TS indicates the vibrational entropy term which is neglected in this study assuming similar entropy contribution for all complexes to the binding free energy.

Analysis of the RMSD trajectories and potential energy plot demonstrated that all systems in the simulation were well-equilibrated and remained stable throughout 10 ns simulation run.
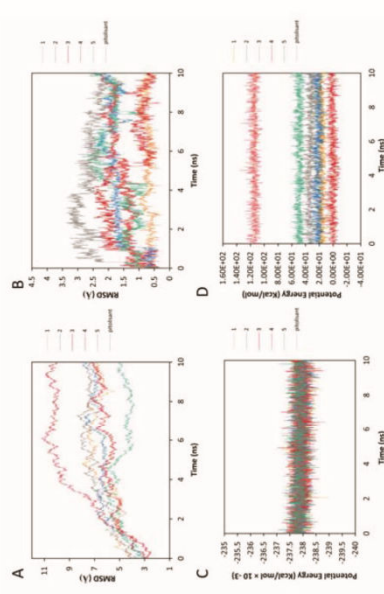
Figure S4.1. The results of molecular dynamics simulation analyses on ligand-H3R complex. Panels A and B show the plot of root mean square deviation (RMSD) fluctuation in a 1 to 10 ns molecular dynamics simulation for ligand-H3R complexes and ligands, respectively. Panels C and D indicate potential energies for the ligand-H3 complexes and compounds 1 to 5 during 10 ns molecular dynamics simulation.

9

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S5. Purity analytics of purchased compounds

Preselected compounds were purchased from various chemical suppliers listed within the MolPort database (Riga, Latvia) to evaluate binding behavior *in vitro*. Purity of supplied compounds was assessed by LC-MS using a Bruker Elute SP HPLC equipped with an Intensity Solo C18 RP column (100 x 2,1 mm) and connected to a Bruker amaZon speedion-trap mass spectrometer (ESI) for detection. Used eluent consisted of water and acetonitrile (1 - 40% acetonitrile, 0.1% formic acid) at a flow of 0.3 ml/min. The purity of the ordered compounds was about 89,0%, expressed as normalized fractional area (Supplementary Material S2).

Table S5.1 Purity of purchased compounds assessed by LC-MS

Compound	$t_R^{a)}$ [min]	m/z	Purity$^{b)}$ [%]
1	23.2	290.17	94.7
2	18.4	338.26	97.7
3	19.1	349.18	93.3
4	20.1	322.13	91.3
5	21.3	284.05	89.0

a) retention time.

b) HPLC analysis (Bruker Elute SP; Column: Intensity Solo C18 RP; column dimensions: 100x2.1 mm; eluent: acetonitrile (1-40%) in water, 0.1% formic acid; flow: 0.3 mL/min) combined with mass spectrometric detection (Bruker amazon speed with ESI; detection: ion-trap; ion-polarity: positive; scan: 100-600 m/z), expressed as normalized peak area (%) as approximated purity.

Fig. S5.3 Compound 2 / LC-MS chromatogram
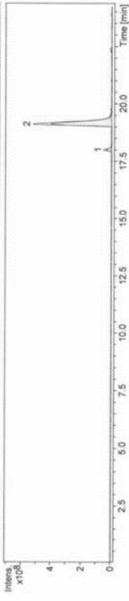
Fig. S5.4 Compound 3 / LC-MS chromatogram
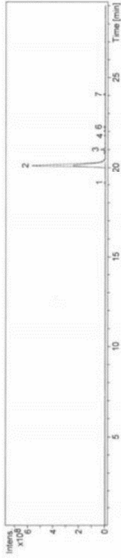
Fig. S5.5 Compound 4 / LC-MS chromatogram
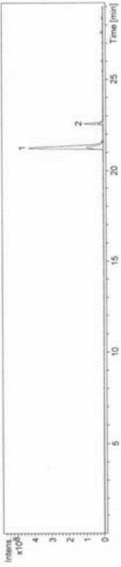
Fig. S5.6 Compound 5 / LC-MS chromatogram

Fig. S5.2 Compound 1 / LC-MS chromatogram

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4.5. In silico and in vitro studies of two non-imidazole multiple targeting agents at histamine H3 receptors and cholinesterase enzymes

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Contribution to research: Co-authorship. DR co-organised shipment of test-ligands, prepared, planned and conducted radioligand displacement experiments, and evaluated corresponding data to determine H3R affinity. DR reviewed the manuscript.

Abstract:

Recently, multi-target directed ligands have been of research interest for multifactorial disorders such as Alzheimer's disease (AD). Since H3 receptors (H3Rs) and cholinesterases are involved in pathophysiology of AD, identification of dual-acting compounds capable of improving cholinergic neurotransmission is of importance in AD pharmacotherapy. In the present study, H3R antagonistic activity combined with anticholinesterase properties of two previously computationally identified lead compounds, that is, compound 3 (6-chloro-N-methyl-N-[3-(4-methylpiperazin-1-yl)propyl]-1H-indole-2-carboxamide) and compound 4 (7-chloro-N-[(1-methylpiperidin-3-yl)methyl]-1,2,3,4-tetrahydroisoquinoline-2-carboxamide), was tested. Moreover, molecular docking and binding free energy calculations were conducted for binding mode and affinity prediction of studied ligands toward cholinesterases. Biological evaluations revealed inhibitory activity of ligands in nanomolar (compound 3: H3R EC_{50} = 0.73 nM; compound 4: H3R EC_{50} = 31 nM) and micromolar values (compound 3: AChE IC_{50} = 9.09 μM, BuChE IC_{50} = 21.10 μM; compound 4: AChE IC_{50} = 8.40 μM, BuChE IC_{50} = 4.93 μM) for H3R antagonism and cholinesterase inhibition, respectively. Binding free energies yielded good consistency with cholinesterase inhibitory profiles. The results of this study can be used for lead optimisation where dual inhibitory activity on H3R and cholinesterases is needed. Such ligands can exert their biological activity in a synergistic manner resulting in higher potency and efficacy.

RESEARCH ARTICLE

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In silico and in vitro studies of two non-imidazole multiple targeting agents at histamine H_3 receptors and cholinesterase enzymes

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Abstract

Recently, multi-target directed ligands have been of research interest for multifactorial disorders such as Alzheimer's disease (AD). Since H_3 receptors (H_3Rs) and cholinesterases are involved in pathophysiology of AD, identification of dual-acting compounds capable of improving cholinergic neurotransmission is of importance in AD pharmacotherapy. In the present study, H_3R antagonistic activity combined with anticholinesterase properties of two previously computationally identified lead compounds, that is, compound **3** (6-chloro-*N*-methyl-*N*-[3-(4-methylpiperazin-1-yl) propyl]-1H-indole-2-carboxamide) and compound **4** (7-chloro-*N*-[(1-methylpiperidin-3-yl)methyl]-1,2,3,4-tetrahydroisoquinoline-2-carboxamide), was tested. Moreover, molecular docking and binding free energy calculations were conducted for binding mode and affinity prediction of studied ligands toward cholinesterases. Biological evaluations revealed inhibitory activity of ligands in nanomolar (compound **3**: H_3R $EC_{50} = 0.73$ nM; compound **4**: H_3R $EC_{50} = 31$ nM) and micromolar values (compound **3**: AChE $IC_{50} = 9.09$ μM, BuChE $IC_{50} = 21.10$ μM; compound **4**: AChE $IC_{50} = 8.40$ μM, BuChE $IC_{50} = 4.93$ μM) for H_3R antagonism and cholinesterase inhibition, respectively. Binding free energies yielded good consistency with cholinesterase inhibitory profiles. The results of this study can be used for lead optimization where dual inhibitory activity on H_3R and cholinesterases is needed. Such ligands can exert their biological activity in a synergistic manner resulting in higher potency and efficacy.

KEYWORDS
anticholinesterase, anti-H_3R agents, histamine H_3 receptor, molecular docking, molecular dynamics simulation, multi-target directed ligands

1 | INTRODUCTION

Although much of medicinal chemistry efforts for drug discovery have been dedicated to "one-molecule-one-target" principle, this approach hardly meets the desired expectation in the case of certain diseases with complex pathophysiology (Chen & Decker, 2013; Geldenhuys & Van der Schyf, 2013; Prati, Uliassi, & Bolognesi, 2014). In this framework, the use of multi-target directed ligands (MTDLs) seems more effective compared to conventional targeting. MTDL is defined as a molecule capable of modulating different targets simultaneously (Chen & Decker, 2013; Geldenhuys & Van der Schyf, 2013; Lazewska et al., 2016). The main advantage associated with a given MTDL relies on enhancing the biological activity through different pharmacophores embedded in the given molecule that are required for interactions with multiple targets (Chen & Decker, 2013). Undoubtedly, such ligands can be beneficial in a more efficient way for the treatment of complex disorders with multifactorial nature. Alzheimer's disease (AD) is classified as neurological disorder with multifactorial etiology, which can be observed with high incidence in aged population. The major features of AD (as the most common form of dementia) are cognitive deficit, loss of memory, and language impairment leading to alterations in behavior and personality. Different abnormal neurological events are responsible for the pathophysiology of AD, such as misfolded and aggregated amyloid, tau protein hyperphosphorylation, oxidative stress, and cholinergic dysfunction (Bajda, Guzior, Ignasik, & Malawska, 2011; Rampa, Belluti, Gobbi, & Bisi, 2011). Based on the amyloid hypothesis, cerebral plaques are formed as a result of β-amyloid peptide aggregation. Formation of intracellular neurofibrillary tangles induced by tau hyperphosphorylation is another pathological initiator for AD development. The second important hypothesis in AD is oxidative stress arising from the accumulation of reactive oxygen species, which can cause cell injury and death. Lastly, cholinergic neurotransmission has a pivotal role in the pathology of AD. In AD patients, the brain acetylcholine levels are significantly reduced in hippocampus and cortex. This neurotransmitter plays a critical role in memory and learning, as well as in cognition. Its activity in the synaptic cleft is controlled by cholinesterase enzymes, responsible for the termination of acetylcholine biological activity through its hydrolysis in cholinergic synapses (Bajda et al., 2011; Darras et al., 2014; Lazewska et al., 2016). Currently, the pharmacotherapy of AD relies on the use of four marketed drugs, namely donepezil, rivastigmine and galantamine, as cholinesterase inhibitors, and the NMDA receptor antagonist memantine (Chen et al., 2012; Lalut et al., 2019; Sadek, Khan, Darras, Pockes, & Decker, 2016). However,

these disease-modifying agents only alleviate temporally the AD clinical symptoms, while their efficacy as monotherapy is one of today's controversial issues in AD.

In recent years, the histamine H₃ receptor (H₃R) has been highlighted as a potential target for the treatment of neurological disorders, including AD (Berlin, Boyce, & Ruiz Mde, 2011; Lazewska & Kiec-Kononowicz, 2014; Vohora & Bhowmik, 2012). These receptors are mainly located in different areas of CNS regulating the release of histamine and other neurotransmitter such as acetylcholine and dopamine through a negative feedback mechanism. The H₃R belongs to the superfamily of G protein-coupled receptors and is coupled to the $G_{i/o}$ class of G proteins, leading to inhibition of the adenylyl cyclase with the subsequent decrease in the level of cAMP, among other actions (Nieto-Alamilla, Marquez-Gomez, Garcia-Galvez, Morales-Figueroa, & Arias-Montano, 2016). There are lines of evidence indicating that H₃R blockade enhances acetylcholine release, thereby leading to improved cognitive and behavioral symptoms (Brioni, Esbenshade, Garrison, Bitner, & Cowart, 2011; Medhurst et al., 2007; Raddatz, Tao, & Hudkins, 2010; Sadek, Saad, Sadeq, Jalal, & Stark, 2016).

According to the complexity and multifactorial characteristics of AD, the use of MTDLs may provide more promising results in terms of cognition enhancement in a more specific way. There are several reports in the literature introducing dual-acting ligands capable of improving cholinergic neurotransmission via simultaneous antagonistic activity at H₃Rs and cholinesterase inhibitory activity (Bajda et al., 2012; Bembenek et al., 2008; Incerti et al., 2010; Khanfar et al., 2016; Morini et al., 2008; Sadek, Khan, et al., 2016). In a previous study, we used in silico virtual screening for the identification of novel ligands at the H₃R from ZINC database. A variety of molecular modeling techniques such as molecular docking and dynamics simulation, as well as drug-likeness filtering criteria, were employed to select the candidate molecules. The selected compounds were experimentally evaluated using radioligand displacement studies, and the results revealed that two of them, obtained from the ligand-based search method, were capable of binding to H₃Rs in the sub-micromolar K_i range (Ghamari et al., 2018). In the current work, we aimed to evaluate H₃R antagonist/inverse agonist and anticholinesterase activity of the identified ligands with improved efficacy as a result of their synergistic activity.

2 | METHODS AND MATERIALS

2.1 | Chemicals and reagents

Compounds with ZINC IDs Zinc69700808 and Zinc90563066 were purchased from chemical suppliers of MolPort database

(Riga, Latvia). Purity of the supplied compounds was evaluated by LC-MS as described previously (Ghamari et al., 2018). Cyclic AMP XP Assay Kit (cat no. 4339) was obtained from Cell Signaling Technology. 3-isobutyl-1-methylxanthine (IBMX), (R)-α-methylhistamine, thioperamide, forskolin, G418 (Geneticin), acetylcholinesterase (AChE, E.C. 3.1.1.7, Type V-S, lyophilized powder, from electric eel), butyrylcholinesterase (BuChE, E.C. 3.1.1.8, from equine serum), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich. CHO-K1 cells were purchased from the National Cell Bank of Iran, Pasteur Institute of Iran. Dulbecco's modified Eagle medium (DMEM) with L-glutamine and fetal bovine serum (FBS) were from Gibco® Life Technologies. Donepezil was purchased from Darou Pakhsh Pharma Chem Co. Acetylthiocholine iodide and 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) from Sigma-Aldrich were kind gifts from Dr. Azarmi, Tabriz University of Medical Sciences.

2.2 | Cell culture and transfection

CHO-K1 cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C and 5% CO₂ in a humidified incubator. CHO-K1 cells were grown to 50%–70% confluence and stably transfected with mammalian expression vector of pCI-neo encoding the full length of human H₃R (1,335 bp, 445 aa; Osorio-Espinoza, Escamilla-Sanchez, Aquino-Jarquin, & Arias-Montano, 2014) by electroporation. Briefly, after harvesting, cells were resuspended in 400 μL DMEM and transferred to an electroporation cuvette with a 0.4-cm gap between electrodes. DNA (10 μg/ml) was added and electroporation was conducted according to the Bio-Rad's preset protocol programmed in Gene Pulser Xcell. After electroporation, cells were transferred to a 6-well plate and incubated for 48 hr in complete media. Thereafter, the transfected cells were selected with geneticin (G-418; 600 μg/ml). Stable transfectants were maintained in the presence of 400 μg/ml geneticin.

2.3 | cAMP accumulation assay

CHO-K1 cells stably expressing the H₃R were plated in a 96-well plate (60,000 cells/well) and incubated overnight at 37°C. The medium was removed, and the cells were rinsed with 200 μl prewarmed phosphate-buffered saline (PBS). The cells were then incubated with serum-free medium containing IBMX (1 mM) for 10 min at 37°C. The previously identified compounds (i.e., compounds **3** and **4**) and/or reference compounds ((R)-α-methylhistamine and thioperamide) were added at increasing concentrations (0.1 nM–10 μM) and incubated for 5 min. Subsequently, forskolin (1 μM) was added and incubated for additional 30 min at 37°C. Where required, for evaluating the effect

of the studied compounds in the presence of agonist, the fixed concentration of 15 nM was used for (R)-α-methylhistamine. The cyclic AMP XP Assay Kit (Cell Signaling Technology) was utilized for the determination of intracellular cAMP accumulation. For this purpose, the cells were rinsed twice with 200 μl ice-cold PBS, followed by the addition of 100 μl/well of 1X lysis buffer with an incubation on ice for 10 min. To the cAMP assay plate, 50 μl of the HRP-linked cAMP solution and 50 μl of the sample were added and plates were incubated at room temperature for 3 hr on a horizontal orbital plate shaker. The contents of the plates were discarded, and wells were washed four times with 200 μl 1× wash buffer. TMB substrate (100 μl) was added, and the wells were incubated for 30 min at room temperature. Finally, the reaction was terminated by adding of 100 μl STOP solution and the absorbance was measured at 450 nm using ELISA reader (BioTek, US).

2.4 | Acetylcholinesterase and butyrylcholinesterase inhibition assay

In order to determine the inhibitory activity of the studied ligands, the spectrophotometric Ellman's test was conducted using AChE and BuChE from electric eel and horse serum, respectively (Ellman, Courtney, Andres, & Feather-Stone, 1961). The stock solutions of the tested compounds were prepared in DMSO. For each compound, different concentrations ranging from 0.1 nM to 100 μM were prepared in 100 mM sodium phosphate buffer (pH = 8.0). The assay solution for the preincubation (5 min at room temperature) was sodium phosphate buffer containing 0.25 U/ml AChE or BuChE, 0.5 mM DTNB, and different concentrations of the tested compounds. The reaction was started by the addition of acetylthiocholine iodide (1 mM) as substrate, followed by absorbance measurement after 10 min at a wavelength of 412 nm. Donepezil was used as positive control. The percentage of inhibition was calculated using the following equation: % inhibition = $100 \times [(A_{\text{sample}} - A_{\text{Blank}})/(A_{\text{Max}} - A_{\text{Blank}})]$, where A_{sample} is the absorbance of each sample including the test compound, A_{Blank} denotes the absorbance without enzyme, and A_{Max} shows the absorbance without the test compound. Each concentration was examined in triplicate.

2.5 | Cell viability assay

For evaluation of the effects of the studied ligands on in vitro cell viability, the MTT assay was used (Mosmann, 1983). CHO-K1-hH₃R cells were seeded in triplicate in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 hr at 37°C under a humidified atmosphere and 5% CO₂ saturation. Stock solutions of the studied compounds were prepared in DMSO, diluted in fresh medium and added to the wells in a range 0.1 nM–100 μM. After 72 hr of incubation, the medium was discarded, followed by the addition

of 100 µl of filtered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide (MTT) solution (in a final concentration of 0.5 mg/ml) and incubation for 4 hr at 37°C in 5% CO_2 atmosphere. Then, the culture medium was carefully removed by tapping on paper towel followed by the addition of 100 µl solubilizing buffer containing Sorensen's phosphate buffer (12.5%) and DMSO (87.5%) for dissolving the produced formazan crystals. The plate was incubated for 40 min at room temperature with gentle shaking. Finally, the absorbance was measured at 570 nm. In this assay, doxorubicin (DX) was used as positive control. The percentage of cell viability was calculated using the following formula: % cell viability = 100 × [(A_{sample} − A_{Blank})/($A_{Control}$ − A_{Blank})]; in this equation, A_{sample} refers to the absorbance of tested compound, A_{Blank} shows well containing only medium and $A_{Control}$ demonstrates untreated cells. All the experiments were conducted in triplicate. GraphPad Prism software (version 6.01) was utilized for the analysis of the results and determination of GI_{50} (concentration required for a 50%-inhibition of cell growth) based on a sigmoidal model using nonlinear regression analysis.

2.6 | Molecular docking and binding free energy calculation for ligand–enzyme complex

Molecular docking experiment was carried out to predict the mode of interactions between the ligands and cholinesterase enzymes. The experimental co-ordinates for AChE (PDB ID: 4EY7) (Cheung et al., 2012) and BuChE (PDB ID: 4TPK) (Brus et al., 2014) were retrieved from Protein Data Bank at the Research Collaboratory for Structural Bioinformatics (http://www.RCSB.org; Berman et al., 2000). Flexible docking of ligands into the binding site of enzymes was performed using GOLD program (version 5.0; CCDC Inc., Cambridge, UK) running under LINUX operating system. In order to determine the binding site, a geometric center was assigned based on the important residues in the active site of enzyme considering all atoms within a radius of 10 Å. By applying the default settings in GOLD, the best scoring function was selected based on re-docking experiment of donepezil into the binding site of AChE. Top-ranked docking pose in complex with corresponding enzyme was subjected to molecular dynamics (MD) simulation. Assisted Model Building with Energy Refinement (AMBER) suite of programs (version 14; Case et al., 2005; Pearlman et al., 1995) was used for MD calculation operating on a Linux-based (Centus 6.8) GPU work station consisting of four NvidiaK40M (each has 12 GB RAM and 2880 CUDA cores), 2X Intel Xeon E5–2697 v2, 2.7 GHz (total of 48 cores), total RAM = 128 GB. AMBER usable co-ordinate files (in the format of *.prmtop and *.inpcrd) were generated using *leap* module. Then, an appropriate number of counterions were added to the system for neutralizing the total charge and immersed into a rectangular box of explicit TIP3P water

molecules by the buffering distances set to 12 Å in all directions. Prior to MD simulation, the solvated system was energy minimized by 500 steps of steepest descent followed by 500 steps of conjugated gradient using *Sander* module of AMBER package. Subsequently, the system was heated for 50 ps with gradual increase in temperature from 0 to 300 K. Density equilibration was performed for additional 50 ps followed by 500 ps of constant pressure equilibration at 300 K with a time step of 2 fs. By applying SHAKE algorithm, all bonds involving hydrogen atoms were constrained. A 10 ns MD simulation was carried out using Particle Mesh Ewald method for calculation of long-range electrostatic interactions. Periodic boundary conditions were used for all the calculations without applying any constraint to either the protein or the ligand molecules. The trajectory of MD simulation was achieved by writing out the co-ordinates every 10 ps. Postprocessing the trajectory resulted to dynamically sampling of snapshots from simulation trajectory with an interval of 10 ps. The dielectric constant values for the interior of solute and the surrounding solvent were set to 1.0 and 80, respectively. Finally, the binding free energy was calculated for the complex of ligand–enzyme complex using molecular mechanics Poisson–Boltzmann surface area (MM-PBSA)/generalized Born–surface area (GBSA) methods implemented in the AMBER package (Case et al., 2005; Kollman et al., 2000). For obtaining binding free energy, sum of molecular mechanics energies, solvation free energies, and entropic terms were considered which was subsequently averaged over a series of equilibrated snapshots derived from MD simulation trajectory. For calculation of interaction energies, water molecules and counterions were excluded and presented as the average value.

For obtaining the binding free energy (ΔG_{bind}), the following equation was used:

$$\Delta G_{bind} = G_{water(complex)} - G_{water(protein)} - G_{water(ligand)}$$

where $G_{water(complex)}$, $G_{water(protein)}$, and $G_{water(ligand)}$ are the free energies of the complex, protein, and ligand, respectively. Free energy for each species is calculated as follows:

$$G = E_{gas} + \Delta G_{solvation} - TS$$

$$\Delta G_{solvation} = \Delta G_{polar} + \Delta G_{non-polar}$$

$$E_{gas} = E_{int} + E_{vdw} + E_{elec}$$

$$E_{int} = E_{bond} + E_{angle} + E_{tors}$$

G shows the calculated average free energy, and E_{gas} is the standard force-field energy, including internal energy (E_{int}) in the gas phase as well as non-covalent van der Waals (E_{vdw}) and electrostatic (E_{elec}) energies. E_{int} is composed of E_{bond}, E_{angle}, and E_{tors}, which are the contributors of internal energy originated

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from the strain caused by deviation of the bonds, angle, and torsion angle from their equilibrium values. $\Delta G_{\text{solvation}}$ consists of ΔG_{polar} and $\Delta G_{\text{non-polar}}$ demonstrating the polar and non-polar contributions to the solvation free energy. The polar contribution is calculated by Poisson–Boltzmann or generalized Born model, while the non-polar contributions are achieved by the calculation of solvent accessible surface area using the linear combinations of pairwise overlaps method (Weiser, Shenkin, & Still, 1999). TS refers to the vibrational entropy term which is ignored in this study assuming similar entropy contribution for all complexes to the binding free energy.

3 | RESULTS

3.1 | H₃R antagonism in the cAMP accumulation assay

Functional properties of the selected compounds (i.e., compounds **3** and **4**) were determined in the forskolin-stimulated cAMP accumulation assay using hH₃R-expressing CHO-K1 cells. As shown in Figure 1a, the reference agonist (*R*)-α-methylhistamine decreased cAMP formation and when the cells were co-treated with the compounds and the reference agonist, the agonistic effect of (*R*)-α-methylhistamine was suppressed. Compounds **3** and **4** enhanced in a concentration-dependent manner cAMP levels in CHO-K1 expressing human H₃Rs that were incubated in the presence of (*R*)-α-methylhistamine and forskolin (Figure 1a), indicating the antagonistic activity of these compounds. Such antagonistic activity was more pronounced for compound **3** compared to compound **4** by means of changes in intrinsic activity of the receptor. Antagonistic potencies of compounds determined from sigmoidal dose–response curves in the functional assay are shown in Table 1. Moreover, in this cell system, a titration of these compounds in the absence of the reference agonist was performed and the results demonstrated that both compounds increased cAMP formation by themselves with maximal effect comparable to thioperamide as standard H₃R antagonist (Figure 1b). From similar concentration–response patterns in the literature and compared to thioperamide, it can be concluded that compounds **3** and **4** behave as inverse agonists at the human H₃R (Kuder et al., 2016; Lazewska et al., 2017; Sors et al., 2017).

3.2 | Cholinesterase inhibitory activity

The anticholinesterase activity of compounds **3** and **4** were evaluated according to the Ellman's method using AChE and BuChE from electric eel and horse serum, respectively. Donepezil was used as the reference compound in this assay, and the results are demonstrated in Table 1. As shown in Table 1, the compounds exhibited inhibitory activity on both AChE and BuChE in the micromolar range, indicative of moderate inhibition toward both cholinesterase enzymes. As the level of acetylcholine is of great importance in AD,
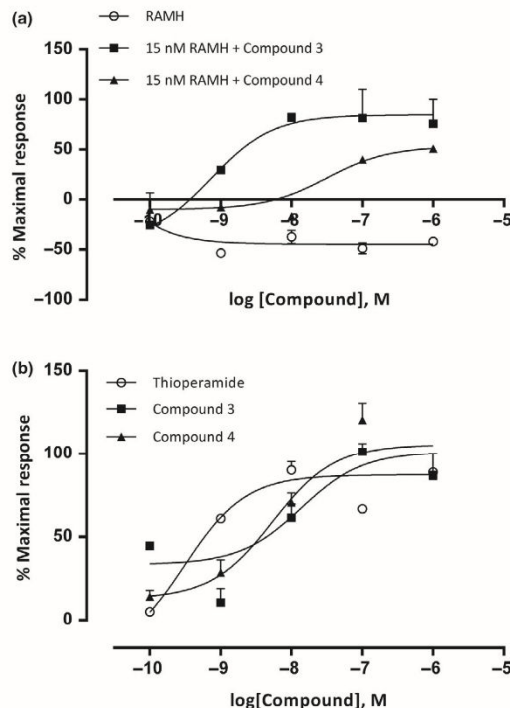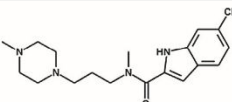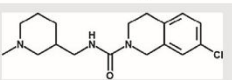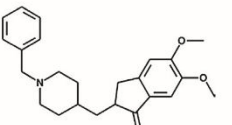
FIGURE 1 cAMP accumulation studies in CHO-K1 cells expressing human histamine H₃ receptor co-treated with forskolin and the studied compounds in the presence (a) and absence (b) of (*R*)-α-methylhistamine (RAMH). The response was calculated with the following formula: % maximal response = $100 \times [(A - A_{\text{basal}})/(A_{\text{max}} - A_{\text{basal}})]$ where A is the absorbance of the sample, A_{max} is the absorbance at maximum stimulation, and A_{basal} is the absorbance at basal level. Data are represented as mean $\pm$ *SE* from duplicate determinations

non-selective inhibition of both AChE and BuChE would therefore be beneficial from a therapeutic point of view. Based on the obtained results, the IC₅₀ values for inhibition of AChE were 9.09 and 8.40 μM for compounds **3** and **4**, respectively, whereas in the case of BuChE, these values were achieved 21.10 and 4.93 μM for compounds **3** and **4**, respectively. The results of cholinesterase inhibition assays are comparable with previously reported studies in which the inhibitory activity of the compounds and reference molecule (i.e., donepezil) is in micromolar and submicromolar concentration range for BuChE and AChE, respectively (Lalut et al., 2019; Lazewska et al., 2016; Li et al., 2014).

3.3 | Toxicity evaluation

To estimate potential cytotoxic effects of studied compounds and determine their influence on the viability of

TABLE 1 Chemical structures, antagonistic potencies to the human histamine H_3 receptor, and cholinesterase inhibitory activities of tested compounds

Compound	Structure	$H_3R\ EC_{50} \pm SE$ [nM]	$AChE\ IC_{50} \pm SE$ [μM]	$BuChE\ IC_{50} \pm SE$ [μM]
3		0.73 ± 0.016	9.09 ± 2.09	21.10 ± 4.8
4		31 ± 1.95	8.40 ± 1.93	4.93 ± 0.68
Donepezil		nt.	0.024 ± 0.002	2.50 ± 0.35

nt.: not tested.

hH₃R expressing CHO-K1 cells, the MTT assay was employed. Judged among the concentration corresponding to a 50% inhibition of growth (GI_{50}), the tested compounds did not exhibit significant cytotoxic influence on cell viability of the examined cell line in a concentration range from 0.1 nM to 100 μM compared to doxorubicin (DX) as reference compound (compound **3**, $GI_{50} = 8.38 \pm 0.43$ μM; compound **4**, $GI_{50} \approx 100$ μM, and DX, $GI_{50} = 2.1 \pm 0.26$ μM). As shown in Figure 2, following incubation of the studied compounds with hH₃R expressing CHO-K1 cells for 72 hr, the viability of the cells was intact up to 1 μM, whereas the cytotoxic effect is observed at higher concentrations of compounds (i.e., >10 μM). Based on the obtained results, it seems that they have no significant impact on cell viability which is more noticeable for compound **4** inferred from the corresponding GI_{50}. The results are comparable with those published previously in terms of toxicity of reference compound and selected compounds (Lazewska et al., 2018; Morini et al., 2008).

