

Aminergic Ligands for Multitargeting in Central Nervous System Disorders

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To my parents

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ABSTRACT

Central nervous system (CNS) disorders, such as depression, Alzheimer's disease (AD), and schizophrenia, continue to present major clinical challenges due to their complex nature. Despite intensive research, drug development has progressed slowly, and current pharmacotherapies often fail to adequately address core symptoms or are limited by side effects. Multitargeting ligands (MTDLs), designed to simultaneously and synergistically modulate multiple biological targets, represent a promising strategy to enhance treatment efficacy. This cumulative dissertation describes the pharmacological characterisation of novel aminergic ligands that, within the multitargeting framework, target dopamine D₂ (D2R), dopamine D₃ (D3R) and/or histamine H₃ receptor (H3R), in combination with serotonin receptors or enzymes such as acetylcholinesterase (AChE) and monoamine oxidase B (MAO-B). Radioligand binding assays, enzyme assays, and in vivo mouse models were employed to assess affinity, selectivity, and physiological effects. Additionally, fluorescent D2R/D3R ligands were developed as potential tools for bioimaging applications and fluorescence assays.

Several novel MTDLs were characterised within the scope of this work. The pimavanserin derivative ST-2300 combines H3R and serotonin receptor subtype 5-HT_{2A} antagonism and demonstrated antidepressant- and anxiolytic-like effects (Publication 1). In a series of benzothiazole derivatives, promising H3R ligands were identified that also inhibit AChE and MAO-B, making them potential drug candidates for AD (Publication 2). Publication 3 showed that the H3R/D2R/D3R-targeting ligand ST-2223 reduces autism-like repetitive behaviours in a mouse model of autism spectrum disorder (ASD). Publications 4 – 6 focus on the pharmacology of dopamine receptors. An extensive library of bitopic ligands was designed based on the antipsychotic drug cariprazine and pharmacologically characterised. Insights were gained regarding D2R/D3R/5-HT_{2A} selectivity, affinity, and the influence of orthosteric and secondary receptor-ligand interactions (Publication 4). In Publication 5, arylpiperazine derivatives were synthesised and tested pharmacologically, combining dopaminergic and serotonergic activity with antioxidant properties. While these compounds showed promising affinities for potential use in psychoses and ASD, they exhibited limited metabolic stability. Finally, in Publication 6, BOPPY-based fluorescent ligands were developed as fluorescent probes for D2R/D3R.

Overall, these results demonstrate that MTDLs targeting histaminergic and dopaminergic systems can make substantial advances in the treatment of CNS disorders. This work highlights the therapeutic and translational potential of the described aminergic ligands and provides a significant contribution to future drug development in neuropsychiatric and neurodegenerative diseases.

Zusammenfassung

ZUSAMMENFASSUNG

Erkrankungen des zentralen Nervensystems wie Depressionen, Morbus Alzheimer und Schizophrenie stellen aufgrund ihrer Komplexität nach wie vor große klinische Herausforderungen dar. Trotz intensiver Forschung schreitet die Entwicklung von Medikamenten nur langsam voran, und die derzeitigen medikamentösen Therapien können die Kernsymptome oft nicht ausreichend behandeln oder sind durch Nebenwirkungen eingeschränkt. Multitargeting-Liganden (MTDLs), die mehrere biologische Ziele gleichzeitig modulieren, bieten einen innovativen Ansatz zur Verbesserung von Wirksamkeit und Verträglichkeit zukünftiger Wirkstoffe.

Diese kumulative Dissertation beschreibt die pharmakologische Charakterisierung neuartiger aminerg wirkender Liganden im Rahmen des MTDL-Konzepts. Zielstrukturen waren Dopamin-D₂/D₃- (D2R/D3R) und Histamin-H₃-Rezeptoren (H3R), ergänzt durch Serotoninrezeptoren und Enzyme wie Acetylcholinesterase (AChE) und Monoaminoxidase B (MAO-B). Zur Bewertung der Affinität, Selektivität und physiologischen Wirkungen wurden Radioligandenbindungs- und Enzymassays sowie ausgewählte in-vivo-Modelle verwendet. Zusätzlich wurden fluoreszierende D2R/D3R-Liganden für mögliche Imaging-Anwendungen entwickelt.

Im Rahmen dieser Dissertation konnten mehrere aminerge MTDLs charakterisiert werden. Das Pimavanserin-Derivat ST-2300 kombiniert H3R mit Serotonin (5-HT_{2A})-Antagonismus und zeigte in vivo antidepressive und angstlösende Eigenschaften (Publikation 1). In einer Reihe an Benzothiazol-Derivaten wurden vielversprechende MTDLs identifiziert, die zusätzlich AChE und MAO-B inhibieren (Publikation 2). In Publikation 3 konnte gezeigt werden, dass der Ligand ST-2223, der als H3R/D2R/D3R MTDL designed wurde, autismus-ähnliche Verhaltensweisen in einem Mausmodell mildert. Eine umfangreiche Bibliothek an bitopischen Cariprazin-Derivaten wurde im Rahmen der Publikation 4 pharmakologisch charakterisiert. So konnten Erkenntnisse über D2R/D3R/5-HT_{2A} Rezeptorselektivität, Affinität und über den Einfluss von orthosterischen/sekundärer Rezeptor-Liganden-Wechselwirkungen erhalten werden. In Publikation 5 wurden Arylpiperazin-Derivate charakterisiert, die dopaminerge und serotonerge Aktivität mit antioxidativen Eigenschaften kombinieren, jedoch nur eine geringe metabolische Stabilität aufweisen. Zuletzt wurden in Publikation 6 BOPPY-basierte Fluoreszenzliganden für D2R/D3R entwickelt, die als Werkzeug für die Bioimaging dienen könnten.

Die vorgestellte Arbeit verdeutlicht das therapeutische und anwendungsbezogene Potenzial von MTDLs, die ein aminerges Wirkspektrum vereinen und liefert einen wichtigen Beitrag zur zukünftigen Arzneimittelentwicklung im Bereich neuropsychiatrischer und neurodegenerativer Erkrankungen.

1 INTRODUCTION

1.1 THE CONCEPT OF MULTITARGETING DRUGS

Modern drug discovery has come a long way since Paul Ehrlich proposed his 'magic bullet concept' in the late 19th century. In this idealised theory, he envisions a highly specific drug that selectively targets a particular disease or pathogen, destroying the disease-causing source while leaving the healthy tissue unaffected. This was based on the idea that diseases have a distinct molecular target that can be exploited for therapeutic reasons. Nowadays, we know that this 'one disease – one target – one drug' paradigm cannot meet the requirements of complex, multifactorial diseases. Still, Ehrlich's drug discovery programme marked a significant milestone in pharmaceutical research as it pioneered the systematic chemical modifications of lead compounds to optimise their biological activity [2]. In opposition to Ehrlich's idea, the concept of multitargeting drugs and polypharmacology has gained attention over the last decades. Multitargeting drugs (MTDs) or multitarget-directed ligands (MTDLs) are therapeutic agents that interact with multiple targets or pathways simultaneously. By engaging multiple targets, MTDs aim to achieve a beneficial polypharmacology with additive or even synergistic therapeutic efficacy [4].

Complex, multifactorial diseases could benefit significantly from MTDs. By definition, complex diseases result from the interplay of various genetic, environmental, and lifestyle factors [5], in contrast to single-gene disorders, such as sickle cell anaemia, which are primarily characterised by a malfunction or mutation in a single gene. Multifactorial diseases, including cancer, neurodegenerative and psychiatric disorders, infectious diseases, and metabolic and cardiovascular conditions, face a tough challenge in efficacious drug development. Despite the emergence of highly potent single-target drugs in recent years, preclinical efficacy could not be sufficiently reproduced in human subjects [6,7]. Given the involvement of multiple signalling pathways or dysregulation of different physiological processes, a modulation of multiple biological targets is a promising approach to restore physiological conditions and achieve higher therapeutic efficacy in complex diseases. Addressing multiple targets may even necessitate lower drug doses due to additive and synergistic effects, facilitating adequate therapeutic outcomes at reduced drug doses and thereby enlarging the therapeutic window [8,9].

The multitargeting strategy can be achieved through either polypharmacy, which involves the combination of several target-specific drugs (combination therapies), or through polypharmacology, which utilises a single drug molecule that addresses multiple targets in a balanced manner [10]. The potential benefits of MTD therapy, as compared to a combination therapy, include a more predictable pharmacokinetic and safety profile

1.1 The Concept of Multitargeting Drugs

(unified onset time, half-life, and metabolism), reduced risk of drug-drug interactions, and lower chances of adverse effects and negative synergistic effects [10]. Additionally, MTDs have a statistically lower probability of developing target-based resistance and offer a reduced treatment complexity due to simplified dosing schedules, greatly enhancing patient compliance. Furthermore, MTDs offer a more straightforward development path, as regulatory requirements for combination therapies are more stringent, often necessitating separate assessments of individual drugs and their combinations, which can delay the evaluation of new therapies and uncover new toxicities [4].

Early MTD discovery took great benefit from serendipitous discoveries. For example, long-established drugs such as aspirin, paracetamol, and metformin exhibit multiple target activities by coincidence. Modern drug discovery approaches intentionally aim to design molecules with multitargeting activity. Morphy and Rankovic have conducted fundamental research in the classification of multitarget agents and the design strategies, as outlined in their pioneering publications [11–13], followed by several other reviews on multitargeting [4,10,14,15].

The key factor of successful MTDL design and development relies on the strategic selection of target combinations. Although in theory, any combination of targets is feasible, multitarget molecules are more readily developed when the targets share close relationships. For example, enzymes belonging to the same protein family, various G protein-coupled receptors with structural similarities, or targets that bind structurally alike ligands present feasible options for target selection. Furthermore, the consideration of cellular and tissue localisation of these targets is crucial, as different localisations introduce additional complexity and challenges to the development process [10]. There are several strategies for identifying suitable target combinations for MTDL: One rational approach involves utilising insights from clinical data. An exemplary case is the development of antipsychotic drugs, where clinical data from dopamine D₂ receptor antagonists, coupled with observations on anti-serotonergic effects, led to the creation of drugs with a functional profile at dopaminergic and serotonergic receptors, minimising side effects and enhancing efficacy [16,17]. Another strategy employs high-content screening or phenotypic screening of target combinations. However, this strategy harbours the impracticality and ethical concerns associated with testing numerous compound combinations in vivo. Instead, utilising cellular, tissue, or non-mammalian animal models proves more convenient, allowing the assessment of compound combinations for synergistic effects [10]. Furthermore, in silico techniques, including statistical approaches, machine learning, and pathway/network pharmacology analyses, represent a third strategy [18,19]. While these in silico techniques are generating

acceptable hypotheses, in vitro or in vivo validations remain irreplaceable. A multidisciplinary approach combining in vitro and in silico methods can provide valuable data on synergy while remaining ethical and cost-effective [10].

Finding a suitable lead structure is another crucial aspect in MTD discovery. Two major strategies are employed in this context. The screening-based approach starts with a single ligand exhibiting noticeable activity for one target and at least one additional, albeit weak activity for another target [15]. Subsequently, in the optimisation process, efforts are directed towards harmonising the affinities for the two (or more) targets. This strategy is particularly useful when knowledge about the pharmacophore of a specific target is limited. The second strategy is a knowledge-based approach that commences with two molecules possessing known target-specific pharmacophores [12,20]. There are three options for designing an MTDL via a knowledge-based approach (Figure 1): linking, fusing, or merging pharmacophores. Linking pharmacophores involves the straightforward connection of pharmacophores through stable or cleavable linkers [21]. However, this method has its limitations, as it often results in the formation of large molecular weight molecules. The potential consequences include diminished bioavailability and a failure to reach desired cellular compartments, and the linker itself may disturb the ligand's interaction with the target, posing challenges to achieving optimal binding [10]. In the fused pharmacophores approach, two pharmacophores are directly combined without the use of a linker [12]. Despite its simplicity, this method gives rise to larger molecules characterised by high lipophilicity. The merging strategy is the most complicated approach, as it requires the amalgamation of two features from different pharmacophores into a single, unified molecule. This results in an overlap that produces a smaller molecule with a lower molecular weight. Notably, this approach aims to satisfy Lipinski's rule of five [22]. Although the rule has limitations and an increasing number of approved drugs fall outside of it, it still provides a useful starting point, particularly in early-stage screenings, for assessing drug-likeness. Despite its complexity, merging pharmacophores holds promise as a strategic avenue for designing multitarget ligands that not only exhibit favourable drug-like properties but also have the potential for enhanced therapeutic efficacy.

1.1 The Concept of Multitargeting Drugs

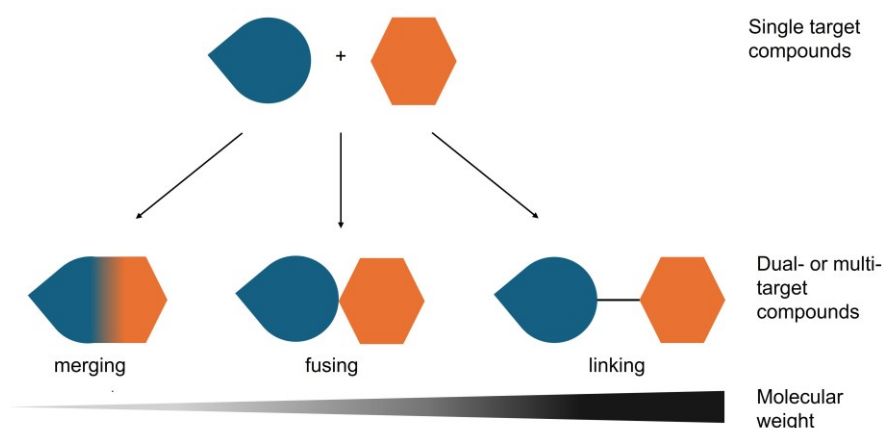


Figure 1: Knowledge-based strategy of designing dual or multitargeting compounds by merging, fusing, or linking pharmacophores of single target compounds, adapted from [13] and [23]. Merging compounds results in lower molecular weights and higher drug-likeness.