3.4 | Molecular docking and calculation of binding free energy for ligand–enzyme complex

In order to predict the mode of interactions between studied compounds and the cholinesterases, the molecular docking experiment was conducted. To this end, flexible docking of compounds **3** and **4** into active site of enzymes was performed using GOLD program based on the optimized condition set for re-docking of co-crystallized donepezil into active site of enzymes. Figure 3 and Table 2 illustrate the key residues in the active site of enzymes interacting with studied compounds. The main interactions observed for compound **3** bound to the active site of AChE include a π–π stacking, two
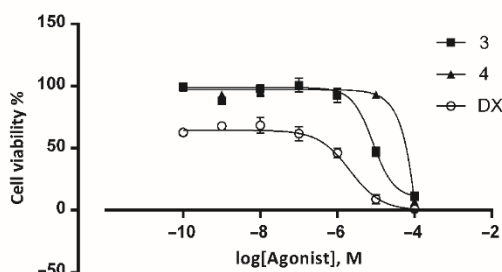
FIGURE 2 MTT cytotoxicity assay. Anti-proliferative effects of the studied compounds on histamine H_3 receptor expressing CHO-K1 cells are shown as percentage of cell viability. Data are expressed as mean ± SE from a representative experiment performed in triplicate

hydrophobic pockets, and a hydrogen bond interaction. The π–π stacking is established between indole ring of compound **3** and Trp[286]. One of the hydrophobic interactions is formed between the alkyl part of the linker in compound **3** and Tyr[337] and Tyr[341] from AChE, while the other hydrophobic interactions are observed between methylpiperazine moiety of the compound **3** and Trp[86] and His[447] from the enzyme. Besides, Tyr[124] of the AChE is engaged in the hydrogen bond with nitrogen of indole ring in compound **3**. In case of molecule **4**, the main interactions are similar to those observed for compound **3** with the phenyl ring of tetrahydroisoquinoline moiety being involved in a π–π stacking interaction with Trp[286]. Additionally, the tetrahydroisoquinoline part hydrophobically interacts with Tyr[341] of enzyme. Furthermore, a hydrophobic pocket composed of Trp[86], Tyr[337], and His[447] surrounds the methylpiperidine at the endmost part of molecule **4**. A hydrogen bond is also seen between Tyr[124] and nitrogen of tetrahydroisoquinoline. Such similar interactions
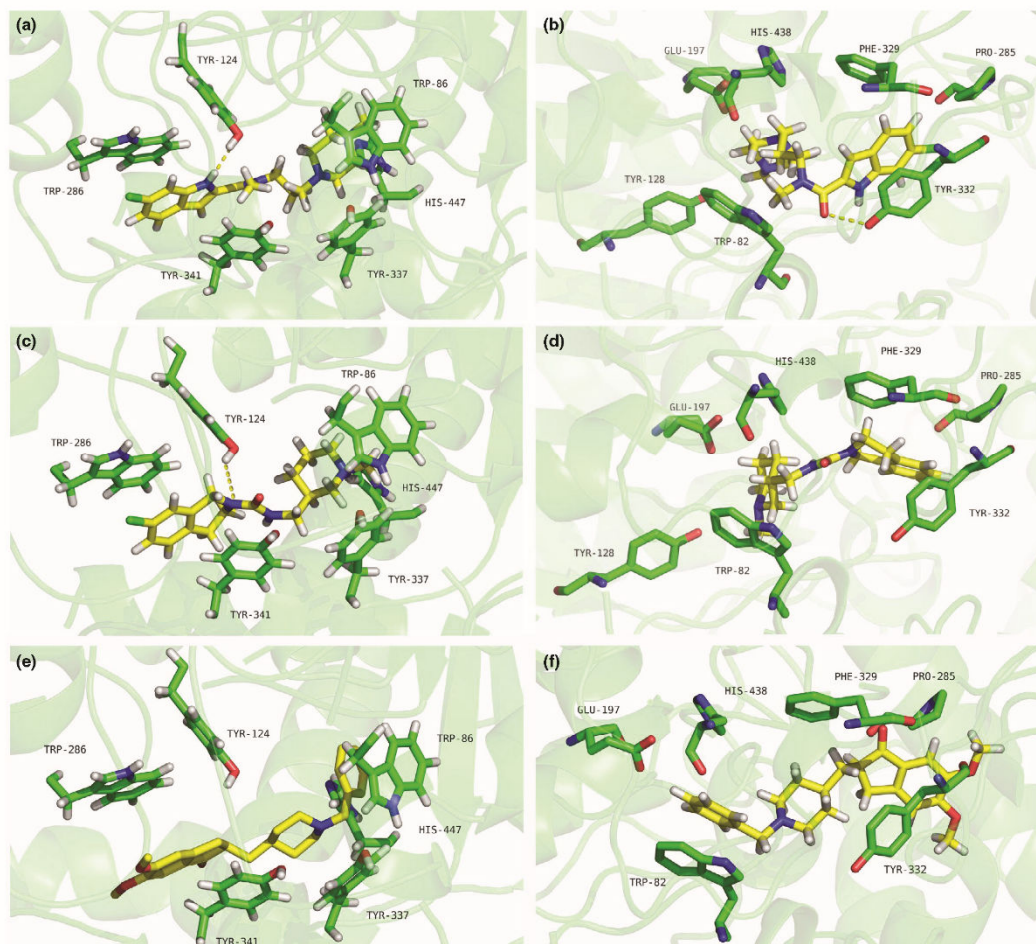
FIGURE 3 3D representation of compounds **3**, **4**, and donepezil (Panels (a) to (f)) docked onto the binding site of cholinesterase enzymes generated by PyMOL program (version 1.7.x). Panels (a), (c), and (e) show the interactions of the compounds **3**, **4**, and donepezil with AChE, respectively. Panels (b), (d), and (f) indicate the interactions of compounds **3**, **4**, and donepezil in complex with BuChE, respectively. The ligands and the main interacting residues are shown as sticks. Only the side chains of the interacting residues from the receptor are shown for further clarity [Colour figure can be viewed at wileyonlinelibrary.com]

have been detected for the complex of donepezil and AChE enzyme.

The interactions observed for the complex of inhibitor BuChE resemble those seen for AChE. The identified interactions for compound **3** consist of hydrophobic interactions and a hydrogen bond. A hydrophobic pocket formed by Trp[82], Tyr[128], and Glu[197] of the BuChE accommodates the methylpiperazine part of compound **3**. Alkyl part of linker in compound **3** makes hydrophobic interaction with His[438]. Furthermore, the other hydrophobic interactions are observed between indole ring of compound **3** and Pro[285], Phe[329], and Tyr[332]. Moreover, oxygen of amide linker in compound **3**

makes a hydrogen bond with Tyr[332]. Compound **4** binds into the active site of BuChE predominantly via hydrophobic interactions. One of the hydrophobic interactions is established by contribution of Trp[82], Tyr[128], Ser[198], and His[438] from the enzyme and methylpiperidine part of compound **4**, whereas the tetrahydroisoquinoline part of compound **4** is surrounded by a hydrophobic pocket formed by Pro[285], Phe[329], and Tyr[332] of the enzyme.

In the current study, binding free energy for the ligand–enzyme complexes was calculated using AMBER package. To this end, MD simulation was performed for the best docked pose for 10 ns. The snapshots were extracted from the MD

trajectory every 10 ps to be used for calculation of binding free energy using MM-PBSA/GBSA methods. The analysis of the MD trajectory based on root-mean-square deviation (RMSD) and potential energy indicated that all systems in the simulation process were well equilibrated and stable during the simulation period (Figure S1). Table 3 provides the results of binding free energy calculations for the complexes with respect to donepezil (as reference compound).

4 | DISCUSSION

The complex and multifaceted pathophysiological nature of AD necessitates the identification and development of novel therapeutic agents. For several decades of research, H₃R antagonists have gained much attention in the field of neurological disorders (Brioni et al., 2011; Esbenshade et al., 2008; Sadek, Saad, et al., 2016; Shan, Bao, & Swaab, 2015; Tiligada, Kyriakidis, Chazot, & Passani, 2011; Vohora & Bhowmik, 2012). There are some H₃R antagonists in clinical trial studies for which their effectiveness in AD is being evaluated as mono- or add-on therapy. The examples of such clinical candidates are ABT-288, AZD-5213, GSK-239512, GSK-1004723, MK-0249, and S 38093 (for more details, see Ghamari et al., 2019). Recently, several studies have focused on developing ligands targeting both H₃Rs and cholinesterase enzymes (Bajda et al., 2012; Bembenek et al., 2008; Darras et al., 2014; Incerti et al., 2010; Lazewska et al., 2016; Sadek, Khan, et al., 2016), since the synergistic effect of these agents leads to enhance cholinergic neurotransmission through different mechanisms.

Previously, we identified novel anti-H₃R agents using in silico virtual screening methods. Briefly, a combination of structure- and ligand-based approaches was applied to search compounds from ZINC library on the homology model of the human H₃R. Following the screening, the molecules were also inspected for drug-likeness and ADME properties. Furthermore, the results of binding assays on selected compounds demonstrated that two compounds (i.e., compounds **3** and **4**) were capable of binding to histamine H₃Rs with K_i values in submicromolar concentration range (Ghamari et al., 2018). In the current study, dual inhibitory activity of these compounds on H₃Rs and cholinesterase enzymes was investigated. The analysis of cAMP accumulation within CHO-K1 cells expressing recombinant hH₃Rs revealed that compounds **3** and **4** elevate cAMP levels by themselves and antagonize the effect (R)-α-methylhistamine, implying the compounds to be classified as H₃R antagonists/inverse agonists.

The anticholinesterase activity of such compounds was also assessed, showing moderate inhibition on both, AChE and BuChE, with IC_{50} values in the micromolar range. Both compounds were approximately equipotent toward AChE, whereas compound **4** exhibited higher potency on BuChE compared to compound **3**. In addition to AChE, targeting BuChE is of great importance in AD pharmacotherapy as several studies indicate that the activity and levels of acetylcholine in the CNS are modulated by AChE as well as by BuChE (Darvesh, Hopkins, & Geula, 2003; Mesulam et al., 2002). Furthermore, BuChE activity is enhanced in hippocampus and cerebral cortex along AD progression (Perry, Perry, Blessed, & Tomlinson, 1978). Therefore, this can be a driving force for designing non-selective inhibitors with superior efficiency to selective AChE inhibitors in advanced stages of AD. In this context, compounds **3** and **4** were able to target both of cholinesterase enzymes. Similar to the ligands presented herein, there are several reports in which dual-acting cholinesterase inhibitors and H₃R antagonists were designed and experimentally evaluated. Darras et al. (2014) designed and synthesized the hybrid molecules containing piperdinylpropoxyphenyl moiety and related H₃R pharmacophores with nitrogen-bridgehead moieties as AChE inhibition pharmacophore. The most potent compound of this study, that is, compound **41** (named UW-MD-71), showed high potency at both targets (hH₃R: K_i = 76.2 nM and hAChE: IC_{50} = 33.9 nM) (Darras et al., 2014). Subsequently, the effect of this compound on memory retrieval and precognitive enhancement was also assessed in an in vivo study. The findings of this investigation demonstrated promising

TABLE 2 The key amino acids of cholinesterase enzymes involved in the interactions with the studied compounds

Enzyme	Amino acids
AChE	Trp^{86}, Tyr^{124}, Trp^{286}, Tyr^{337}, Tyr^{341}, His^{447}
BuChE	Trp^{82}, Tyr^{128}, Glu^{197}, Ser^{198}, Pro^{285}, Phe^{329}, Tyr^{332}, His^{438}

TABLE 3 Calculated mean values of binding free energies for the complex of docked lead molecules using generalized Born, $\Delta G_{Binding\,(GB)}$, and Poisson–Boltzmann, $\Delta G_{Binding\,(PB)}$, methods for 10 ns MD simulation

Compound	AChE		BuChE	
	$\Delta G_{Binding\,(GB)}$	$\Delta G_{Binding\,(PB)}$	$\Delta G_{Binding\,(GB)}$	$\Delta G_{Binding\,(PB)}$
3	−37.11 (±2.41)	−23.93 (±3.75)	−29.91 (±3.84)	−25.75 (±4.34)
4	−30.4 (±4.72)	−24.24 (±4.46)	−30.85 (±2.47)	−26.92 (±3.97)
Donepezil	−42.6 (±3.06)	−28.34 (±6.14)	−35.98 (±3.06)	−26.17 (±3.95)

Note: Energy values are dimensioned as kcal/mol. Standard deviations are shown in parentheses.

results in improving retrieval processes in rat memory models (Khan et al., 2016). The similar in vivo results were also observed for another, structurally related compound named UW-MD-72 (Sadek, Khan, et al., 2016). In another study, the dual inhibitory activities of diether derivatives of homo- or substituted piperidines were investigated. The most potent compound of this series (i.e., compound **13**) exhibited high affinity to H₃R (K_i = 3.48 nM) and moderate inhibitory activity to cholinesterase enzymes (EeAChE: IC$_{50}$ = 7.91 μM and EqAChE: IC$_{50}$ = 4.97 μM; Bajda et al., 2012). Extending the previous work, a novel series of compounds was designed with chlorophenoxyalkylamine scaffold inspired from the structure of compound **13**. This structural modification led to identification of the most potent compound of this set (1-(7-(4-chlorophenoxy)heptyl)homopiperidine) with increased affinity toward cholinesterase enzymes (EeAChE: IC$_{50}$ = 1.93 μM and EqAChE: IC$_{50}$ = 1.64 μM); however, the antagonistic potency of H₃R was not improved (hH₃R: K_i = 203 nM; Lazewska et al., 2016). More structurally different compounds as dual-acting cholinesterase inhibitors and H₃R antagonists were designed based on a 4,4′-biphenyl scaffold with mono- and dibasic moieties at distal ends. The findings of this study showed a dibasic piperidine containing compound (1-[2-(4′-piperidinomethyl-biphenyl-4-yl) ethyl]piperidine), revealing high H₃R binding affinity (hH₃R: K_i = 1.9 nM) and moderate inhibition on rat cholinesterase (rAChE: IC$_{50}$ = 1.096 μM; Morini ct al., 2008). Morcover, among a series of piperidinoalkyl derivatives with different length of linker and terminal heterocyclic ring, an analogue containing a tetrahydroaminoacridine moiety at distal end of molecule showed promising results with H₃R affinity corresponding to K_i = 1.8 nM and inhibitory activity of IC$_{50}$ = 0.26 nM at rat cholinesterase (Incerti et al., 2010). In a recent study, a series of MDTL's acting at H₃Rs and cholinesterases as well as on monoamine oxidases was developed (Bautista-Aguilera et al., 2017). Based on a MTDL with AChE and BuChE targeting properties (ASS234), the author demonstrates that a 1-Benzylpiperidin-4-yl alkyl motif as found as pharmacophore in donepezil can be shifted toward a 1-piperidinylalkyl pharmacophore leading to a pharmacophoric overlap of H₃R and AChE/BuChE targeting motif. Collectively, by structural inspection of mentioned dual inhibitors and the ligands disclosed here, it seems that the presence of basic moiety such as piperidine ring connecting to aromatic/heteroaromatic moiety through a linker is necessary for exerting inhibitory activity on either targets. The results of such studies can motivate medicinal chemists to rational design of novel dual-acting ligands of H₃R and cholinesterase enzymes.

The cell viability estimation of the studied compounds showed no significant cytotoxic effect on CHO-K1 cells up to micromolar concentrations. According to the GI_{50} values, the cytotoxic effect is more evident for compound **3** compared to the reference compound DX and therefore in agreement with the previously predicted in silico toxic potential. Based on the scale of toxicity alert, compound **4** was predicted to be a safe compound in comparison with compound **3** (Ghamari et al., 2018).

In order to gain insight into the possible mode of interactions between ligands and cholinesterase enzymes, docking studies were performed. The analysis of the proposed interactions shows similarity in the binding mode to that of donepezil co-crystallized with AChE (Cheung et al., 2012). The indole and tetrahydroisoquinoline rings of compounds **3** and **4** can be considered to be equivalent to indanone ring of donepezil in establishing π–π interaction with Trp[286] of AChE. Similarly, methylpiperazine and methylpiperidine moieties of compounds **3** and **4** resemble the benzyl ring of donepezil which stack against Trp[86]. The Tyr[337] and Tyr[341] from AChE are additional key residues for the interactions with the studied ligands as observed in donepezil–AChE complex. Such interactions were also predicted for the complex of inhibitor BuChE due to high similarity of the residues located in the active sites of both enzymes. For example, amino acids Trp[82], Tyr[332], and His[438] from BuChE are equivalent to Trp[86], Tyr[341], and His[447] from AChE in terms of interactions with the ligand. The results of molecular docking studies are in line with formerly published findings (Bajda et al., 2012; Darras et al., 2014; Jang et al., 2018; Lazcwska ct al., 2016; Morini ct al., 2008). In addition, the results of binding affinity prediction for the complexes of ligand-cholinesterase are consistent with the experimentally determined anticholinesterase activity of compounds **3** and **4**. The calculated binding energy for donepezil demonstrated that this therapeutic agent strongly inhibits the cholinesterase enzymes (Table 3) which is in close agreement with the experimental results (Table 1). Although in the case of AChE inhibition, there is no significant difference in the inhibitory potency of compounds **3** and **4**, the results of binding affinity calculations correlate well with IC$_{50}$ values. It can be deduced from comparison of ΔG values obtained from MM-PBSA and MM-GBSA methods. This consistency implies the reliability of MM-PBSA/GBSA methods in predicting binding affinities of studied ligands to cholinesterase enzymes.

Although compounds **3** and **4** showed antagonistic activity at the human H₃R combined with cholinesterase inhibitory properties, compound **3** was more potent in terms of H₃R blockade with approximate equipotency in cholinesterase inhibition compared to compound **4**. On the other hand, by considering the results obtained from toxicity evaluation, it seems that compound **4** has greater safety compared to compound **3**. Moreover, it appears that structural modification of both compounds can be beneficial for developing dual-acting compounds with improved efficacy and lower toxicity. To reach this goal, the use of medicinal chemistry-oriented

strategies such as scaffold hopping or bioisosteric replacement is highly recommended. Additionally, more preclinical biological assays both in in vitro and in vivo paradigms are needed to elucidate the potential effect of the designed compounds.

5 | CONCLUSIONS

According to the special place of in silico methods in modern drug discovery, remarkable advances have been achieved in the last decades at the early stages of the drug design and discovery pipeline. In this study, the anti-H₃R ligands identified through previous in silico virtual screening approaches were functionally evaluated and found as dual-acting H₃R antagonists and cholinesterase inhibitors. Collectively, the presented lead compounds can serve as promising starting points for further development of novel anti-AD agents through medicinal chemistry aided structural modification and optimization.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Supplementary Material

In Silico and In Vitro Studies of Two Non-Imidazole Multiple Targeting Agents at Histamine H3 Receptors and Cholinesterase Enzymes

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S1. The results of molecular dynamics simulation analyses on ligand-cholinesterase complexes
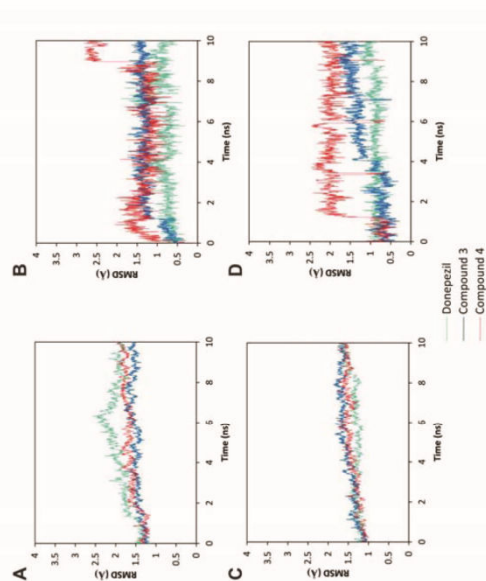
Figure S1.1. The results of molecular dynamics simulation analyses on ligand-cholinesterase complexes. Panels A to D show the plots of root mean square deviation (RMSD) fluctuation in a 1 to 10 ns molecular dynamics simulation for ligand-cholinesterase complexes (panels A and C) and ligands (panels B and D). Panels A and B refer to the RMSD results when AChE used as cholinesterase enzyme while C and D indicate RMSD results where BuChE was used during molecular dynamics simulation.

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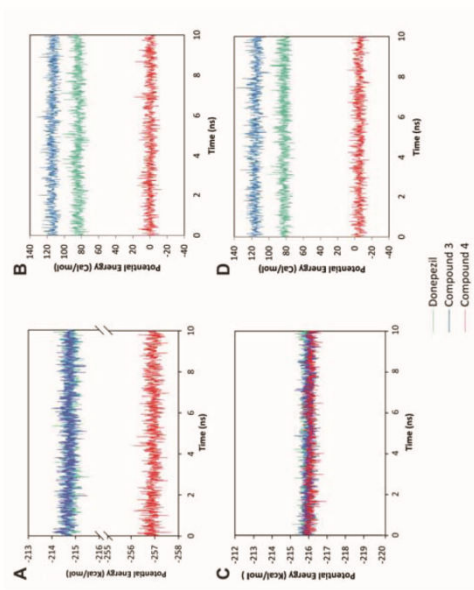
Figure S1.2. The results of molecular dynamics simulation analyses on ligand-cholinesterase complexes. Panels A to D show the plots of potential energies fluctuation in a 1 to 10 ns molecular dynamics simulation for ligand-cholinesterase complexes (panels A and C) and ligands (panels B and D). Panels A and B refer to the potential energy results when AChE used as cholinesterase enzyme while C and D indicate potential energy results where BuChE was used during molecular dynamics simulation.

4.6. The dual-active histamine H3 receptor antagonist and acetylcholine esterase inhibitor E100 ameliorates stereotyped repetitive behavior and neuroinflammmation in sodium valproate-induced autism in mice

Nermin Eissa[1], Sheikh Azimullah[1], Petrilla Jayaprakash[1], Richard L. Jayaraj[1], <u>David Reiner</u>[2], Shreesh K. Ojha[1], Rami Beiram[1], Holger Stark[2], Dorota Łażewska[3], Katarzyna Kieć-Kononowicz[3], Bassem Sadek[1]

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<u>Contribution to research:</u> Co-authorship. DR co-organised shipment of test-ligands, prepared, planned and conducted radioligand selectivity screenings at H_1R and H_4R, and evaluated corresponding data. DR proof-edited the manuscript.

<u>Abstract:</u>

Postnatal exposure to valproic acid (VPA) in rodents induces autism-like neurobehavioral defects which are comparable to the motor and cognitive deficits observed in humans with autism-spectrum disorder (ASD). Histamine H3 receptor (H3R) and acetylcholine esterase (AChE) are involved in several cognitive disorders such as Alzheimer's disease, schizophrenia, anxiety, and narcolepsy, all of which are comorbid with ASD. Therefore, the present study aimed at evaluating effect of the novel dual-active ligand E100 with high H3R antagonist affinity and balanced AChE inhibition on autistic-like repetitive behavior, anxiety parameters, locomotor activity, and neuroinflammation in a mouse model of VPA-induced ASD in C57BL/6 mice. E100 (5, 10, and 15 mg/kg) dose-dependently and significantly ameliorated repetitive and compulsive behaviors by reducing the increased percentages of nestlets shredded (all $P < 0.05$). Moreover, pretreatment with E100 (10 and 15 mg/kg) attenuated disturbed anxiety levels ($P < 0.05$) but failed to restore the hyperactivity observed in the open field test. Furthermore, pretreatment with E100 (10 mg/kg) the increased microglial activation, proinflammatory cytokines and expression of NF-κB, iNOS, and COX-2 in the cerebellum as well as the hippocampus (all $P < 0.05$). These results demonstrate the ameliorative effects of E100 on repetitive compulsive behaviors in a mouse model of ASD. To our knowledge, this is the first in vivo demonstration of the effectiveness of a potent dual-active H3R antagonist and AChE inhibitor against autistic-like repetitive compulsive behaviors and neuroinflammation, and provides evidence for the role of such compounds in treating ASD.

neuroinflammmation in sodium valproate-induced autism in mice, *Chem.-Biol. Interact.*, 2019, 312:108775, with permission for personal use from Elsevier.

The dual-active histamine H3 receptor antagonist and acetylcholine esterase inhibitor E100 ameliorates stereotyped repetitive behavior and neuroinflammmation in sodium valproate induced autism in mice

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ARTICLE INFO

ABSTRACT

Postnatal exposure to valproic acid (VPA) in rodents induces autism-like neurobehavioral defects which are comparable to the motor and cognitive deficits observed in humans with autism spectrum disorder (ASD). Histamine H3 receptor (H3R) and acetylcholine esterase (AChE) are involved in several cognitive disorders such as Alzheimer's disease, schizophrenia, anxiety, and narcolepsy, all of which are comorbid with ASD. Therefore, the present study aimed at evaluating effect of the novel dual-active ligand E100 with high H3R antagonist affinity and balanced AChE inhibition on autistic-like repetitive behavior, anxiety parameters, locomotor activity, and neuroinflammation in a mouse model of VPA-induced ASD in C57BL/6 mice. E100 (5, 10, and 15 mg/kg) dose-dependently and significantly ameliorated repetitive and compulsive behaviors by reducing the increased percentages of nestlets shredded (all $P < 0.05$). Moreover, pretreatment with E100 (10 and 15 mg/kg) attenuated disturbed anxiety levels ($P < 0.05$) but failed to restore the hyperactivity observed in the open field test. Furthermore, pretreatment with E100 (10 mg/kg) the increased microglial activation, proinflammatory cytokines and expression of NF-κB, iNOS, and COX-2 in the cerebellum as well as the hippocampus (all $P < 0.05$). These results demonstrate the ameliorative effects of E100 on repetitive compulsive behaviors in a mouse model of ASD. To our knowledge, this is the first in vivo demonstration of the effectiveness of a potent dual-active H3R antagonist and AChE inhibitor against autistic-like repetitive compulsive behaviors and neuroinflammation, and provides evidence for the role of such compounds in treating ASD.

1. Introduction

Autism spectrum disorder (ASD) is a common neurobehavioral disorder with limited treatment options [1,2]. Despite its increasing prevalence, the pathophysiological background of ASD is not fully understood [3]. Accordingly, no specific treatment can have potential effect for all autistic children [4,5]. Consequently, recent developments of new agents with multiple pharmacological effects have become promising strategy for novel treatment options of multifactorial disorders such as ASD [6–8]. In search of studying the etiology of ASD, the role of several central neurotransmitters, e.g., serotonin (5-HT), acetylcholine (ACh), dopamine (DA), γ-aminobutyric acid (GABA),

glutamate (Glu), and histamine (HA), in early brain development encourage to be a significant area of research, and accumulation of evidences suggest that several numerous neurotransmitters such as ACh, 5-HT, DA, GABA, Glu, and HA are involved in the onset and progression of ASD [9–19]. The involvement of histamine receptors (HRs) in the cognitive functions has long been confirmed, notably of H3Rs which are mainly expressed in the CNS [20–23]. While activation of H1R and H2R facilitates slow excitatory postsynaptic potentials, H3Rs are coupled to Gi/Go-proteins that control the biosynthesis and release of histamine with high constitutive activity, as an auto-receptors. Moreover, H3Rs functioning as hetero-receptors can also regulate the release of other numerous brain neurotransmitters like ACh, Glu, GABA, 5-HT, DA in

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various brain regions [24,25]. Accordingly, blocking central H3Rs has been projected to improve the cortical fast rhythms closely associated with cognitive behaviors [25], and numerous preclinical studies indicated particularly that H3R antagonists/inverse agonists have been found to exhibit a unique feature by their potential cognition-enhancing property [22,23,26,27]. Furthermore, several drug candidates with H3R antagonist properties have shown to increase the release of brain ACh, therefore alleviating symptomatic features and progression of Alzheimer's disease (AD) in clinical trials [28,29]. In addition, a previous report demonstrated lower ACh level in the prefrontal cortex region of mice which showed attention deficit and impulsive behavior [30]. Interestingly, cholinergic deficit was, also, found in BTBR T + tf/J mice, an animal model that displays behaviors consistent with the three diagnostic categories for ASD, namely impaired social interaction and communication as well as increased repetitive behaviors [31–35]. Based on the high level of attention generated by these preclinical experimental outcomes, the central H3Rs are an attractive target for developing novel H3R antagonists/inverse agonists with the potential role in neuropsychiatric multi-neurotransmitter disorders, e.g., Alzheimer's disease, cognitive deficit associated with schizophrenia and recently on ASD [1,22,27,36–39]. In view of that both acetylcholine esterase (AChE) and H3Rs (auto- and heteroreceptors) are involved in the regulation of numerous brain neurotransmitters including ACh and HA, dual-active AChEIs and H3R antagonists have been developed by several groups [40–43]. Therefore, in present study we describe the effects of a recently developed dual-active AChEI and H3R antagonist, namely E100 [1-(7-(4-chlorophenoxy) heptyl) homo-piperidine] with histamine H3 receptor (H3R) antagonist affinity (hH3R K_i = 203 nM) and acetylcholine esterase inhibitory effect (EeAChE IC$_{50}$ = 2 µM and Eq-BuChE IC$_{50}$ = 2 µM) in male C57BL/6 mice model of ASD-like repetitive compulsive behaviors induced by prenatal exposure to valproic acid (VPA, 500 mg/kg, i.p.). The effects of E100 on locomotor activity and anxiety-like behaviors of the same animals were evaluated in open field test, since anxiety and motor activity could misperceive performance of animals in behavioral tests [44]. Moreover, neuroinflammation was assessed in cerebellum and hippocampus, as several studies showed that cerebellar inflammation may alter social behavior in adult mice, as cerebellum is involved in executive and cognitive functions [45–48], whereas hippocampus is involved in memory, learning and recently in cognition including social cognition [49]. In addition, abrogative studies were carried out co-administering CNS penetrant H1R antagonist mepyramine (MPA), H2R antagonist zolantidine (ZLT), H3R agonist (R)-α-methyl histamine (RAM), and cholinergic muscarinic antagonist scopolamine (SCO) to test whether brain histaminergic as well as cholinergic neurotransmission are involved in the effects provided by E100.

2. Materials and methods

2.1. Animals

C57BL/6 mice (aged 8–12 weeks, weighing 20–25 g) (Jackson Laboratory, Bar Harbor, USA) bred in the local central animal facility of the College of Medicine and Health Sciences, United Arab Emirates University [50] were used in this study. All mice were housed in plastic cages under a standard light/dark cycle (12-h light/dark cycle, lights on at 6 a.m.) at a constant temperature of 22–25 °C, with free access to tap water and a standard rodent chow diet. Mice were kept in separate cages for mating. Female mice were observed daily and placed in a separate cage when pregnancy was confirmed by the presence of a vaginal plug. This day was considered embryonic day 0 (E0). On E12.5, pregnant females were i.p. injected with 500 mg/kg VPA dissolved in isotonic 0.9% sodium chloride solution, and control mice were injected with saline, as described previously [51,52]. The day of delivery was defined as postnatal day 0 (P0). All offspring were weaned and sex-grouped (5–6 mice/cage) at P21. Pups from VPA-exposed mothers were

considered the VPA-exposed model mice for ASD, and were used for the experiments at the age of 8 weeks. On the other hand, the pups of mothers treated with saline were used as control mice. All procedures were carried out in accordance with the recommendations of the European Communities Council Directive of 24 November 1986 (86/609/EEC), and were approved by the Institutional Animal Ethics Committee in the College of Medicine and Health Sciences/United Arab Emirates (Approval No. ERA-2017-5603). All authors confirm that all methods were carried out in accordance with relevant guidelines and regulations.

2.2. Drugs and biochemical reagents

The H3R antagonist E100 was designed and synthesized in the Department of Technology and Biotechnology of Drugs, Krakow, Poland, according to a previously described procedure [53,54].

The H3R antagonist E100 belongs to the chlorophenoxyalkylamine derivatives which were synthetically achievable through two-step synthetic pathway. In the first step, the precursor 1-(7-bromoheptyl)-4-chlorobenzene was obtained in simple one-step alkylation of 4-chlorophenol with 1,7-dibromoheptane refluxed in sodium propanolate. Obtained 1-(7-bromoheptyl)-4-chlorobenzene was then reacted with homopiperidine in the mixture of ethanol/water with powdered potassium carbonate and a catalytic amount of potassium iodide. The desired product E100 was obtained as a free base and crystallized as salt of oxalic acid. The structure of E100 was confirmed by several elemental and spectral analyses. For estimation of the levels of proinflammatory cytokines (IL-1β, IL-6, TNF-α, and TGF-β), commercially available enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, USA). Rabbit anti–NF–κB p65 antibody was purchased from Abcam (Cambridge, MA, USA) and Rabbit anti-COX2 and rabbit anti-iNOS antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-actin antibody was obtained from MERCK (Millipore, USA), while the goat anti-mouse and goat anti rabbit secondary immunoglobulin G antibodies conjugated to horse-radish peroxidase were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Iba-1 polyclonal rabbit was purchased from Wako Chemicals (Richmond, VA, USA). Alexa Fluor 488- conjugated secondary goat anti-rabbit antibodies were purchased from Life Technologies (Grand Island, NY, USA). Protease and phosphatase inhibitors were obtained from Thermo Scientific (Waltham, MA, USA). The Pierce® BCA Protein Assay Reagent Kit and the SuperSignal® West Pico PLUS chemiluminescence substrate kit were purchased from Thermo Fisher Scientific, Inc. (Rockford, IL, USA). Mini-Protean TGX® precast electrophoresis gels were obtained from Bio-Rad Laboratories, Inc. (USA). Vectashield® fluorescent mounting media was procured from Vector Laboratories (Burlingame, CA, USA). All the reagents used in the study were of analytical grade and were purchased from Sigma-Aldrich. All test compounds were suspended in 1% aqueous solution of Tween 80. All mice were i.p. injected at a volume of 10 mL/kg, and all doses are expressed in terms of the free base.