MTDLs emerge as valuable choices for the treatment of various multifactorial diseases. Especially in the field of central nervous system (CNS) disorders like neurodegenerative diseases, the discovery and development of new drugs have encountered significant challenges, leading to high failure rates both preclinically and clinically. These failures often stem from inadequate evidence of clinical efficacy, incomplete understanding of disease pathophysiology, and the lack of suitable predictive animal models [7]. Given the limited success of single-agent treatments, there is a growing consensus that combination therapies or MTDLs might offer the best hope for CNS conditions resistant to current therapeutic interventions.

Despite progress in elucidating disease mechanisms, our understanding of the roles of specific targets in neuronal systems remains incomplete [7]. The complexity of CNS diseases and the frequent clinical trial failures underscore the need for a deeper insight into the brain's biochemical milieu. Neurotransmitters are crucial for neuronal development, differentiation, and survival [24]. Therefore, imbalances in neurotransmitter levels result in disrupted brain functions, manifesting in a range of neurological and psychological disorders. Histamine, a neurotransmitter whose important role in brain physiology was frequently overlooked, may offer new therapeutic avenues. In contrast, dopamine, a neurotransmitter involved in several executive and behavioural functions, has been extensively studied, yet treatments targeting its pathways remain suboptimal. The following chapters will further explore both neurotransmitter systems, their roles in disease, and the potential of MTDLs as innovative therapeutic strategies.

1.2 THE HISTAMINERGIC SYSTEM

Histamine (HA) is a biogenic amine that is produced in various cells, including mast cells, parietal cells, and neurons. Originally identified as a hormone and tissue mediator, HA's potential for neuronal activity was under debate but hinted at through the sedative side effects of antihistamines such as the early first-generation H_1 receptor antagonists. In the 1970s, emerging evidence began to highlight the presence of HA and histaminergic neurons within the brain. However, it was not until 1984 that the neuronal histaminergic system gained widespread acceptance [25]. This recognition came with the immunohistochemical validation of the tuberomammillary nucleus (TMN) in the hypothalamus as the sole source of histaminergic neurons [26,27]. These neurons project widely throughout the brain, indicating the extensive role of HA in various cellular, synaptic, and behavioural functions. Histaminergic innervations in mammalian brain reach the cerebral cortex, amygdala, striatum, substantia nigra, hippocampus, thalamic areas, cerebellum, the posterior pituitary gland, retina, and spinal cord with different densities depending on the brain area [25] (Figure 2).

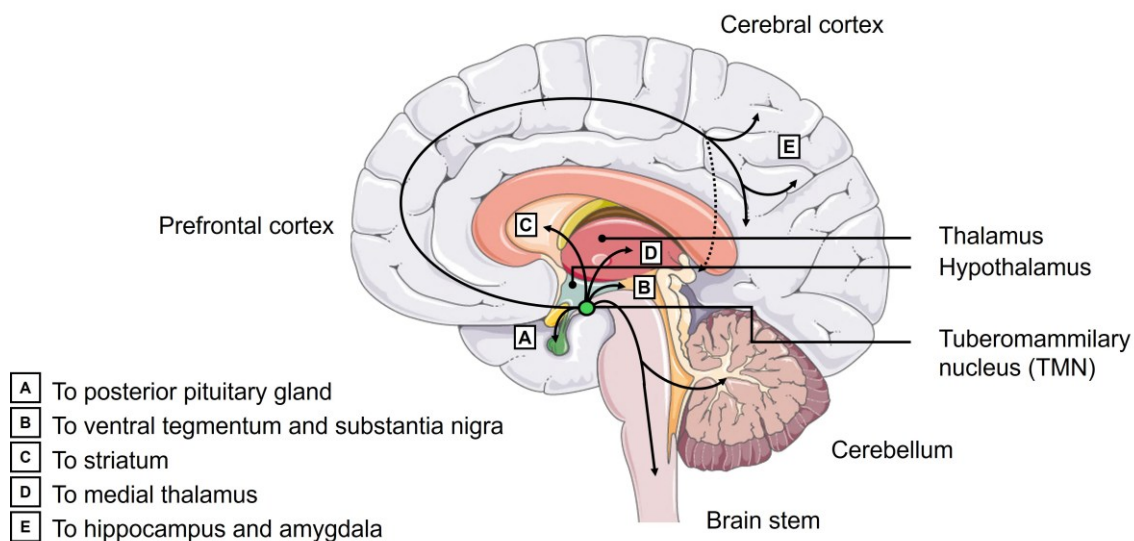


Figure 2: Histaminergic projection throughout the brain: Histaminergic neurons originate from the tuberomammillary nucleus, projecting to several brain regions with varying density. Moderate to dense projection pathways reach the posterior pituitary gland (A), the ventral tegmentum (B), the striatum (C), the medial thalamus (D), hippocampus and amygdala (E), as well as the brain stem and cerebellum (adapted from [28]).

Within the brain, HA's biosynthesis occurs in histaminergic neurons and mast cells [29]. HA is synthesised from L-histidine via decarboxylation by histidine decarboxylase (HDC) (Figure 3). It is stored in vesicles until release into the synaptic cleft upon calcium-dependent depolarisation of nerve terminals. Within the synaptic cleft, the degradation of HA involves the enzyme HA *N*-methyltransferase (HNMT), which converts HA into *N*^l-methylhistamine [30]. This metabolite is subsequently oxidised by monoamine oxidase B (MAO-B) to 1-methyl-4-imidazoleacetic acid. In the periphery, HA

1.2 The Histaminergic System

is converted to imidazole acetaldehyde by diamine oxidase (DAO). The activity of DAO in the brain is relatively low [31].

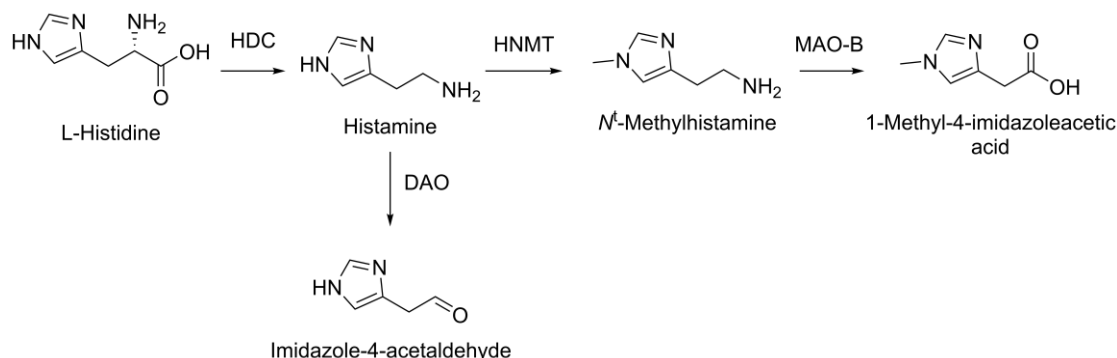


Figure 3: Histamine (HA) biosynthesis and degradation: HA is synthesised from L-histidine by histidine decarboxylase (HDC). Within the synaptic cleft, HA is degraded either by HA *N*-methyltransferase (HNMT) or in the extracellular space by diamine oxidase (DAO).

The neurophysiological and hormonal effects of HA are mediated through the activation of four G protein-coupled receptors (GPCRs). GPCRs are a huge and diverse class of membrane proteins that play a crucial role in signal transduction and are involved in a variety of physiological processes [32,33]. Their structural hallmarks include seven transmembrane helical domains, three extracellular loops, three intracellular loops, an extracellular N-terminal region, and an intracellular C-terminal region. GPCRs possess an extracellular/transmembrane binding site for ligands and an intracellular binding site for GTP-binding proteins (G proteins). Depending on the type of G protein ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$), different cell signalling processes are activated or inhibited.

Among GPCRs, HA receptors belong to the class A, rhodopsin-like GPCR. The first identified HA receptor, the histamine H_1 receptor ($H1R$), was discovered in the early 20th century, leading to the subsequent discovery of H_2 , H_3 , and H_4 receptors ($H2R$, $H3R$, $H4R$). The four receptors differ in tissue localisation, HA affinity, and signalling mechanisms (Figure 4). The $H1R$ is implicated in allergic responses and the regulation of sleep-wake cycles, while the $H2R$ is enriched in the stomach, where it stimulates gastric acid secretion. The most recently identified $H4R$ [34] has been found in the periphery on hematopoietic cells, i.e., mast cells, eosinophils, or leukocytes, playing a role in immune responses and inflammation. The presence of the $H4R$ in the brain remains a subject of debate. To date, there is no conclusive evidence of its neuronal presence, although some studies suggest its existence in the mouse brain [35]. While $H1R$, $H2R$, and $H4R$ mediate diverse peripheral and central effects of HA, the $H3R$ occupies a unique position in the CNS with its distinct localisation and signalling properties. The following chapter will focus on the $H3R$, its pharmacological profile, and the ligands developed to modulate its function.

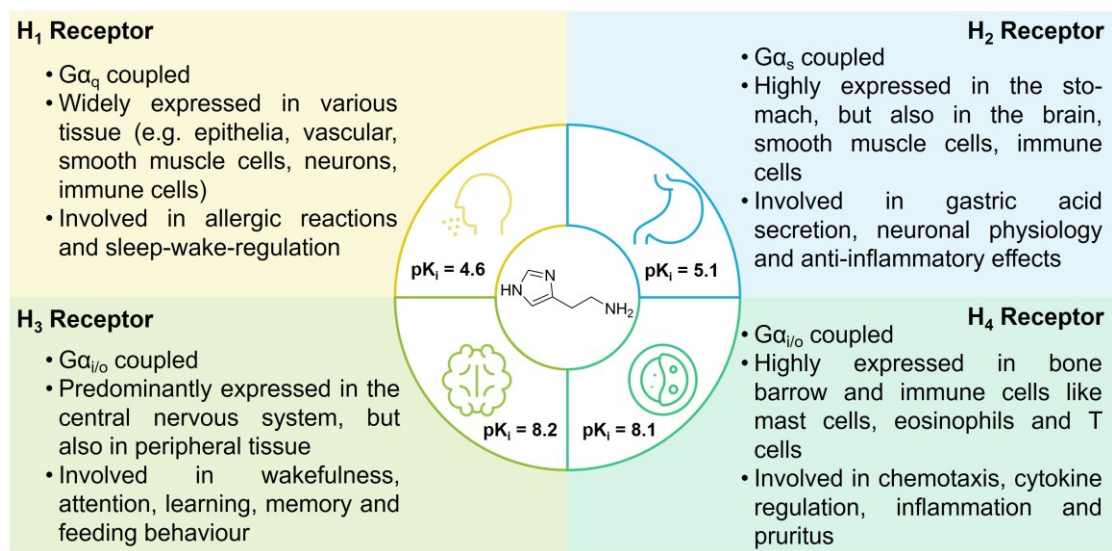


Figure 4: HA and its receptors: HA is a crucial biogenic amine involved in various physiological functions, including immune responses, gastric acid secretion, and neurotransmission. The effects of HA are mediated through its interaction with four types of receptors, each with unique functions and distribution throughout the body. H1R and H2R have a lower affinity for the endogenous ligand HA compared to H3R and H4R [36].

1.3 THE HISTAMINE H₃ RECEPTOR AND ITS LIGANDS

The H3R, discovered in 1983 by Arrang et al. [37], added a new dimension to the existing knowledge of the H1R and H2R. The H3R is predominantly expressed in the CNS, particularly on neuronal cells, with varying expression levels reported in the thalamus, different parts of the basal ganglia, cortex, and hippocampus. Due to its expression in relevant brain regions, it plays a central role in modulating the sleep-wake cycle, food intake, and cognitive processes [38]. The H3R can be found on presynaptic nerve terminals of histaminergic neurons (autoreceptor function), regulating the synthesis and release of HA into the synaptic cleft through a negative feedback mechanism [39,40] (Figure 5). On the other hand, as a presynaptic heteroreceptor on non-histaminergic neurons, H3R activation reduces the release of other neurotransmitters, including dopamine, γ -aminobutyric acid (GABA), serotonin (5-HT), noradrenaline, and acetylcholine [36]. The H3R shows a high degree of constitutive activity. Ligands exerting antagonism or inverse agonism promote HA release and thereby postsynaptic HA signalling events, leading to several biological effects like wakefulness, improved cognition, memory, and learning. Understanding the role of the H3R in modulating neuronal networks and neurotransmitter release marked a key step toward exploring its potential as a therapeutic target in neurological and psychiatric disorders.