2.3. Study design and drug treatment

Pregnant C57 mice were monitored and day of birth was recorded as postnatal day 0 (P0). All offspring were weaned, sex-grouped at P 21 (Scheme 1). Then only male offspring were divided into 2 groups where Group A served as prenatally VPA-exposed mice and Group B served as prenatally saline-exposed mice. All mice started receiving treatments daily from P44 for 21 days. Group A were further subdivided where Group I received only 1% aqueous solution of Tween 80, i.p., Group II received E100 5 mg/kg, i.p, Group III received E100 10 mg/kg, i.p, Group IV received E100 15 mg/kg, i.p, Group V received donepezil (DOZ, 1 mg/kg, i.p.), the acetylcholine esterase inhibitor as a reference drug, Group VI were VPA-exposed mice co-administered with E100 10 mg/kg, i.p and RAM 10 mg/kg, i.p, Group VII were VPA-exposed

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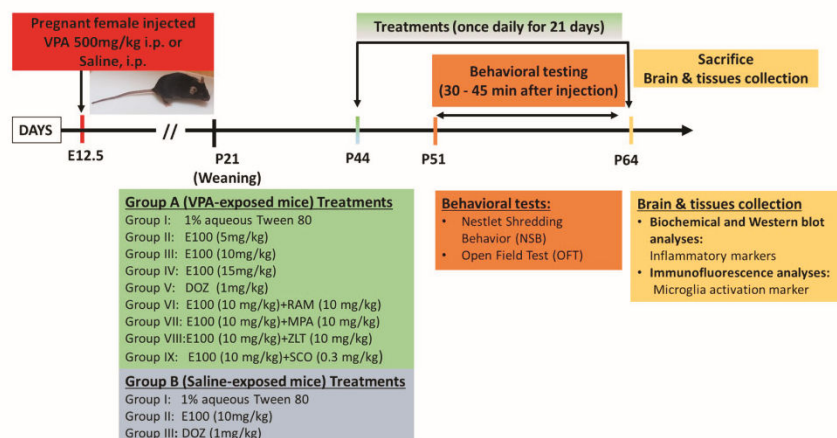
Scheme 1. Schematic illustration of prenatally VPA-induced ASD, drug treatments, behavioral studies, and biochemical assessments with VPA mice. Pregnant mice were administered with VPA (500 mg/kg, i.p.) at embryonic day 12.5 (E12.5). Systemic treatments started from postnatal day (P44). Daily injections continued for a duration of 21 days until P64. Behavioral studies were conducted starting from P51. All mice were then sacrificed at P64 for biochemical, western blot and immunofluorescence analyses. Modified after Eissa et al., 2018 [58].

mice co-administered with E100 10 mg/kg, i.p. and MPA 10 mg/kg, i.p., Group VIII were VPA-exposed mice co-administered with E100 10 mg/kg, i.p and ZLT 10 mg/kg, i.p and Group IX were VPA-exposed mice co-administered with E100 10 mg/kg, i.p and SCO 0.3 mg/kg, i.p. Group B were subdivided where Group I received only 1% aqueous solution of Tween 80, i.p., Group II received E100 10 mg/kg, i.p and Group III received DOZ 1 mg/kg, i.p. One week after starting the treatments, that is from P51, mice were subjected to behavioral testing in a sequence to assess stereotyped repetitive and compulsive like-behaviours, locomotion, and anxiety, by conducting nestlet shredding behavioral test (NSB) and open field test (OFT) (Scheme 1). On P64 all the animals were sacrificed; brains were isolated for biochemical estimations (IL-1β, IL-6, TNF-α, TGF-β), Western blot analyses (NF-κB, iNOS and COX-2) and Immunofluorescence analyses for microglial activation. All doses were selected based on the results of our previous studies on strongly related dual-active compounds, and are expressed in terms of the free bases [39,55] (Scheme 1). E100 and DOZ or 1% aqueous of Tween 80 were administered 30–45 min before each behavioral test. All the behavioral studies were performed in the light phase between 09:00 and 15:00. To reduce the number of animals used, the biochemical analysis, western blotting, and immunofluorescence staining were performed in the same group of animals that were subjected to behavioral tests (Scheme 1). The doses of MPA, ZLT, and SCO were injected 30–45 min before behavioral assessments, while the CNS penetrant H3R agonist RAM was administered 15–20 min before the start of behavioral tests to ensure its presence in the CNS, as RAM was described to show fast metabolism [56]), and were selected according to previous studies [55,57–60].

2.4. Brain collection and tissue preparation for biochemical studies

For further biochemical assessments, brain collection and tissue preparation experiments were carried out following 21 days of drug treatment (Scheme 1). The animals were deeply anesthetized with pentobarbital (40 mg/kg, i.p.), and cardiac perfusion was performed using 1 × PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) at pH 7.4 to wash out the blood. The animals were injected with the inflammatory stimulus lipopolysaccharide (LPS, 25 μg/kg, i.p., from *E. coli* serotype 0111:B4) 2 h before the sacrifice. Accordingly, the inflammatory stimulus LPS demonstrate a significant LPS-induced exacerbated rise in the expression of

proinflammatory cytokines (IL-1β, IL-6, TNF-α, and TGF-β) in cerebellum of VPA-exposed mice [47,58]. The brains were then quickly removed and placed on an ice plate, where the two hemispheres were separated. The cerebellum and hippocampus were excised from the brain and snap-frozen in liquid nitrogen for biochemical tests [61]. On the day of assay, the tissues were homogenized on ice in the extraction buffer recommended by the manufacturer, radioimmunoprecipitation assay buffer (50 mM Tris HCl, pH 7.4, 140 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and 0.5% sodium deoxycholate) with protease and phosphatase inhibitors. The homogenates were sonicated and centrifuged for 30 min at 14000 rpm at 4 °C to remove tissue debris, and the resulting supernatant was used for the pro-inflammatory cytokine assessment and Western blot analysis. After being transcardially perfused with PBS, four animals from each group were further perfused with 4% paraformaldehyde (cold) in 0.1 M phosphate buffer (pH 7.2). After removing the brains from the skulls, they were post-fixed in the same fixative (4% paraformaldehyde) for 48 h at 4 °C. The brains were then immersed in 10% sucrose solution for three consecutive days at 4 °C. Finally, the brains were stored at −80 °C for cryostat sectioning [61,62]. All experimenters who performed the behavioral, biochemical tests, and immunofluorescence analysis were blinded to the experimental groups.

2.5. Behavioral tests

2.5.1. Nestlet shredding behavior (NSB)

The test to evaluate NSB was performed by placing commercially available cotton fiber (nestlets) (5 cm × 5 cm, 5-mm thick, ~ 2.5 g each), after weighing it on an analytical balance, into a cage (19 cm × 29 cm × 13 cm) filled with fresh, unscented mouse bedding material to a depth of 0.5 cm. One nestlet was placed on top of the bedding in each test cage, and the filter-top cover was placed on the cage. No food and water were provided during the test. One mouse was added to each cage and left undisturbed with the nestlet for 30 min. After test completion, the remaining intact nestlet was removed from the cage using forceps and allowed to dry overnight. The remaining unshredded nestlet was weighed, and then the percentage of nestlet shredded was calculated. The remaining shredded nestlet material and bedding were discarded [58,63].

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2.5.2. Open field test (OFT)

Open field test (OFT) is an exploratory activity performed in a novel environment, assessed in an open field box (45 × 45 × 30cm) [47]. This test systematically assesses novel environment exploration, general locomotor activity, and anxiety-related behavior in rodents. The centre region was defined as the central 23 × 23 cm area. Mice were introduced into the centre area of the arena and given 5 min habituation before actual behaviors recording. The total distance moved in the whole arena, time spent in the centre and periphery were recorded for 10 min using CCD camera-assisted motion tracking apparatus and software (EthoVision 3.1, Noldus Information Technology, the Netherlands). After each trial, apparatus was cleaned using 70% ethanol and allowed to dry [64,65]. When evaluating the results, longer time spent in the centre indicated lower levels of anxiety-like behaviors and total distance travelled reflected locomotor activity.

2.6. Biochemical assessments

2.6.1. Pro-inflammatory cytokine estimations using ELISA

We performed ELISA to quantify the levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, and TGF-β) in the cerebellum. Commercially available ELISA kits for IL-1β, IL-6, TNF-α, and TGF-β and were purchased from R&D Systems. The levels of IL-1β, IL-6, TNF-α, and TGF-β were estimated following the manufacturer's instructions and as described earlier [58,61,62,66]. The optical density was determined at 450 nm using a microplate absorbance reader (Sunrise, TECAN). The results were expressed as pg/mg protein.

2.7. Western blot analysis

Western blot analysis was conducted to measure the levels of expression of COX-2, iNOS, and NF-κB p65 in the cerebellum and hippocampus of different animal groups, as described previously [61,62]. As mentioned earlier, the prepared cell lysates containing the extracted protein from the mouse brain tissues were used. The protein content in the sample was estimated using the Pierce® BCA Protein Assay Reagent Kit. The protein samples containing equal amounts of protein (30 μg), adjusted using radioimmunoprecipitation assay buffer and 4 × Laemmli sample buffer, were loaded and separated in Mini-Protean TGX® precast electrophoresis gels. The proteins were then transferred onto polyvinylidene difluoride membranes that were first activated by soaking in 100% methanol. Subsequently, the membranes were incubated for 1 h with the blocking buffer (non-fat dry milk) and then washed to avoid non-specific binding. The membranes were then incubated overnight at 4 °C with the specific primary rabbit polyclonal antibodies against COX-2 (1:1000), iNOS (1:5000), and NF-κB p65 (1:50000), followed by the horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:20000) on the second day. The bands of proteins detected by the antibodies were visualized using an enhanced chemiluminescence Pico kit as substrate. Subsequently, the blots were stripped and re-probed for actin (1:7000) (used as a loading control) using horseradish peroxidase-conjugated anti-mouse secondary antibody (1:20000). The intensity of the band was measured using densitometry and quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Immunofluorescence labelling of Iba-1

Brains were collected, post-fixed, and stored at −80 °C, as mentioned earlier. On the day of sectioning, the brains were sliced using a cryostat into 20-μm sections. Immunofluorescence staining was performed with the coronal sections of the cerebellum to examine the Iba-1-positive microglia (activated) and according to previously described methods [67]. Brain sections were washed twice with PBS (500 μl/well) and incubated with a blocking reagent (10% normal goat serum in PBS 0.3% Triton-X 100) for 1 h at room temperature. The sections were then washed and incubated with the primary polyclonal rabbit antibody against Iba-1 (1:700) overnight at 4 °C. On the second day, the sections were washed twice with PBS and incubated with fluorescent anti-rabbit secondary antibody (Alexa Flour 488) (1:1000) for 1 h at room temperature. Subsequently, the sections were washed again and mounted using Vectashield® mounting media. The images were then captured using the fluorescent microscope EVOS FL (Thermo Fisher Scientific). A minimum of three sections per brain from four animals were used (three sections per brain) to analyze microglial activation. From each section, the activated microglia were evaluated by measuring the integrated density of the Iba-1 marker signal from three different randomly selected fields of equal areas using the Image J software. The expression levels of Iba-1 were detected by the green fluorescence emitted by Alexa Fluor 488. The total corrected cellular fluorescence (TCCF) was then calculated using the following equation: TCCF = integrated density-(area of selected cell × mean fluorescence of the background) [67]. This TCCF was calculated and normalized against the mean of the control, with results presented as percentage fold increase from the control level.

2.9. Inhibition of radioligand-binding by E100 at H1R and H4R

Inhibition of [³H]-pyrilamine and [³H]-histamine binding to human isoforms of H1R and H4R was determined for E100, as previously described [68,69]. Briefly, binding assays conducted on CHO-K1 cells stably expressing the hH1R were used to determine the antagonist affinity of the respective test compound for hH1Rs. The experimental assays were carried out in triplicates with at least four appropriate concentrations in the range of 100 nM-100 μM of the test compound E100. The resulting nonspecific binding was evaluated in the presence of the standard H1R antagonist chlorpheniramine hydrogenmaleate at a concentration of 10 mM. In addition, the unbound radioligand was removed with four washes of 5 mL of ice-cold HEPES buffer. Liquid scintillation counting using a PerkinElmer MicroBeta Trilux scintillation counter was used to determine the amount of radioactivity collected on the filter used in the current experiment. Accordingly, competition binding data were analyzed using the software GraphPad Prism 5.01 (GraphPad Software, Inc.) using nonlinear least squares fit, and K_i values were calculated from the IC_{50} values according to the Cheng—Prusoff equation [68–70]. For the measurement of antagonist affinity of the test compound E100 to hH4Rs and as described previously, binding assay experiments were carried out [68,69]. Briefly, the competition binding experiments were run on incubating membranes, 35 μg/well (prepared from Sf9 cells expressing hH4R, coexpressed with G protein Gγi2 and Gβ1γ2 subunits) in a final volume of 0.2 mL containing binding buffer and [³H]histamine (10 nM, 15.3 Ci/mmol) in a 96-well microtiterplate. Assays were conducted in triplicates with seven appropriate concentrations in the range of 0.1 nM–100 μM of E100. Nonspecific binding, bound radioligand, and free radioligand were determined as described previously [68,69], and competition binding data were analyzed using the software GraphPad Prism 5.01 (GraphPad Software, Inc.) using nonlinear least squares fit. K_i values were calculated from the IC_{50} values according to the Cheng-Prusoff equation [68–70].

2.10. Statistical analysis

For the NSB and OFT tests, statistical significance was assessed with a group (Control, VPA) × drug (SAL, E100) analysis of variance. The data were analyzed for normality by assessing the sample distribution or skewness (- 1.5 to + 1.5 considered normally distributed). The source of the detected significances was determined by Bonferroni's multiple comparison post hoc test. *P* values less than 0.05 were considered statistically significant. The number of mice per group is indicated in the figures. Statistical significance for Western blot and immunofluorescence analyses was calculated using one-way analysis of

4

variance followed by post hoc Tukey's multiple comparison test. For statistical comparisons, the software package SPSS 25.0 (IBM Middle East, Dubai, UAE) was used. The results are expressed as the means and standard errors of the means (SEM).

3. Results

3.1. In Vitro affinities at hH1Rs, hH3Rs, and hH4Rs

The novel dual-active ligand E100 was tested for its H3R affinity by [^{3}H]N^α-methylhistamine displacement assays on membrane preparations of HEK-293 cells, stably expressing the $hH3R$. The results showed that E100 displayed histamine H3 receptor (H3R) antagonist affinity with a Ki value of 203 ± 48 at $hH3Rs$. E100 was further evaluated for its affinity at human histamine H1 ($hH1R$) and H4 ($hH4R$) receptors. The results showed that test compound E100 exhibited (> 10,000 nM for H1R, 203 nM for H3R, > 10,000 nM for H4R) a selectivity profile toward H3Rs with at least 10-fold lower affinity at $hH1$- and H4Rs.

3.2. In Vivo behavioral models

3.2.1. Effects of E100 on stereotyped repetitive behavior of VPA-exposed mice in nestlet-shredding behavior test

The effect of subchronic systemic administration of E100 (5, 10, or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) on the percentage increase of shredded nestlet was evaluated in the nestlet-shredding behavior (NSB) test (Fig. 1). Post hoc analyses showed that VPA-exposed mice shredded significantly more nestlets than the saline-exposed mice ($F_{(1,8)} = 44.09$, $P < 0.01$). However, VPA-exposed mice pre-treated with test compound E100 (5, 10, or 15 mg/kg, i.p.) or the reference drug DOZ (1 mg/kg, i.p.) exhibited a significantly lower percentage of shredded nestlets than VPA-exposed mice pre-treated with saline ($F_{(1,8)} = 20.92$, $P < 0.05$; $F_{(1,8)} = 33.26$, $P < 0.01$; $F_{(1,8)} = 38.78$, $P < 0.01$, and $F_{(1,8)} = 42.56$, $P < 0.01$, respectively) (Fig. 1). Moreover, the effects observed with 10 or 15 mg/kg E100 were significantly higher than that observed with 5 mg/kg E100 ($F_{(1,8)} = 6.62$, $P < 0.05$ and $F_{(1,8)} = 9.60$, $P < 0.05$, respectively). Notably, no significant difference in the E100-induced effect on the percentage of shredded nestlets was detected
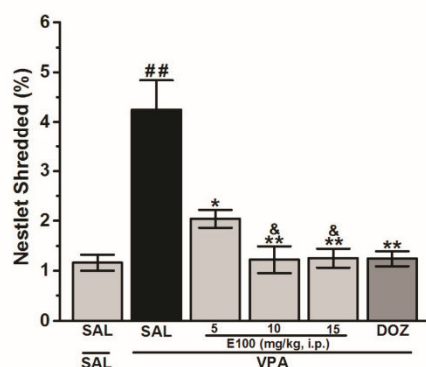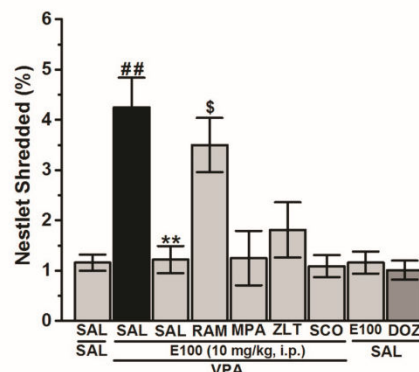

Fig. 2. Effects of RAM, MPA, ZLT, and SCO on E100-provided attenuation of obsessive compulsive behavior of VPA-exposed mice. Obsessive compulsive nestlet shredding behaviour was measured after a 30-min testing session. VPA-exposed mice treated with saline (VPA group) demonstrated elevated stereotyped, repetitive behaviours that were significantly increased compared to Saline-exposed mice (SAL group). E100 (10 mg/kg, i.p) was administered subchronically for 21 days in VPA-exposed mice. Effects of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p. for RAM group), MPA (10 mg/kg, i.p. for MPA group), ZLT (10 mg/kg, i.p., for ZLT group), or SCO (0.3 mg/kg, i.p., for SCO group) on the E100(10 mg/kg)-provided attenuation of stereotyped repetitive and compulsive behavior of VPA-exposed mice were assessed in NSB. Effects of subchronic (21 days) systemic injection of E100 (10 mg/kg) and DOZ (1 mg/kg) on the Saline-exposed mice (SAL group) were assessed in NSB. Data are expressed as the mean ± SEM (n = 5). $^{\#\#}P < 0.01$ vs. Saline-treated Saline-exposed mice. $^{**}P < 0.01$ vs. Saline-treated VPA-exposed mice. $^{\$}P < 0.05$ vs. E100(10 mg)-treated VPA-exposed mice.

between the 10 mg/kg and 15 mg/kg doses ($F_{(1,8)} = 0.01$, $P = 0.93$) (Fig. 1). Also, no significant difference in the E100-induced effect on the percentage of shredded nestlets was detected between the 10 mg/kg and 15 mg/kg doses and DOZ (1 mg/kg), with ($F_{(1,8)} = 0.003$, p = 0.96) and ($F_{(1,8)} = 0.002$, p = 0.95), respectively (Fig. 1).

3.2.2. Effects of RAM, MPA, ZLT, and SCO on E100-provided attenuation of stereotyped repetitive behavior in VPA-exposed mice in nestlet-shredding behavior test

Subchronic systemic administration of E100 (10 mg/kg, i.p.) significantly lowered the percentage of shredded nestlets in VPA-exposed mice ($F_{(1,8)} = 33.26$, $P < 0.01$). As shown in Fig. 2 and observed in the post hoc analyses, the E100 (10 mg/kg)-induced decrease in the percentage of shredded nestlets was countered following subchronic systemic co-administration of RAM (10 mg/kg, i.p.) ($F_{(1,8)} = 11.45$, $P < 0.05$) compared with E100 (10 mg)-treated VPA-exposed mice (Fig. 2). However, MPA, ZLT, and SCO failed to reverse this E100-induced decrease in the percentage of shredded nestlets, as they showed no significant effect compared with VPA-exposed mice treated with E100 (10 mg/kg, i.p.) ($F_{(1,8)} = 0.003$, $P = 0.96$; $F_{(1,8)} = 0.74$, $P = 0.41$, and $F_{(1,8)} = 0.11$, $P = 0.75$, respectively) (Fig. 2).

3.2.3. Effects of E100 on anxiety levels and locomotor activity of VPA-exposed mice in open field test

The observed effects of subchronic systemic injection of saline or E100 (5, 10, or 15 mg/kg, i.p.) on the time spent in the centre (Fig. 3A), time spent in the periphery (Fig. 3B) and total distance travelled (Fig. 3C) of VPA-exposed mice tested in the OFT are shown in Fig. 3A–C. As shown in Fig. 3B&C, there were no significant effects of subchronic systemic exposure of VPA-exposed mice to E100 (5, 10, and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) on time spent in the periphery and total distance travelled vs. saline-treated VPA-exposed mice (all

Fig. 1. E100 attenuated increased obsessive compulsive features in nestlet shredding test. Obsessive compulsive nestlet shredding behavior was measured after a 30-min testing session. VPA-exposed mice treated with saline (VPA group) demonstrated elevated stereotyped, repetitive behaviors that were significantly increased compared to Saline-exposed mice (SAL group). E100 (at a dose of 5, 10, or 15 mg/kg, i.p) or DOZ (1 mg/kg, i.p.) were administered subchronically for 21 days in VPA-exposed mice. Data are expressed as the mean ± SEM (n = 5). $^{\#\#}P < 0.01$ vs. Saline-treated Saline-exposed mice. $^{*}P < 0.05$ vs. Saline-treated VPA-exposed mice. $^{**}P < 0.01$ vs. Saline-treated VPA-exposed mice. $^{\&}P < 0.05$ vs. E100(5 mg)-treated VPA-exposed mice.
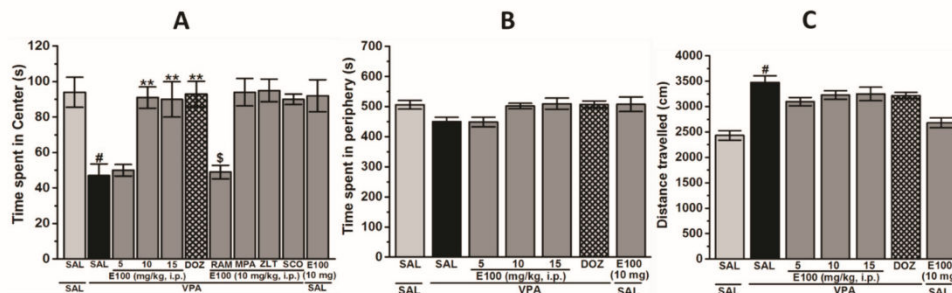
Fig. 3. E100 ameliorated fear-related behavior without affecting locomotor activity in open field test. VPA-exposed mice demonstrated elevated anxiety and deficits in cognition behaviours that were significantly increased compared to Saline-exposed mice. E100 (5, 10 or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) was administered subchronically for 21 days in VPA-exposed mice. E100 (10 and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) attenuated the decreased time spent in the central arena (Fig. 3A), however, failed to modify the decreased time spent in the periphery (Fig. 3B) as well as the increased total distance travelled (Fig. 3C) in the OFT in VPA-exposed mice. Effects of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p. for RAM group), MPA (10 mg/kg, i.p. for MPA group), ZLT (10 mg/kg, i.p., for ZLT group), or SCO (0.3 mg/kg, i.p., for SCO group) on the E100(10 mg)-provided amelioration of time spent in the centre as well as time spent of VPA-exposed mice were assessed in OFT (Fig. 3A). Data are expressed as the mean ± SEM (n = 5). $^\#P < 0.05$ vs. Saline-treated saline exposed mice. $^{**}P < 0.01$ vs. Saline-treated VPA-exposed mice. $^\$P < 0.05$ vs. E100(10 mg)-treated VPA-exposed mice.

$P > 0.01$) (Fig. 3B&C). In contrast, analysis of variance demonstrated that VPA-exposed mice pretreated with E100 (10 and 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) spent a significantly higher percentage of time in the centre of the arena, with $[F_{(1,8)} = 19.95;\ P < 0.01]$, $[F_{(1,8)} = 10.31;\ P < 0.05]$, and $[F_{(1,8)} = 18.24;\ P < 0.01]$, respectively (Fig. 3A). As shown in Fig. 3A and observed in the post hoc analyses, the E100 (10 mg)-provided increase in the time spent in the centre of arena was abrogated following subchronic systemic co-administration of RAM (10 mg/kg, i.p.), with $[F_{(1,8)} = 28.93;\ P < 0.05]$ compared with E100 (10 mg)-treated VPA-exposed mice. However, MPA, ZLT, and SCO failed to counteract this E100-provided increase in time spent in central arena, as they had no significant effect compared to VPA-exposed mice treated with E100 (10 mg/kg, i.p.), with $[F_{(1,8)} = 0.05;\ p = 0.82]$, $[F_{(1,8)} = 0.18;\ p = 0.69]$, and $[F_{(1,8)} = 0.03;\ p = 0.86]$, respectively (Fig. 3A). Notably, subchronic treatment of Saline-exposed mice with E100 (10 mg/kg, i.p.) had no significant influence on time spent in the central arena, time spent in the periphery, and total distance travelled when compared to saline-pretreated Saline-exposed mice, with $[F_{(1,8)} = 0.01;\ p = 0.92]$, $[F_{(1,8)} = 0;\ p = 0.95]$, and $[F_{(1,8)} = 2.65;\ p = 0.14]$, respectively (Fig. 3A–C).

3.3. Biochemical assessments

3.3.1. Effects of E100 pre-treatment on the levels of proinflammatory cytokines in the brain tissue of VPA-exposed mice

The effects of E100 on the exacerbated levels of proinflammatory cytokines (interleukin [IL]-1β, IL-6, tumor necrosis factor [TNF]-α, and tumor growth factor [TGF]-β) following lipopolysaccharide (LPS) challenge in the brain tissue of VPA-exposed mice were evaluated (Fig. 4A–D). The results revealed that a systemic prenatal injection of VPA (500 mg/kg, i.p.) induced ASD-like behaviors in VPA-exposed mice and significantly increased the levels of IL-1β (Fig. 4A), IL-6 (Fig. 4B), TNF-α (Fig. 4C), and TGF-β (Fig. 4D) compared with the saline-exposed mice (all $P < 0.001$). However, subchronic systemic administration of E100 (10 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) significantly mitigated the increase in the levels of these proinflammatory cytokines in VPA-exposed mice (all $P < 0.01$) (Fig. 4A–D). Notably, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) partially abrogated ($P < 0.01$) the protective effects of E100 (10 mg/kg, i.p.) against VPA-induced elevation of IL-1β (Fig. 4A) and TNF-α (Fig. 4C) levels, but entirely reversed the E100 (10 mg/kg)-induced protection against increase in IL-6 (Fig. 4B) and TGF-β (Fig. 4D) levels (all $P < 0.01$).

3.3.2. Effects of E100 on the protein expression of NF-kB p65, iNOS, and COX-2 in the brain tissue of VPA-exposed mice

The expressions of nuclear factor kappa-light-chain-enhancer of activated B cells p65 subunit (NF-κB p65), induced nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) were also tested using western blotting of lysates from the cerebellum (Fig. 5A) and hippocampus (Fig. 5B). A significant increase in the expression of NF-κB p65 ($P < 0.01$), iNOS ($P < 0.001$), and COX-2 ($P < 0.01$) was observed in the cerebellum of VPA-exposed mice compared with that of the saline-exposed mice (Fig. 5AI-III). However, following subchronic systemic pretreatment of VPA-exposed mice with E100 (10 mg/kg), a remarkable reduction in the levels of NF-κB p65, iNOS, and COX-2 was observed compared with the VPA-exposed mice (all $P < 0.05$) (Fig. 5AI-III). Similarly, hippocampal tissues of VPA-exposed mice also showed a significant increase in the expression of NF-κB p65 ($P < 0.001$), iNOS ($P < 0.01$), and COX-2 ($P < 0.01$) (Fig. 5BI-III) compared with the saline-exposed mice. However, following subchronic systemic pretreatment of VPA-exposed mice with E100 (10 mg/kg), a remarkable reduction in the levels of NF-κB p65, iNOS, and COX-2 was observed compared with the VPA-exposed mice (all $P < 0.05$) (Fig. 5BI-III). Interestingly, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) reversed the mitigating effects of E100 (10 mg/kg, i.p.) on the expression of NF-κB p65, iNOS, and COX-2 in the cerebellum. Similarly, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) reversed the mitigating effects of E100 (10 mg/kg, i.p.) on the expression of NF-κB p65, iNOS and COX-2 in the hippocampus of VPA-exposed mice (all $P < 0.05$).

3.4. Immunoflourescence

3.4.1. Effects of E100 on the activation of Iba-1 in the cerebellum of VPA-exposed mice

Activation of glial cells (microglia) in VPA-exposed mice is considered an index of inflammatory responses [62], and was also observed in this study (Fig. 6A&B). The observed results showed that VPA-exposed mice exhibited a significant increase in the expression of ionized calcium-binding adaptor molecule-1 (Iba-1) in microglia, which is a marker of their activation ($P < 0.05$) (Fig. 6A&B). Immunofluorescence staining revealed a significant increase ($P < 0.05$) in the number of activated microglia in VPA-exposed mice compared with the saline-exposed mice (Fig. 6A&B). However, subchronic systemic treatment of VPA-exposed mice with E100 (10 mg/kg, i.p.) significantly decreased the number of activated microglia compared with the saline-
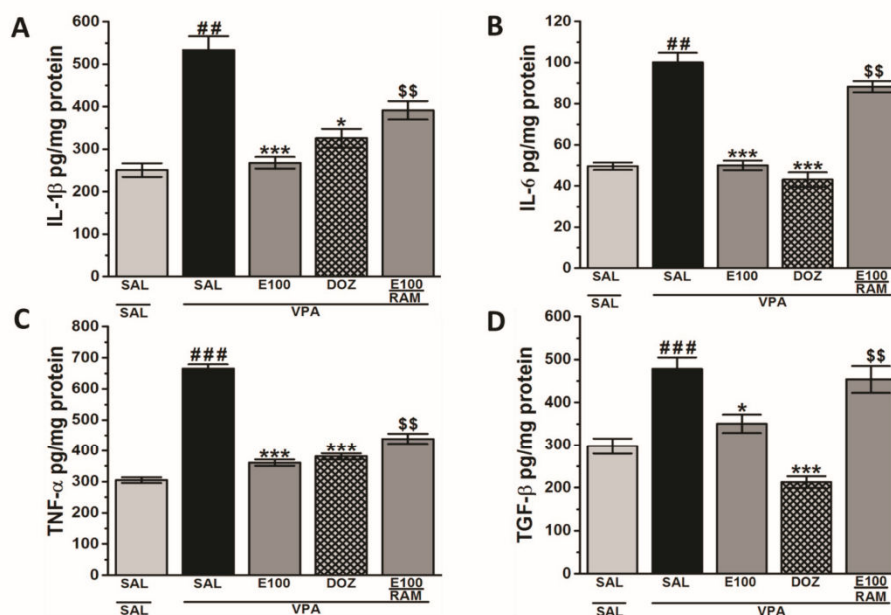
Fig. 4. E100 restored levels of proinflammatory cytokines in the cerebellum of the experimental animals. Modulated Interleukines (IL-1β, IL-6), Tumor Necrosis Factor (TNF-α), and Transforming Growth Factor (TGF-β) were assessed. VPA-exposed mice showed a significant increase in IL-1β (Fig. 4A), IL-6 (Fig. 4B), TNF-α (Fig. 4C), and TGF-β (Fig. 4D), compared to Saline-exposed mice. E100 (5, 10, or 15 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) were administered subchronically for 21 days in VPA-exposed mice. E100 (10 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) significantly decreased IL-1β, IL-6, TNF-α, TGF-β (Fig. 4A–D). Effects of subchronic (21 days) systemic co-injection RAM (10 mg/kg, i.p.) on E100(10 mg)-provided modulation of proinflammatory cytokines levels were assessed (Fig. 4A–D). Saline-exposed mice were injected with saline. Data are expressed as the mean ± SEM (n = 5). $^{###}P < 0.001$ vs. SAL-exposed mice. $^{*}P < 0.05$ vs. Saline-treated VPA-exposed mice. $^{***}P < 0.001$ vs. Saline-treated VPA-exposed mice. $^{$$}P < 0.01$ vs. E100 (10 mg)-treated VPA-exposed mice.

exposed mice ($P < 0.05$) (Fig. 6C). Moreover, subchronic systemic co-administration of RAM (10 mg/kg, i.p.) entirely reversed the effects of E100 (10 mg/kg, i.p.) on the number of activated microglia compared with the E100 (10 mg)-treated VPA-exposed mice ($P < 0.05$) (Fig. 6D).