1.3 The Histamine H3 Receptor and Its Ligands

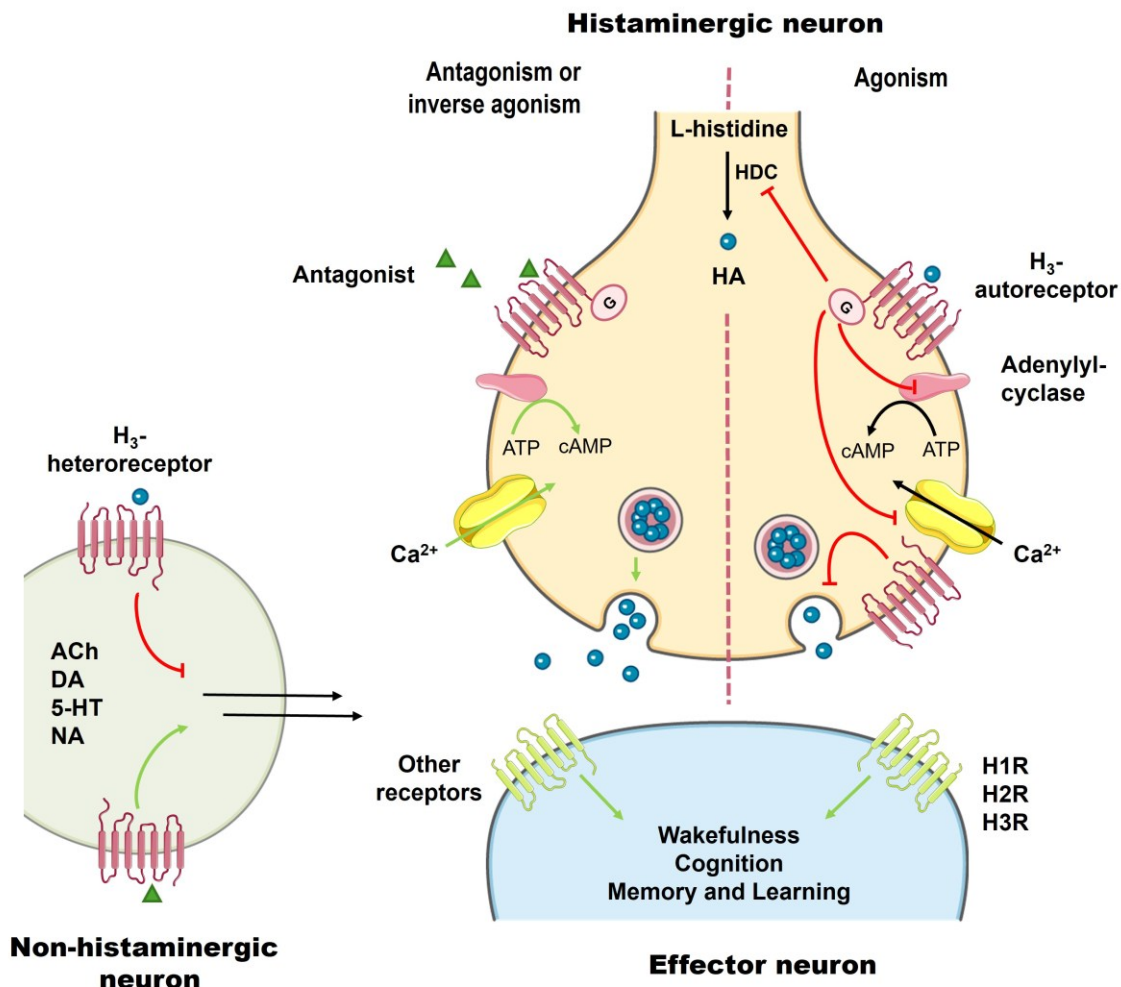


Figure 5: HA is biosynthesised from L-histidine by histidine decarboxylase (HDC) and stored in vesicles. The H3R can be expressed pre- or postsynaptically. Pre-synaptic H3R on histaminergic neurons, also known as H₃-autoreceptor, can modulate histaminergic neurotransmission dependent on the ligand binding to it. HA binding to H3R activates G protein-dependent events, including inhibition of HDC, adenylyl cyclase, Ca²⁺-influx, and HA release into the synaptic cleft. This effect is called a negative feedback mechanism. Pre-synaptic H3R on non-histaminergic neurons, so-called H₃-heteroreceptors, promote the release of other neurotransmitters like acetylcholine (ACh), dopamine (DA), serotonin (5-HT), or noradrenaline (NA) when bound by antagonists/inverse agonists. Modified from [28].

Genomic Organisation and Isoforms

The human H3R gene (HRH3, GenBank ID 11255) is located on chromosome 20q13.33. This gene comprises three introns and four exons [41], a structure that is relatively uncommon among GPCRs. The H3R shares a low sequence identity with H1R and H2R but an overall sequence identity of 38% with H4R, with up to 54% within the transmembrane [36,42]. The H3R gene transcription results in multiple isoforms due to alternative splicing. The full-length receptor is composed of 445 amino acids. To date, at least 20 human isoforms have been identified, not all of which are functional. The functional hH3R isoforms with a classic 7-transmembrane structure, including hH3R-453, -445, -415, -413, -373, -365, and -329, show alterations in key areas like the intracellular loop 3 (IL3) and C-terminal tail, crucial for signalling and regulation [43].

Changes in IL3 are linked to autoreceptor function (e.g., hH3R-413) [44] and constitutive activity (e.g., hH3R-365) [45] of the H3R. Co-expression of the H3R isoforms occurs in the human brain but shows region-specific expression patterns at the transcriptome level. hH3R isoforms 445 and 365 are the most expressed isoforms within the human brain [46]. hH3R-445 is highly expressed in the thalamus, caudate nucleus, and cerebellum, with moderate levels in the amygdala, hippocampus, cerebral cortex, and substantia nigra. While hH3R-415 and hH3R-365 have similar high expression in these areas, they have low expression in regions like the amygdala, hippocampus, substantia nigra, and cerebral cortex [41]. The physiological implications of these expression patterns across brain regions are still not fully understood. The study of H3R heterogeneity is an active field of research [47]. This interest is driven by the observation that shorter, functional isoforms exhibit differences in their pharmacological properties, ligand potencies, signalling events, and expression patterns [36]. Isoform bias is a form of differential signalling, where different receptor isoforms selectively activate or attenuate signalling pathways. For the hH3R, isoform bias has been proven [43,46,47]. Additionally, differential ligand affinity and constitutive affinity among hH3R isoforms were observed. From a therapeutic point of view, the broad modulation of hH3R isoforms in the CNS by H3R-targeting drugs might lead to on-target side effects, affecting normal physiology. Targeting specific hH3R isoforms or pathways could offer a more targeted approach.

H3R Signal Transduction

The H3R is a GPCR linked to a $G_{\alpha_{i/o}}$ protein. When activated by HA or other agonists, the H3R undergoes a series of conformational changes. These changes facilitate the exchange of GDP for GTP within the associated G protein, leading to the subsequent dissociation of the G_{α} subunit from the $G\beta/\gamma$ dimer and the receptor. The GTPase activity inherent to the G_{α} subunit catalyses the hydrolysis of GTP to GDP as a form of self-regulation, reverting the subunit to its inactive state, which then reassociates with the receptor and $G\beta/\gamma$ subunits. Following receptor activation, the ligand-bound GPCRs are phosphorylated by G protein-coupled receptor kinases (GRKs), which promotes the recruitment and binding of β -arrestins. Bound β -arrestins reduce G protein re-association, thereby desensitising the receptor. Additionally, it initiates clathrin-mediated receptor internalisation, which can either lead to receptor recycling after dephosphorylation or receptor degradation. Both processes are part of the downregulation of receptor signalling and availability.

1.3 The Histamine H3 Receptor and Its Ligands

After receptor activation, the dissociated subunits initiate distinct cellular signalling pathways (Figure 6). The activated $G\alpha_{i/o}$ inhibits the enzyme adenylate cyclase (AC), thereby reducing the synthesis of cyclic AMP (cAMP) from ATP. Given that cAMP is an allosteric activator of protein kinase A (PKA), the inhibition by $G\alpha_{i/o}$ results in reduced activation of this secondary effector protein. PKA itself is involved in a variety of biological functions, influencing synaptic plasticity, gene expression, and behavioural processes [48]. Activation of the H3 receptor inhibits the Na^+/H^+ exchange (NHE) in sympathetic nerve terminals, a mechanism involved in restoring intracellular pH [49]. This effect is thought to be $G\alpha_{i/o}$ -dependent [50], although the underlying mechanism remains to be fully elucidated.

Besides $G\alpha_{i/o}$ mediated effects, the heterodimer $G\beta/\gamma$ initiates several other signalling events. The $G\beta/\gamma$ subunit mediates a decrease in Ca^{2+} influx through voltage-gated calcium channels (VGCC) and stimulates the opening of G protein-gated inwardly rectifying potassium channels (GIRK), thereby attenuating neurotransmitter exocytosis and decreasing neuronal excitability [49,51]. $G\beta/\gamma$ also activates the PI3K/AKT/GSK3 β (phosphatidylinositol 3-kinases/ protein kinase B/ glycogen synthase kinase-3 beta) pathway, influencing neuronal migration, neuroprotection, and cell survival [49,52]. Activation of the mitogen-activated protein kinase (MAPK) pathway, which plays a role in cell growth, differentiation, neuronal plasticity, and memory, occurs upon H3R stimulation and is believed to depend on the $G\beta/\gamma$ complex, although β -arrestins might also participate in MAPK activation [53]. Activation of H3R stimulates phospholipase A₂ (PLA₂) via a $G\beta\gamma$ -dependent pathway. In cardiac sympathetic nerves, H3R-triggered $G\beta\gamma$ release activates MAPK, leading to PLA₂ phosphorylation and arachidonic acid (AA) release [54]. Additionally, direct stimulation of PLA₂ by $G\beta\gamma$ subunits was previously demonstrated [55]. Activation of PLA₂ also releases docosahexaenoic acid and lysophospholipids. Those are known precursors to lipid mediators like cytotoxic 4-hydroxynonenal, which is known to be involved in apoptotic neuronal death and is elevated in disorders such as Alzheimer's and Parkinson's disease [56]. Furthermore, PLC pathway activation has been observed post-H3R activation, leading to inositol 1,4,5-trisphosphate (IP3)-dependent intracellular calcium release from the endoplasmic reticulum. This effect is typically associated with $G\alpha_q$ -coupled receptors but is also partially $G\beta/\gamma$ dependent. Notably, this pathway's activation is highly dependent on the isoform variation, with calcium mobilisation observed in H3R-445 but not H3R-365 isoforms (isoform bias) [51].

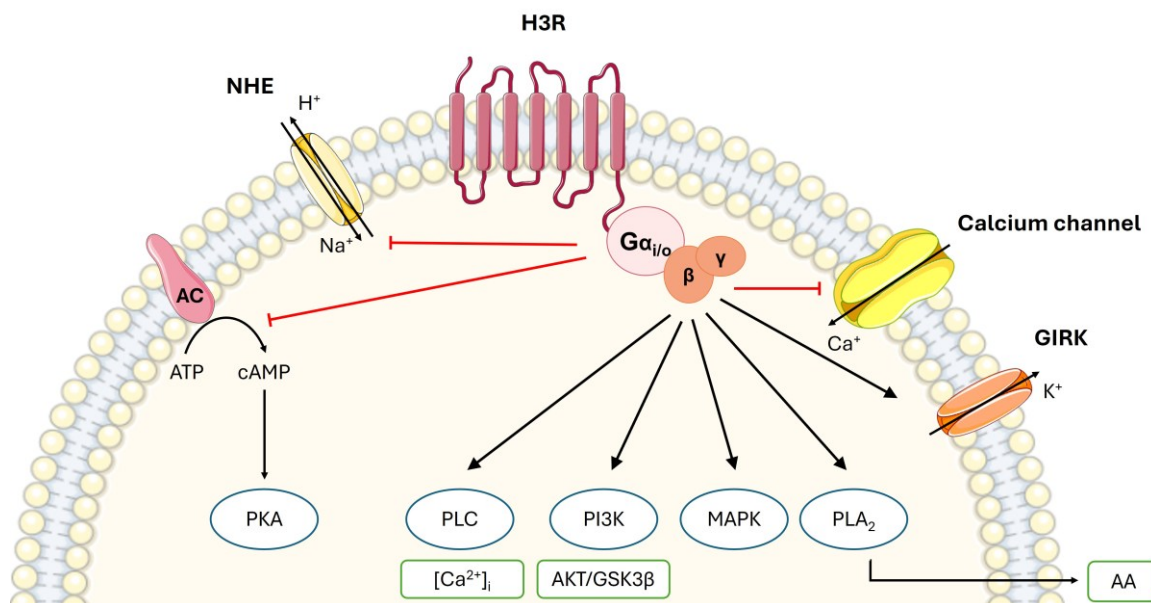


Figure 6: Signalling pathways: H3R activation after binding of HA leads to the dissociation of the G-protein from the receptor. The heterotrimeric protein dissociates into two subgroups, $G\alpha$ and $G\beta\gamma$, both involved in different signalling pathways. $G\alpha_{i/o}$ mediated signalling includes the inhibition of AC, leading to downregulation of cAMP-induced PKA activation, and the inhibition of Na^+/H^+ exchange. On the other hand, the $G\beta\gamma$ subunit activates different pathways, including PLC, PI3K, MAPK, PLA_2 , as well as the inhibition of calcium influx and the stimulation of potassium efflux via GIRK. PLC activation initiates intracellular Ca^{2+} influx, while PI3K activation leads to downstream activation of the AKT/GSK3 β pathway. PLA_2 activation increases the release of lipid second messengers like AA. Modified from [28]. Abbreviations: AC: adenylyl cyclase, AKT: protein kinase B, AA: arachidonic acid, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, GIRK: G protein-coupled inwardly rectifying potassium channel, GSK3 β : glycogen synthase kinase 3 beta, NHE: Na^+/H^+ exchange, MAPK: mitogen-activated protein kinase, PI3K: phosphoinositide 3-kinase, PKA: protein kinase A, PLA_2 : phospholipase A2, PLC: phospholipase C.

In addition to $G\alpha$ - and $G\beta\gamma$ -mediated signalling, GPCRs can also signal through β -arrestin-dependent mechanisms. This topic has been extensively explored by the research group of Lefkowitz et al. [57,58]. The β -arrestin-dependent pathways have been proven for several 7-TM receptors, including angiotensin type 1A [59] and β_2 -adrenergic receptor (β_2AR) [60,61]. For the H3R, desensitisation and internalisation occur in a GRK- and clathrin-dependent manner [62], and β -arrestin2 recruitment has been confirmed across various H3R isoforms [46,47]. However, β -arrestin-mediated signal transduction has not yet been demonstrated for this receptor.

An important characteristic of the H3R is its constitutive activity, meaning it can transition to an active state and activate second messengers independently of agonist binding. True antagonists are ligands that bind to the receptor without influencing this basal activity. In contrast, ligands that decrease the receptor's basal activation state are termed inverse agonists. Some signalling events, such as cAMP production, AA release [63], and the PI3K pathway [48], are more prone to constitutive receptor activation. In contrast, events like β -arrestin recruitment or MAPK activation exhibit no or a low degree of constitutive activity [47,48]. The basal activity of H3R makes it an intriguing target for diseases that are characterised by abnormal neurotransmitter concentrations.