4. Discussion

In search of sensitive and specific markers of ASD as well as potential therapeutic interventions, the study of brain neurotransmitters has attracted considerable attention. Along with the genetic and environmental factors, accumulated evidence suggests that a variety of several brain neurotransmitters, such as ACh, 5-HT, DA, GABA, Glu, and HA, are implicated in the onset and progression of ASD, substantiating the significance of this research area in the study of ASD etiology [9–14,16–19,71]. As alterations in histaminergic as well as cholinergic neurotransmission are thought to play a crucial role in the phenotypic outcomes of ASD-related behavioral features [1,72,73], the aim of the current study was to examine the effects of the pharmacological modulation of brain HA and ACh using the novel dual-active AChEI and H3R antagonist E100 on a mouse model of ASD-like stereotyped repetitive behaviors induced by prenatal exposure to VPA, as stereotypy, repetitive behavior, and restricted interests are considered core features of ASD. Moreover, recent studies have suggested that histaminergic signaling abnormalities may contribute to rare diseases such as Tourette syndrome [74], a condition reported to be among the most prevalently comorbid neurodevelopmental disorders with ASD and characterized by stereotypies [75–77]. Furthermore, Rapanelli et al. have reported the implication of H3R in repetitive behavior-related pathology [77]. In the current study, VPA-exposed mice

subchronically pretreated with E100 (10 or 15 mg/kg, i.p.) or with the reference drug DOZ (1 mg/kg, i.p.) showed comparable reduction in stereotyped repetitive behavior in the NSB test (Fig. 1). Numerous previous studies showed that H3Rs functioning as hetero-receptors can also regulate the release of several other brain neurotransmitters, e.g. ACh, Glu, GABA, 5-HT, DA in different brain regions [24,25]. Consequently, blocking central H3Rs has been projected to improve the cortical fast rhythms closely associated with cognitive behaviors [25], and numerous preclinical studies indicated particularly that H3R antagonists exhibited a unique feature by their potential cognition-enhancing property [22,23,26,27]. Furthermore, several drug candidates with H3R antagonist properties have shown to increase the release of brain ACh, therefore alleviating symptomatic features and progression of Alzheimer's disease (AD) in clinical trials [28,29]. In addition, a recent report demonstrated lower ACh level in the prefrontal cortex region of mice which showed attention deficit and impulsive behavior [30]. Interestingly, deficit in cholinergic neurotransmission was, also, found in BTBR T + tf/J mice, an idiopathic animal model with the three diagnostic categories for ASD, namely impaired social interaction and communication as well as increased repetitive behaviors [31–35]. Additionally, the E100 (10 mg/kg)-induced effects observed in the NSB test were nullified when mice were administered RAM, but not MPA, ZLT, or SCO (Fig. 2). The mechanism by which the repetitive/compulsive behavior is improved following systemic administration with E100 may involve its capability, as a potent dual-active H3R antagonist and AChE inhibitor, to modulate the release of different neurotransmitters besides HA and ACh, such as DA and 5-HT, in several specific brain areas. Interestingly, the results observed for E100 in NSB are in agreement with previous studies in which subchronic systemic
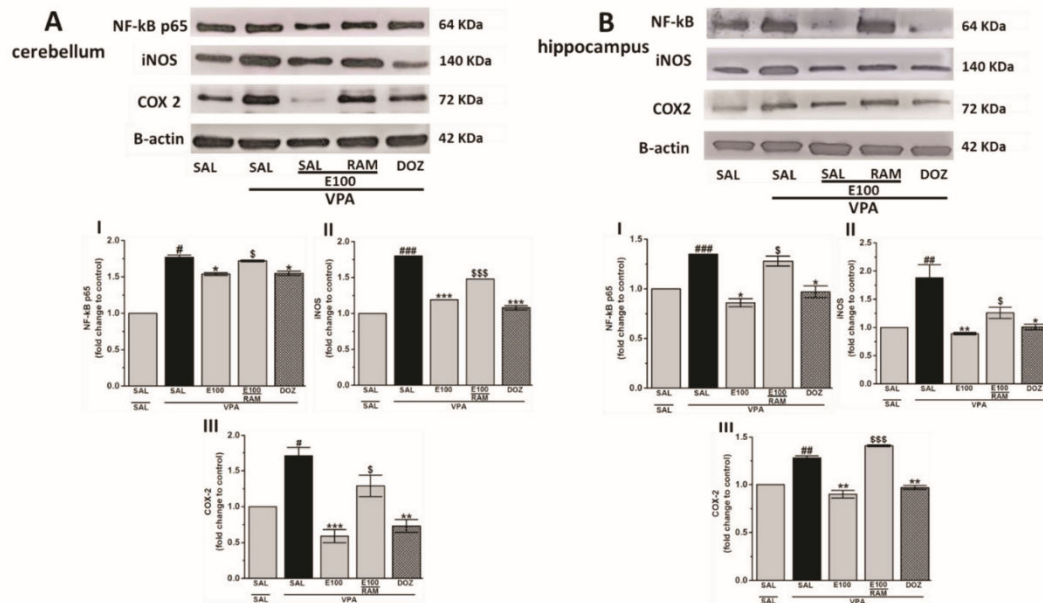
Fig. 5. Representative immunoblots of NF-kB p65, iNOS & COX-2 expression in cerebellum and hippocampus of the experimental animals. NF-kBp65, iNOS and COX-2 are modulated by subchronic treatment with E100 (10 mg) in cerebellum and hippocampus of prenatally VPA treated mice. (A) Cerebellum tissues of VPA-exposed group were subjected to immunoreactions with anti–NF–kB p65 (I), anti-iNOS (II), and anti-COX2 (III). (B) Hippocampal tissues of VPA-exposed group were subjected to immunoreactions with anti–NF–kB p65 (I), anti-iNOS (II), and anti-COX2 (III). Expression levels were determined using western blotting in both the cerebellum and hippocampus. The VPA-exposed mice showed significant increase in NF-kBp65, iNOS and COX-2 compared to Saline-exposed mice in both brain regions (Fig. 5A&B I-III). Subchronic treatment with E100 (10 mg/kg, i.p.) or DOZ (1 mg/kg,i.p.) to VPA-exposed mice remarkably decreased the expression levels of NF-kBp65, iNOS and COX-2 in both brain regions (Fig. 5A&B I-III). However, subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p.) reversed the E100(10 mg)-provided suppressed expression in NF-kBp65, iNOS and COX-2 in cerebellum (Fig. 5AI-III), and in NF-kBp65, iNOS and COX-2 in hippocampus (Fig. 5B I-III). Blots were quantified using Image J and corresponding results were represented as fold change of control. Data are expressed as mean ± SEM (n = 3). #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs Saline-exposed mice; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. VPA-exposed mice and $P < 0.05$, $$P < 0.01$, $$$P < 0.001$ vs. E100(10 mg)- treated VPA-exposed mice.

administration of the non-imidazole H3R antagonist DL77 significantly and dose-dependently (5, 10, 15 mg/kg, i.p.) mitigated the increased percentage of shredded nestlet in adult male Tuck-Ordinary mice of VPA-induced ASD features, comprehending the current observations in NSB with E100 capability to facilitate the release of several brain neurotransmitters besides HA and ACh that are of crucial function in repetitive/compulsive behaviors in different animal species [58]. Consequently, assessing the levels of different brain neurotransmitters, including HA and ACh, in different brain areas of the VPA-exposed mice with ASD-like behaviors as well as after pre-treatment with E100 would further help to understand which neural circuits may be involved in this observed behavioral improvement.

The effects of E100 on locomotor activity as well as anxiety levels were examined as ligands modulating anxiety levels or locomotion may give rise to a false-positive effect in these behavioral tests. Therefore, locomotor activity was examined to simultaneously rule out possible intrinsic impairment of spontaneous locomotor activity since reduced activity could otherwise wrongfully be interpreted as increased measures of anxiety hence give rise to a false-positive effect in behavioral tests. The results observed indicated that E100 (10 and 15 mg) significantly increased time spent in the central area, confirming the ability of E100 to modulate anxiety-associated fear levels. In contrast, E100 failed to restore hyperactivity, as no effect was exhibited with all doses on total distance travelled (Fig. 3). Thus, the improvements in repetitive/compulsive behaviours observed for E100 in NSB appeared

unlikely to be connected with a modulating effect in locomotor activity of the tested mice. Moreover, the failure of E100 (at all doses) and DOZ in alleviating the hyperactivity observed in VPA-exposed mice may have been due to the well-known existing imbalance of excitatory (Glu) and inhibitory (GABA) neurotransmitters, as such an imbalance was observed in several clinical trials in patients with ASD [78–81].

Previous studies have found that several proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and TGF-β, are elevated in the autistic brain [82–85]. Consistent with these findings, our study showed that the levels of TNF-α, IL-1β, IL-6, and TGF-β were significantly increased in the cerebellum of VPA-exposed mice compared with age-matched control mice (Fig. 4A–D). However, systemic administration of E100 (10 mg/kg, i.p.) significantly modulated the levels of the proinflammatory cytokines in VPA-exposed mice (Fig. 4A–D). In addition, when co-administered with E100, the CNS-penetrant H3R agonist abrogated the E100-induced protective effects against increased levels of proinflammatory cytokines (Fig. 4), indicating the involvement of brain HA in facilitating the neuroprotective action of E100 in VPA-exposed mice with ASD-like features. The regulation of immune response is mediated by NF-κB via induction of the expression of inflammatory cytokines and chemokines, establishing a feedback mechanism that can produce chronic or excessive inflammation. Activation of NF-κB is significantly increased in children with ASD [86,87]. Young et al. also observed elevated levels of NF-κB in the blood and brain of patients with ASD [88]. Moreover, neuroinflammation is characterized by
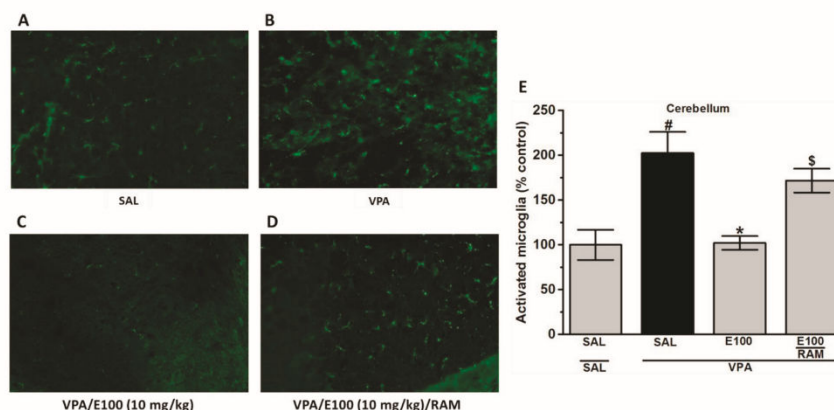
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Fig. 6. Effects of E100 on iba-1 in cerebellum tissues of VPA-exposed mice. Profound expression of iba-1-positive microglia (Fig. 6B) was found in the VPA-exposed mice compared to the Saline-exposed mice (Fig. 6A). In contrast, subchronic treatment with E100 (10 mg/kg, i.p.) to the VPA-exposed mice showed significantly lower staining of iba-1 compared to the VPA-exposed mice (Fig. 6C). Subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p) counteracted the E100(10 mg)-provided amelioration of iba-expression of VPA-exposed mice ($^{\$}P < 0.05$) (Fig. 6D) (scale bar 1000 μm). Quantitative analysis of activated microglia (Fig. 6E) revealed a significant increase ($^{\#}P < 0.05$) in the number of activated microglia in the VPA-exposed mice compared to the Saline-exposed mice. However, subchronic treatment with E100 (10 mg/kg, i.p.) to the VPA-exposed mice significantly reduced ($^{*}P < 0.05$) the number of activated microglia in the E100-treated VPA-exposed mice compared to the saline-treated VPA-exposed mice group. $^{\$}P < 0.05$ *vs.* E100(10 mg)- treated VPA-exposed mice. Data are expressed as the percent mean $\pm$ SEM (n = 3).

reactivity of microglia and astrocytes, activation of iNOS signaling, and increased expression and release of cytokines and chemokines [89]. Evidence indicates that patients with ASD exhibit ongoing neuroinflammatory processes in various regions of the brain involving microglial activation [82,90]; consequently, aberrant expression of cytokines and their signaling intermediaries are often observed. Furthermore, recent findings have shown that mice exhibiting disrupted COX-2 signaling might serve as a novel animal model to assess ASD [91]. In the current study, the expression of NF-κB, iNOS, and COX-2 were significantly higher in the cerebellum as well as the hippocampus of VPA-exposed mice compared with saline-exposed mice (Fig. 5A&B). However, systemic administration of E100 (10 mg/kg, i.p.) significantly reduced the increased levels of NF-κB, iNOS, and COX-2, and the CNS-penetrant H3R agonist, when co-administered with E100, reversed the E100-induced neuroprotection in both brain regions (Fig. 5A&B). The latter observations indicate that brain HA is strongly involved in facilitating the neuroprotective action of E100 in VPA-exposed mice with ASD-like features.

Growing evidence suggests a crucial role for immune dysregulation in patients with ASD. Several ASD risk-related genes are linked to the immune system- and maternal immune system-related risk factors of ASD [92,93]. Microglia, a representative immune cell in the brain, plays an important role in synaptic refinement [92,93], and are also supposed to be associated with the pathogenesis of ASD [92,93]. Moreover, previous reports have also shown changes in the activation, number, and distribution of microglia in the brains of patients with ASD [94,95]. In the current study, the results showed that expression of Iba-1-positive microglia was significantly higher in the cerebellum of VPA-exposed mice than in that of the saline-exposed control mice (Fig. 6A–D). Further, immunofluorescence staining revealed a significant increase in the number of activated microglia in VPA-exposed mice compared with the saline-exposed mice (Fig. 6A&B). However, systemic pre-treatment of VPA-exposed mice with E100 (10 mg/kg, i.p.) significantly decreased the number of activated microglia compared with the saline-exposed control mice (Fig. 6C), and the E100-induced effect on activated microglia was entirely counteracted by co-administration of RAM (Fig. 6D). The latter result signifies the involvement of brain HA in facilitating the neuroprotective effects of E100 against the disrupted microglia of VPA-exposed mice.

5. Conclusion

The novel dual-active H3R antagonist and AChE inhibitor E100 ameliorates ASD-like stereotyped repetitive behaviors induced by prenatal exposure of C57BL/6 mice to VPA. Moreover, E100 modulates the levels of central HA and ACh in an inflammatory context, mitigating the increase in the levels of proinflammatory cytokines and expression of NF-κB, iNOS, and COX-2 in the cerebellum as well as the hippocampus. To our knowledge, this is the first in vivo demonstration of the effectiveness of a potent dual-active H3R antagonist and AChE inhibitor in palliating stereotypies of ASD-like features induced by prenatal exposure to VPA, and provides evidence for the role of such compounds in treating ASD.

Conflicts of interest

The authors declare no competing interests.

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Supplementary Information

The dual-active histamine H3 receptor antagonist and acetylcholine esterase inhibitor E100 ameliorates stereotyped repetitive behavior and neuroinflammation in sodium valproate-induced autism in mice

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S.1) Supplementary Methods

S.1.1) Cell culture & membrane preparations of histamine H1- and H4-receptor expressing cells

Membrane preparations from Sf9-insect cells transiently expressing the human H4 receptor were carried out as described previously [1, 2].

For generating cell membrane preparations of histamine H1-receptors (H1R), CHO-cells stably expressing the human isoform of the H1R were cultured in Dulbecco's Modified Eagles Medium (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10% fetal bovine serum (PAN Biotech, Aidenbach, Germany), 1% L-Glutamine solution (200 mM, Sigma-Aldrich), Penicillin-Streptomycin (100 units/0.1 mg/mL; Sigma-Aldrich) and 1% non-essential aminoacid solution (100x) on cell-culture flasks (175 cm², Sarstedt, Nuembrecht, Germany) upon confluence, maintaining the culture flasks at 37 °C in atmosphere at absolute humidity and 5% CO_2. Upon confluence, medium was removed and cells were detached using membrane buffer (HEPES 20 mM, pH = 7.4; $MgCl_2$ 10 mM; NaCl 100 mM). The suspension was sonicated for three cycles (15 sec, each) and centrifugated for 30 min at 50,000 x g at 4 °C. The remaining pellet was re-suspended in fresh membrane buffer and homogenized using a hand-potter. Protein content was determined by the method of Bradford [3].

S.1.2) Inhibition of radioligand-binding by E100 at histamine H1 and H4 receptors

Activity of E100 at the H1R and H4R was determined by incubation of 10 µM or 1 µM of compound for either 120 min with [³H]-pyrilamine and H1R-membrane preparations (40 µg/well) or for 60 min with [³H]-histamine dihydrochloride and H4R-membrane preparations (40 µg/well), respectively. Radiolabeled ligands were purchased from PerkinElmer (Boston, MA; USA).

Therefore, E100 was diluted from DMSO-based stock solution (10 mM) in either membrane

buffer for H₃R (as described above) or in membrane buffer for H₄R (TRIS 75 mM, EDTA 1 mM, pH = 7.4; MgCl₂ 12.5 mM). The procedure for harvesting and scintillation counting was as described previously [4]. Percentage of inhibition was calculated based on total (TB) and non-specific (NSB) binding levels of radioligand, according to:

$$\text{Inhibition}(\%) = [1 - (\text{remained binding} - \text{NSB})/(\text{TB} - \text{NSB})] * 100\%$$

S.2) Results

S.2.1) Inhibition of radioligand-binding by E100 at histamine H1 and H4 receptors

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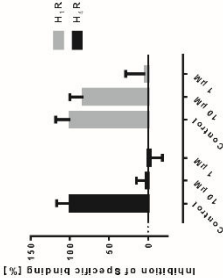
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## 4.7.	The dual-active histamine H₃ receptor antagonist and acetylcholine esterase inhibitor E100 alleviates autistic-like behaviors and oxidative stress in valproic acid induced autism in mice

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Contribution to research: Co-authorship. DR co-organised shipment of test-ligands, prepared, planned and conducted radioligand selectivity screenings at H₁R and H₄R, and evaluated corresponding data. DR proof-edited the manuscript.

Abstract:

The histamine H3 receptor (H3R) functions as auto- and hetero-receptors, regulating the release of brain histamine (HA) and acetylcholine (ACh), respectively. The enzyme acetylcholine esterase (AChE) is involved in the metabolism of brain ACh. Both brain HA and ACh are implicated in several cognitive disorders like Alzheimer's disease, schizophrenia, anxiety, and narcolepsy, all of which are comorbid with autistic spectrum disorder (ASD). Therefore, the novel dual-active ligand E100 with high H3R antagonist affinity (hH3R: K_i = 203 nM) and balanced AChE inhibitory effect (EeAChE: IC_{50} = 2 M and EqBuChE: IC_{50} = 2 M) was investigated on autistic-like sociability, repetitive/compulsive behaviour, anxiety, and oxidative stress in male C57BL/6 mice model of ASD induced by prenatal exposure to valproic acid (VPA, 500 mg/kg, intraperitoneal (i.p.)). Subchronic systemic administration with E100 (5, 10, and 15 mg/kg, i.p.) significantly and dose-dependently attenuated sociability deficits of autistic (VPA) mice in three-chamber behaviour (TCB) test (all $p < 0.05$). Moreover, E100 significantly improved repetitive and compulsive behaviors by reducing the increased percentage of marbles buried in marble-burying behaviour (MBB) (all $p < 0.05$). Furthermore, pre-treatment with E100 (10 and 15 mg/kg, i.p.) corrected decreased anxiety levels ($p < 0.05$), however, failed to restore hyperactivity observed in elevated plus maze (EPM) test. In addition, E100 (10 mg/kg, i.p.) mitigated oxidative stress status by increasing the levels of decreased glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT), and decreasing the elevated levels of malondialdehyde (MDA) in the cerebellar tissues (all $p < 0.05$). Additionally, E100 (10 mg/kg, i.p.) significantly reduced the elevated levels of AChE activity in VPA mice ($p < 0.05$). These results demonstrate

the promising effects of E100 on in-vivo VPA-induced ASD-like features in mice, and provide evidence that

a potent dual-active H3R antagonist and AChE inhibitor (AChE-I) is a potential drug candidate for future

therapeutic management of autistic-like behaviours.

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The Dual-Active Histamine H₃ Receptor Antagonist and Acetylcholine Esterase Inhibitor E100 Alleviates Autistic-Like Behaviors and Oxidative Stress in Valproic Acid Induced Autism in Mice

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Abstract: The histamine H3 receptor (H3R) functions as auto- and hetero-receptors, regulating the release of brain histamine (HA) and acetylcholine (ACh), respectively. The enzyme acetylcholine esterase (AChE) is involved in the metabolism of brain ACh. Both brain HA and ACh are implicated in several cognitive disorders like Alzheimer's disease, schizophrenia, anxiety, and narcolepsy, all of which are comorbid with autistic spectrum disorder (ASD). Therefore, the novel dual-active ligand E100 with high H3R antagonist affinity (hH3R: K_i = 203 nM) and balanced AChE inhibitory effect (EeAChE: IC_{50} = 2 μM and EqBuChE: IC_{50} = 2 μM) was investigated on autistic-like sociability, repetitive/compulsive behaviour, anxiety, and oxidative stress in male C57BL/6 mice model of ASD induced by prenatal exposure to valproic acid (VPA, 500 mg/kg, intraperitoneal (i.p.)). Subchronic systemic administration with E100 (5, 10, and 15 mg/kg, i.p.) significantly and dose-dependently attenuated sociability deficits of autistic (VPA) mice in three-chamber behaviour (TCB) test (all $p < 0.05$). Moreover, E100 significantly improved repetitive and compulsive behaviors by reducing the increased percentage of marbles buried in marble-burying behaviour (MBB) (all $p < 0.05$). Furthermore, pre-treatment with E100 (10 and 15 mg/kg, i.p.) corrected decreased anxiety levels ($p < 0.05$), however, failed to restore hyperactivity observed in elevated plus maze (EPM) test. In addition, E100 (10 mg/kg, i.p.) mitigated oxidative stress status by increasing the levels of decreased glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT), and decreasing the elevated levels of malondialdehyde (MDA) in the cerebellar tissues (all $p < 0.05$). Additionally, E100 (10 mg/kg, i.p.) significantly reduced the elevated levels of AChE activity in VPA mice ($p < 0.05$). These results demonstrate the promising effects of E100 on in-vivo VPA-induced ASD-like features in mice, and provide evidence that a potent dual-active H3R antagonist and AChE inhibitor (AChEI) is a potential drug candidate for future therapeutic management of autistic-like behaviours.

Keywords: VPA-induced autism-like behaviors; mice; sociability; repetitive behaviors; anxiety; histamine H3R; antagonist; acetylcholine esterase inhibitor; E100; oxidative stress; cerebellum

1. Introduction

Autistic spectrum disorder (ASD) is a neurodevelopmental disorder with a large population prevalence, characterized by impairments in social interaction and restricted/repetitive behavioral pattern or interest [1,2]. Despite its increasing prevalence, the pathophysiology of ASD is still poorly understood [3,4]. The difficulty in understanding the pathophysiology of ASD lies in the complex involvement of several clinical and behavioral symptoms, making clinically accessible specific treatments for ASD often less effective [5,6]. Recent advances in drug developments focus on novel agents with multiple pharmacological effects for multifactorial diseases, such as ASD [7–9]. In search of sensitive and specific markers of ASD, numerous research efforts have focused on the study of various brain neurotransmitters [4]. Hence, assessment of the function of numerous brain neurotransmitters, e.g., histamine (HA), acetylcholine (ACh), serotonin (5-HT), dopamine (DA), γ-aminobutyric acid (GABA), and glutamate (Glu) in initial brain growth encourages to be an important area of research in the field of developing newer therapeutics [4,10–20]. Accordingly, the brain cholinergic neurotransmitter system with ACh has an essential role in controlling ASD-related behavioral features including attention [19], cognitive flexibility [20], social interaction [21], and stereotypical behaviors [9,16,18,22]. Preclinical as well as clinical evidences reveal the involvement of cholinergic system dysfunction in the phenotypic outcomes of ASD-related behavioral features, in both humans and animal models [23]. In ASD patients, there are significant irregularities in the brain cholinergic system. Anatomically there is abnormality in the number and structure of neurons in a basal forebrain cholinergic nucleus of patients diagnosed with ASD [24]. Additionally, a remarkable reduction in the level of choline, a precursor of the neurotransmitter ACh and agonist for nicotinic-cholinergic receptor, was reported in individuals diagnosed with ASD [25]. In addition, abnormalities in the levels of nicotinic ACh receptors were observed in several brain regions, e.g., neocortex, cerebellum, thalamus, and striatum, of patients diagnosed with ASD, with the chief abnormalities being the reduced levels of muscarinic receptors (M1 type) [26–28].

Several essential physiological functions e.g., sleep–wake cycle, energy and endocrine homeostasis, sensory and motor functions, cognition, and attention, are controlled by the brain histaminergic system, and as such, are all severely affected in neuropsychiatric disorders [4,29–32]. Histamine mediates its effects through binding to four known histamine receptor (HR) subtypes belonging to the family of G-protein-coupled receptors, and designated H1 to H4 receptors (H1R–H4R). The histamine H3 receptor (H3R) initially described in 1983 was found to be a constitutively active receptor mostly expressed in the brain and was evaluated pharmacologically to negatively regulate histamine synthesis and release, acting as presynaptic auto-receptors [33,34]. In addition, H3Rs functioning as hetero-receptors can also control the release of other neurotransmitters like ACh, Glu, GABA, 5-HT, and DA in various brain regions [35–39]. Moreover, it has been revealed that H3Rs are predominantly expressed in the central nervous system (CNS), while activation of H1R and H2R mediates slow excitatory postsynaptic potentials. Interestingly, few studies projected the use of HR antagonists in the therapeutic management of autistic behavior. Consequently, famotidine (a histamine H2R antagonist) was projected to be a possible treatment for ASD children [12], since famotidine was revealed to alleviate sociability deficits in a patient with schizophrenia [13], a brain disorder that shares various genetic factors and symptoms with ASD [14,15]. In addition, niaprazine (a histamine H1R antagonist) has weakened features such as unbalanced attention, resistance to alteration, and frustration in patients with ASD [40]. Furthermore, it has been proposed that histamine H3R antagonists are of potential therapeutic future for the treatment of several brain disorders, e.g., Alzheimer's disease (AD), schizophrenia, and narcolepsy [29–32]. Accordingly and in an animal model of schizophrenia, H3R antagonist was found to ameliorate behavioral deficiencies, including spatial working memory deficit, an abnormality also found in ASD patients [32]. Besides, antagonism of histamine H3Rs was found to reduce social behavior deficits in rodents exposed to phencyclidine, signifying the promising potential use of H3R antagonist in the therapeutic management of ASD [31,32]. Additionally, ciproxifan, an old-generation H3R antagonist,

was found to attenuate impaired sociability and stereotypics in animal model of ASD in which rodents were exposed to valproic acid (VPA) [1].

During pregnancy, environmental risk factors may affect the inflammatory response of new-borns, hence altering postnatal brain development [41]. VPA as an environmental risk factor showed activation in different brain regions, with evidence of long-lasting glia activation in the hippocampus and the cerebellum [42], which are two brain regions associated with autism-related features, namely social interaction and repetitive behaviours [41,43]. Moreover, numerous preclinical experiments indicated that inflammation in the cerebellum may change social behaviours in adult mice, since cerebellum was found to be involved in executive and cognitive behavioural functions [43–45]. Interestingly and based on neuropathological findings of several studies on autism post-mortem brains, cerebellum has been identified as one of the key brain regions that can play role in autistic features [46], and substantial accumulating evidence has linked the cerebellum with higher cognitive functions [47]. Moreover, the cerebellum is being considered a key structure within the social circuitry [48]. Furthermore, valproic acid as an environmental risk factor showed activation in different brain regions, with evidence of long-lasting glia activation in the hippocampus and the cerebellum [49], which are two brain regions linked to autism-related behaviour, namely social interaction and repetitive behaviours [46–52].

Considering the aforementioned preclinical as well as clinical results, H3Rs represent a promising target for developing new dual-active compounds with the potential role in neuropsychiatric multi-neurotransmitter disorders, e.g., AD, cognitive deficit accompanying schizophrenia and ASD [1,29,53–55]. Given the involvement of AChE and H3-auto- and hetero-receptors in the modulation of several central neurotransmitters including ACh and HA, dual-active AChE inhibitors (AChEIs) and H3R antagonists have been developed by several groups [56–62]. Therefore, we describe the effects of a novel dual-active AChE inhibitor and H3R antagonist E100 (1-(7-(4-chlorophenoxy) heptyl) homo-piperidine) with balanced acetylcholine esterase inhibitory effect (*Ee*AChE: IC$_{50}$ = 2 μM and *Eq*BuChE: IC$_{50}$ = 2 μM), histamine H3 receptor (H3R) antagonist affinity (*h*H3R K_i = 203 nM), and high selectivity profile towards H3R subtype (Figure 1) in male C57BL/6 mice model of ASD, induced by prenatal exposure to VPA (500 mg/kg, i.p.). Moreover, the effects of E100 on locomotor activity and anxiety-like behaviors of the same animals were tested in the elevated plus-maze (EPM), since anxiety and motor activity can influence the performance of animals [62]. Furthermore, the effects of E100 on AChE activity and oxidative stress markers were assessed in the cerebellum, as it is involved in executive and cognitive functions and exaggerated oxidative stress may alter social behavior in adult mice [4,46]. In addition, the ability of the H1R antagonist mepyramine (MPA), H2R antagonist zolantidine (ZLT), H3R agonist (*R*)-α-methylhistamine (RAM), and cholinergic muscarinic antagonist scopolamine (SCO) to reverse the effects provided by E100 were evaluated to clarify whether brain HA and ACh are involved in the effects exhibited by E100.

E100

K_i (*h*H3R)[a] = 203 nM
K_i (*h*H1R)[b] > 1000 nM
K_i (*h*H4R)[c] > 10000 nM
IC$_{50}$ *Ee*AChE[d] = 2 μM
IC$_{50}$(*Eq*BChE)[e] = 2 μM
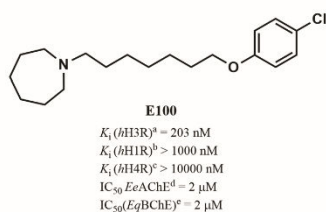
Figure 1. Chemical structure of the dual-acting human H3R (*h*H3R) antagonist and AChE inhibitor E100 and in vitro data with regard to *h*H1-, *h*H3-, and *h*H4R, *Ee*AChE, and *Eq*BuChE. [a,b,c] Binding assays to determine affinity to H1-, H3-, and H4Rs were performed in differently expressed cells as previously described n = 3 [59]. [d] AChE: Acetylcholine esterase; *Ee*: electric eel; [e] BuChE: Butyrylcholinesterase; *Eq*: equine [63–65].

2. Results

2.1. Effects of E100 on Sociability Impairments in Three-Chamber Behaviour (TCB) Test and Stereotyped Repetitive Behavior in Marble-Burying Behaviour (MBB)

The effect of systemic injection of E100 (5, 10, and 15 mg/kg, i.p.) and donepezil (DOZ; 1 mg/kg, i.p.) on ASD-like sociability impairments in the three-chamber behaviour (TCB) task and stereotyped repetitive behavior in marble burying behavior (MBB) in VPA-exposed mice (VPA mice) are shown in Figure 2A,B. There was a statistically significant difference between groups as determined by statistical analyses ($F_{(7,48)} = 5.118$, $p < 0.01$). As observed in the Tukey post hoc analyses, VPA mice exhibited significantly lower sociability expressed as sociability index (SI) when compared to saline-exposed control mice (CNT), with SIs of (-0.07 ± 0.05) and (0.40 ± 0.07), respectively ($p < 0.01$) (Figure 2A). However, E100 (5, 10, and 15 mg/kg) significantly increased SI of VPA mice with SI values of (0.22 ± 0.08), (0.44 ± 0.08), and (0.40 ± 0.09) when compared to VPA mice with a SI of (-0.07 ± 0.05) (all $p < 0.05$) (Figure 2A). The results revealed that the enhancement in SI observed with E100 (10 mg/kg, SI = 0.44 ± 0.08) was statistically comparable to that shown with DOZ (1 mg/kg, SI = 0.39 ± 0.07, $p = 0.99$) (Figure 2A). Moreover, there was a statistically significant difference between tested groups in the abrogative study ($F_{(10,66)} = 5.454$, $p < 0.01$) (Figure 2B). Interestingly, the E100-provided improvement of sociability was counteracted following co-administration with RAM (10 mg/kg, i.p.), ZLT (10 mg/kg, i.p.), or SCO (0.3 mg/kg, i.p.), with SI values of (0.05 ± 0.04, $p < 0.05$), (0.16 ± 0.03, $p < 0.05$), and (0.18 ± 0.05, $p < 0.05$), respectively (Figure 2B). However, co-administration of the CNS-penetrant H1R antagonist MPA (10 mg/kg, i.p.) with SI value of (0.40 ± 0.10, $p = 1.00$) failed to reverse the E100 (10 mg/kg)-provided sociability enhancement observed in the E100 (10 mg/kg)-treated VPA animals (Figure 2B). Notably, subchronic treatment of CNT mice with E100 (10 mg/kg, i.p.), RAM (10 mg/kg, i.p.), MPA (10 mg/kg, i.p.), ZLT (10 mg/kg, i.p.), or SCO (0.3 mg/kg, i.p.) had no significant influence on SI compared to saline-pretreated CNT mice, with SI values of (0.36 ± 0.06, $p = 1.00$), (0.42 ± 0.08, $p = 1.00$), (0.38 ± 0.07, $p = 1.00$), (0.39 ± 0.07, $p = 1.00$), and (0.35 ± 0.06, $p = 1.00$), respectively (Figure 2B).

In the marble-burying behaviour (MBB) and as determined by statistical analyses, there was a significant difference between all groups assessed ($F_{(7,32)} = 5.797$, $p < 0.01$). Statistical post hoc analyses showed that VPA mice ($58.00 \pm 4.81\%$, $p < 0.01$) buried significantly more marbles compared to the CNT animals ($26.00 \pm 4.55\%$) tested in MBB (Figure 2C). However, E100 (10 or 15 mg/kg, i.p.) and DOZ (1 mg/kg, i.p.) significantly reduced the increased percentage of marbles buried by VPA mice when compared to saline-treated VPA mice, with ($27.00 \pm 4.60\%$, $p < 0.01$), ($27.00 \pm 3.34\%$, $p < 0.01$), and ($37.00 \pm 4.37\%$, $p < 0.01$), respectively (Figure 2C). Moreover, there was a statistically significant difference between tested groups in the abrogative study with ($F_{(10,44)} = 5.935$, $p < 0.01$) (Figure 2D). The E100 (10 mg)-provided a decrease in the percentage of buried marbles ($27.00 \pm 4.60\%$) was entirely abrogated by co-administration of RAM ($60.00 \pm 5.28\%$, $p < 0.05$), ZLT ($53.00 \pm 8.30\%$, $p < 0.05$), and SCO ($52.00 \pm 7.94\%$, $p < 0.05$), respectively (Figure 2D). However, MPA ($29.00 \pm 3.84\%$, $p = 1.000$) failed to counteract the E100 (10 mg)-provided effect ($27.00 \pm 4.60\%$) on VPA (Figure 2D). Notably, subchronic treatment of CNT mice with E100 (10 mg/kg, i.p.), RAM (10 mg/kg, i.p.), MPA (10 mg/kg, i.p.), ZLT (10 mg/kg, i.p.), or SCO (0.3 mg/kg, i.p.) had no significant influence on percentage of buried marbles compared to saline-pretreated CNT mice, with ($24.00 \pm 2.96\%$, $p = 1.00$), ($27.20 \pm 4.63\%$, $p = 1.00$), ($25.80 \pm 4.22\%$, $p = 1.00$), ($28.40 \pm 5.04\%$, $p = 1.00$), and ($27.40 \pm 3.00\%$, $p = 1.00$), respectively (Figure 2D).
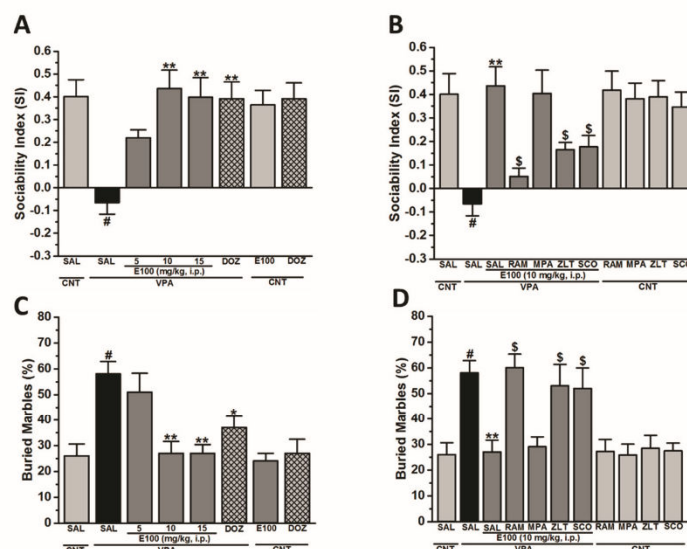
Figure 2. E100 improved sociability in three-chamber behaviour (TCB) and repetitive behavior in marble-burying behaviour (MBB) paradigms. (**A,B**) Following acclimatization for a duration of 10 min, male mice were allowed to explore all three chambers for 10 min. The obtained results were expressed in form of Sociability index (SI). Control (CNT) mice received systemic injections of saline (group 1), E100 (10 mg/kg) (group 7), and donepezil (DOZ) (1 mg/kg) (group 8), whereas VPA mice were injected with saline (group 2), E100 (5, 10, and 15 mg/kg) (groups 3–5), or DOZ (1 mg/kg, i.p.) (group 6) subchronically for 21 days (**A**). Abrogative studies of subchronic (21 days) systemic co-injection of RAM (10 mg/kg, i.p. for group 9), mepyramine (MPA) (10 mg/kg, i.p. group 10), zolantidine (ZLT) (10 mg/kg, i.p., for group 11), or scopolamine (SCO) (0.3 mg/kg, i.p., for group 12) on the E100 (10 mg)-provided improvement of sociability of VPA mice were assessed (**B**). Marble-burying behavior (MBB) was measured after a 30-min testing session applying the same treatments. VPA mice treated with saline (group 2) displayed significantly increased repetitive behaviors when compared to CNT mice (group 1). E100 (5, 10, or 15 mg/kg, i.p) or DOZ (1 mg/kg, i.p.) were injected systemically and subchronically for 21 days in VPA mice (**C**). Effects of subchronic (21 days) systemic co-administration of RAM (10 mg/kg, i.p., group 9), MPA (10 mg/kg, i.p., group 10), ZLT (10 mg/kg, i.p., group 11), or SCO (0.3 mg/kg, i.p., group 12) on the E100(10 mg)-provided attenuation of stereotyped repetitive behavior of VPA mice were assessed MBB (**D**). CNT mice were injected with saline, E100 (10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.). RAM (10 mg/kg, i.p.), MPA (10 mg/kg, i.p.), ZLT (10 mg/kg, i.p.), or SCO (0.3 mg/kg, i.p.) (**D**). Data are expressed as the mean ± standard errors of the means (SEM) (n = 7 for TCB and n = 5 for MBB). 8 groups of 7 mice per group in TCB (**A,B**) and 8 groups of 5 mice per group in MBB (**C,D**) were used. The effects of E100 were analyzed using two-way analysis of variance (ANOVA) with dose of drugs and animals (either VPA or CNT mice) as the between-subjects factor, and post hoc comparisons were performed with Tukey's test in case of a significant main effect. # $p < 0.05$ vs. CNT mice. ** $p < 0.01$ vs. saline-treated VPA mice. $ $p < 0.05$ vs. E100 (10mg)-treated VPA mice.

2.2. Effects of E100 on Anxiety Levels and Locomotor Activity of Valproic Acid (VPA)-Exposed Mice in Elevated Plus Maze (EPM) Test

Figure 3A–F shows the observed effects of E100 (5, 10, or 15 mg/kg, i.p.) on the anxiety levels (the time spent (Figure 3A) and the number of entries into open arms (Figure 3B) of VPA mice assessed in the EPM test. Additionally, the locomotor activity expressed as the number of entries into closed arms (Figure 3C) was simultaneously evaluated in the same EPM test. Moreover, the abrogative effects of systemic co-administration of RAM, MPA, ZLT, or SCO on the E100-provided effects were tested

(Figure 3D–F). The studies have been conducted for 5 min each. The results observed for the time spent and number of entries into open arms revealed a statistically significant difference between groups with ($F_{(10,55)} = 5.505$, $p < 0.01$) for time spent and ($F_{(10,55)} = 7.339$, $p < 0.01$) for number of entries (Figure 3A,B). Statistical post hoc analyses showed that VPA mice spent significantly less time (19.00 ± 4.46 s, $p < 0.05$) and displayed a lower number of entries (1.60 ± 0.37, $p < 0.01$) in open arms when compared to CNT mice with time spent (60.00 ± 7.02 s) and number of entries (5.50 ± 0.66) (Figure 3A,B). However, subsequent post hoc analyses revealed that E100 when administered at 10 or 15 mg/kg significantly altered the time spent exploring the open arms of the maze during a 5 min session compared to saline-treated VPA mice, with (50.17 ± 4.21 s, $p < 0.05$) and (54.67 ± 8.00 s, $p < 0.05$), respectively (Figure 3A). Moreover, post hoc evaluation revealed that E100 when administered at 10 or 15 mg/kg i.p. significantly increased the number of entries into the open arms of the maze during a 5 min session compared to saline-treated VPA mice, with (3.67 ± 0.54, $p < 0.05$) and (4.33 ± 0.51, $p < 0.05$), respectively (Figure 3B). However, E100 (5 mg/kg) failed to alter time spent and number for entries into open arms of VPA mice, with (20.98 ± 3.00 s, $p = 0.57$) and (2.41 ± 0.37), respectively, and as compared with VPA mice with (19.00 ± 4.46 s) and (1.60 ± 0.37) for time spent and number of entries, respectively (Figure 3A,B). Interestingly, VPA mice pretreated with DOZ (1 mg) spent significantly longer time exploring the open arms compared to saline-treated VPA mice, with (47.17 ± 4.46 s, $p < 0.01$) (Figure 3A). Further analyses of data describing the number of entries into the open arms of the maze yielded practically the same results for DOZ (1 mg/kg, i.p.), with (4.67 ± 0.73, $p < 0.01$) (Figure 3B). On the other hand, VPA mice entered the closed arms significantly more often than CNT mice, with (11.83 ± 1.22, $p < 0.01$) (Figure 3C). Besides, subchronic treatment of CNT mice with E100 (10 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) had no significant influence on time spent in open arms (Figure 3A), number of entries into open (Figure 3B) or into closed arms (Figure 3C) compared to saline-pretreated CNT mice (all $p > 0.05$). Interestingly, the E100 (10 mg)-provided increase in the time spent in open arms (Figure 3D), and the number of entries into open arms (Figure 3E) was entirely abrogated by co-administration of RAM ($p < 0.05$). However, MPA, ZLT, and SCO failed to counteract the E100 (10 mg)-provided effect on VPA mice (Figure 3D,E). Notably, the number of closed arm entries following subchronic systemic injection of E100 (5, 10, or 15 mg/kg) and DOZ (1 mg/kg, i.p.) was not significantly different as compared to saline-treated VPA mice, with (10.50 ± 0.75, $p = 0.99$), (9.50 ± 0.81, $p = 0.88$), (10.50 ± 1.45, $p = 0.99$), and (11.33 ± 1.24, $p = 1.00$), respectively (Figure 3C,F).

2.3. Effect of E100 on Oxidative Stress Levels in Cerebellar Tissues of VPA-Exposed Mice

As determined by statistical analysis, there was a significant difference between all groups assessed on the levels of MDA (malondialdehyde, $F_{(4,20)} = 5.505$, $p < 0.01$), GSH (glutathione, $F_{(4,20)} = 7.399$, $p < 0.01$), SOD (superoxide dismutase, $F_{(4,20)} = 8.586$, $p < 0.01$), and CAT (catalase, $F_{(4,20)} = 8.057$, $p < 0.01$) of VPA mice and following subchronic systemic administration of saline, E100 (10 mg/kg, i.p.), or DOZ (1 mg/kg, i.p.) (Figure 4A–D). The results showed that MDA was significantly increased (109.14 ± 10.84 µg/mg protein, $p < 0.05$) and GSH (16.16 ± 1.19 µg/mg protein, $p < 0.05$), SOD (8.66 ± 1.43 U/mg protein, $p < 0.05$), and CAT (13.24 ± 0.96 nmol/min/mg protein, $p < 0.05$) were significantly reduced in the cerebellum of VPA mice compared to CNT mice, with (57.66 ± 8.79 µg/mg protein) for MDA, (54.30 ± 5.59 µg/mg protein) for GSH, (35.72 ± 3.44 U/mg protein) for SOD, and (20.01 ± 0.37 nmol/min/mg protein) for CAT (Figure 4A–D). However, cerebellum of VPA mice pretreated with E100 (10 mg/kg, i.p.) displayed a significant reduction of MDA (39.69 ± 3.89 µg/mg protein, $p < 0.001$) as well as significant elevation of GSH (57.88 ± 8.23 µg/mg protein, $p < 0.001$), SOD (28.15 ± 4.82 U/mg protein, $p < 0.01$), and CAT (20.51 ± 0.73 nmol/min/mg protein, $p < 0.05$), and as compared with saline-treated VPA mice (Figure 4A–D). Similarly, cerebellum of VPA mice pretreated with DOZ (1 mg/kg, i.p.) showed a significant reduction of MDA (58.43 ± 14.00 µg/mg protein, $p < 0.05$) as well as significant increase of GSH (51.59 ± 7.51 µg/mg protein, $p < 0.001$), SOD (23.12 ± 3.66 U/mg protein, $p < 0.05$), and CAT ($18.77 + 0.84$ nmol/min/mg protein, $p < 0.05$), and as compared with saline-treated VPA mice (Figure 4A–D). Moreover, systemic co-administration of RAM (10 mg/kg,

i.p.) partially abrogated the E100 (10 mg)-provided decrease against the VPA-induced increase in the level of MDA (82.26 ± 10.80 µg/mg protein, $p = 0.01$) (Figure 4A), and it also partially reversed the E100 (10 mg)-provided increases in GSH (32.18 ± 3.22 µg/mg protein, $p = 0.03$), SOD concentrations (12.32 ± 3.08 U/mg protein, $p = 0.04$), and CAT (15.05 ± 1.68 nmol/min/mg protein, $p = 0.02$) in VPA mice (Figure 4B–D).
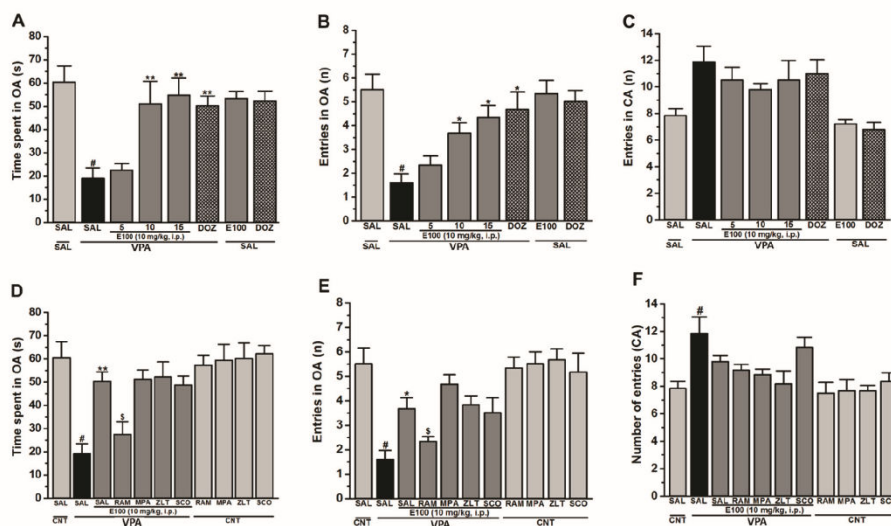
Figure 3. E100 ameliorated fear-related behavior without affecting locomotor activity in elevated plus maze (EPM). VPA mice injected with saline (group 2) displayed significantly increased deficits in cognitive behaviors compared to CNT mice (group 1). Test compound E100 (5, 10, or 15 mg/kg, i.p.) or DOZ (1 mg/kg) were injected for 21 days to VPA mice (subchronic). E100 (10 and 15 mg/kg, groups 4 and 5) and DOZ (1 mg/kg, group 6) attenuated the decreased time spent on the open arms, however, failed to modify the increased number of entries into the closed arms of the EPM (**A–C**). Abrogative effects of subchronic (21 days) systemic co-administration of RAM (10 mg/kg, group 9), MPA (10 mg/kg, group 10), ZLT (10 mg/kg, group 11), or SCO (0.3 mg/kg, group 12) on the E100 (10 mg)-provided improvement in number and time spent for open arms of VPA mice were measured (**D,E**). Number of entries into closed arms was elevated in saline-treated VPA mice (group 2) when compared to saline-treated CNT mice (group 1). E100 (5, 10, and 15 mg/kg) and DOZ failed to modulate the increased number of entries into closed arms (**C,F**). Additionally, CNT mice treated with E100 (10 mg/kg, group 7) did not show significant difference in number of entries into closed arms when compared with saline-treated CNT mice (group 1). Data are expressed as the mean ± SEM (n = 6). # $p < 0.05$ vs. CNT mice. * $p < 0.05$ vs. Saline-treated VPA mice. ** $p < 0.01$ vs. saline-treated VPA mice. $ $p < 0.05$ vs. E100 (10mg)-treated VPA mice. In the EPM test, 8 groups of 6 mice per group were used. The effects of E100 were analyzed using two-way analysis of variance (ANOVA) with dose of drugs and animals (either VPA or CNT mice) as the between-subjects factor, and post hoc comparisons were performed with Tukey's test in case of a significant main effect (**A–D**).
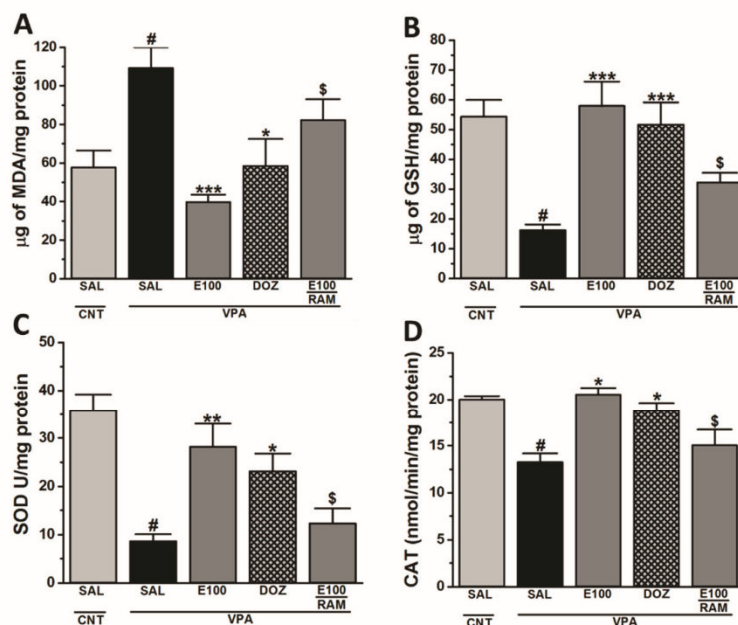
Figure 4. E100 restored levels of oxidative stress markers in the cerebellum. Modulated malondialdehyde (MDA), glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) were assessed. VPA mice showed a significant increase in MDA (**A**) and significant decrease in GSH (**B**), SOD (**C**), and CAT (**D**) compared to CNT mice. Subchronic systemic administration of E100 (10 mg/kg) or DOZ (1 mg/kg) were assessed in VPA mice. E100 (10 mg/kg) or DOZ (1 mg/kg) significantly reduced the increased levels of MDA (**A**) and significantly increased the reduced levels of GSH, SOD and CAT (**A–D**). Abrogative effects of subchronic (21 days) systemic co-administration with RAM (10 mg/kg) on modulation of oxidative stress levels provided by E100 (10 mg) were assessed (**A–D**). Data are expressed as the mean ± SEM (n = 5). $^{\#}$ $p < 0.05$ vs. VPA mice. * $p < 0.05$ vs. VPA mice. ** $p < 0.01$ vs. VPA mice. *** $p < 0.001$ vs. VPA mice. $^{\$}$ $p < 0.01$ vs. E100 (10 mg)-treated VPA mice. In biochemical assessments, 5 groups of 5 mice per group were used. The effects of E100 were analyzed using two-way analysis of variance (ANOVA) with dose of drugs and animals (either VPA or CNT mice) as the between-subjects factor, and post hoc comparisons were performed with Tukey's test in case of a significant main effect (**A–D**).

2.4. Effect of E100 on Acetylcholine Esterase Activity in Cerebellum Tissues of VPA-Exposed Mice

As determined by statistical analyses, there was a significant difference between groups assessed on the levels of AChE ($F_{(3,15)}$ = 4.176, $p < 0.05$) (Figure 5). The observed results showed that VPA mice exhibited a significant increase in the activity of AChE enzyme in cerebellum (426.72 ± 31.20 nmol/min/mg protein, $p < 0.05$) when compared to CNT mice (291.87 ± 33.34 nmol/min/mg protein) (Figure 5). However, subchronic systemic treatment of VPA mice with E100 (10 mg/kg, i.p.) significantly decreased the AChE activity of VPA mice (282.87 ± 36.13 nmol/min/mg protein, $p < 0.05$) when compared with the saline-treated VPA mice (426.72 ± 31.20 nmol/min/mg protein) (Figure 5). Similarly, subchronic systemic pretreatment with the reference drug DOZ (1 mg/kg, i.p.) significantly decreased the AChE activity of VPA mice (326.16 ± 24.09 nmol/min/mg protein, $p < 0.05$) when compared with the saline-treated VPA mice (426.72 ± 31.20 nmol/min/mg protein) (Figure 5).
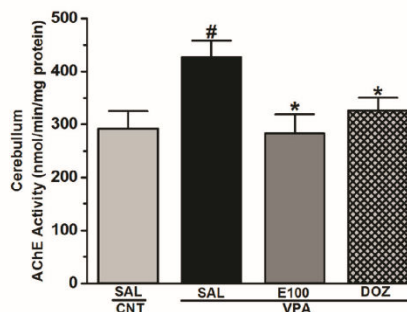
Figure 5. Effects of E100 on acetylcholine esterase activity in cerebellum tissues of valproic acid (VPA)-exposed mice. Inhibitory effects of E100 (10 mg/kg, i.p.) on acetylcholine esterase enzyme in the cerebellum of VPA mice. Quantitative analysis revealed a significant increase ($^{\#}$ $p < 0.05$) in the acetylcholine esterase enzyme activity in cerebellum of VPA mice compared to the CNT mice. However, subchronic treatment with E100 (10 mg/kg, i.p.) or DOZ (1 mg/kg, i.p.) to the VPA mice significantly reduced (* $p < 0.05$) this activity compared to the VPA mice. Values are expressed as the percent mean ± SEM. For assessment of AChE activity 4 groups were used. 4 CNT mice were used for saline group and 5 VPA mice were used for each treatment group. The effects of E100 were analyzed using two-way analysis of variance (ANOVA) with dose of drugs and animals (either VPA or CNT mice) as the between-subjects factor, and post hoc comparisons were performed with Tukey's test in case of a significant main effect.

3. Discussion

Alteration in brain histaminergic and cholinergic neurotransmissions is supposed play a critical role in the clinical results of ASD-related behavioural features [1,25,66]. Consequently, the aim of the current study was to assess the modulating effects of the novel dual-acting AChEI and H3R antagonist E100 on brain HA and ACh applying a mouse model VPA-induced ASD-like behaviors. Our findings show that E100 significantly and dose-dependently improved sociability deficits in TCB paradigm and stereotypies in MBB paradigm in VPA mice. In the TCB paradigm, systemic pretreatment with E100 ameliorated the impairment in sociability demonstrated by VPA mice, since these animals, when pretreated with E100, showed significantly higher SI, with observed levels similar to the CNT mice. Various previous studies have focused on the procognitive effects of several H3R antagonists on social memory [67–70], a behavioral feature that is also altered in ASD [69]. Moreover, previous reports suggested that an impaired cholinergic system causes cognitive problems that may include social problems, which were reversed by donepezil treatments [71,72]. Importantly, the sociability-enhancing effect observed for E100 was dose-dependent, since E100 (10 mg/kg) showed an optimum effect comparable to that provided by the reference drug DOZ. Contrarily, a dose of 15 mg/kg E100 did not further improve upon the E100 (10mg)-provided sociability enhancement. The observations for the dose-dependent effects of E100 are in line with our recent studies observed with a non-imidazole-based H3R antagonist on VPA-induced ASD in Tuck-Ordinary mice [73], and an imidazole-based H3R antagonist in preclinical experiments in different rodents [1]. Moreover, the observations of sociability-enhancing effects for E100 aligns with earlier experimental results observed with the imidazole based H3R antagonist ciproxifan in Swiss mice [1], and comprehend our previously observations in which E100 enhanced social novelty in mice [63]. The mechanism behind E100-provided sociability improvement is unclear. Still, it might be explained by its dual action with the capability of H3R antagonists to mediate the release of different neurotransmitters other than histamine, such as DA, 5-HT, and ACh, in specific brain regions [74], together with its AChE inhibitory property that results in correction of abnormal cholinergic transmission. Interestingly, the E100-provided enhancing effects on sociability were reversed when mice were co-administered the H3R agonist RAM, the H2R antagonist ZLT, or with muscarinic

cholinergic antagonist SCO, but not with the centrally acting H1R antagonist MPA, indicating that HA and ACh, through activation of postsynaptically located H2Rs (but not H1Rs) and muscarinic cholinergic receptors, respectively, obviously contribute to neuronal pathways important for alteration of sociability processes in the TCB paradigm in VPAmice. Therefore, considering the levels of different brain neurotransmitters, including HA and ACh, in various brain areas of the VPA-exposed mice with ASD-like behaviors as well as when pretreated with E100 would further assist in understanding the neural intersections involved in the observed behavioral enhancement. This is in vivo evidence that a simultaneous interaction with the above two targets leads to symptomatic in vivo enhancements of behavioral autistic-like parameters in mice. However, the mitigating effects observed for the dual-acting compound E100 are not due to either its AChE-inhibiting or to its H3R-blocking properties alone, since it neither acts purely as an H3R antagonist DL77 (non-imidazole based H3R antagonist [73]) or ciproxifan (an imidazole-based H3R antagonist [1]) nor as an AChEI such as DOZ (used as a reference drug in the current study). Notably, the postulated advantage of a dual-acting compound (e.g., E100) with combined affinities at the required targets over co-administration of two drugs are the straightforward single-compound pharmacokinetics. Thereby, putative drug–drug interactions occurring with combination therapy might be avoided. Conclusively, the dose-finding for co-application of two different drugs can be bypassed which would become necessary since the effective doses might be considerably different from the ones applied in the case of monotherapy, especially in multifactorial disorders like ASD. Whether the above mitigation of autistic-like behaviors is also induced after administration of H3R antagonist or co-administration of an H3R antagonist and an AChEI was beyond the scope of this project and will require dose-finding experiments for several ratios of the combination of AChEIs and H3R antagonist.

Repetitive behavior and restricted interests are considered as core features of patients with ASD [75,76]. In previous preclinical studies, abnormalities in histaminergic signalling was found to contribute to rare diseases such as Tourette syndrome [77], a condition featured by stereotypies and described to be among the most commonly comorbid neurodevelopmental disorders with ASD [1,63,73]. The results observed in the present study showed that VPA mice pretreated with E100 (10 or 15 mg) or with the reference drug DOZ (1 mg) demonstrated similar decreases in repetitive behavior when tested in MBB test, and the E100 (10 mg)-provided effects in MBB were nullified when mice were co-administered with RAM, ZLT, or SCO, but not with MPA. The mechanism by which the repetitive/compulsive behavior is improved following systemic administration with E100 could be explained with the capability of E100 to modulate the brain levels of different neurotransmitters in several specific brain areas besides HA and ACh, such as DA and 5-HT, through antagonist interaction of E100 with histamine H₃ heteroreceptors expressed on dopaminergic and serotonergic neurons [78–80]. Furthermore, the results observed for E100 in MBB mirror a previous study in which acute systemic administration of the non-imidazole H3R antagonist DL77 significantly decreased the number of buried marbles in adult male Tuck-Ordinary mice of VPA-induced ASD features [73]. Interestingly, the results observed for E100 on repetitive behavior in MBB comprehend our previously observations for E100 in nestlet shredding behavioral test, a test that also evaluates repetitive/obsessive compulsive paradigm in rodents [63].

The effects of systemic administration with E100 on locomotion as well as anxiety levels were tested as pharmacological compounds that are able to modulate anxiety levels or locomotor activity may give rise to a false-positive effect in these behavioral paradigms. Therefore, locomotor activity was assessed simultaneously to exclude possible intrinsic deficits of spontaneous locomotor activity. Consequently, the numbers of entries into the closed arms were used as indicators of locomotor activity, while time spent and number of entries into open arms provided indications about anxiety levels in the EPM test. The results showed that E100 (10 or 15 mg) reduced elevated anxiety levels in VPA mice, similarly as DOZ (1 mg), measured by the time spent in open arms and number of open arms entries. However, pretreatment of VPA mice with E100 (5, 10, or 15 mg) or DOZ (1 mg/kg) did not alter a locomotor activity as measured by number of entries into closed arms. Moreover,

subchronic co-administration of VPA mice with the most promising dose E100 (10 mg/kg, i.p.) and RAM, MPA, ZLT, or SCO had no significant influence on the number of closed arm entries compared to saline-pretreated CNT mice. Furthermore, subchronic systemic injection of CNT mice with the most promising dose E100 (10 mg/kg, i.p.) failed to modify locomotor activity of CNT mice in EPM test. These results comprehend our previously observations for E100 in open field assessment [63]. Thus, the improvements in sociability and repetitive/compulsive behaviors observed for E100 in TCB and MBB, respectively, appear unlikely to be associated with a modulating effect in locomotor activity of the tested mice. Moreover, the E100 (10 mg)-provided effects on anxiety-like behaviors of treated mice were nullified when mice were co-administered with RAM, but not with MPA, ZLT, or SCO, indicating that postsynaptic histaminergic receptor subtypes (H1Rs and H2Rs) and postsynaptic muscarinic cholinergic receptors are not involved in the E100-provided effects on anxiety-like behavior of VPA mice. However, the H3R agonist RAM abrogated the effects provided by E100, demonstrating that E100 may exerted its effects on anxiety-like behaviors VPA mice through modulation of several other neurotransmitters, such as serotonin [80,81], glutamate, and GABA [82–84], that are reported to be imbalanced in ASD patients. These results were in accordance with previous results that revealed anxiolytic-like effects of a non-imidazole-based H3R antagonist as well as UW-MD-71 with no differences in spontaneous locomotor activity [56,57,85].