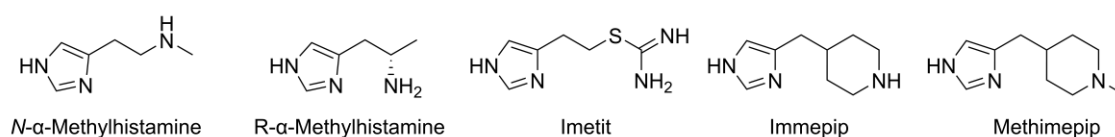
1.3 The Histamine H3 Receptor and Its Ligands

GPCR ligands may demonstrate selectivity with respect to the signalling cascades they stimulate or reduce, which is referred to as biased signalling [64,65]. Currently, it is generally recognised that GPCRs can adopt different receptor conformations depending on which ligand has bound, which can cause a bias for certain signalling pathways or an attenuation of others. Biased ligands can be used to selectively modulate signalling pathways that are abnormal in disease states without affecting clinically unaffected signalling pathways.

H3R Ligands

For over four decades, the field of H3R ligands has experienced a high research intensity, which culminated in the approval and marketing of Pitolisant (Wakix®), the pioneering H3R antagonist/inverse agonist for the treatment of narcolepsy. In the early stages of H3R ligand discovery, HA was used as the structural template. The first H3R agonists were structurally similar due to the retained imidazole ring, e.g., R- α -methylhistamine and *N*- α -methylhistamine (Figure 7). [3H]-*N*- α -methylhistamine is a frequently used radiolabelled ligand for competitive binding experiments due to its high affinity for rat H3R (rH3R) [66]. Further drug development led to the discovery of imetit and immepip with improved lipophilic properties. They are both characterised by a high affinity for rH3R, which is why they have established themselves as a practical pharmacological tool [66]. Nevertheless, they all have a low HA receptor selectivity and a low oral bioavailability and are rapidly metabolised [67]. This limits their in vivo practicability; however, they were historically useful in vitro tools. After the discovery of the H4R, some H3R ligands previously thought to be selective had to be reclassified. Interestingly, the methylated derivative of immepip, methimnepip, shows excellent potency and also selectivity to H3R (>2000 fold selectivity over H1R, H2R, and H4R) [36]. Currently, there are no clinically relevant H3R agonists, but possible future indications are pain, inflammation, migraine, or insomnia [67].

Imidazole-containing H3R agonists



Imidazole-containing H3R antagonists

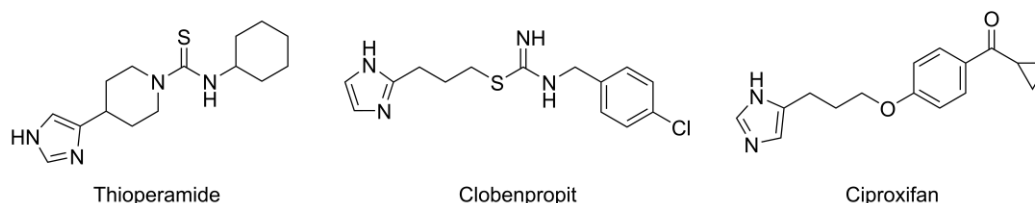


Figure 7: Early imidazole-containing H3R ligands.

The first H3R antagonists were imidazole-based ligands, with the most prominent examples being thioperamide, clobenpropit, or ciproxifan (Figure 7). The imidazole ring was maintained due to the fact that initial studies had shown a drastic drop in potency upon imidazole replacement [68,69]. Thioperamide was broadly used as an H3R antagonist in behavioural animal models, as it is particularly potent on rH3R (rH3R K_i = 4.3 nM). The same applies to clobenpropit, which found application in different in vivo models of, e.g., Alzheimer's disease, working memory, or seizure [70–72]. However, both compounds have a profound activity at H4R [73] (Table 1). Ciproxifan, though, is more selective towards H3R and has a good oral bioavailability; thus, it was extensively used as a reference compound in preclinical animal models. However, these ligands have the same disadvantages as other imidazole-containing compounds: CYP450 inhibition, low CNS penetration, off-target activity, and low HA receptor subtype selectivity [74].

Table 1: Binding affinities (pKi) for human HA receptor subtypes (hH1–4R) for selected agonists and antagonists. Data from [36].

Ligand	hH1R pK _i	hH2R pK _i	hH3R pK _i	hH4R pK _i
Imetit	4.30	5.10	9.29	8.48
Immepip	4.79	<3.5	9.44	7.79
Methimipip	-	-	9.00	5.70
Thioperamide	3.88	4.27	7.6	7.04
Clobenpropit	5.45	5.47	9.0	7.98
Ciproxifan	<5	<5	7.1	5.73

A milestone was the replacement of imidazole with bioisosteric structural motifs like pyrrolidine and piperidine rings. Those non-imidazole-containing ligands display a wide range of functional groups, but they share certain similarities within their structure.

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This general blueprint of an H3R antagonist/inverse agonist was identified by several structure-activity relationship (SAR) studies [75,76]. The blueprint can be divided into four parts (Figure 8): The western part contains a basic moiety, often a tertiary amine, that is crucial for receptor binding [77]. This basic structure is connected to an aliphatic linker that links the basic moiety with the core region. The core often contains a lipophilic/aromatic structure and a heteroatom connecting the linker and core. In the eastern part of the pharmacophore, several substitutions with different functionalities are feasible, e.g., lipophilic, basic, polar, or even acidic groups. The eastern part has been frequently used to incorporate different moieties with the aim of enhancing H3R affinity [76] and fine-tune pharmacological properties.

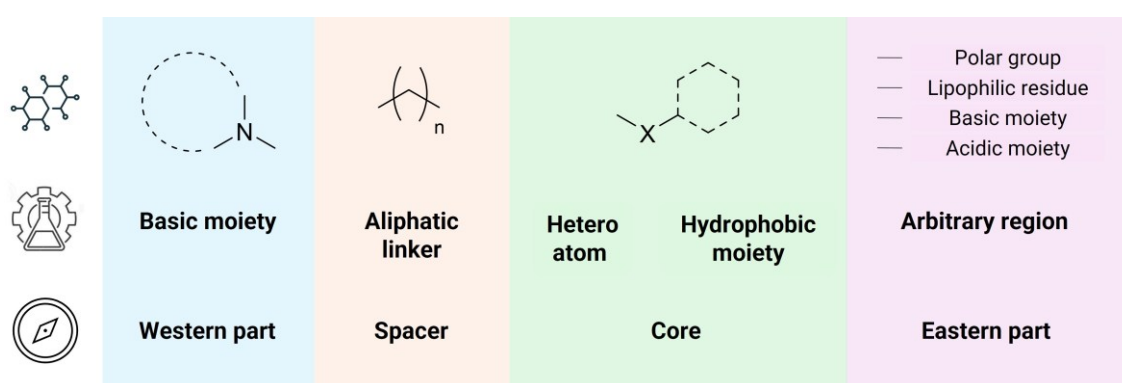


Figure 8: Blueprint of typical structural components of non-imidazole H3R antagonists, illustrating the western basic moiety, aliphatic spacer, central core with heteroatoms and hydrophobic groups, and a variable eastern region. Modified from [76].

Detailed insights into receptor-ligand interactions and ligand orientation within the binding pocket can be obtained through X-ray crystallography or cryo-electron microscopy (Cryo-EM), but such data were long unavailable for the H3R. The crystal structure of the H3R was published by Peng et al. [78] only recently. In 2024, cryo-EM structures (Figure 9) of HA receptors in complex with various G-proteins and ligands were reported by Shen et al. [79] and Zhang et al. [80]. Prior to these advances, knowledge on receptor-ligand interaction was primarily predicted through homology modelling and molecular docking studies [77,81–84]. These computational methods are valuable tools for studying proteins with little experimental data. However, their accuracy is limited, and the use of different homology models and docking procedures may produce variable or even contradictory information on ligand binding. Nonetheless, evidence from crystal structure, cryo-EM, and homology modelling has highlighted key features of ligand interactions, including: (i) a crucial ionic salt bridge between aspartic acid (Asp114/D_{3.32}) and/or glutamic acid (Glu206/E_{5.56}) and protonated basic groups of the ligand, (ii) hydrophobic interactions (π -stacking, CH- π , cation- π) with aromatic amino residues facing the binding site, and (iii) hydrogen bonds between hetero atoms in the linker or arbitrary region with, e.g., tyrosine side chains.

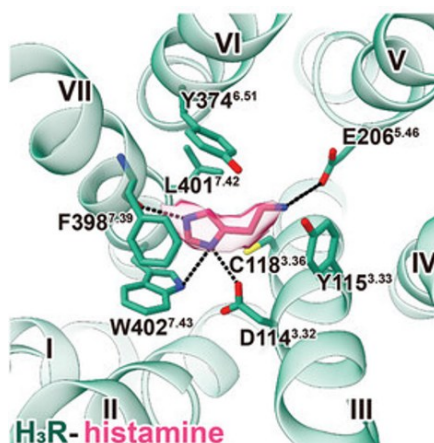
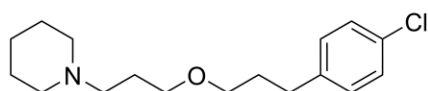


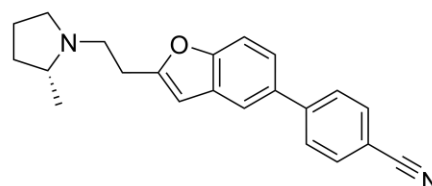
Figure 9: Cryo-EM structure of the H3R bound to HA (magenta), showing key interactions with Asp114 (D3.32) and Glu206 (E5.46), supported by aromatic residues that stabilise ligand orientation in the binding pocket. Picture from Shen et al. [79] Copyright © 2024 The Authors. Advanced Science published by Wiley-VCH GmbH CC4.0 <https://creativecommons.org/licenses/by/4.0/>

Following the first potent non-imidazole H3R antagonists developed in 1998 [85], the number of compounds with pyrrolidine, piperidine, piperazine or diazepine scaffolds expanded steadily in the early 2000s [86–88], with several entering clinical trials for sleep and cognitive disorders, attention deficit hyperactivity disorder (ADHD), Alzheimer's disease, and allergic rhinitis [36] (Figure 10).

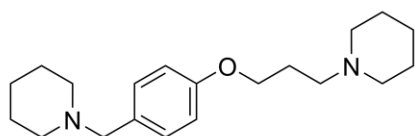
Non-imidazole containing H3R antagonists



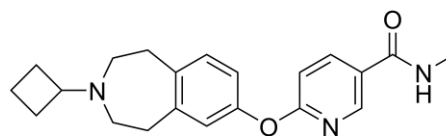
Pitolisant
hH3R K_i = 5 nM



ABT-239
hH3R K_i = 0.45 nM



JNJ-5207852
hH3R K_i = 0.6 nM



GSK-189254
hH3R K_i = 0.7 nM

Figure 10: Prominent examples of clinically relevant non-imidazole H3R antagonists [36].

Among them, Pitolisant – also known as tiprolisant or BF-2649 – remains the most prominent, and is to date the only marketed H3R drug, approved for narcolepsy with or without cataplexy (Wakix®) and for obstructive sleep apnoea in adults with excessive daytime sleepiness (Ozawade®). Pitolisant promotes its wake-promoting effects through blockage of presynaptic auto- and heteroreceptors, enhancing histaminergic neurotransmission as well as release of other neurotransmitters. Lately,

1.3 The Histamine H3 Receptor and Its Ligands

Pitolisant was tested in a Phase III study for safety and efficacy in paediatric narcolepsy (age 6-17) with/without cataplexy, with a beneficial outcome on EDS and cataplexy symptoms [89]. The safety profile was in accordance with that observed in adult patients. Furthermore, Pitolisant is also undergoing or has recently completed trials for idiopathic hypersomnia (Phase III, NCT05458128), restless-legs syndrome (Phase IV, NCT05581576), autism spectrum disorder (ASD) (Phase II, NCT05953389), Prader-Willi syndrome (Phase II, NCT04257929), and alcohol use disorder (Phase I, NCT04596267). Additionally, the effects of Pitolisant on cognitive and emotional processing in healthy individuals are being explored in an academic study at the University of Oxford (NCT05849675).

Beyond Pitolisant, numerous preclinical studies have demonstrated that H3R antagonists/inverse agonists can improve memory and learning in animal models [28,74] and elevate neurotransmitter levels across different brain regions [90,91]. Given this broad therapeutic potential, HA neurotransmission and the H3R have emerged as promising targets in neuropsychiatric and neurodegenerative diseases, which will be discussed in the following two chapters.

1.3.1 DEPRESSION AND ANXIETY

MAJOR DEPRESSIVE DISORDER

Major depressive disorder (MDD) is a well-documented mental health condition marked by significant disturbances in mood, affect, neurovegetative functions, cognition, and psychomotor activities [92]. MDD is not only a leading problem in public health globally but also the primary cause of mental-health-related disability [93]. Its global prevalence is around 4.4% [94], illustrating its widespread impact. MDD exhibits a higher occurrence in women than in men and is more common in individuals with other medical conditions than in those without [95]. For both genders, the typical onset age for MDD is around 25, with peak risk spanning from mid-adolescence to the early 40s [3]. The major symptoms include depressed mood and anhedonia, a certain state of despair, hopelessness, fatigue, and loss of concentration. In extreme cases, suicidal ideation may occur [96]. The disorder significantly undermines personal relationships, employment opportunities, and cognitive and educational ability, affecting various facets of an individual's life. The pathophysiology of MDD is not yet fully understood; however, it is widely accepted that MDD cannot be explained by a single mechanism but rather arises from a multifactorial, complex cause. Several factors might contribute to disease onset, including genetic predisposition (heritability), environmental and epigenetic influences (childhood trauma, epigenetic changes), neurobiological changes (monoaminergic system disturbances,

neuroinflammation, impaired neurogenesis), and structural or functional brain changes (hippocampal volume reduction, altered brain networks) [3].