Previous studies revealed that imidazole-based H3R antagonists, namely clobenpropit and ciproxifan, mitigated several oxidative stress markers (e.g., MDA and GSH) in amphetamine- or dizocilpine-augmented oxidative stress in a preclinical mice model of schizophrenia, signifying the protective effects of H3R antagonists in such conditions [86,87]. In the current study, the results showed that VPA mice with ASD-like behavioral features displayed significant increase in MDA, with a concomitant decline in GSH, SOD, and CAT in the cerebellum tissues, and several previous studies showed that cerebellum is significantly involved in executive and cognitive functions [39,42,43,83]. The observed results for E100 (10 mg/kg) and DOZ (1 mg/kg) showed a significant reduction of MDA as well as a significant elevation of GSH, SOD, and CAT. Moreover, systemic co-administration with RAM (10 mg) reversed the E100 (10 mg)-provided modulating effects on MDA, GSH, SOD, and CAT in VPA mice. The latter results indicate that modulation of brain histamine provided by E100 may have contributed to the correction of an unbalanced ratio of radical oxygen species through the generation of endogenous cellular antioxidant defensive mechanisms.

The ability of E100 to enhance cholinergic activity in VPA mice and to exert its potential effect on cognitive deficit associated with sociability impairments was confirmed by measuring AChE activity in the cerebellum. The results revealed that the activity of AChE in E100 (10 mg)-treated mice was significantly reduced compared to VPA mice, and comparable to the DOZ. Considering the role of this enzyme which is responsible in degrading the ACh, it has been reported in a previous study that AChE inhibition augmented ACh in the synapse, therefore, relieving the cognitive rigidity and ameliorating the social deficiency in VPA mice [25].

4. Materials and Methods

4.1. Animals

Bred in the local central animal facility of the College of Medicine and Health Sciences, United Arab Emirates University, C57BL/6 (C57) mice (aged 8–12 weeks, weighing 20–25 g) (Jackson Laboratory, Bar Harbor, ME, USA) were used in this study [88]. All mice were kept in plastic cages under a standard light/dark cycle, namely 12 h light cycle, and lights were switched on at 6:00 a.m. Additionally, animals were housed at constant temperature 22–25 °C, and with free access to tap water and a standard rodent chow diet. For mating, male and female mice were housed together, and female mice were observed daily. The day was considered as embryonic day 0 (E0) on which the vaginal plug was detected. Each pregnant female was then kept in a separate cage until delivery, the day of delivery was defined as postnatal day 0 (P0). The Institutional Animal Ethics Committee in the College of Medicine and

Health Sciences/United Arab Emirates (Approval No. ERA-2017-5603) approved all procedures that were carried out in accordance with the recommendations of the European Communities Council Directive of 24 November 1986 (86/609/EEC).

4.2. Drugs

The dual-active AChE inhibitor and H3R antagonist E100, namely 1-(7-(4-chlorophenoxy) heptyl)azepane, was designed and synthesized in the Department of Technology and Biotechnology of Drugs Kraków, Poland, according to previously published procedures [65]. All chemical reagents used in the current study, including sodium valproate (VPA) (500 mg/kg, i.p.), donepezil hydrochloride (DOZ, 1 mg/kg, i.p.), the CNS-penetrant H3R agonist (R)-α-methylhistamine (RAM, 10 mg/kg, i.p.), the CNS-penetrant H1R antagonist pyrilamine (MPA) (10 mg/kg, i.p.), the CNS-penetrant H2R antagonist zolantadine (ZLT) (10 mg/kg, i.p.), and Scopolamine hydrochloride (SCO) (0.3 mg/kg, i.p.) were obtained from Sigma-Aldrich (St. Louis, MO, USA).The assay kit for reduced glutathione (GSH, Assay Kit, Lot no: 095M4114V, Product code: 1002170877) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The lipid peroxidation assay kit for estimation of malondialdehyde (MDA, Lot no: MDA-2409, Product code: NWK-MDA01) was purchased from North West Life Science (Vancouver, WA, USA). The assay kits for superoxide dismutase (SOD, Batch no: 0538703, Item: 706002) and catalase (CAT, Batch no: 0539007, Item: 707002) were purchased from Cayman chemical (Ann Arbor, MI, USA). Acetylcholinesterase activity colorimetric assay kit (Lot no: GR 3295454-2, Product: ab65345) was purchased from BioVision (Milpitas, CA, USA). All the reagents used in the study were of analytical grade and were dissolved in 1% aqueous Tween 20 solution (saline) and administered intraperitoneally (i.p.) at a volume of 10 mL/kg adjusted to body weight, and all doses are expressed in terms of the free base. All the reagents used in the experiments were of analytical grade.

4.3. Study Design and Treatments

4.3.1. Prenatal Treatment

On E12.5, pregnant females were intraperitoneally (i.p.) injected with either VPA 500 mg/kg [89,90], or saline and returned to their home cages, as described previously [63,73,89,90]. After the injection of VPA few pregnant mice died, and some gave still birth or underwent desorption. From the successfully delivered pups, only male offspring were used in the study. Pups delivered from VPA-exposed mothers were considered as VPA mice and were used for the experiments when they reached age of 8 weeks. On the other hand, pups delivered from mothers exposed to saline were used as CNT mice. All obtained offspring were weaned and gender-grouped (5–6 mice/cage) at P21.

4.3.2. Postnatal Treatments

On P21, male offspring (VPA mice with autistic features) from VPA-exposed mothers and from mothers that received saline (CNT, control mice) were divided into 16 subgroups (number of mice per group is provided in the following respective experimental sections), and received intraperitoneally (i.p.) the following treatment groups and as shown in experimental design (Figure 6): 1: CNT mice injected with saline, 2: VPA mice injected with saline (1% aqueous Tween 20), 3: VPA mice injected with E100 (5 mg/kg, i.p.), 4: VPA mice injected with E100 (10 mg/kg, i.p.), 5: VPA mice injected with E100 (15 mg/kg, i.p.), 6: VPA mice injected with DOZ (1 mg/kg, i.p.), 7: CNT mice injected with E100 (10 mg/kg, i.p.), 8: CNT mice injected with DOZ (1 mg/kg, i.p.), 9: E100 (10 mg/kg, i.p.) was co-administered with RAM (10 mg/kg, i.p.), 10: E100 (10 mg/kg, i.p.) was co-administered with MPA (10 mg/kg, i.p.), 11: E100 (10 mg/kg, i.p.) was co-administered with ZLT (10 mg/kg, i.p.), 12: E100 (10 mg/kg, i.p.) was co-administered with SCO (0.3 mg/kg, i.p.), 13: CNT mice injected with RAM (10 mg/kg), 14: CNT mice injected with MPA (10 mg/kg, i.p.), 15: CNT mice injected with ZLT (10 mg/kg, i.p.), and 16: CNT mice injected with RAM (10 mg/kg, i.p.). All co-administrations were carried out as separate injections with 5-min interval following administration of the test compound

(E100). E100 (5, 10, and 15 mg/kg), or DOZ 1 mg/kg or vehicle (saline) were injected once daily for 21 days, from postnatal day (P44). All doses were selected based on the results of our previous studies of strongly related dual-active compounds and are expressed in terms of the free bases [57,85]. E100 and DOZ or saline were administered 30–45 min before each behavioral test, followed by a series of behavioral tests which began one week after starting the treatments and according to previously published works [63,73]. Doses for RAM, MPA, ZLT, and SCO were carefully selected according to previous experimental protocols from our laboratories. The behavioural experiments of the study were carried out between 9:00 a.m. and 3:00 p.m., and were conducted in the following sequence once the animals were 50 days old: three-chamber behaviour test (TCB), marble burying behaviour (MBB), and elevated plus maze (EPM). The behavioural assessments were carried out in the morning (8:00 a.m. and 12:00 p.m.) in a calm and sealed off area that was illuminated with four 60 V light-emitting diodes (LEDs). Before starting the behavioural tests, animals were habituated in the study place at least for one hour. To reduce the number of animals used, the levels of oxidative stress and AChE activity were studied in the same groups of animals that were subjected to behavioral tests.
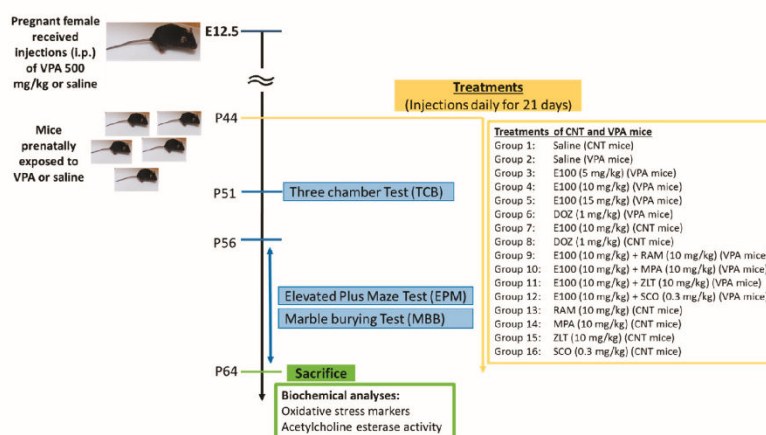
Figure 6. Schematic illustration of systemic treatments, behavioral experiments, and biochemical measurements with VPA and CNT mice. At embryonic day 12.5 (E12.5), pregnant mice were administered intraperitonially with VPA (500 mg/kg) or Saline. After delivery of pups and starting from postnatal day (P44), treatments of VPA mice and CNT mice were carried out. The systemic administrations continued for 21 days until VPA and CNT mice reached P64. Starting from P51, behavioral assessments were conducted. Following behavioral assessments, all mice were sacrificed at P64 for biochemical and immunofluorescence analyses. VPA and CNT treatment groups (8–12 mice/group) were subdivided into 16 subgroups and received intraperitoneally (i.p.) the following treatments and as shown in experimental design: VPA offspring (mice with autistic features; VPA) Group 1: CNT mice injected with saline, group 2: VPA mice injected with saline, group 3: VPA mice injected with E100 (5 mg/kg, i.p.), group 4: VPA mice injected with E100 (10 mg/kg, i.p.), group 5: VPA mice injected with E100 (15 mg/kg, i.p.), group 6: VPA mice injected with DOZ (1 mg/kg, i.p.), group 7: CNT mice injected with E100 (10 mg/kg, i.p.), group 8: CNT mice injected with DOZ (1 mg/kg, i.p.), group 9: E100 (10 mg/kg, i.p.) was co-administered with RAM (10 mg/kg, i.p.), group 10: E100 (10 mg/kg, i.p.) was co-administered with MPA (10 mg/kg, i.p.), group 11: E100 (10 mg/kg, i.p.) was co-administered with ZLT (10 mg/kg, i.p.), group 12: E100 (10 mg/kg, i.p.) was co-administered with SCO (0.3 mg/kg, i.p.), group 13: CNT mice injected with RAM (10 mg/kg), group 14: CNT mice injected with MPA (10 mg/kg, i.p.), group 15: CNT mice injected with ZLT (10 mg/kg, i.p.), and group 16: CNT mice injected with SCO (0.3 mg/kg, i.p.). All co-administrations were carried out in separate injections with 5-min interval following the test compound (E100) administration. CNT; control mice delivered from saline-exposed mice. VPA; autistic mice delivered from VPA-exposed mice.

4.4. Behavioral Tests

4.4.1. Three-Chamber Behavior (TCB)

As previously described, the sociability test was performed [63,73,91,92]. It is a rectangular three chambered transparent polycarbonate cage (homemade), with one center chamber (40 cm × 20 cm × 22 cm) and two side chambers (40 cm × 20 cm × 22 cm) separated by two sliding doors. In the first session, a test mouse was habituated for 5 min in the center chamber with the two side doors closed. In the second session and following habituation, the doors were opened to allow the test mouse to explore all three chambers for a duration of 5 min. Before starting the third session, a stranger mouse of similar age, gender, and strain with no previous contact with the test mouse (referred to as a novel mouse (NM)), was positioned in a small plastic cage in the either left or right chamber, chosen randomly to avoid side preference, while the other cage was kept empty in the opposite chamber and was referred to as a novel object (NO). In the third session, the test mouse was allowed to explore all three chambers and cages for 10 min sociability test, and the time spent exploring the NM and NO (sniffing) was was automatically recorded during the experiment using EthoVision® Software (Noldus, Netherlands). Finally, the time spent in the chamber with NM and around the cage was compared with the time spent in the chamber with NO. Eight groups of 7 mice/group were used for the TCB assessment. As previously described, sociability index (SI) was calculated by applying a mathematical equation to allow the direct comparison of social behavior of the treated groups [63,73], and was calculated with the following formula:

$$SI = \frac{\text{Time exploring novel mouse 1} - \text{Time exploring novel object}}{\text{Time exploring novel mouse 1} + \text{Time exploring novel object}}.$$

4.4.2. Marble Burying Behavior (MBB)

The test was performed as previously reported with slight modifications [63,93–96]. Briefly, each mouse was individually kept in a polycarbonate cage (26 cm × 48 cm × 20 cm) with fitted filter-top covers, and filled with fresh, unscented mouse bedding material to a depth of 5 cm, for habituation. After habituation for 10 min, the mouse was removed, and 20 glass marbles (15 mm diameter) were carefully overlaid equidistantly in a 4 × 5 arrangement in the cage. Each mouse was returned to its designated test cage and was allowed to explore for a duration of 30 min. The percentage of marbles buried (a marble was considered to be covered when >50% was covered by the bedding) was recorded and calculated as previously described [63]. Eight groups of 5 mice/group were used for the MBB assessment.

4.4.3. Elevated Plus Maze (EPM) Test

The EPM test was performed as previously described with slight modifications [97–100]. Briefly, the maze is composed of two opposite open arms (30 cm × 6 cm), two opposite closed arms (30 cm × 6 cm × 15 cm) and a central area (6 cm × 6 cm) constructed from plexiglas. An animal was placed in the center of the maze facing an open arm. Mice entries as well as time spent into each arm were measured for 5 min using EthoVision® Software (Noldus, Netherlands). The maze was carefully cleaned using a, with alcohol dampened, tissue (70%, *v/v*) to eliminate the odor of the previously assessed mouse. Eight groups of 6 mice/group were used in the EPM test.

4.5. Biochemical Assessments

4.5.1. Brain Collection and Tissue Preparation for Biochemical Studies

Following behavioral assessments, the animals were sacrificed according to previously published protocols [63,73]. Deep anesthesia of the treated animals was achieved with pentobarbital (40 mg/kg, i.p.). Cardiac perfusion was carried out using 1× PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) at pH 7.4 to wash out the blood. The perfusion

was carried out manually by using a 50 mL syringe with 20 G needle. The optimal pressure was obtained by slowly flowing 1× PBS (approximately 5 mL/minute). The mice were observed until liver, heart, and kidney were blood free and gave a whitish color, an indication of blood removal. The brains were quickly removed and placed on an ice plate. The cerebellum was excised from the brain and snap-frozen in liquid nitrogen for further use in biochemical tests [63,73]. On the day of biochemical assessment, the tissues were homogenized and placed on ice in the extraction buffer recommended by the manufacturer, radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl, pH 7.4, 140 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and 0.5% sodium deoxycholate))with protease and phosphatase inhibitors. The homogenates were sonicated and centrifuged for a duration of 30 min at 14,000 rpm and at 4 °C to eliminate tissue debris, and the resulting supernatant was used for the assessments of oxidative stress levels and AChE activity [88,89]. Five groups of 5 mice/group were used for oxidative stress marker estimations.

4.5.2. Oxidative Stress Marker Estimations

Lipid Peroxidation Estimation

Malondialdehyde (MDA) detection kit was used to estimate the amount of lipid peroxidation after the manufacturer's instructions, as previously described in our laboratories [63,101,102]. Briefly, samples or calibrators (250 μL) were incubated in the presence of acid reagent and thiobarbituric acid (250 μL). Then butylated hydroxytoluene in ethanol (10 μL) was added and vortexed vigorously. Samples were then incubated for 60 min at 60 °C and centrifuged at 10,000× g for 2–3 min. The reaction mixture was transferred to a cuvette aseptically and the absorbance was measured at 532 nm using VersaMax™ Microplate Reader (Molecular devices, San José, CA, USA). The ELISA reader was used from, tunable Microplate Reader with a SoftMax Pro reading software, wavelength range 340 nm to 850 nm. The protein estimation was performed by using BCA 96 well microplate method. Kits from Thermo Fisher Scientific (Waltham, MA, USA) (product number #23225) were obtained and the kits protocols were followed. Nunc MaxiSorp™ high protein-binding capacity 96 well ELISA plates were purchased from Thermo Fisher Scientific (product number #439454). The results are expressed as μM MDA/mg protein.

Glutathione (GSH) Estimation

The estimation of GSH levels were carried out according to the manufacturer's instructions of the commercially available GSH kit purchased, and as reported earlier [63,101,102]. The reduced glutathione was estimated in the samples that were first deproteinized with 5% 5-sulfosalicylic acid solution and centrifuged to remove the precipitated protein. The obtained supernatant was used to assess the levels of GSH by measuring the absorbance of test samples at 412 nm with the kinetics for 5 min applying the microplate reader. The results are expressed as μM GSH/mg protein.

Estimation of Antioxidant Enzymes Activity

For estimation of the activity of antioxidant enzymes Superoxide dismutase (SOD) and Catalase (CAT), manufacturer's instructions of commercially available kits were followed, and as previously reported [101,102]. CAT absorbance was read using a micro plate reader at 540 nm, and activity was expressed as nmol/min/mg protein. The protein estimation was carried out using the same devices and in similarity to the experimental protocol described under the Lipid Peroxidation Estimation Section. SOD absorbance was read at 450 nm using a microplate reader and activity was expressed as unit/mg protein.

Determination of Acetylcholinesterase (AChE) Activity in VPA-Exposed Mice Cerebellum

The acetylcholine assay kit was used, and the procedure followed was according to the manufacturer. The assay relates the hydrolysis of ACh to choline by AChE enzyme. Briefly, 5 μL of supernatant of homogenate (cerebellum tissue) was placed into the plate. Then, 45 μL of working reagent that consists of AChE assay buffer, and 50 μL reaction mix were added into each well.

After incubation for 20–30 min at 37 °C, absorbance was read in a kinetic mode, and choosing two time points in a linear range to calculate the AChE activity of the sample, using VersaMax™ MicroplateReader with a tunnelable reader ranged from 340–850 nm wavelength.

4.6. Statistics

For behavioral studies and biochemical assessments, data were expressed as means ± SEM. The data were analyzed for normality by assessing the sample distribution or skewness (−1.8 to +1.8 considered normally distributed). After the results had passed the tests for normality, the effects of E100 were analyzed using two-way analysis of variance (ANOVA) with dose of drugs and animals (either VPA or CNT mice) as the between-subjects factor, and post hoc comparisons were performed with Tukey's test in case of a significant main effect. For statistical comparisons, the software package SPSS 25.0 (IBM Middle East, Dubai, UAE) was used. The p values less than 0.05 were considered statistically significant.

5. Conclusions

The novel dual-active H3R antagonist and AChE inhibitor E100 alleviated sociability deficits in TCB and stereotypies in MBB. In addition, E100 reduced elevated AChE activity and mitigated oxidative stress levels in cerebellum of mice with ASD-like behaviors induced by prenatal exposure to VPA (Figure 7). These results demonstrate the alleviating effects of E100 in different behavioural and biochemical assays following in vivo VPA-induced ASD in mice, and are to our knowledge the first in vivo demonstration that a potent dual-active H3R antagonist and AChE inhibitor is effective in improving sociability deficits and stereotypies of ASD-like features induced by prenatal exposure to VPA, and provide evidence to such dual-active compound to be used as a potential template for further drug design towards novel therapeutic entities for the treat ASD. However, additional trials with newer developed agents of this class and in different autistic animal models and in other species are warranted to clarify the pharmacological profile of the current class to develop proper, clinically potential candidates with balanced inhibitory affinities at both targets, namely the AChE and the H3Rs. Additionally, further investigations assessing pharmacokinetics/pharmacodynamics analysis for E100 are warranted to comprehend the provided ameliorating effects on ASD-like features and to exclude possible off-target effects.
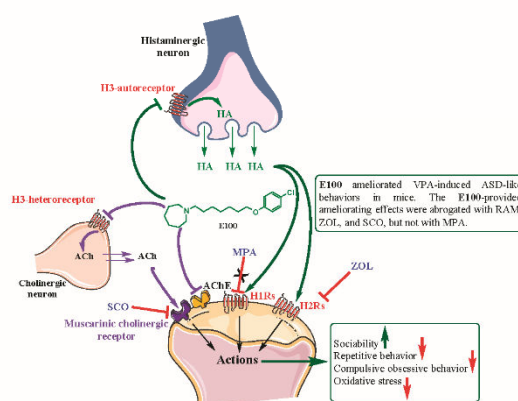

Figure 7. Putative mode of action of E100 by blocking the acting auto- and hetero histamine H3 receptors (H3Rs) and inhibition of the acetylcholine esterase enzyme (AChE). Regulating the release of brain histamine (HA) and acetylcholine (ACh), respectively and inhibiting the metabolism of ACh.

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4.8. Epigenetics meets GPCR – Inhibition of histone H3 methyltransferase (G9a) and histamine H₃ receptor for Prader-Willi syndrome

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Contribution to research: DR designed, planned, and conducted radioligand displacement assays and functional studies. DR organised CRE-Luc cell lines, organised and established functional assays and conducted cell-culture. DR organised the shipment of test-ligands, and evaluated corresponding data. DR wrote the manuscript and was involved in processing the review.

Abstract:

The role of epigenetic regulation is in large parts connected to cancer, but additionally, its therapeutic claim in neurological disorders has emerged. Inhibition of histone H3 lysine N-methyltransferase, especially G9a, has been recently shown to restore candidate genes from silenced parental chromosomes in the imprinting disorder Prader–Willi syndrome (PWS). In addition to this epigenetic approach, pitolisant as G-protein coupled histamine H3 receptor (H3R) antagonist has demonstrated promising therapeutic effects for Prader–Willi syndrome. To combine these pioneering principles of drug action, we aimed to identify compounds that combine both activities, guided by the pharmacophore blueprint for both targets. However, pitolisant as selective H3R inverse agonist with FDA and EMA-approval did not show the required inhibition at G9a. Pharmacological characterization of the prominent G9a inhibitor A-366, that is as well an inhibitor of the epigenetic reader protein Spindlin1, revealed its high affinity at H3R while showing subtype selectivity among subsets of the histaminergic and dopaminergic receptor families.

This work moves prominent G9a ligands forward as pharmacological tools to prove for a potentially combined, symptomatic and causal, therapy in PWS by bridging the gap between drug development for G-protein coupled receptors and G9a as an epigenetic effector in a multi-targeting approach.

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Epigenetics meets GPCR: inhibition of histone H3 methyltransferase (G9a) and histamine H₃ receptor for Prader–Willi Syndrome

David Reiner[1], Ludwig Seifert[2], Caroline Deck[2], Roland Schüle[3], Manfred Jung[2] & Holger Stark[1]✉

The role of epigenetic regulation is in large parts connected to cancer, but additionally, its therapeutic claim in neurological disorders has emerged. Inhibition of histone H3 lysine *N*-methyltransferase, especially G9a, has been recently shown to restore candidate genes from silenced parental chromosomes in the imprinting disorder Prader–Willi syndrome (PWS). In addition to this epigenetic approach, pitolisant as G-protein coupled histamine H₃ receptor (H₃R) antagonist has demonstrated promising therapeutic effects for Prader–Willi syndrome. To combine these pioneering principles of drug action, we aimed to identify compounds that combine both activities, guided by the pharmacophore blueprint for both targets. However, pitolisant as selective H₃R inverse agonist with FDA and EMA-approval did not show the required inhibition at G9a. Pharmacological characterization of the prominent G9a inhibitor A-366, that is as well an inhibitor of the epigenetic reader protein Spindlin1, revealed its high affinity at H₃R while showing subtype selectivity among subsets of the histaminergic and dopaminergic receptor families. This work moves prominent G9a ligands forward as pharmacological tools to prove for a potentially combined, symptomatic and causal, therapy in PWS by bridging the gap between drug development for G-protein coupled receptors and G9a as an epigenetic effector in a multi-targeting approach.

Prader–Willi syndrome (PWS) is a rare neurogenetic disorder that affects approximately 1 of 15,000–30,000 newborn infants[1,2]. Clinically, the disease manifests in a marked hypotonia that presents as earliest symptoms in reduced fetal movement, in sucking weakness of neonates and further limits motoric development in early childhood[3]. Following a period of reduced nutrition due to decreased muscle tone, the disease proceeds with a bland feeling of satiety, leading to a massive urge for eating (hyperphagia). If not controlled by exogenic dietary limitation through caregivers, PWS leads to obesity during adolescence and adulthood, that is the common reason for increased morbidity and mortality of such patients[1,3]. Next to behavioral disorders, patients often show mild mental retardation such as restraints in executing complex tasks and/or mildly reduced intelligence, short stature, hypogonadism, a general delayed development and sleeping issues that demonstrate as hypersomnia and excessive daytime sleepiness (EDS)[3–5].

PWS is referred to as a neurogenetic disorder that has been associated with a loss of genetic information between loci q11 and q13 on the chromosome 15 where several SNORD clusters and the genes SNURF-SNRPN, NDN, MKRN3 and MAGEL2 are located[6]. However, it is hard to correlate their loss with specific symptoms of the phenotype. On the one hand, their specific functions have not been elucidated yet, on the other hand, not many PWS or PWS-like phenotypes could be attributed to the loss of a single of such genes[7]. The function of the small nucleolar RNA (snoRNA) expressed by SNORD116 has not been elucidated yet, though, the deletion of this cluster suggests a critical role for determining the PWS phenotype[8,9]. Progress of knowledge about the organization of genes led to an understanding of the molecular origin of PWS. It is caused by a loss of the paternally inherited genes within the depicted loci, either due to deletion or uniparental disomy. At the same

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	G9a methyltransferase inhibition at 10 µM[a] (n)	Spindlin1 inhibition[b]	hH₃R affinity K_i [CI$_{95\%}$][c] (n)
Ciproxifan	− 1.9 ± 4.9% (2)	No inhibition	320 [250–430] nM[47] (3)
UCL-2190	13.9 ± 9.3% (2)	No inhibition	11 [3.5–33] nM[48] (3)
Pitolisant	− 2.6 ± 12.2% (4)	No inhibition	12 [11–13] nM[47] (5)
A-366	100.00 ± 0.04% (6) IC_{50} = 2.5 nM[28]	IC_{50} = 182.6 nM[37]	17 [8–37] nM (6)
UNC-0642	99.90 ± 0.09% (4)	IC_{50} = 2.7 ± 6.7 µM	1.8 [0.6–5.5] nM (4)

Table 1. Representative ligands and their G9a inhibition, Spindlin1 inhibition and H₃R affinity. [a]AlphaLISA based CLOT (Chemiluminescence-based oxygen tunnelling) assay; results are expressed as means ± s.d. from the indicated number of replicates (n). [b]Screening for inhibition of the epigenetic reader protein Spindlin1 in a fluorescence polarization-based approach. [c]Affinity to the human isoform of histamine H3 receptor (hH₃R) as determined by [³H]N$^\alpha$-methylhistamine displacement studies.

time, the copy of information remains on the corresponding maternal chromosome[10]. However, the genes on this opposite parental chromosome are silenced by epigenetic mechanisms, such as DNA methylation or post-translational histone modifications that lead to imprinting of the corresponding alleles. Thus, PWS is referred to as an "imprinting disorder", a group of disorders that shares many clinical manifestations such as affected growth, development, metabolism or behavior[11].

The current pharmacotherapeutic interventions in PWS involve substitution of Growth Hormone that has shown to improve body composition and motoric strength. It can and should be applied before the first birthday of infants[1]. Additionally, the application of sexual hormones, antipsychotics and antidepressants in the disease is reported in the literature[1]. Among the psychiatric drugs, modafinil demonstrated effectiveness to relieve the impulsive behavior of PWS patients and has been approved for the treatment of EDS or narcolepsy[12]. Similarly, application of pitolisant as novel inverse agonist/antagonist at the G-protein coupled histamine H₃ receptor (H₃R) by children suffering from PWS is known to us[13–15]. The drug obtained market-approval by the European Medicines Agency of the European Union (EMA) in 2016 for narcolepsy with or without cataplexy, recently followed by the FDA (U.S. Food and Drug Administration) approval. In clinical studies, the drug displayed significant improvement of EDS determined by the Epworth Scale of Sleepiness (ESS) and non-inferiority towards the therapeutically established modafinil[16]. Additionally, pitolisant is currently examined for effects in pediatric narcoleptic patients (ClinicalTrials.gov database of the U.S. National Institutes of Health, Identifier: NCT02611687, https://www.clinicaltrials.gov/). Though highly significant clinical studies for pitolisant in PWS patients are missing to date, recent patient-based case reports suggest benefits of this H₃R targeting drug. It shows improved activity of patients, reduction of daytime-sleepiness as well as improvements in mental clarity and processing speed[13–15]. Moreover, preclinical in vivo examination in SNORD116-deficient PWS mice showed abolished baseline changes in REM sleep after administration of pitolisant[17] that has emphasized the role of H₃R in the pathophysiology of PWS.

The outlined therapeutic options are mainly linked to decrease behavioral and endocrinal symptoms; however, without clear evidence for each of them. Therefore, appropriate and causal pharmacotherapy for PWS is still demanded.

On this search, the demonstration of an epigenetic approach to PWS by Kim and co-workers in 2017 has got our attention[10,18]. The group shows that at least two inhibitors of the histone H3 lysine-9 (H3K9) methyltransferase G9a (syn. Euchromatic histone N-methyltransferase 2, EHMT-2) are capable of restoring the expression of candidate PWS genes from the maternally inherited chromosome. While the group found no alterations in the level of DNA-methylation within the imprinted region and genes were still restored, the role of methylation for gene silencing seems less important in PWS. Therefore, the relevance of histone H3 methylation as a regulator of the expression of the imprinted genes during imprinting has been highlighted[18]. Further unknown roles of G9a to gene expression may contribute[19], and involvement of additional regulators of gene expression seems likely. For example, some G9a inhibitors have shown inhibition of Spindlin1 that belongs to the epigenetic "reader" proteins and has been studied for its role in cancer progression[20]. It can detect H3K4 trimethylation (H3K4me3) and trigger downstream signalling[21] as well as the expression of rRNA genes[22].

Inspired by the recent progress of pitolisant in PWS, we aimed to accelerate the preclinical and clinical investigation by the discovery of further H₃R inverse agonists/antagonists with improved profiles. The reported potential of the G9a inhibitors UNC-0642 and UNC-0638 to restore the expression of candidate genes in PWS prompted us to identify H₃R antagonists among compounds with inhibitory activity for G9a. Additionally, we took identified lead-compounds for a selectivity screening among histamine H₄ receptors that possess high structural similarity to H₃R[23] as well as towards dopaminergic receptor subtypes that have been associated with the regulation of food intake[24]. Finally, relevant queries were made for Spindlin1 inhibition to identify congeners for further pharmacological elucidation of involvement of this target in PWS.

Results

Cross-over screening of H₃R ligands and dual G9a/Spindlin1 inhibitors.

Testing of the H₃R ligands pitolisant and ciproxifan did not reveal remarkable inhibition of G9a and Spindlin1. UCL-2190 showed only slight G9a inhibition when compared to negative control (buffer only, $P = 0.035$). In contrast, known G9a inhibitors potently diminished H3K9 dimethylation (Table 1). Additionally, UNC-0642 inhibited Spindlin1 to interact
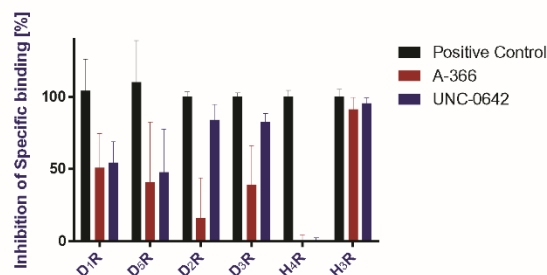
Figure 1. Screening for selectivity of G9a-inhibitors at 1 µM among dopamine D_1, D_5, D_2 and D_3 receptors (D_1R, D_5R, D_2R, D_3R, respectively) and at the histamine H_4 receptor (H_4R). For comparison, the figure depicts the inhibition of specific binding to H_3R that was extracted from affinity screening data. Bars represent means ± s.d. of the inhibition of radioligand binding to the respective receptor by either A-366, UNC-0642 or control compound (100 µM fluphenazine for D_1R and D_5R, 10 µM haloperidol for D_2R and D_3R, 100 µM JNJ-7777120 for H_4R or 10 µM pitolisant for H_3R). [³H]-SCH23390, [³H]-spiperone, [³H]-histamine and [³H]N^α-methylhistamine were used as radiolabelled tracers at D_1R/D_5R, D_2R/D_3R, H_4R and H_3R, respectively, each at approx. $1 \times K_D$.

with trimethylated H3K4 at 10 µM. Interestingly, such dual G9a/Spindlin1 inhibitors were as well able for potent displacement of [³H]N^α-methylhistamine from human isoform of H_3R (hH_3R) in the nanomolar concentration range. While A-366 exerted its action at a concentration similar to the prominent H_3R inverse agonist/antagonist pitolisant, UNC-0642 was more active by about an order of magnitude.

Selectivity screening of G9a inhibitors at other GPCR subtypes.
The G9a inhibitors A-366 and UNC-0642 were screened for their ability to inhibit binding of radiolabeled ligands to dopamine D_1, D_2, D_3, D_5 receptors (D_1R, D_5R, D_2R, D_3R) and histamine H_4 receptor (H_4R) (Fig. 1). For both compounds, significant differences to the respective positive controls were observed ($P < 0.05$; positive controls: 10 µM haloperidol for dopaminergic receptor subtypes, 100 µM JNJ-7777120 for histamine H_4 receptor, 10 µM pitolisant for H_3R). Such differences were slight with regards to H_3R (A-366: Δbetween means = 9%, UNC-0642: Δbetween means = 5%). However, differences in inhibition were significantly more pronounced when comparing their activity between H_3R and the other GPCR subtypes ($P < 0.02$), suggesting lower receptor affinity for the latter. A-366 and UNC-0642 did not differ from each other for their exerted radioligand displacement at H_3R, H_4R, D_1R and D_5R ($P > 0.22$). In contrast, a higher susceptibility to displace [³H]-spiperone from D_2R and D_3R was observed for UNC-0642 than for A-366 ($P < 0.01$).

Mode of antagonism of A-366 at rat isoform of H_3R.
Additionally, A-366 was investigated in a cAMP-response element driven luciferase reporter gene (CRE-Luc) assay at another isoform of H_3R. At the *Rat Norvegicus* isoform of H_3R (rH_3R), A-366 potently shifted receptor activation by histamine ($EC_{50} = 2.3$ [0.4–14.9] nM). As determined from fitting data of Fig. 2a, the resulting affinity was in line with the observations above that used the human isoform of H_3R (hH_3R, $K_B = 15$ [2–150] nM). Subsequent Schild-plot showed that A-366 shifts the affinity of histamine in a rather equipotent manner (Fig. 2b) with a slightly reduced slope (0.79 ± 0.45, mean ± 95% confidence interval) but not significantly different from unity.

Discussion
Among our search for novel H_3R ligands with combined G9a inhibitory activity, relevant progress could be made in this study to define a novel mode of action in the pharmacotherapy of PWS. Particularly guided by the recent clinical effects of pitolisant, we started with the search for a potential epigenetic mechanism of action for pitolisant as well as ciproxifan and UCL-2190. However, such could not be delineated based on our data. Ciproxifan serves as an advanced pharmacological tool on preclinical investigation stage and a standard tool in various rodent models[25], despite that included imidazole moiety[26]. Some drawbacks associated with the susceptibility of imidazole to inhibit CYP enzymes led to the derivative UCL-2190 that belongs to the second, nonimidazole-based generation of H_3R antagonists[27]. Whereas a slight H_3R inhibition in low percentile range was observed for UCL-2190, the corresponding affinity estimate would be far apart from such observed for potent G9a inhibitors. Crystal structures of some G9a inhibitors in complex with the enzyme suggest the necessity of protonated heterocyclic element for ionic interaction with the Asp1088 residue of the enzyme[28–30]. We attribute the lack of G9a inhibition by our scrutinized H_3R inverse agonists to the absence of this structural feature.

In contrast, we could identify potent H_3R ligands among G9a inhibitors. Therefore, we examined UNC-0642, bearing a quinazoline-core motif and A-366 as a spirocyclic 2-amino-3H-indole-based G9a-pharmacophore. The latter is suggested to be protonated at physiological pH-value due to variation towards an inherent amidine or aromatic guanidine functionality[30,31]. Further aliphatic and amino group-containing moieties are tolerated. Interestingly, for some G9a inhibitors, the core bears substituents like a 3-pyrrolidinopropoxy moiety as present in
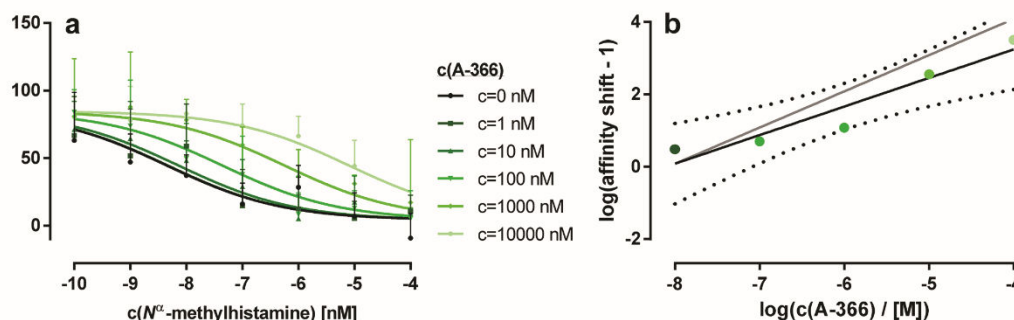
Figure 2. Schild-assay revealing antagonist properties of A-366 at rat isoform of H_3R. (**a**) Effects of the H_3R agonist N^α-methylhistamine on formation of cAMP concentrations were studied in a cAMP response element-driven luciferase reporter gene (CRE-Luc) assay in HEK-293 T cells that were stably transfected with the receptor as described by Nordemann et al.[44,45] Evaluated data originated from two independent experiments performed in duplicate and are stated as means ± s.d. (**b**) Data from panel a were transformed to a Schild-plot that resulted in a regression of $R^2 = 0.91$ (black line, with 95% confidence band depicted with small dots). The slope was not different from unity (grey line).

A-366 and UNC-0642. As enlightened from the crystal structures, the latter motif could be linked to an increased potency at G9a due to substrate mimicking of lysine in position 9 of histone H3K9 and therefore a blocking of the lysine binding tunnel in the histone H3 binding pocket[30]. Additionally, this variation draws the basis for an H_3R pharmacophore that can constitute of a basic moiety, linked by an alkyl-spacer towards a substitutable aromatic central core[32]. The high binding affinity of the G9a inhibitors can be explained as such features have already been incorporated into UNC-0642 and A-366. For the former tool compound, the findings are in line with its previous characterization as a G9a inhibitor with selectivity over a broad range of kinases, transporters, ion channels as well as GPCR's, except an affinity at histamine H_3R[28].

To search for potential discriminants between both pharmacological tools at GPCRs, we extended our in vitro profiling with selectivity studies against a small set of dopamine receptors (D_1R, D_5R, D_2R, D_3R) as well as the histamine H_4 receptor that shows the highest structural similarity to H_3R among GPCRs[23]. In all cases, inhibition of radioligand binding to the off-targets was lower for UNC-0642 and A-366 when compared with the respective positive controls at such receptors and also lower when compared to their inhibitory activity at H_3R. In essence, one could hypothesize an additional action of agonists at D_2R would have beneficial effects for PWS due to a suppressed food-intake in vivo[24]. Consequently, antagonists could compromise such an effect[33]. Thus, we see the selectivity against D_2R and D_3R, that was slightly more pronounced for A-366 than for UNC-0642, as an essential property for our desired pharmacological tools.

As a consequence of the well-documented interspecies differences of H_3R affinity, we decided to determine A-366 binding at the rH_3R. Due to the $G_{i/o}$ coupling nature of H_3R, agonists as N^α-methylhistamine lead to a reduced intracellular cAMP content compared to untreated cells[34]. In a Schild-based[35] characterization of A-366 as depicted in Fig. 2, we observed a potency that was consistent with such at hH_3R and an equipotent affinity shift of agonist with increasing antagonist concentrations. This result creates a basis for exploitability of H_3R mediated effects in preclinical PWS in vivo studies, although we are aware that mouse models have been predominantly used in the past. However, some reports move for extended usage of animal models other than PWS mouse models as such do usually not present obesity and hyperphagia simultaneously[36].

Together with the previously presented data[30,37], our results indicate that both G9a standard ligands have low nanomolar H_3R binding affinities with required selectivity among further GPCR subtypes and that they exert potent inhibition of G9a and Spindlin1. Besides the effectiveness of UNC-0642 in PWS mice that was mentioned previously, this compound has already been subjected for further neurological examination, showing amelioration of autism-like social deficits in Shank3-deficient mice[38] and reduction of anxiety-related behavior in adult mice[39]. In the latter study, effects similar to those of UNC-0642 could be demonstrated for A-366[39]. This implies both compounds to be tolerated in mouse or rat models and that they possess essential features for neurological drugs such as blood–brain barrier permeability and metabolic stability[18,28,30,40]. Therefore, both ligands will be suitable pharmacological tools for potential in vivo investigation.

Concluding our search for potential dual G9a inhibitors/H_3R antagonists for the treatment of PWS in future, significant prerequisites for applying the preclinical candidate A-366 in PWS studies could be identified. With the identification of H_3R antagonizing properties of A-366, our in vitro characterization presents this compound as a multi-target ligand that has a high potential to show symptomatic effects in the neurogenetic PWS, congruent to those described for pitolisant (Fig. 3). Secondly, the recently demonstrated gene restoration from maternal chromosomes by UNC-0642 mediated G9a inhibition should allow for a potential causal intervention by A-366. Besides, the advanced preclinical development stage of this drug makes it very attractive for further clinical characterization, promising a symptomatic and causal approach in the pharmacotherapy of PWS.
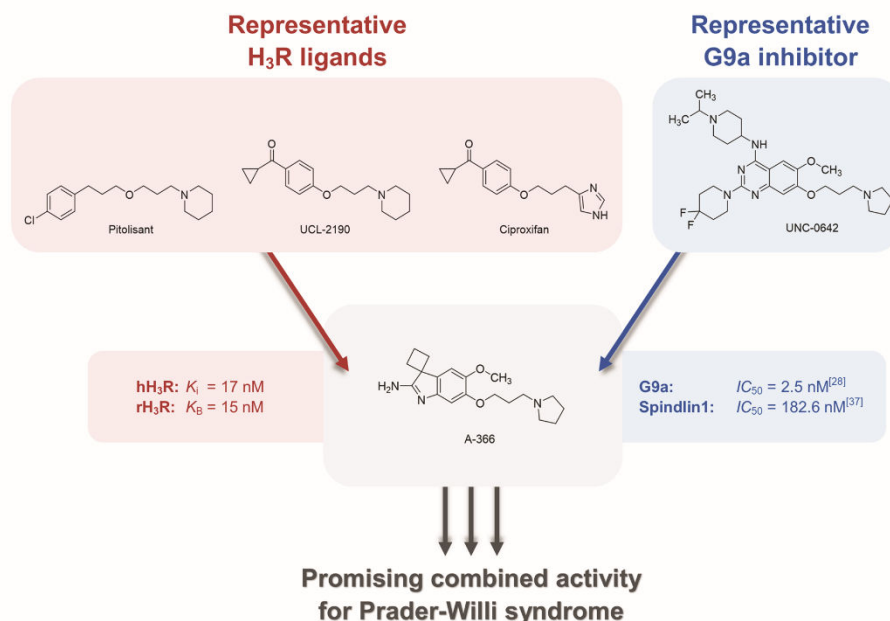
Figure 3. Promising combined H₃R antagonist, G9a- and Spindlin1-inhibitor activity of A366 for pharmacotherapy of Prader–Willi syndrome (PWS).

Methods

Materials.   UCL-2190[41], Ciproxifan[42] and Pitolisant[27] were from own laboratory stocks of which synthesis and analytics have been described previously. G9a-inhibitors A-366 and UNC-0642 as well as G9a enzyme, S-adenosylmethionine (SAM, (2S)-2-amino-4-[[(2S,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytet-rahydrofuran-2-yl]methyl-methylsulfonio]butanoate), biotinylated histone H3 (1–21) fragment and Dulbecco´s modified eagle medium (DMEM, article no. D5671) were purchased from Sigma-Aldrich, Taufkirchen, Germany. Fetal bovine serum albumin (FBS Good-Forte) and Dulbecco´s Phosphate Buffered Saline (DPBS) were provided by PAN biotech (Aidenbach, Germany). The radioligands [³H]N$^{\alpha}$-methylhistamine, [³H]histamine, [³H]spiperone and [³H]SCH23390 were purchased from PerkinElmer (Rodgau, Germany) as well as AlphaLISA materials such as AlphaLISA H3K9me2 acceptor beads, streptavidin-coated donor beads, detection buffer (5x) and white 384-well microplates (OptiPlate). Human or animal blood/tissue/cell samples have not been used in this study.

Cell culture and membrane preparations.   Cell culture and membrane preparations for radioligand displacement assays were performed according to the protocols provided by Bautista-Aguilera et al.[43].

HEK-293T cells were used for cAMP-response element driven luciferase reporter gene (CRE-Luc) assays, that were stably transfected with cDNA of the H₃R isoform of *Rattus Norvegicus* (rH₃R, NCBI sequence code: NC_005102.4) and a vector containing the *Photinus pyralis* luciferase with a cAMP-response element in its promotor region[44]. Cells were cultured in DMEM supplemented with 1% FBS in the presence of hygromycin (250 µg/mL) and geneticin (1,000 µg/mL) under culture conditions of 37.0 °C, 5.0% CO₂-saturation and 95.0% humidity (for source of cell lines see Supplementary Information).

Radioligand displacement assays at GPCRs.   The affinity of A-366 and UNC-0642 at human isoform of H₃R (NCBI sequence code: NM_007232.3) was determined in radioligand displacement studies at membrane preparations of transfected HEK-293 T cells. Therefore, titration schemes ranging from 0.003 to 1,000 nM were prepared in duplicates and incubated with 20 µg/200 µL protein and [³H]N$^{\alpha}$-methylhistamine (c = 2 nM) for 90 min. To determine non-specific binding, additional samples of pitolisant 10 µM were prepared. For off-target activity screenings, 1 µM of G9a inhibitors were incubated with receptors at the conditions that are described in Table 2. Therefore, triplicates were examined in the case of dopaminergic or histaminergic receptor subtypes, respectively.

The workflow to terminate incubation and measurement of bound radioligand was identical for both experimental set-ups. Briefly, samples were filtrated from microplates onto GF/B filters presoaked with 0.3% poly-ethyleneimine solution using a 96-well cell harvester. Filter mats were washed three times with water at 4 °C,

Receptor NCBI sequence code (protein content)	Cell line	Radioligand (concentration)	Control (concentration)	Incubation time
Dopamine D₁ NM_000794.5 (10 μg/200 μL)	HEK-293 T	[³H]SCH23390 (0.3 nM)	Fluphenazine (100 μM)	120 min
Dopamine D₂ NM_016574.3 (25 μg/200 μL)	CHO-K1	[³H]spiperone (0.2 nM)	Haloperidol (10 μM)	120 min
Dopamine D₃ NM_000796.6 (20 μg/200 μL)	CHO-K1	[³H]spiperone (0.2 nM)	Haloperidol (10 μM)	120 min
Dopamine D₅ NM_000798.5 (5 μg/200 μL)	HEK-293 T	[³H]SCH23390 (0.3 nM)	Fluphenazine (100 μM)	120 min
Histamine H₄ NM_021624.4 (60 μg/200 μL)	Sf9	[³H]histamine (10 nM)	JNJ-7777120 (100 μM)	60 min

Table 2. Conditions for screening of A-366 for off-target activity (dopamine D_1, D_2, D_3, D_5 and histamine H_4 receptors).

dried for 60 min (54 °C), soaked with scintillation liquid (Betaplate Scint, PerkinElmer), sealed and subjected to scintillation counting.

G9a-inhibition screening. Inhibition of G9a was examined in an AlphaLISA based format with protocols provided by PerkinElmer. In brief, compounds were incubated for 30 min on white 384-well microplates at the indicated concentration and with 5 nM G9a (Supplementary Information, Figure S1), 100 nM histone H3 (1–21) fragment and 15 μM SAM in assay buffer (50 mM Tris–HCl (pH = 9.0); 50 mM NaCl, 1 mM dithiothreitol, 0.01% Tween-20). Incubation was terminated by addition of anti-H3K9me2 acceptor beads in provided detection buffer. After incubating the mixture for 60 min, streptavidin-coated donor beads were added to the mix for additional 30 min. Luminescence was then measured using the AlphaLISA luminescence filter of an Infinite M1000pro multiplate reader (Tecan, Maennedorf, Switzerland) for 1,000 ms (integration time).

Spindlin1 inhibition screening. Spindlin1 inhibition was determined using the fluorescence polarization displacement assay described by Wagner et al.[37] For the IC_{50} values, 12 concentrations were measured in triplicates.

CRE-Luc assays at rH₃R. CRE-Luc assays were conducted by following the protocol provided by Nordemann et al.[44,45], with slight modifications: For functional-based Schild[46] studies in HEK-293T cells, such were seeded into polyethyleneimine-coated 96-well tissue culture plates (TPP) at 2·10⁵ cells/200 μL/well in assay medium (DMEM without phenol-red, 1% FBS) and allowed to attach for 24–48 h. Afterwards, forskolin (c_{final} = 3 μM) and serially-diluted N^{α}-methylhistamine (10,000–0.01 nM) were added to the reaction cells in absence or presence of A-366 (10–100,000 nM) using a Freedom EVO® liquid handling robot (Tecan). The mixture was incubated for 5 h under culture conditions.

Subsequently, the medium was removed and replaced by 80 μL lysis buffer (25 μM tricine, 10% glycerol, 2 μM egtazic acid, 1% Triton X-100, 5 μM MgSO₄-7H₂O and 1 μM dithiothreitol) for 30 min while shaking at 300 rpm. Lysed homogenate was transferred into white microplates. Luminescence was recorded using an Infinite M1000pro multiplate reader (Tecan) in luminescence mode (3,000 ms integration time, no filter) immediately after addition of 40 μL assay-buffer (25 mM glycylglycine, 15 mM MgSO₄-7H₂O, 15 mM KH₂PO₄, 4 mM egtazic acid, 2 mM dithiothreitol, 1 mM ATP, 50 μM coenzyme A, 0.02 mg/mL D-luciferin potassium salt) by the injector module.

Data handling and statistics. For experiments employing radiolabeled ligands, raw data that were measured as counts-per-minute [c.p.m.] were reduced by non-specific binding. For affinity measurements, such results were fitted to least-squares method "One site competition" of GraphPad Prism version 7.0 (La Jolla, CA, United States) and final values were calculated as means [95% confidence interval]. In case of selectivity experiments, inhibition of specific binding [%] was calculated from raw data according to [1- (SM – NSB)/(TB – NSB)]*100%, where SM, NSB and TB refer to binding in the presence of ligand, non-specific binding and total binding, respectively. Data were stated as means ± s.d. For G9a inhibition studies, results were calculated from luminescence according to: 100% * [1-(SM-NC)/(PC-NC)], where SM, NC and PC refer to luminescence in samples including test compound, water and A-366 at 10 μM, respectively. Data were stated as means ± s.d. with the indicated number of replicates. For CRE-Luc assays, data were normalized to luminescence derived by forskolin containing samples (= 100%) and minimum luminescence measured in samples containing forskolin + N^{α}-methylhistamine (10 μM) (= 0%). Data from both experiments were globally fit to the "Gaddum/Schild EC_{50} shift" model of GraphPad Prism and were stated as means [95% confidence interval].

Where appropriate, non-parametric tests or parametric t-tests were conducted utilizing GraphPad Prism to test for differences between data, while assuming significance if $P < 0.05$.

Data availability
Data for the conducted studies will be provided by the corresponding author upon reasonable request.

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Author contributions

D.R. designed, conducted and evaluated radioligand-based affinity and selectivity experiments, G9a inhibition screening, CRE-Luc assays and drafted the manuscript. L.S. & C.D. designed, conducted and assessed relevant Spindlin1 inhibition studies. R.S., M.J. & H.S. provided materials. M.J. & H.S. created and supervised the project, and they maintained the collaboration. All authors thoroughly reviewed and accepted the manuscript.

Competing interests

H.S. contributed to the discovery of pitolisant. The authors declare to have no financial conflicts of interest.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-70523-y.

Correspondence and requests for materials should be addressed to H.S.

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Supplementary Information

Epigenetics meets GPCR –
Inhibition of histone H3 methyltransferase (G9a) and histamine H₃ receptor for Prader-Willi Syndrom

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AlphaLISA™ based G9a inhibition
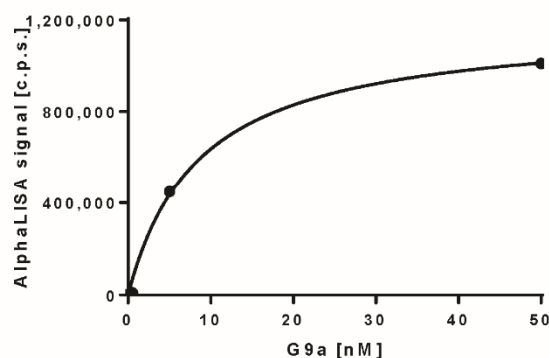
Figure S1. G9a enzyme titration (0.05, 0.5, 5 and 50 nM) at fixed concentrations of recombinant histone H3 fragment 1-21 (c = 100 nM) and *S*-adenosylmethionine (SAM, c = 15 μM). Incubation was carried out once for 30 min. Data represent means ± s.d. from an initial experiment performed in duplicates. For further tests, 5 nM G9a were used.

5. Summary & perspectives

Tremendous efforts have been made in molecular pharmacology within the last decades, aiming for identification of novel potent H_3R inverse agonists with effectiveness on clinical investigation stage. Despite the multiple involvements of H_3R in physiological regulation of not only the histaminergic neurotransmitter system, but as well of GABAergic, glutamatergic, dopaminergic and adrenergic ones, only one H_3R inverse agonist has succeeded on its way throughout the drug development, with market authorisation in the European Union and, in 2019, within the U.S.A (Syed, 2016; Kumar et al., 2019). Given this discrepancy between multiple efforts in drug design and drugs approved after almost 40 years of H_3R research, this research aimed to identify potential pitfalls in the molecular pharmacology of H_3R ligands. The second objective was dedicated to the identification of novel hit and lead structures with multitarget directed profile for complex neurogenetic disorders such as schizophrenia and Gilles de la Tourette syndrome, Alzheimer´s disease and Parkinson´s disease, ASD and the PWS.

In order to increase throughput in an automated screening environment, an FP-based homogenous method to investigate drug-target binding kinetics was developed. Bodilisant was chosen due to the outlined initial considerations of fluorescent, non-imidazole-based antagonists as chemical probes and to elucidate their binding behaviour (Reiner et al., 2019). Another reason in favour of bodilisant was its detectability in even nanomolar concentration range. This observation was a practical prerequisite to obtain a signal in FP, which requires binding of a significant amount of bodilisant. Furthermore, employing novel fluorescence-labelled chemical probes in screening methods can unveil pharmacologic features of the target in interest. The observations made in the study in section 3.1 highlights the importance of reaching out for novel tracers and to motivate further exploration of receptor binding behaviour of H_3R ligands (Reiner et al., 2019). Most attractively, the method enabled for distinguishing association kinetics of the standard antagonists ciproxifan, clobenpropit, thioperamide, pitolisant (cf. page 29) and the agonist NAMH (cf. page 24) while measuring comparable dissociation kinetics. This observation points towards differences in the mechanism of receptor binding but similarities in unbinding events, the latter being an inherent property of the drug-target complex. Besides, the observed affinities were in marked contrast to such observed with the standard-tracer [³H]NAMH, for all investigated ligands except for pitolisant. Employing a classical molecular pharmacological model, the displacement behaviour of [³H]NAMH against bodilisant was characteristic for a competitive binding behaviour including secondary binding sites for NAMH and consequently for bodilisant. However, the location of this secondary binding site could not be elucidated to date. Certain probability for this binding occurring elsewhere could not be ruled out. However, it was considered low as a result of study design, that relied on experimentation with purified membrane extracts derived from a receptor-overexpressing recombinant cell line. Furthermore,

non-specific binding in such measurements was determined with the H₃R antagonist pitolisant, representing a drug which underwent thorough selectivity screening during development. However, comparison with screening techniques that consider the spatial proximity between the receptor and a given ligand (such as F/BRET techniques) will help to rule out this uncertainty, while deciphering the receptor structure in complex with bodilisant will provide final evidence for the binding mode (Goulding et al., 2018).

As an orthogonal method to [³H]NAMH displacement assays, it was subsequently used in an attempt to characterise receptor binding of the novel non-imidazole-based H₃R agonist **6-k** (cf. page 26) that was previously reported by Ghoshal et al. (2018b). This group identified 6-k in a functional assay, applying a biosensor consisting of an engineered luciferase, which restores luciferin-oxidation upon conformational shift after cAMP-complexation. The effects were indirectly attributed to H₃R, upon antagonism of signal in the presence of a selective H₃R antagonist (Ghoshal et al., 2018b), while the determination of receptor binding was not performed. The non-imidazole nature which was apart from previously known H₃R pharmacophores prompted us for further elucidation on level of receptor binding. As within our [³H]NAMH displacement assay, an unambiguous receptor binding was not observed for 6-k, the novel FP-method was employed to screen the agonist against potential secondary binding sites. However, even this method did not show displacement of bodilisant (section **3.2**, (Reiner et al., 2020b)). As such behaviour might not only occur from off-target activity but as well occur for partial agonists in an overexpressing system, a subset of consecutive functional assays within the Gα$_i$/AC/cAMP cascade were performed by our collaboration partners, each without effect that was in accordance with previously published results.

In conclusion, the novel FP-based methods delivered important hints for the molecular pharmacology of H₃R ligands and was able to differentiate between distinct receptor binding modes of potent receptor ligands. Additionally, it was able to identify compounds with H₃R affinity while not showing results for compounds with an apparent off-target activity.

Given such insights, using drug-target binding experiments were chosen as a suitable strategy for all subsequent projects to identify novel hit and lead compounds with multitargeting properties, in order to expand pharmacotherapeutic strategies for neurogenetic disorders. While the novel FP-based method using bodilisant will be an element of future characterisation but not the desired substitute for radioligand displacement assays, the latter were chosen for further purposes in order to achieve comparability of my results with the literature and functional assays were considered as a second-line option for more detailed characterisation.

The purpose of the work described in section 3.3, was to explore novel H₃R ligands based on five-membered multi-heterocycles such as oxadiazoles and thiazole (Khanfar et al., 2018). We have focussed on the introduction of heteroaromatic elements into the standard pharmacophore of H₃R ligands, proving for bioisosteric replacement of the central aromatic core. This design was rationalised by improved drug-like properties that focussed such nuclei within several recent drug development campaigns and resulted in lead structures against a plethora of disorders (Saha et al., 2013; Chhabria et al., 2016). However, the previously outlined pharmacophore model for H₃R ligands (Figure 8) was not uniformly applicable to draft potent 1,2,4- or 1,3,4-oxadiazole-based H₃R ligands.

For ligands incorporating 1,3-thiazoles, an easier strategy was chosen based on previous works that presented a highly affine H₃R ligand (Figure 18). ST-979 can be seen as a result of bioisosteric replacement of the central phenyl core within the compound 1-(3-([1,1'-biphenyl]-3-yloxy)propyl)piperidine, by a 5-methyl-1,3-thiazole core. Interestingly, the corresponding ligand ST-2088, which was derived by the same strategy but showed a different orientation of the central methyl thiazole, showed an essential drop in affinity as compared to that of ST-979 (Figure 18, upper panel). This observation might be rationalised mainly for two reasons. First, as of the substituents, only the methyl scaffold attached to the central nucleus is changing its spatial orientation, this observation might be due to a steric repulsive effect. Secondly, if this discrepancy in affinity may be due to thermodynamic stability of a rotamer of ST-2088 different to that as drawn in Figure 4, the interactions of the functionalised thiazole with the receptor may change additionally.

Within our studies, we have shown that derivatisation of the substituents was suitable to obtain highly affine H₃R ligands. Concomitantly, a series of 38 derivatives with affinities ranging from micromolar to single-digit nanomolar concentration range were characterised in this project. The amelioration of the impaired affinity of ST-2088 was mainly attributable to two strategies,

- i. functionalisation of the phenyl-moiety with H-bond acceptors within the "arbitrary region" (see ST-2114 and MAK-84, Figure 18), or
- ii. rigidification of the linker to the alicyclic amine leading to a spatial constraint in the eastern part of the pharmacophore (see ST-2111 and MAK-84, Figure 18).

Interestingly, ABT-239 (cf. page 32) represents a well-characterised drug candidate that underwent clinical investigation and which contains both design features as well.

Regarding potential lead structures with applicability as H₃R inverse agonists, the group consists of easily accessible H₃R ligands with promising lead-like properties. They comply to Lipinski´s and Veber´s rules, and they possess good toxicity profile according to structure-based prediction (Khanfar et al., 2018).

1-(3-([1,1'-biphenyl]-4-yloxy)propyl)piperidine
hH$_3$R: K_i = 76 nM

ST-979
hH$_3$R: K_i = 41 nM

ST-2088
hH$_3$R: K_i = 870 nM

ST-2111
hH$_3$R: K_i = 5.4 nM

ST-2114
hH$_3$R: K_i = 4.2 nM

MAK-84
hH$_3$R: K_i = 7.0 nM
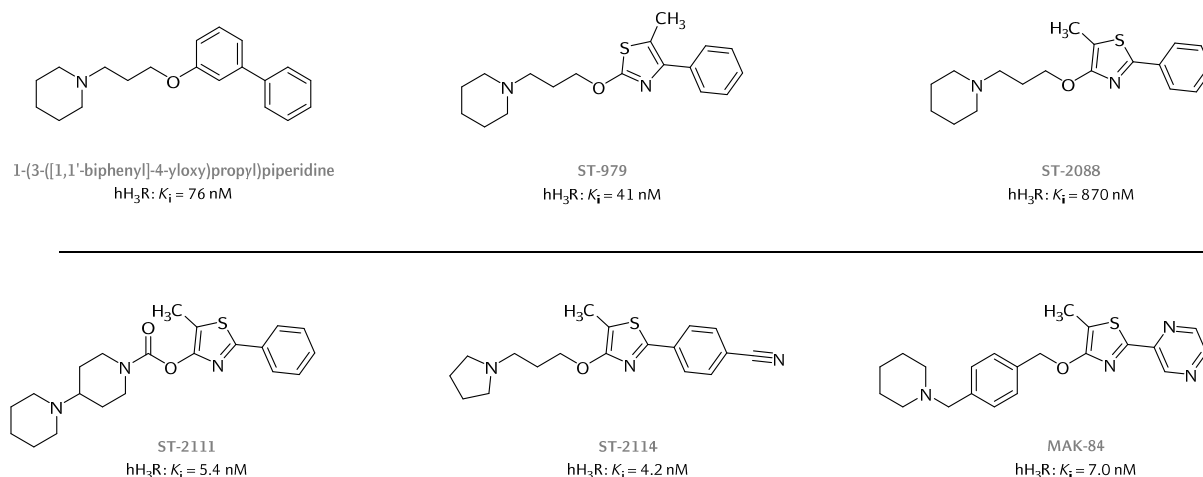
Figure 18. Novel 1,3-thiazole-based H₃R ligands, taken from Khanfar et al. (2018) (section 3.3). All affinity estimates were derived within the same experimental setup.