Depression is a prevalent comorbidity among chronic medical conditions including neurological, metabolic, cardiovascular disorders, and cancer. This comorbidity brings pronounced clinical implications due to the additive or synergistic negative impact on overall health [97]. Notably, the presence of comorbid depression is often associated with a worse prognosis and an increase in mortality rates. The mechanisms underlying this co-occurrence are multifaceted, involving coincidental overlaps, therapy-related effects, behavioural factors, and shared biological processes [95]. Conversely, patients with MDD are at higher risk of developing cardiovascular, metabolic diseases, and dementia, including Alzheimer's disease [98,99] (). Cognitive impairment is a significant concern in patients with MDD, commonly affecting executive functions, memory, and

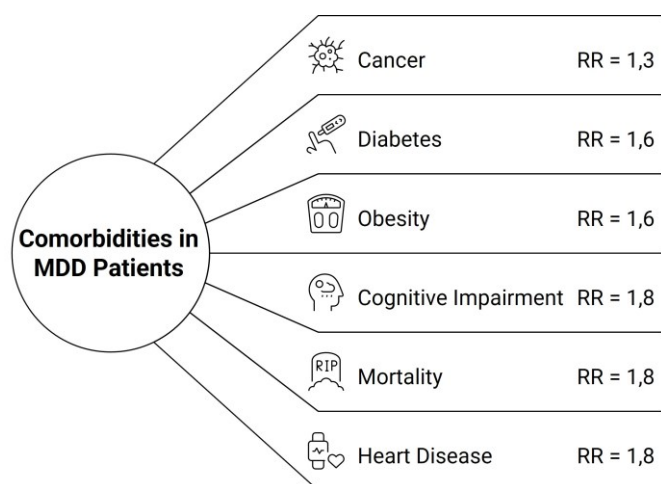


Figure 11: Comorbidities of MDD patients: The relative risk (RR) of developing one of the above-mentioned comorbidities is increased in MDD patients compared to those without depression, which is the result of a meta-analysis from longitudinal studies [1]. Modified from [3].

attention. These neurocognitive impairments play a crucial role in the psychosocial functioning and overall quality of life for affected individuals [3]. Even after achieving remission, patients may continue to experience subtle impairments in executive control and memory, highlighting the need for comprehensive treatment strategies that address both the emotional and cognitive aspects of depression.

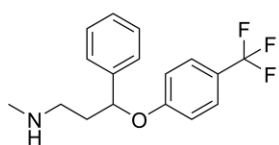
Disease Management

Treatment of MDD involves a psychosocial approach, encompassing psychotherapy, social support, and/or pharmacotherapy. However, for moderate to severe cases of depression, medication is considered necessary [100]. In terms of pharmacotherapy, the main goal is to enhance neurotransmission, especially serotonergic and/or noradrenergic neurotransmission by inhibition of reuptake transporters or certain receptor blockage. First-line treatments for depression include selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), bupropion (a norepinephrine-dopamine reuptake inhibitor), or vortioxetine (a multimodal receptor antidepressant) [101] (Figure 12). SSRIs and SNRIs are generally well-tolerated; however, they are not devoid of adverse effects like headaches, nausea, dizziness, gastrointestinal symptoms, and long-term effects like

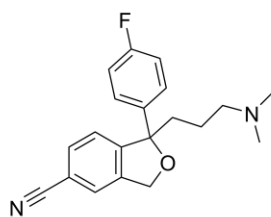
1.3 The Histamine H3 Receptor and Its Ligands

sexual dysfunction, loss of libido, anxiety, sleep disturbances, weight gain, or agitation [3,100]. Furthermore, these medications often require time to manifest benefits, and achieving full recovery might necessitate a combination of multiple drugs.

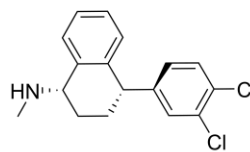
Tricyclic antidepressants, such as amitriptyline or imipramine (Figure 12), have been the gold standard of antidepressant treatment for a long time. This class of antidepressants inhibits the uptake of serotonin, norepinephrine, or both, but also blocks histaminergic, cholinergic, and adrenergic receptors, leading to a profound side effect profile and possible lethal poisoning by overdose [102,103]. It is noteworthy that a significant proportion of MDD patients, ranging from a third to a half, exhibit resistance or only partial responses to antidepressants [100]. This situation underscores an urgent need not only for the development of new and effective drugs but also for a deeper understanding of their modes of action.

SSRI

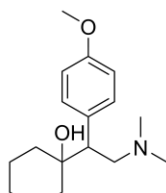
Fluoxetine



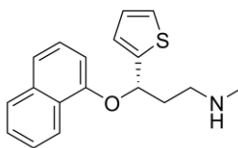
Citalopram



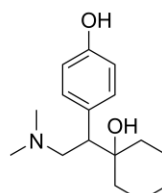
Sertraline

SNRI

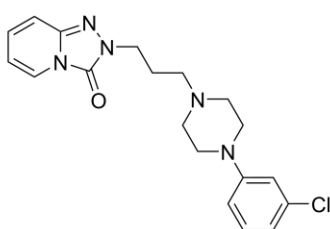
Venlafaxine



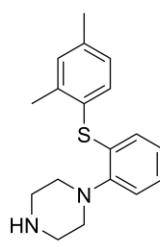
Duloxetine



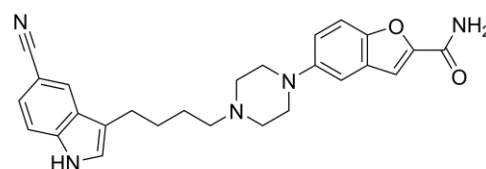
Desvenlafaxine

Serotonin modulators

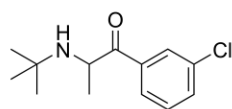
Trazodone



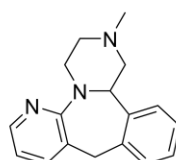
Vortioxetine



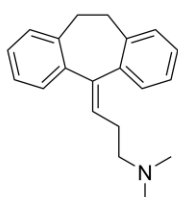
Vilazodone

Atypical antidepressants

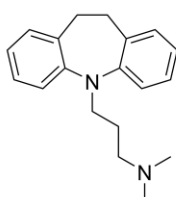
Bupropion



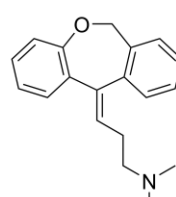
Mirtazapine

Tricyclic antidepressants

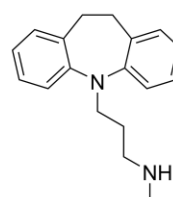
Amitriptyline



Imipramine



Doxepin

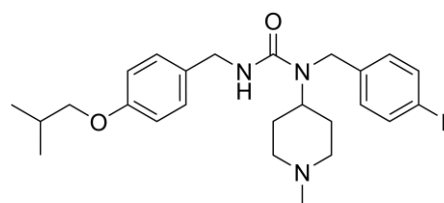


Desipramine

Figure 12: Representative chemical structures of clinically relevant antidepressants categorised by pharmacological class: selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), serotonin modulators, atypical antidepressants, and tricyclic antidepressants.

1.3 The Histamine H3 Receptor and Its Ligands

Pimavanserin (PIM) has been a compound of interest for its potential in addressing depressive and associated symptoms in patients with MDD, particularly those unresponsive to standard antidepressants [104]. It was approved in the United States in 2016 for treating psychosis in Parkinson's disease patients. PIM is a small molecule (Figure 13) that modulates serotonergic neurotransmission through its antagonistic/inverse agonistic functionality on serotonin receptor



Pimavanserin

h5-HT_{2A} pK_i = 9.3

h5-HT_{2C} pK_i = 8.8

Figure 13: Chemical structure of pimavanserin, a 5-HT_{2A} receptor antagonist/inverse agonist. In vitro affinity data from [105].

subtype 5-HT_{2A}, and to a lesser extent, on serotonin receptor subtype 5-HT_{2C} [105]. It shows negligible affinity for other aminergic receptors or monoamine transporters [106], distinguishing it from traditional antipsychotics that act on dopamine receptors and from first-line antidepressants targeting serotonin transporters. PIM has been tested as an adjunctive treatment for MDD in patients who have shown inadequate responses to conventional antidepressants. In a phase 2 double-blind, placebo-controlled clinical trial (CLARITY, NCT03018340) [107], PIM significantly improved depression severity and showed benefits for associated MDD symptoms such as anxiety, sleep disturbances, irritability, and treatment-related sexual dysfunction. However, the outcome of two combined phase 3 clinical trials (NCT03968159 in the US; NCT03999918 in Europe) [108] did not meet the primary endpoint, as PIM failed to exhibit a significant improvement in depression symptoms when compared to placebo. Despite its promising pharmacological profile and initial positive outcomes in reducing depressive and associated symptoms, the effectiveness of PIM in treating MDD as an adjunctive therapy remains uncertain. Beyond these clinical outcomes, the structural features that define PIM's selective serotonergic activity also make it an attractive building block for multitarget drug design.

GENERALISED ANXIETY DISORDER

In Western societies, anxiety disorders represent the largest group of mental diseases, with over 60 million Europeans diagnosed with such a condition in 2010 [109]. The hallmark features of anxiety include an excess of fear and anxiety, along with an avoidance of perceived threats and occasional panic attacks. This group of disorders is rather diverse, including but not limited to generalised anxiety disorder, separation anxiety, social anxiety, panic disorder, and specific phobias. Despite significant research, the neuropathophysiological mechanisms remain largely unknown, although it is

hypothesised that alterations in the limbic system, the hypothalamic-pituitary-adrenal axis, and genetic predispositions may play crucial roles [109].

Generalised anxiety disorder (GAD) is characterised by a persistent state of worry and anxiety across various contexts outside the patient's control. It is often accompanied by physical symptoms such as tension, restlessness, fatigue, concentration difficulties, or sleep disturbances [110]. Additionally, autonomic arousal may show as hyperventilation or tachycardia [109]. The lifetime prevalence of anxiety disorders seems to be influenced by geographic and socioeconomic factors [111], with significant differences between the studied countries, ranging from estimates of <5% to >30 [112]. The variability of prevalence throughout the world is not fully understood yet. Further research is needed to investigate whether these variations are true or result from invalid diagnosis criteria for different cultural backgrounds and the lack of measurement equivalence [111,113].

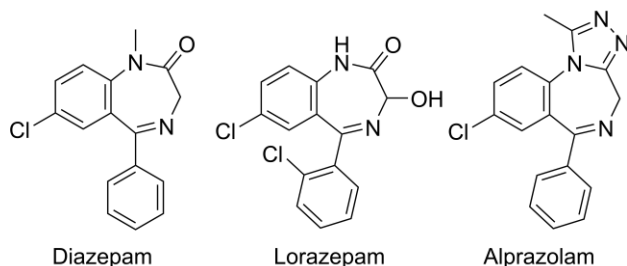
Disease Management

When it comes to the treatment of anxiety disorders, the options are categorised into psychological and pharmacological interventions. Cognitive-behavioural therapy (CBT) often stands as the first-line psychological treatment for certain anxiety disorders, recommended by health practice guidelines across numerous countries. CBT covers a variety of therapeutic techniques, including psychoeducation, cognitive restructuring, exposure to feared stimuli, breathing, and relaxation exercises. All of them have been found effective in relieving symptoms in individuals of all ages suffering from anxiety disorders [109]. Pharmacological treatment might serve as an alternative or supplement to psychological methods, with antidepressants, specifically SSRIs and SNRIs, being the primary choice of medical treatment. These medications have demonstrated efficacy in improving several anxiety disorders, with the exception of certain phobias, and are favoured due to their safety profile and minimal risk of abuse [109]. The downside is that anxiolytic effects often occur with a latency period of 2 to 6 weeks, and side effects such as jitteriness and a temporary increase in anxiety can occur at the beginning [114]. Tricyclic antidepressants have a well-proven efficacy against certain anxiety disorders, but have a higher risk of adverse effects compared to SSRIs/SNRIs, and should be avoided in patients with suicidal tendencies [114]. Benzodiazepines are considered second- or third-line treatments due to their efficacy against a variety of anxiety disorders (Figure 14). They are acting as positive allosteric modulators on GABA-A receptors, enhancing their response to the inhibitory neurotransmitter GABA [115]. However, their potential for abuse, withdrawal symptoms, and dependency restricts their usage, especially in individuals with a history of alcohol or drug abuse [109]. Buspirone, a non-benzodiazepine anxiolytic, has shown effectiveness in treating GAD and is

1.3 The Histamine H3 Receptor and Its Ligands

recommended as a secondary option when SSRIs are ineffective or cause adverse effects. Buspirone acts as a high-affinity 5-HT_{1A} partial agonist, with a lower affinity for 5-HT₂ receptor subtypes and the dopamine D₂ receptor [116]. In general, the effectiveness of current anxiolytic treatment typically reaches an upper threshold, with treatment response rates of approximately 50% and the possibility of relapses [109]. Therefore, there is an ongoing need for novel, safe, and effective drugs.

Benzodiazepines



Non-benzodiazepine anxiolytic

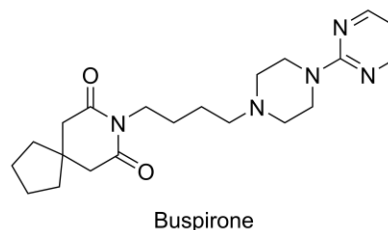


Figure 14: Typical representatives of clinically used anxiolytics categorised by pharmacological class: benzodiazepines and non-benzodiazepine anxiolytics.

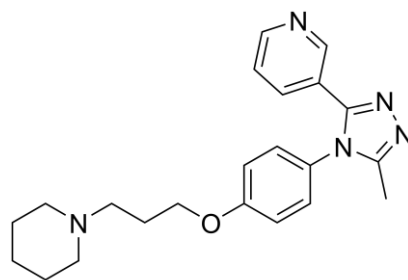
Therapeutic Potential of H3R Antagonists for MDD and GAD

In the preclinical stage of innovative, anxiolytic, and antidepressant research, H3R antagonists have shown quite promising results, especially in improving cognition, mood, and wakefulness [98]. An important aspect of these compounds is their potential for a lower risk of abuse and to cause fewer side effects than the conventional pharmacological treatments. This could be an important advancement in the therapeutic strategies for managing depressive and anxious symptoms, offering a balance between efficacy and a more favourable safety profile.