Overall, the derived compound series will be of importance and a guide for future chemico-biological efforts to 1,3-thiazole-based H₃R ligands and the understanding of drug-target interaction with the H₃R. This study constrains the simplicity of ligand design guided by the general H₃R pharmacophore, in particular, constrained the replaceability of the central aromatic core by a 5-methyl-1,3-thiazole moiety in an attempt to improve drug-likeness.

MTDLs have emerged as promising drug candidates with superior activity in multifactorial diseases such as neurogenetic disorders (section 1.6). Given the numerous involvements of H₃R in neurological processes and the corresponding value for not only a single but two or more diseases, the search was conducted among targets with numerous pharmacotherapeutic implications. Thereby, the multitargeting concept could be expanded from the 'one disease – multiple targets' paradigm to a 'multiple targets – multiple diseases' strategy.

Thus, the scope of the first approach was the identification of novel hit compounds among pharmacophore fragments with known H₃R affinity from recent studies, but with potential usefulness for future medicinal chemistry efforts for drugs against **schizophrenia** and **Gilles de la Tourette syndrome**. Both represent diseases of high inheritability but without clear genetic aetiology (section 1.5) and in both, DA receptor antagonists have provided some therapeutic effects, but not uniformly. Therefore, combined **H₃R/D₂R/D₃R ligands** may have superior effects. Currently available H₃R ligands with D₂ and D₃ antagonism are of large molecular size. Therefore, I have focussed on the characterisation of the LINS01 series with previously published affinities at HA receptor subtypes H₁, H₂ and H₃ (Corrêa et al., 2019). The compounds were subjected for [³H]-spiperone displacement at the short and full-length isoform of D₂R and D₃R, respectively. As depicted in Table 9, LINS01004, LINS01005 and LINS01012 fulfilled the desired

properties with affinities in (sub)micromolar concentration range, while showing comparability among their D_3R affinity measures (Corrêa et al., 2020)(section 4). Only LINS01004 showed slight discrepancies in receptor binding to each investigated receptor subtype (affinity profile: $hH_3R > hD_3R > hD_2R$), while LINS01012 was showing higher affinity at hH_3R without differentiation between hD_2R and hD_3R. Interestingly, all compounds were different in terms of their H_3R affinity (Corrêa et al., 2019), suggesting further exploration of modifications of the motif attached to the piperazine and 5-position of dihydro benzofuran moiety. The latter insights will, therefore, be crucial in hit-to-lead optimisation of this series of MTDLs to candidate structures for the application in **schizophrenia** and likewise, in **GTS**.

Table 9. Hit compounds with combined activity at either HA hH_1R or hH_3R from (Corrêa et al., 2019), or at hH_3R, $hD_{2,s}R$ and hD_3R from (Corrêa et al., 2020).

	R^1	R^2	$hH_1R^{1)}$ K_D [µM]	$hH_2R^{1)}$ K_D [µM]	$hH_3R^{1)}$ K_D [µM]	$hD_{2,s}R$ K_i [µM]	hD_3R K_i [µM]
LINS01001	H	H	5.4 [2.5;11.2]	> 10	> 10	> 10	> 10
LINS01003	Me	H	2.2 [1.1;4.6]	> 10	0.21 [0.13;0.33]	> 10	> 10
LINS01004	Allyl	H	> 10	> 10	0.14 [0.09;0.21]	5.5 [5.0;6.0]	1.5 [0.8;3.0]
LINS01005	Ph	H	> 10	> 10	2.5 [0.9;6.4]	2.4 [2.0;2.9]	0.89 [0.45;1.75]
LINS01007	Me	Cl	> 10	> 10	0.50 [0.35;0.68]	> 10	> 10
LINS01008	Me	Me	> 10	> 10	0.16 [0.09;0.29]	> 10	> 10
LINS01010	Me	*t*Bu	> 10	> 10	0.10 [0.09;0.11]	> 10	> 10
LINS01012	Ph	Me	> 10	> 10	0.51 [0.44;0.60]	2.4 [1.1;5.3]	1.5 [0.4;4.8]

all affinity values are stated as means [95 % confidence-interval]
[1)] Affinities converted from pK_i values, taken from (Correa et al., 2017; Corrêa et al., 2019)

The discovery of H_3R ligands with putative modulation of striatal DA levels aiming for application in PD models, prompted screening of previously designed **H_3R ligands with combined MAO B inhibition**. A first study with the previously known H_3R antagonist, with the capability to enhance cortical HA turnover, **DL76** (Lazewska et al., 2006), revealed potent MAO B inhibition (Łażewska et al., 2020)(section 4.2).

Concerning the previously made assumption for the responsible MAO B pharmacophore in ciproxifan and UCL-2190 (section 1.6), the *tert*-butyl substituted phenyl scaffold elicited a considerably higher enzyme inhibition compared to the cyclopropylmethanone substituted one. These findings point towards a preference of MAO B for unpolar groups in the eastern part of the H₃R pharmacophore.

Proving for a pharmacophoric relevance of the piperidinoalkoxy scaffold on the MAO binding capacities, the results from collaborators from the Medical College of Jagiellonian University Krakow (Poland) pointed more towards a direct involvement of the whole H₃R pharmacophore into MAO B binding. Intolerability towards mono- and dimethylation of piperidine in 3-,4- and 5-position as well as towards increased linker length was demonstrated. In contrast, monomethylation in 2-position of piperidine seemed to improve MAO B binding for the piperidinopropoxy-based ligand. Screening for the H₃R affinities of this compound series showed some, but less sensitivity towards dimethylation in combination with increased linker length, compared to the behaviour against MAO B. Such results from a first larger series of dual H₃R/MAO B ligands provide evidence for a complete overlap of H₃R and MAO B pharmacophore. Thus, it prompts for including MAO B assay into the standard selectivity screening of future H₃R ligands. With a focus on the identification of a combined H₃R/MAO B inhibitors within this series, DL76 depicted the most interesting candidate within this series, not representing a novel ligand but being re-characterised as a dual-targeting ligand with good preclinical profile. Fulfilling the aim to prove for potential applicability in an *in vivo* model of PD, DL76 exhibited profound and dose-dependent reduction of neuroleptics-induced catalepsy in Cross Leg Position test, besides showing ambiguous results in the bar test (section 4.2).

ST-1930[1]

hH₃R: $K_i = 160$ nM
MAO B: Inhibition < 50 % (@10 µM)

ST-1931[1]

hH₃R: $K_i = 300$ nM
MAO B: Inhibition = 72 % (@10 µM)

ST-2328

hH₃R: $K_i = 6.7$ nM
MAO B: $IC_{50} = 10$ µM

ST-2035

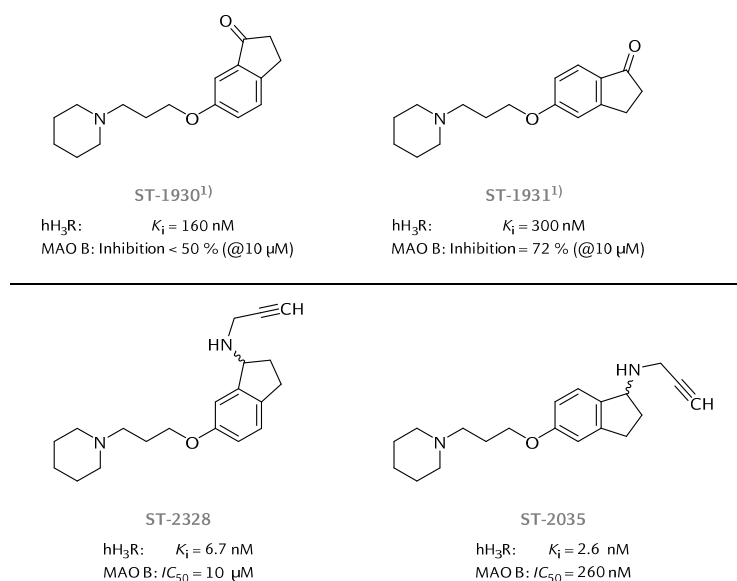hH₃R: $K_i = 2.6$ nM
MAO B: $IC_{50} = 260$ nM

Figure 19. Rasagiline inspired MAO B inhibitors with H₃R affinities from section 4.3 (Lutsenko et al., 2019). [1]Depicted indanone-derivatives (ST-1930, ST-1931) were taken from a previous study (Affini et al., 2018).

Furthermore, H₃R binding properties were investigated for two derivatives of the previously published H₃R/MAO B inhibitors ST-1930 and ST-1931 (Figure 19), with structural resemblance to the approved irreversible and selective MAO B inhibitor rasagiline (Lutsenko et al., 2019)(section 4.3). Within this study, the propargyl aminoindane derivative ST-2328 showed less potent and reversible MAO B inhibition compared to the 5-substituted congener ST-2035, which irreversibly and selectively inhibited MAO B in the nanomolar concentration range. In contrast, both candidates did not differ in their low nanomolar K_i for H₃R (section 4.3). This affinity pattern emphasised the assumption that was raised for the DL76 congeners (section 4.2), that whole H₃R pharmacophore is involved in MAO B binding. The latter is supported by the binding behaviour, where MAO B showed a higher tolerability of a contralateral substitution at the indane scaffold than for an ipsilateral one.

With regards to extensive studies by Yogev-Falach et al. (2002; 2003; 2006), both drug-like give rise for a probable MAO-independent neuroprotective mechanism as shown by rasagiline, ladostigil and even propargylamine. This structural feature is not included in DL-76 (Łażewska et al., 2020)(section 4.2). Therefore, they may suit as promising lead candidates for further exploration in models of AD and PD.

The discovery of MAO B inhibition exerted by the pharmacophore of H₃R was a motivator for a novel approach towards **combined H₃R inverse agonist/ChE inhibitors** in order to achieve additive effects by enhancement of ACh release and decelerated degradation. In contrast to previous approaches (e.g., by Petroianu et al. (2006) or Bautista-Aguilera et al. (2018)), this search was not conducted upon rational design using the piperidinoalkoxyphenyl pharmacophore for H₃R, but among databases of commercially available ligands with unknown binding properties towards the receptor and the enzymes of interest (section 4.4 and 4.5). Therefore, a set of computational screening methods was set up by our collaborators from Tabriz University of Medical Sciences (Iran) with subsequent application towards libraries of commercially available ligands. In more detail, they consisted of either ligand-based structural similarity approach guided by the structure of pitolisant among the SwissSimilarity database (Zoete et al., 2016), or a search method using a pharmacophore model that was based on a generated H₃R homology model in complex with pitolisant among the ZINCPharmer dataset (Koes et al., 2012). Both methods could be used for screening among smaller libraries of < 11 Mio and < 23 Mio compounds.

Moreover, a hybrid approach consisting of both, pitolisant structure-based library generation and subsequent pharmacophore-based virtual screening was undertaken among the larger ZINC15 database (>210 Mio compounds, (Sterling et al., 2015)). In summary, four commercially available ligands were derived from ligand-based structural similarity approach, one from the hybrid-method. In contrast, no structure from the pharmacophore-based approach was able to pass the preset parameters for drug-
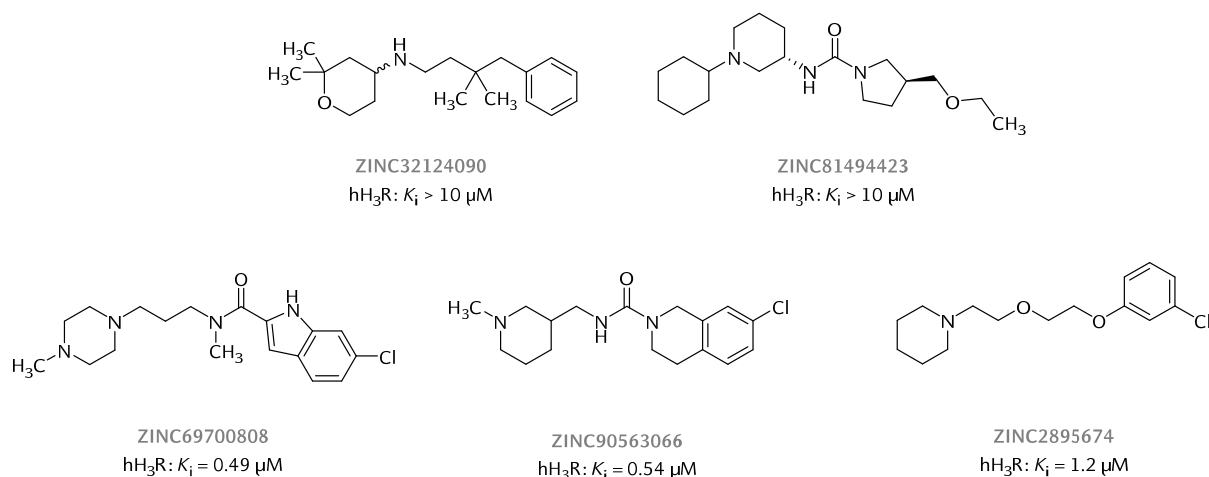
Figure 20. Novel H₃R pharmacophores identified from combined virtual screening and molecular pharmacological analysis, taken from (section 4.4)(Ghamari et al., 2019b).

likeness and predicted absorption, distribution, metabolism and excretion (ADME) properties (Ghamari et al., 2019b). The affinity at H₃R was determined again using the [³H]NAMH displacement approach to rule out false positives among such screening results (Figure 20). Thereby, three out of five screenings hits resulted in novel H₃R ligands in micromolar and submicromolar concentration range. While ZIN2895674 resemble fragments of pitolisant but consisting of a more hydrophilic linker, ZINC69700808 and ZINC90563066 compose of methyl piperazine and methyl piperidine as basic moieties, and bear 6-chloro-1*H*-indole and 7-chloro-1,2,3,4-tetrahydroisochinoline as aromatic core moieties, respectively.

Subsequent functional investigation of the latter two compounds revealed H₃R inverse agonist efficacy, and both were even more potent than expected from H₃R affinity alone. These findings argue for a strong decoupling of the Gα$_{i/o}$/AC/cAMP pathway, exerted by such compounds. Concerning our search for combined H₃R/AChE inhibitors, both compounds were able to inhibit cholinesterases in micromolar concentration range, slightly less active than the previously presented contilisant, which was yield by rational drug design approach. Both lead compounds were similar in their capabilities to inhibit AChE and differed marginally in their preference for BuChE, with ZINC69700808 showing somewhat lower and ZINC90563066 somewhat higher affinity in comparison to AChE (Ghamari et al., 2020) (section 4.5). This combined strategy of computational and molecular pharmacological methods for the discovery of potent MTDL with H₃R inverse agonism/AChE provided us with two lead structures based on novel pharmacophore motifs to serve as a starting point for future lead optimisation on the one hand, but for the application in *in vivo* models of neurogenetic disorders such as Alzheimer´s disease on the other.

Not only AD represents a condition where combined H₃R inverse agonist/ChE inhibitors emerged as potential drugs to ameliorate decreased cortical AChE levels. Similar aberrations have been found in the BTBR mouse models for ASD. Consequently, this was a motivator for testing the effects of a known H3R

Table 10. Changes in behavioural paradigms of autism in C57BL/6 mice with or without valproate-induced autistic-like behaviours, upon several pharmacotherapeutic interventions (E100 or donepezil), and reversal tests by BBB-permeable H_1R, H_2R, H_3R and M_1R-M_5R antagonists (mepyramine, zolantadine, RAMH and scopolamine). Each drugs were administered i.p. (section 4.6 and 4.7).

| Phenotype | Test | Healthy (controls) | | | | | | | Autism-like deficits (valproate-induced) | | | | | | |
		C	E100	donepezil	mepyramine	zolantadine	RAMH	scopolamine	(intervention) donepezil	E100	mepyramine	zolantadine	RAMH	scopolamine
Anxiety	Open-field test[1]	C	~	nd	nd	nd	nd	nd	↑ sC / ↓	↓ sC	~	~	↑	~
	Elevated-plus maze[2]	C	~	~	~	~	~	~	↑ sC / ↓	↓ sC	~	~	↑	~
Sociability	Three-chamber behaviour[2]	C	~	~	~	~	~	~	↓ sC / ↑	↑ sC	~	↓	↓	↓
Repetitive behaviour	Nestlet-shredding behaviour[1]	C	~	~	nd	nd	nd	nd	↑ sC / ↓	↓ sC	~	~	↑	~
	Marble-burying behaviour[2]	C	~	~	~	~	~	~	↑ sC / ↓	↓ sC	~	↑	↑	↑
Locomotion	Elevated-plus maze[2]	C	~	~	~	~	~	~	↑ sC / ~	~	~	~	~	~

C = control; sC = subgroup control; nd = not determined; ↑ = increased; ~ = not altered; ↓ = decreased.

[1] Ref. (Eissa et al., 2019), section 4.6.

[2] Ref. (Eissa et al., 2020), section 4.7.

inverse agonist/AChE & BuChE inhibitor in C57BL/6 mice with induced autism-like behaviour upon prenatal exposure to valproate. On the one hand, valproate treatment resulted in neuroinflammation that was characterised by increased levels of proinflammatory cytokines in cerebellum and hippocampus, both areas involved in either executive or cognitive tasks, E100 was able to ameliorate such levels, to decrease elevated expression of enzymes linked to inflammation processes (Eissa et al., 2019), and to reduce increased markers of oxidative stress (Eissa et al., 2020).

Moreover and focussing on behavioural effects, E100 (10 mg/kg or 15 mg/kg) was efficient in abrogation of valproate-induced autistic deficits in terms of anxiety, sociability and repetitive or compulsive-obsessive behaviour. As depicted in Table 10, such effects were comparable with such exerted by the approved AChE inhibitor donepezil. Determination of involved neuronal circuitries was conducted by abrogation groups, where each effect was diminished upon H₃R agonism (RAMH), but not by H₁R antagonism (mepyramine). This prompted for *in vitro* selectivity screening to exclude inherent H₁R antagonism of E100. This was confirmed for concentrations up to 1 μM while some inhibition was seen at 10 μM. Thus, a contribution of H₁R to the observed effects seems unlikely but cannot be completely ruled out. H₄R-mediated distortions in inflammatory processes, however, seem not to participate in the observed effects due to unambiguous absence of effect in a physiologic concentration range.

Moreover, H₂R receptors (as indicated by zolantadine-antagonism), together with muscarinergic receptors (as indicated by scopolamine-antagonism), showed involvement in sociability and the marble-burying paradigm for repetitive behaviour but not in the nestlet shredding paradigm (Eissa et al., 2019; 2020). At first sight, the results obtained from donepezil might be surprising, which indicate a cholinergic triggered but non-muscarinergic effect. However, this can be rationalised by a potential involvement of nicotinic ACh receptors, of which α4β2 and α7 heteromers in the forebrain, have shown a contribution to cognitive performance (Dineley et al., 2015) and of which the α7 receptor has shown to activate histaminergic TMN neurons. Thus, E100 joins the group of combined H₃R inverse agonists/ChE inhibitors as a drug candidate with promising *in vivo* effects for the complex ASD.

My work on H₃R inhibitors with combined effects at other targets terminates with some outlook on the problem of whether modulating the genetic basis might provide us with better strategies for neurogenetic disorders. Fortunately, auspicious efforts in the field of G9a inhibitors have been recently dedicated to neurogenetic disorders such as AD, ASD and PWS (section 1.6). This progress supports multitargeting properties apart from specific neurotransmitter levels and moves towards the most intracellular source of neurologic pathophysiology, that usually falls out of sight in the daily business of a histaminologist. A straightforward approach to a multi-faceted neurogenetic disease will be an MTDL combining targets with

numerous physiological indications. This theorem was the motivator for the last project, dedicated to the rare-disorder PWS and novel MTDLs for the rare-disorder PWS. More precisely, the H_3R and the histone H3 methyltransferase G9a were chosen not due to their coincidental linguistic comparability but due to their apparent engagement in various neurogenetic disorders.

Initially, we could rule out inherent G9a inhibition properties of H_3R ligands that often seem to bind desired co-targets, as shown previously (section 4.8)(Reiner et al., 2020a). Conversely, the study revealed potent H_3R antagonism of A-366, which was exclusively investigated in models of cancer so far, and UNC-0642 which evolved as a candidate drug for neurogenetic diseases. Whereas some H_3R affinity was ascribed for UNC-0642 before (Liu et al., 2013), this important property was neglected in the interpretation of recent results from mouse models of AD, ASD and PWS (Kim et al., 2017; Griñán-Ferré et al., 2019; Wang et al., 2019). Also, A-366 was identified as an MTDL with previously known high potency at G9a (Sweis et al., 2014). The compound displayed high affinity at hH_3R, high selectivity over other dopaminergic and histaminergic receptor subtypes, fulfils multiple drug-like properties due to exceptionally low molecular weight of a MTDL and shows no affinity difference to a rodent H_3R isoform (Figure 21). Such prominent drug candidates with extensive preclinical characterisation have appeared as potent H_3R ligands, which is
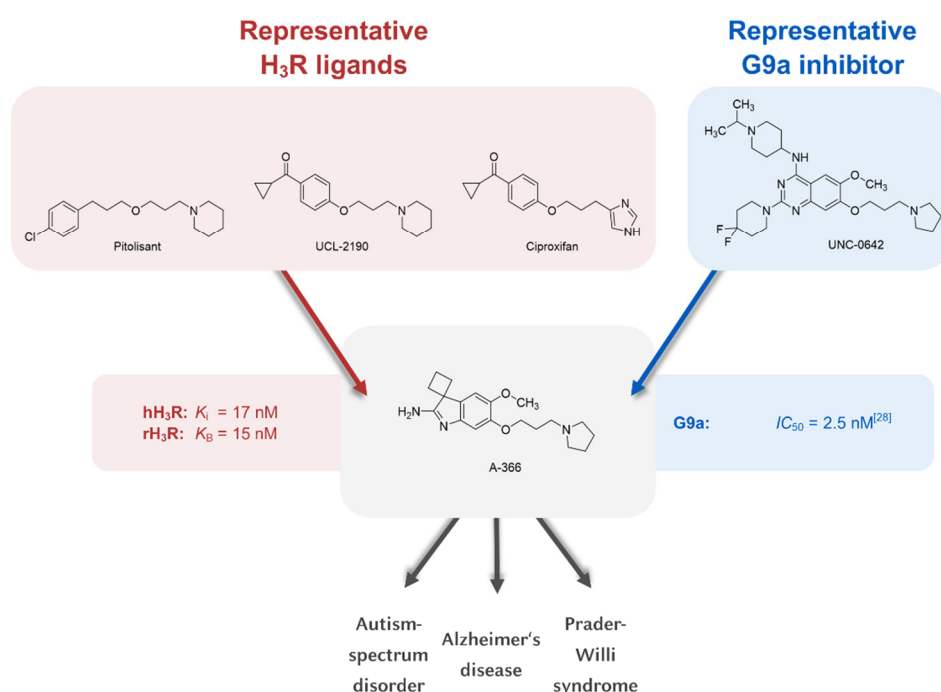
Figure 21. A-366 as MTDL with G9a inhibitory and H_3R-antagonist properties as a paradigmatic shift of pharmacotherapy in neurogenetic disorders (section 4.8). Figure is a derivative from Figure 3 in (Reiner et al., 2020a), used under CC BY 4.0.

**Molecular pharmacology of histamine H₃ receptor ligands
and their implications in neurogenetic disorders**
David Reiner-Link (2020)

attributable to an inherent H₃R pharmacophore of such ligands.

Concluding such findings, A366 and UNC-0642 are promising tools of which a pivotal mode of action can be expected. Due to the involvement of G9a in many neurogenetic disorders where H₃R inverse agonists have emerged as well, these drug candidates will be of relevance for the future pharmacotherapy of neurogenetic disorders. Thus, combined H₃R antagonists/G9a inhibitors represent MTDLs with an excellent profile, which strike the path from a neurotransmission-focussed strategy outlined in the anterior studies of this thesis, towards a single but versatile pharmacotherapeutic approach to numerous neurogenetic disorders (Figure 21).

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7. List of abbreviations and synonyms

AAS	ascending arousal system
Aβ1-i	β-amyloid fragment 1-i
(B/F)RET	(bioluminescence/förster) resonance energy transfer
5-HT	5-hydroxytryptamine, serotonin
5-HT2CR	serotonin 5-HT2C receptor
5-HT3R	serotonin 5-HT3 receptor
5-MH	5-methylhistamine
A2AR	adenosine A2A receptor
A2AR	adenosine A2A receptor
aa	amino acid
AAS	ascending arousal system
AChE	acetylcholinesterase
AChE	acetylcholine esterase
ACS	American Chemical Society
AD	Alzheimer's disease
AD	Alzheimer´s disease
ADHD	attention-deficit / hyperactivity disorder
ADME	absorption, distribution, metabolism and excretion
ADMET	ADME and toxicology
APP	amyloid precursor protein
ArA	arachidonic acid
ArcN	arcuate nucleus
AS	Alzheimer Syndrom
ASD	autism-spectrum disorder
ASS	Autismus-Spektrum-Störung
AUCc-plasma(t)	area under the plasma-concentration-time curve
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BTBR	Black and Tan Brachyury, T+ tf/J
BuChE	butyrylcholine esterase
cAMP	cyclic adenosine monophosphate
cDNA	copy DNA
CFP	cyan fluorescent protein
ChE	cholinesterase
CNS	central nervous system
CRE	cAMP response element
CRH	corticotropin-releasing hormone
CYP	cytochrome P450
DA	dopamine
DAO	diamine oxidase
DBB	diagonal band of Broca
DNA	deoxyribose nucleic acid

DSM-5	diagnostic and statistic manual of mental disorders, edition-5
ECL	extracellular loop
EDS	excessive daytime sleepiness
EPM	elevated plus maze
ERK-1/2	extracellular signal-regulated kinase 1/2
ERNEST	European Research Network on Signal Transduction
EST	expressed sequence tag
F-BODIPY	boron-dipyrromethene-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
FDA	Food and Drug Administration of the United States of America
FP	fluorescence polarisation
G protein	guanosine triphosphate-binding protein
GABA	γ-amino butyric acid
GABAA/BR	GABAA/B receptor
GBD	global burden of diseases
GDP	guanosine diphosphate
GIRK	G protein-coupled inwardly rectifying potassium channels
GPCR	G protein-coupled receptor
GRAFS	glutamate, rhodopsin, adhesion, frizzled/Taste2 and secretin families
GSH	glutathione
GSK3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
GTPγS	guanosine 5'-O-[gamma-thio]triphosphate)
GTS	Gilles de là Tourette syndrome
GWAS	genome-wide association study
H1R/H2R/H4R	histamine H1/H2/H4 receptor
H3R	histamine H3 receptor
HA	histamine
HDC	l-histidine decarboxylase
hH3R	human (Homo sapiens) isoform of H3R, GPCR-97,
HLA	human leukocyte antigen
HNMT	histamine-N-methyl transferase
i.c.v.	intra-cerebroventricular
ICL	intracellular loop
iodoproxyfan	FUB 249
IUPHAR	International Union of Basic and Clinical Pharmacology
LC	locus caeruleus
LHA	lateral hypothalamic area
LTR	lifetime risk
M1R/M2R/M3R	muscarinergic acetylcholine M1/M2/M3 receptor
MAO A/B	monoamine oxidase A/B
MAP	mitogen-activated protein
MAPK	MAP kinase

MBB	marble-burying behaviour
MCH	melanin-concentrating hormone
MDA	malondialdehyde
mean$_{geom}$	geometric mean
Mepyramine	pyrilamine
mH3R	mouse (Mus musculus) isoform of H3R, [UniProtKB identifier: P58406]
MPT	mesopontine tegmentum
mRNA	messenger RNA
MTDL	multitarget-directed ligand
MTL	Multi-Targeting-Ligand
NAMH	N^{α}-methylhistamine
NET	norepinephrine transporter
NMDAR	N-methyl-d-aspartate receptor
ORL1	Opioid receptor-like 1 receptor
ORX	orexin
ORX1R/ORX2R	orexin-1/-2 receptors
ORX-A/ORX-B	orexin-A (hypocretin-1)/orexin B (hypocretin-2)
PAG	periaqueductal grey
PCR	polymerase chain reaction
PD	Parkinson´s disease
pD2	negative logarithm of EC50
PFA	perifornix / perifornical area
PI3K	phosphoinositide 3 kinase
PKA	protein kinase A
PKB	protein kinase B
PPI	prepulse inhibition
PS	Parkinson Syndrom
PWS	Prader–Willi syndrome/Prader-Willi-Syndrom (de.)
RAMH	*(R)*-α-methylhistamine
REM	rapid eye movement
rH3R	rat (Rattus norvegicus) isoform of H3R, [UniProtKB identifier: Q9QYN8]
RNA	ribose nucleic acid
SCN	suprachiasmatic nucleus
SERT	serotonin transporter
SN	substantia nigra
SNc	SN, pars compacta
SNCA	α-synuclein
SNORD	snoRNA cluster
snoRNA	small nucleolar RNA
SNP	single-nucleotide polymorphism
SNr	SN, pars reticulata
SNRPN	small nucleolar riboprotein-N

SNURF	SNRPN upstream reading frame
SOD	superoxide dismutase
SWS	slow-wave sleep
TCB	three-chamber behaviour
teMH	tele-methylhistamine, N$^\tau$-methylhistamine
TM	transmembrane
TMD/H	transmembrane domain/helix
TMN	tuberomammillary nucleus
U.S.A.	United States of America
VLPO	ventrolateral preotic nucleus
VPA	valproate
YFP	yellow fluorescent protein
ZINC	ZINC is not commercial
µOR	µ-opioid receptor
α_2AR	α_2 adrenoceptor
α-FMH	α-fluoromethylhistidine
β_2AR	β_2 adrenoceptor
κOR	κ opioid receptor
σ_1R	σ_1 receptor

Presentation of aminoacids: Throughout this thesis, amino acids within peptide sequences are depicted by one-letter code followed by the numeric index of consecutive position in the peptide chain as recommended by the Commission on Biochemical Nomenclature of International Union of Pure and Applied Chemistry (IUPAC-IUB) (Nomenclature, 1984). Within this scheme, the numbering of histamine follows the recommendations for histidine. Numeric indices of amino acids in transmembrane regions of GPCRs are followed by superscript indices according to Ballesteros & Weinstein while following the recommendations for most conserved aminoacids in TMHs by Baldwin (Baldwin, 1993; Ballesteros et al., 1995).

Appendix

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2019 – 2020	Board-Member of the Organisation Committee
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	ERNEST - European Research Network on Signal Transduction
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2013, May - 2014, November	President
	German Pharmaceutical Students' Association (BPhD e.V.)
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Screening techniques	fluorescent & radiometric approaches, label-free techniques

Düsseldorf, den 01. September 2020   David Reiner-Link

List of publications & presentations

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Metabolic Enzymes Simultaneously to Fight Neurodegenerative Diseases - Histamine H3
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Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf" erstellt worden ist. Weiterhin versichere ich, dass diese Dissertation nicht bereits einer anderen Fakultät vorgelegt worden ist und bisher keine erfolglosen und auch keine erfolgreichen Promotionsverfahren für mich eröffnet wurden.

Düsseldorf, den 01. September 2020   David Reiner-Link

Erklärungen zum Promotionsgesuch

Im Rahmen des Antrags auf Zulassung zum Promotionsverfahren erkläre ich entsprechend §5, Abs.1 der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine-Universität Düsseldorf vom 15.06.2018, dass das Promotionsgesuch für die Erlangung des Grades „Doktor der Naturwissenschaften" (*doctor rerum naturalium*, Dr. rer. nat.) gestellt wird, dass die Disputation in englischer Sprache abgelegt wird und dass auf den Ausschluss der Öffentlichkeit vom Vortrag und von der Befragung im Rahmen der Disputation verzichtet wird.

Düsseldorf, den 01. September 2020   David Reiner-Link