Several previous studies have illustrated a potential link between histaminergic neurotransmission and the manifestation of depressive states in murine models. In 1998, Lamberti et al. underscored the antidepressant effects of endogenous HA, which is hypothesised to be mediated through the activation of postsynaptic H1Rs [117]. Using the forced swim test (FST) (see excursus 1), an established behavioural test for antidepressant effects, it was demonstrated that administration of either H3R antagonist thioperamide, HNMT inhibitor metoprine, or selective H1R agonists effectively reduced the immobility time. The influence of thioperamide and metoprine was found to be reversible by (R)- α -methylhistamine (RAMH). This suggests that the antidepressant effect is mainly driven by elevated HA levels in the synaptic cleft, either through increased release or reduced breakdown. Importantly, the general mobility of the mice remained

unaffected, supporting the action of these compounds without impairing general motor functions.

The role of the histaminergic system in anxiety has been explored through the study of H3R antagonist ST-1283 by Bahi et al. [118] (Figure 15). Tested in various mouse models, ST-1283 showed effects similar to those of conventional anxiety and depression medications. Like diazepam, ST-1283 increased the time mice spent in the centre of the open field test (OFT) and reduced the number of buried marbles in the marble burying test (MBT). In



ST-1283
hH3R pK_i = 9.62

Figure 15: H3R antagonist ST-1283 [118].

addition, ST-1283 demonstrated antidepressant properties similar to fluoxetine, reducing immobility time in the tail suspension test (TST) and FST, and decreasing feeding latency in the novelty suppressed feeding (NSF) test. Notably, these effects were blocked by RAMH, indicating that the H3R blockade might play a role, although further research is needed to elucidate the specific mechanisms.

1.3 The Histamine H3 Receptor and Its Ligands

Excursus 1: Behavioural animal models for depression and anxiety.

In the field of neuropsychiatric research, the use of in vivo models is important for the understanding and development of treatments for anxiety and depression. Several well-established tests such as the forced swim test (FST), open field test (OFT), tail suspension test (TST), elevated plus maze test (EPM), novelty suppressed feeding (NSF), and marbles burying test (MBT) are frequently employed. These tests are designed to measure the depressive-like and/or anxiety-like behaviours in rodents and are critical for evaluating the potential effectiveness of pharmacological agents.

The FST and TST are designed to assess antidepressant-like activity by recording the immobility time, which reflects the state of depression-like behaviour in the subjects. In FST, rodents are placed in a glass cylinder filled with water, while in TST they are suspended by their tails 50 cm above a table top [213]. Significant reduction of immobility time indicates antidepressant effects of the administered drug substance.

Conversely, EPM and NSF focus on anxiety-related behaviours. EPM evaluates the preference of animals between open and enclosed spaces, by exposing them to an elevated cross-shaped platform with two open and two closed arms. The duration on each arm and the number of entries are measured [213]. An increased time spent on the open arms indicates reduced anxiety-like behaviour. NSF measures the latency to feed in a novel, potentially threatening environment, where food is placed in the centre of an open field. Initially, this test was used to assess anxiety-like behaviour but has now become popular to investigate antidepressant effects.

The OFT is utilized to assess general locomotor activity levels and anxiety. The rodents are placed on a squared open field, which is divided into smaller squares by black lines. Increased time spent in the centre of the arena indicates lower anxiety levels and the total line crossings are measured to assess general locomotor activity.

The MBT measures the compulsion of animals to bury marbles, reflecting their anxiety and obsessive-compulsive tendencies. The number of marbles buried and the time spent burying are related to the anxiety level or compulsive tendencies [213].

These in vivo tests are essential tools as they provide a standardized method to investigate the efficacy of new pharmacological treatments and to understand the underlying neurobiological mechanisms of psychiatric disorders. Despite the simplicity and efficiency of these tests, researchers must interpret the results with caution due to the complex nature of human psychiatric conditions which these models attempt to replicate.

1.3.2 ALZHEIMER'S DISEASE

Alzheimer's disease (AD), an irreversible, neurodegenerative disorder, is the most common cause of dementia that is clinically characterised by cognitive decline, progressive memory loss, and impairments in language and behaviour [119]. According to the World Alzheimer Report, 55 million people were estimated to have suffered from dementia in 2019, with 60-70% cases due to AD [120]. AD is mostly age-related. The prevalence of AD increases with age, affecting about 5% of individuals aged 65–74, 13.1% of those aged 75–84, and one-third of people aged 85 or older [121]. AD is a major burden for public health care; this is fuelled by the fact that between 2015 and 2050, the percentage of people over 60 will approximately double, while the number of people suffering from dementia is expected to triple [122]. AD can be categorised into sporadic and familial AD, where familial cases are associated with inherited genetic mutations. The largest group of patients has sporadic AD without a single genetic cause. Alongside age, various lifestyle, environmental, and metabolic risk factors have been linked to an increased probability of disease development [123,124]. Most forms of AD are late-onset AD, manifesting after the age of 65. Early-onset AD, occurring before age 65, is nearly always familial, while the majority of late-onset AD is sporadic.

Despite extensive research on cellular and molecular mechanisms, the aetiology of AD remains unclear, and there is no cure available. Several pathological mechanisms have been identified that might contribute to the pathogenesis and progression of the disease. The most studied pathological hallmarks are: i) the extracellular aggregation of amyloid beta ($A\beta$) into plaques, and ii) the intracellular formation of neurofibrillary tangles from hyperphosphorylated tau protein. Both are accompanied by synaptic loss and neuronal death (brain atrophy) [125,126]. Other pathological mechanisms include neuroinflammation, oxidative stress, mitochondrial dysfunction, ferroptosis, metal ion accumulation, and CNS hypometabolism [127].

$A\beta$ is a naturally occurring polypeptide mostly secreted by neuronal cells and by other cell types throughout a lifetime. The $A\beta$ monomer is a degradation product of the amyloid precursor protein (APP), which is an integral membrane protein of neuronal synapses. Upon proteolytic breakdown of APP by β -secretase BACE1 (β -site APP cleaving enzyme 1) or γ -secretase, $A\beta$ monomers of varying length between 38 and 43 amino acids are built and secreted into the extraneuronal space. It is suggested that $A\beta$ becomes pathogenic after aberrant accumulation and changes of conformation [128]. $A\beta_{1-42}$ is especially highly prone to aggregate into oligomers. The variety of $A\beta$ species ranges from soluble oligomers and protofibrils to insoluble amyloid plaques, each associated with different neurotoxic effects, including the induction of oxidative stress,

1.3 The Histamine H3 Receptor and Its Ligands

neuroinflammation, synaptic dysfunction, alterations in membrane permeability, or impairment of cell signalling. This can lead to synaptic loss and promote neuronal death [127,129]. The extracellular deposits are resistant to proteolytic cleavage. However, the presence of senile plaques alone is not sufficient to explain AD-related neurodegeneration, since no brain atrophy appears during their maturation [130].

The second most common histopathological hallmark is the accumulation of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein. Tau is a microtubule-associated protein that contributes to the stabilisation and assembly of microtubules, thereby supporting cytoskeletal structures. It is regulated by post-translational modifications like phosphorylation mediated through different kinases, e.g., GSK-3 β [127]. Abnormal hyperphosphorylation of tau leads to a higher affinity for self-aggregation and the formation of filamentous deposits in the soma. The loss of tau's normal function leads to disruptions of the microtubule network, axonal trafficking, and dendritic structures, loss of synapses, neuronal death, and eventually dementia [131]. The concentration of neurofibrillary tangles correlates positively with the progress and severity of AD [132].

Both A β - and tau-associated pathophysiological changes are closely connected to a loss of neurons and neuronal networks. Over the past few decades, numerous studies have emphasised that the severity of AD is closely connected to the disruption of the cholinergic neurons. Cholinergic neurotransmission is widespread in the human CNS, especially in the thalamus, striatum, limbic system, and neocortex. This suggests a critical role in memory, learning, attention, and other higher brain functions. The cholinergic hypothesis in AD suggests that the gradual loss of basal cholinergic neurons is one of the major components responsible for the manifestation of clinical symptoms. This hypothesis is supported by the observation of three early key findings [133]: i) reduced presynaptic cholinergic markers in the cerebral cortex [134,135], ii) severe neurodegeneration of the nucleus basalis of Meynert, which is the origin of cortical cholinergic innervation [136,137], and iii) the detrimental effects of cholinergic antagonists on memory [138]. Additionally, an elevated level of acetylcholinesterase (AChE) has been observed around amyloid plaques and neurofibrillary tangles [139].

Disease Management

The hypothesis was further supported when cholinesterase inhibitors were found to significantly improve symptoms in AD's patients [140], leading to the approval of three AChE inhibitors as treatments for mild to moderate dementia in AD patients, namely donepezil, rivastigmine, and galantamine (Figure 16). Beyond acetylcholine, other neurotransmitters, including serotonin, dopamine, HA, and glutamate (GLU), play crucial roles in cognitive processes. Memantine, a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, represents another neurotransmission-modulating drug in AD treatment. Importantly, these drugs only provide symptomatic improvements. The diverse spectrum of pathological factors makes it difficult to develop disease-modifying drugs that attenuate or reverse the progression of the disease. In recent years, notable progress has been made in the development of disease-modifying treatments.

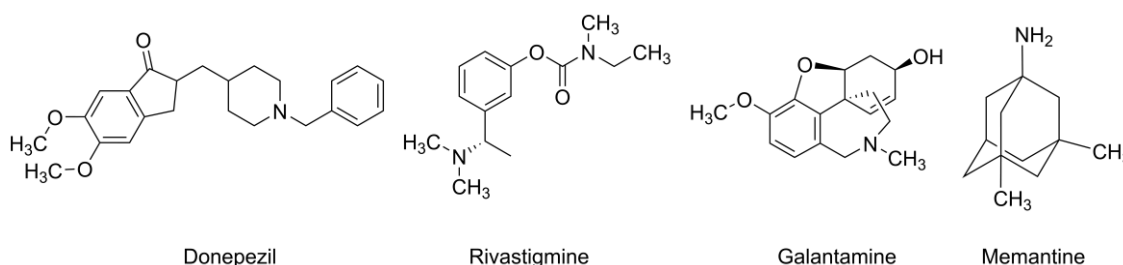


Figure 16: Chemical structures of currently available AD drugs.

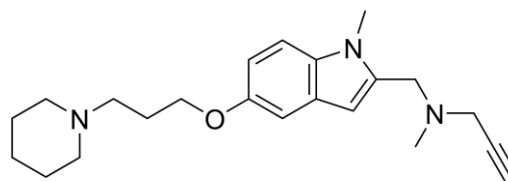
In 2021, the first disease-modifying drug, Aducanumab, was approved by the FDA for AD patients with mild cognitive impairment or mild dementia. Aducanumab is an antibody targeting amyloid fibrils and significantly reduces amyloid plaques in the brain [141]. However, the approval was controversial because two phase 3 studies (EMERGE and ENGAGE) showed contradictory results regarding the efficacy in improving cognitive abilities [142], which is why the European Medicines Agency (EMA) decided to decline the approval. Moreover, frequently appearing side effects, so-called amyloid-related imaging abnormalities (ARIA), raised concerns about the benefit-to-risk ratio [141]. Most of these ARIAs have been asymptomatic, though some life-threatening events have occurred [143]. Donanemab (Kisunla®) and lecanemab (Leqembi®) are next-generation amyloid-targeting antibodies that have been shown to significantly improve cognitive measures, but the risk of ARIAs remains [144], especially within APOE ε4 allele carriers. Lecanemab/Leqembi® has already been approved by the EMA for mild cognitive impairment or early-stage AD [145]. Donanemab/Kisunla® has received a positive recommendation in July 2025 after re-evaluation by the EMA [146]. However, for both antibodies, the patient population is restricted to APOE ε4 non-carriers/heterozygotes, and patients must be frequently monitored to detect adverse events. The unfavourable risk/benefit profile emphasises the need for ongoing research. Although these treatments

1.4 The Dopaminergic System

may slow disease progression, they do not prevent its onset. Targeting amyloid beta alone may therefore be insufficient to address the multifactorial nature of AD.

Therapeutic Potential of H3R Antagonists

The H3R is a promising drug target for AD due to its role in modulating neurotransmitter release. H3R antagonists have been explored as potential treatment options due to their pro-cognitive effects, especially in a multitargeting approach. Contilisant (**Fehler! Verweisquelle konnte nicht gefunden werden.**), a compound that inhibits H3R, AChE, BuChE, and MAO-B simultaneously [147,148], is a promising example of an H3R MTDL for neurodegenerative diseases. In addition, contilisant exhibits neuroprotective and antioxidant qualities that reduce oxidative stress and slow neuronal death. In vivo tests in mice have shown that contilisant significantly improved cognitive function in a model of memory impairment induced by lipopolysaccharide [148], suggesting its potential as a multipurpose therapeutic agent.



Contilisant

hH3R K_i = 10.8 nM
hAChE K_i = 1.69 μ M
hMAO-B IC_{50} = 0.078 μ M

Figure 17: Chemical structure of Contilisant, an MTDL acting on hH3R, hAChE, and hMAO-B. Binding affinity data from [147].

As demonstrated for mood disorders such as MDD and GAD, as well as for neurodegenerative disorders like AD, H3R antagonists have shown promising preclinical effects. Their ability to modulate different neurotransmitter systems underscores the broad therapeutic potential of targeting HA pathways. In combination with additional drug targets, MTDLs hold considerable potential as future treatments of complex, multifactorial disorders. Building on these insights, it is equally important to explore other neurotransmitter systems, such as the dopaminergic system, which plays a central role in other neurodegenerative and psychiatric disorders.

1.4 THE DOPAMINERGIC SYSTEM

Dopamine (DA), is a biogenic amine belonging to the class of catecholamines. Inside the brain, DA acts as a neurotransmitter, responsible for important

neurophysiological functions, such as reward-motivated behaviour, motor control, and cognition. The dopaminergic system operates within a complex homeostatic network, and its dysregulation can lead to severe neurological, psychiatric, and metabolic disorders. The following chapters aim to explore the biological basis of the dopaminergic system in the brain, dopamine receptor involvement in neurodegenerative diseases with special focus on schizophrenia, and evaluate current pharmacological approaches.

DA, like noradrenaline, adrenaline, HA, and 5-HT, belongs to the monoamine neurotransmitters, with DA, noradrenaline, and adrenaline called catecholamines. They share the same structural motif, the catechol nucleus with an ethylamine chain attached to it. The catecholamine neurotransmitters are successive products of a single biosynthetic pathway that starts with the non-essential, proteinogenic amino acid L-tyrosine, which is either absorbed by food intake or can be synthesised from the essential amino acid phenylalanine [149]. Tyrosine is actively transported across the blood-brain barrier (BBB) into the brain. It is taken up into dopaminergic neurons and converted to L-DOPA by the enzyme tyrosine hydroxylase (TH) (Figure 18). In dopaminergic neurons, the aromatic L-amino acid decarboxylase (AADC) catalyses the decarboxylation of L-DOPA to produce DA, the active neurotransmitter. DA is stored in vesicles within dopaminergic neurons and is released upon stimulation, where it binds to postsynaptic DA receptors to mediate various physiological processes. To maintain homeostasis, DA is either reabsorbed into the neuron via DA transporters or degraded by enzymes such as monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) [150].

DA and its precursor L-DOPA can undergo oxidation to form reactive quinones, which contribute to the formation of neurotoxic species and protein adducts. These neurotoxins and the reactive oxygen species (ROS) generated from MAO can lead to neuronal damage and degeneration. Therefore, the DA metabolism greatly affects the redox homeostasis and cell viability [150].

1.4 The Dopaminergic System

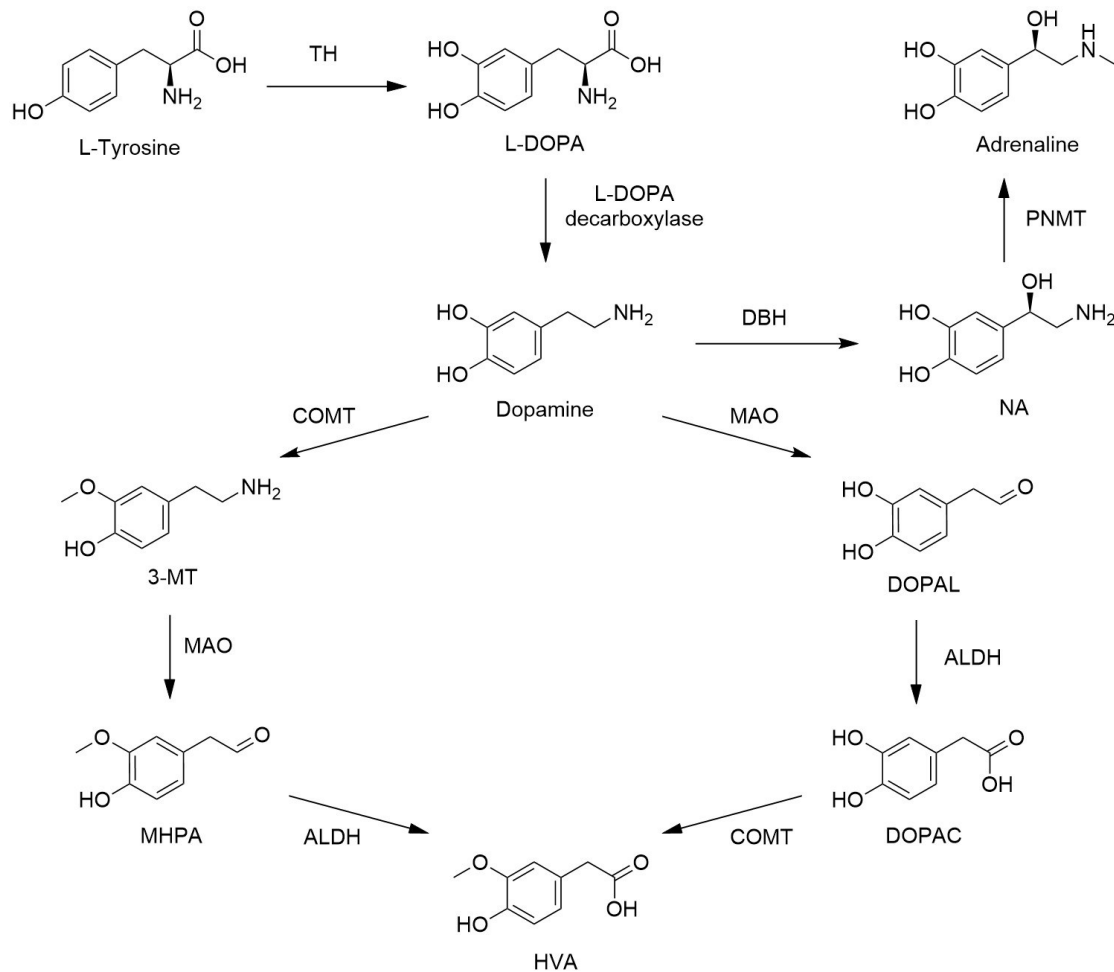


Figure 18: DA is synthesised from L-tyrosine and can be converted to noradrenaline (NA) and adrenaline. DA is degraded via two pathways, both leading to homovanillic acid (HVA). Abbreviations: TH: tyrosine hydroxylase, DBH: DA β-hydroxylase, PNMT: phenylethanolamine *N*-methyltransferase, MAO: monoamine oxidase, COMT: catechol-*O*-methyltransferase, ALDH: aldehyde dehydrogenase, DOPAL: 3,4-Dihydroxyphenylacetaldehyde, DOPAC: 3,4-Dihydroxyphenylacetic acid, 3-MT: 3-Methoxytyramine, MHPA: 3-methoxy-4-hydroxyphenylacetaldehyde.

From the approximately 100 billion neurons in the brain, only about 500,000 are dopaminergic neurons. This limited number of neurons originates from distinct parts of the midbrain. However, they are remarkably organised and project widely into different terminal fields [149]. The dopaminergic system in the brain can be divided into four major pathways (Figure 19), each linked to specific neurophysiological functions. The major pathways include:

1) **Nigrostriatal pathway:** These dopaminergic neurons originate in the substantia nigra pars compacta (SNc) and project to the striatum. Striatal depletion of dopaminergic neurons has been linked early to motor control deficits such as those observed in PD. Nowadays, it is known that the nigrostriatal pathway is crucial for motor control regulating voluntary movement.

2) **Mesolimbic pathway:** Originating in the ventral tegmental area (VTA) of the midbrain, dopaminergic neurons project to the ventral striatum in the forebrain (like the nucleus

accumbens (NAc)). This pathway is often also referred to as the reward pathway, since it is involved in motivation and reward-related behaviour. This pathway is considered to be markedly involved in addictive behaviour in psychiatric disorders.

3) Mesocortical pathway: Also originating in the VTA, mesocortical, dopaminergic neurons project to the prefrontal cortex, influencing executive functions, cognitive control, and decision processes. Irregularities in the mesocortical pathway can contribute to cognitive deficits in diseases like schizophrenia or ADHD.

4) Tuberoinfundibular pathway: This population of DA neurons projects from the arcuate nucleus of the hypothalamus to the median eminence. This pathway regulates prolactin secretion and can prevent hyperprolactinemia.

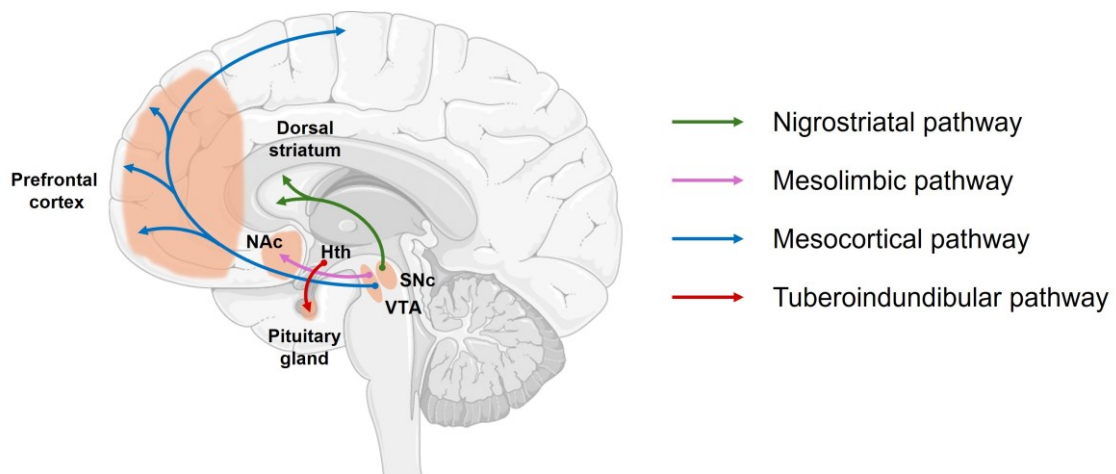


Figure 19: DA plays a significant role in various brain functions, including motor control, motivation, reward, and cognition. Its influence is mediated through several major pathways, each originating from specific brain regions and projecting to different target areas. The four primary dopaminergic pathways are depicted above. NAc: nucleus accumbens, Hth: hypothalamus, SNc: substantia nigra pars compacta, VTA: ventral tegmental area. Modified from [151,152].

1.5 DOPAMINE D₂/D₃ RECEPTORS AND THEIR LIGANDS

DA exerts its effects through five distinct receptor subtypes, all of which belong to the class A G-protein coupled receptors, namely dopamine D₁, D₂, D₃, D₄, and D₅ receptors (D1R-D5R). These five dopamine receptors (DARs) are categorised into two families: D₁-like receptors (D1R and D5R) and D₂-like receptors (D2R, D3R, and D4R).

1.5 Dopamine D2/D3 Receptors and Their Ligands

While D₁-like receptors stimulate cAMP production via G_s-proteins, D₂-like receptors inhibit cAMP production through G_{i/o}-proteins [153].

DARs [154] are widely distributed across the brain and periphery. Within the brain, the D₁R is highly expressed in regions such as the striatum, nucleus accumbens, and frontal cortex, while D₅R expression is more diffusely distributed and at lower densities. The D₂R shows high density in the striatum, olfactory tubercle, and nucleus accumbens, with additional expression in the substantia nigra and hypothalamus. The D₃R is more selectively expressed, predominantly in limbic areas, like the shell of the nucleus accumbens and island of Calleja, with lower levels in the striatum, substantia nigra, ventral tegmental area, cortical regions, and hippocampus. Finally, the D₄R exhibits the lowest overall expression in the brain but was found in the frontal cortex, amygdala, and hippocampus [153]. All DARs are expressed postsynaptically on dopamine-receptive cells, such as GABAergic neurons in the striatum. Notably, D₂R and D₃R also function as presynaptic autoreceptors, regulating DA release, synthesis, and neuronal firing rates through a negative feedback mechanism. While there is limited evidence for the presence of presynaptic D₄R in rat brains [155], this area has not been extensively studied and needs further investigation.

DARs are involved in neurophysiological and pathological processes. The regulation of locomotor activity is predominantly mediated by D₁R, D₂R, and D₃R in the ventral striatum. Depending on the receptor subtype, the localisation (presynaptic or postsynaptic), and the agonist concentration, receptor activation can induce stimulatory or inhibitory effects on locomotor activity. Activation of presynaptic D₂-class autoreceptors reduces DA release, lowering locomotor activity, while activation of postsynaptic D₂R increases movement. Since autoreceptors respond to lower DA levels than postsynaptic receptors, the same DA agonist can decrease activity at low doses but stimulate it at higher doses [153]. D₁R, D₂R, and D₃R in mesolimbocortical regions play a role in reward and reinforcement, contributing to addiction behaviour [153,156]. In the prefrontal cortex, D₁R and D₂R appear to strongly influence memory and learning processes [157]. D₃R is hypothesised to influence cognition by modulating prefrontal cortex function, despite low expression levels in that region. D₃R antagonism has been found to enhance cognition, while agonism impairs it. The pro-cognitive effect may result from mechanisms such as increased acetylcholine release in the prefrontal cortex and activation of CREB signalling in the hippocampus, but further research is necessary [158]. Another well-researched area is the involvement of DARs, mainly D₂R and D₃R, in psychosis, such as observed in schizophrenia and bipolar disorder. This topic will be explored further in Chapter 1.6 Schizophrenia.

Genomic and Structural Aspects

The D₁- and D₂-like receptor families differ significantly in genetic structure, localisation, and pharmacological characteristics. Notably, D₁-like receptors lack introns in their coding regions, whereas D₂-like receptors have several introns, resulting in receptor variants by alternative splicing [159]. For instance, the D2R has two major isoforms: the short form (D2_SR), predominantly presynaptic and functioning as an autoreceptor, and the long form (D2_LR), primarily expressed postsynaptically. These variants differ in structure by 29 amino acids within the third intracellular loop and exhibit distinct physiological, pharmacological, and functional properties [153]. Despite these differences, receptors within the same family share significant sequence homology. D1R and D5R share 80% of their transmembrane (TM) sequence identity. D3R and D4R are 75% and 53% homologous to D2 receptors in their TM domains [160]. This structural similarity makes the development of selective ligands within a receptor family particularly challenging. D2R and D3R share a highly conserved orthosteric binding site (OBS), responsible for primary ligand binding. However, differences in the extracellular loop 2 (ECL2) region and the less-conserved secondary binding site (SBS) contribute to subtype selectivity and functional diversity [161]. The SBS in the extracellular vestibule plays a role in modulating ligand interactions and pharmacological effects [162], e.g., some antipsychotics use SBP binding to improve receptor selectivity while minimising side effects. Bitopic ligands, designed to engage both OBP and SBP, represent a promising approach to refining therapeutic profiles for neuropsychiatric disorders [163].

DAR Signalling

DARs exhibit distinct signalling mechanisms depending on the receptor subtype. The primary signalling mechanism of DA receptors involves the G-protein-dependent regulation of adenylyl cyclase (AC). D₁-like receptors are coupled to G α_s protein, D₂-like receptors are coupled to G $\alpha_{i/o}$ protein. As for GPCR, DARs signal through a variety of G α - or G $\beta\gamma$ -dependent pathways [164]. Additionally, for D1R and D2R, β -arrestin2 signalling events have been documented [165,166]. The detailed description of G-protein-dependent and -independent signalling events was thoroughly provided in Chapter 1.3.

D2R/D3R Ligands

Alterations of the dopaminergic system are involved in a variety of neurological conditions, including PD (PD), Huntington's disease, ADHD, ASD, drug abuse, and schizophrenia. Targeting dopaminergic neurotransmission is a well-established approach for the treatment of neurodegenerative diseases and the management of their associated symptoms. DAR agonists that modulate dopaminergic signalling are widely

1.5 Dopamine D2/D3 Receptors and Their Ligands

used. Several D2R/D3R agonists, including bromocriptine, pergolide, pramipexole, and ropinirole, are applied for the treatment of PD and other disorders, such as restless legs syndrome and sexual dysfunction [167] (Figure 20).

D₂-like receptor agonists

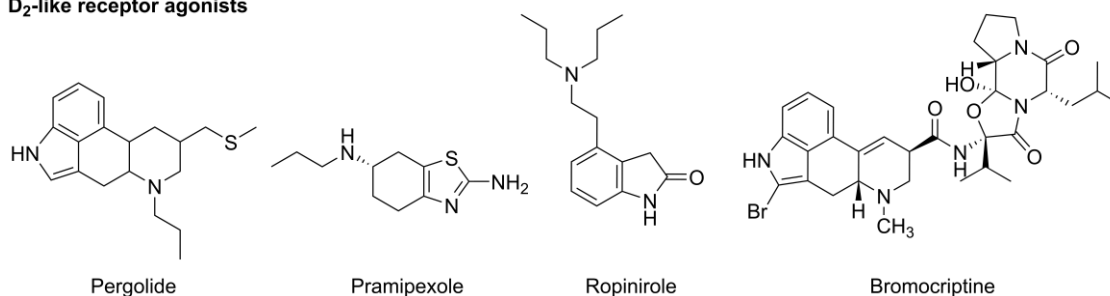
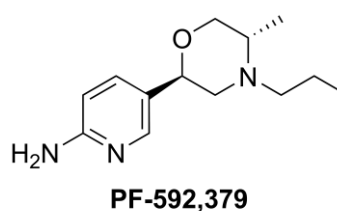


Figure 20: D₂-like receptor agonists for the treatment of Parkinson's disease (PD).

While targeting both D2R and D3R may be beneficial for certain diseases, recent efforts have focused on developing selective ligands – a challenging task due to the high sequence identity between these receptors. Selective D3R agonists have attracted interest because of the receptor's restricted tissue distribution and potential neuroprotective effects [168]. A notable example is PF-592,379 (Figure 21), a highly selective D3R agonist investigated for sexual dysfunction and pain, which demonstrates minimal abuse potential [169].



hD2R IC₅₀ >10 μ M
hD3R K_i = 215 nM

Figure 21: Chemical structure of D3R-prefering agonist PF-592,379. Binding affinity values from [169].

The development of D3R-prefering ligands has been advanced by the introduction of bitopic ligands. Bitopic ligands interact not only with the OBS but also with an allosteric binding site. Some of the most prominent bitopic ligands developed in recent years are D2R/D3R antagonists or partial agonists, including several modern antipsychotics. D2R/D3R ligands with antagonist or partial agonist properties have been characterised through SAR studies, which have identified three structural key features that contribute to receptor affinity and selectivity: a lipophilic aryl amide or ether moiety, a linear alkyl linker, and a basic amine moiety with aryl substitution [170] (Figure 22).

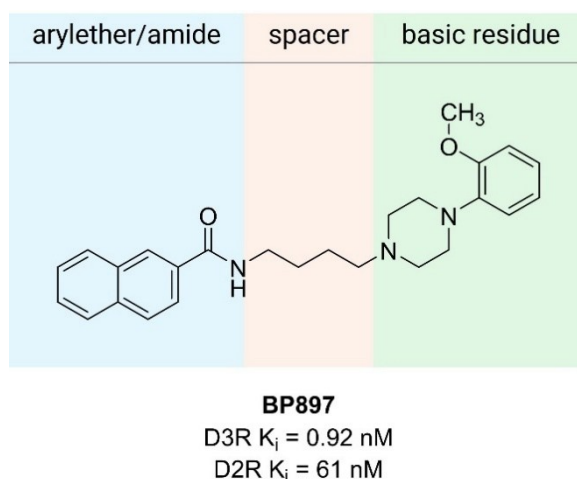


Figure 22: Key structural features of D2R/D3R antagonists/partial agonists, illustrated using the D₃-preferring partial agonist BP897. The molecule comprises three characteristic elements: an aryl amide moiety, a linear alkyl spacer, and a basic amine residue. BP897 shows high affinity for D3R (K_i = 0.92 nM) with 66-fold selectivity over D2R (K_i = 61 nM) [171].

Although modifications can be introduced at various parts of the ligand, certain structural elements have shown particular efficacy in promoting D2R/D3R antagonist activity. For instance, the 4-phenylpiperazine moiety is considered a "privileged scaffold" for D2R/D3R interactions [170]. Other beneficial variations of the basic element include benzazepines, pyrrolidines, tetrahydroisoquinolines, and triazoles [172]. The cationic amine of the basic substructure forms either a hydrogen bond or a salt bridge with Asp110 in TM3 [173]. The amide group acts as a hydrogen bond acceptor, further stabilising ligand binding.

While the arylpiperazine moiety is essential for D2R/D3R affinity, the lipophilic aryl moiety influences receptor subtype selectivity. Modifications in this region allow fine-tuning of binding properties, affinity towards additional drug targets (e.g., used for MTDLs), or optimisation of pharmacokinetic properties [174,175]. A variety of substituents, including heteroaryls and biphenyls, can be tolerated in this region. Structural modifications can also introduce additional functional properties, such as antioxidant activity. Regarding the alkyl spacer, SAR studies have shown that a tetramethylene chain provides optimal D3R affinity and selectivity over D2R [170]. Alternative linker designs, such as phenylmethyl and phenylethyl linkers, have also been explored to refine receptor selectivity [176].

Recent research has highlighted the development of biased ligands as a promising strategy to improve therapeutic outcomes. Biased agonists selectively engage G protein or β -arrestin pathways, optimising efficacy while reducing side effects [177]. Another emerging direction of ligand development involves chemical probes and fluorescent ligands, enabling real-time visualisation of receptor interactions [178].

1.6 Schizophrenia

The pharmacological modulation of D2R and D3R plays a crucial role in neuropsychiatric disorders, particularly schizophrenia. While D2R antagonism remains a cornerstone of antipsychotic treatment, the role of D3R in cognitive and negative symptoms is increasingly being investigated. The following section discusses the pathophysiology of schizophrenia, the role of DA dysfunction, and the therapeutic implications of targeting D2R and D3R in the development of antipsychotic drugs.

1.6 SCHIZOPHRENIA

Schizophrenia (SCH) is a severe psychiatric disorder, with multifactorial causes and a heterogeneous symptom profile, that affects more than 24 million people worldwide [179]. Patients suffer from relapsing psychotic episodes and cognitive disabilities that affect almost all areas of their personal and social lives. The heterogeneous symptoms can broadly be divided into three categories: positive, negative, and cognitive. The positive symptoms refer to events that are not present in healthy individuals, like hallucinations and delusions, which are part of psychotic episodes. Negative symptoms describe the absence or reduction of normal emotional or motivational states, such as anhedonia, social withdrawal, a lack of initiative, and affective flattening. Lastly, cognitive symptoms sum up the range of cognitive dysfunctions commonly observed in schizophrenic patients. Comorbid symptoms like depression, anxiety, or aggressive behaviour are common [180,181]. The aetiology of SCH involves components of genetic and environmental factors that affect early neurodevelopmental states [181]. According to most sources, the onset of SCH typically occurs in late adolescence or early adulthood. However, 18% of individuals experience symptoms before the age of 18. SCH that is diagnosed between the ages of 13 and 17 is referred to as early-onset SCH [182]. During the prodromal phase, the phase before the first psychosis, early signs of cognitive and social impairments begin to emerge [181,183]. Risk factors of SCH include genetic predisposition, prenatal and perinatal complications, paternal age, sex (with men being slightly more affected), urban environment, migration status, or drug abuse [181].

The pathogenesis of SCH is not fully elucidated yet. However, over the last decades, pathological mechanisms and contributing factors have been identified. Among those, irregularities in the neurotransmitter system, especially DA, have been extensively studied. As early as the 1960s, after a serendipitous discovery, chlorpromazine was found to be a potent antipsychotic [184]. In the 1970s, the direct correlation between the therapeutic efficacy of antipsychotic medications and their ability to bind to DARs was made [185–187], which was the beginning of the formulation of the “DA hypothesis of

SCH". The hypothesis was adapted and expanded over the last decades, and new insights keep evolving the theories on the aetiology of SCH.

The DA hypothesis comprises the following assumptions:

1. There is a **hyper**dopaminergic activity in the mesolimbic pathway, predominantly contributing to the positive symptoms.
2. There is a **hypo**dopaminergic activity in the mesocortical pathway, responsible for the negative and cognitive symptoms.

Neurochemical imaging techniques have provided evidence that presynaptic striatal DA synthesis was increased in SCH patients [188], and DA release was elevated [189–191]. D2R/D3R density seems to be 10-20% higher in SCH patients compared to controls, an observation independent from antipsychotic drug effect [192].

Apart from DA, other neurotransmitters seem to be involved. The "glutamate hypothesis" proposes that there is a dysregulation of glutamate, which disrupts the DA balance. This was initially proposed, as it was found that NMDA receptor (NMDAR) antagonists like ketamine and phencyclidine induce psychomimetic symptoms [193,194]. In healthy patients' brains, glutamate excites inhibitory GABA interneurons, maintaining proper signalling balance. However, it is proposed that in SCH, impaired glutamate binding to NMDARs on GABA interneurons reduces inhibitory neurotransmitter release, while excitation from glutamate neurons persists, leading to excessive GLU signalling [195–197] (Figure 23). This disinhibition of cortical GLU neurons leads to excessive GLU release, which overstimulates both striatal DA neurons and inhibitory GABA interneurons. The hyperactivation of DA neurons in the mesolimbic pathway leads to excessive DA release in the subcortical and limbic regions, resulting in hyperdopaminergia and the occurrence of positive symptoms. At the same time, the overstimulation of GABA interneurons results in excessive inhibition of DA neurons that project to the prefrontal cortex, suppressing DA activity in the mesocortical pathway. This contributes to a hypodopaminergic state associated with negative symptoms [198].

1.6 Schizophrenia

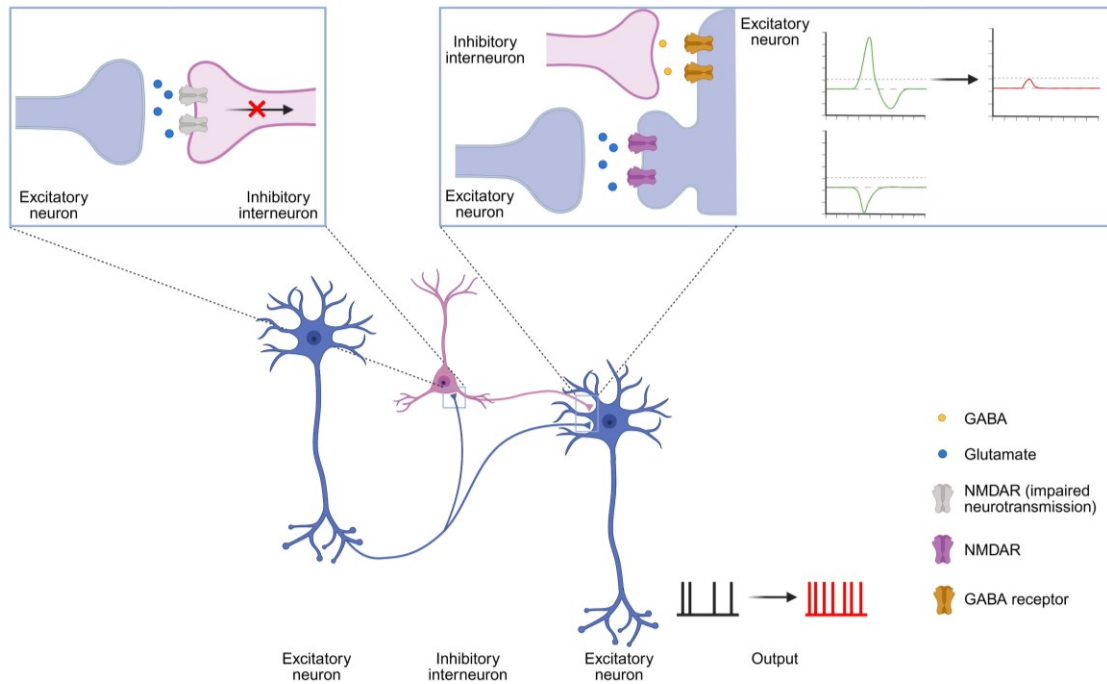


Figure 23: The glutamate (GLU) hypothesis states that in schizophrenia, impaired GLU binding to *N*-methyl-D-aspartate receptors (NMDARs) on γ -aminobutyric acid (GABA) interneurons reduces inhibitory neurotransmitter release, while excitation from glutamatergic neurons persists. This disinhibition leads to excessive cortical GLU release, which overstimulates both striatal DA neurons and downstream inhibitory interneurons. The figure illustrates weakened inhibitory tone (left inset), enhanced excitatory output (right inset), and resulting network hyperactivity. Neurotransmitters and receptor types are indicated: GLU (blue), GABA (yellow), impaired NMDARs (grey), functional NMDARs (purple), and GABA receptors (orange). Figure adapted from [199] and created with <https://BioRender.com>.

In contrast to the DA hypothesis, no glutamate-targeting drugs have been approved to date. While both hypotheses present reasonable arguments, they also have their limitations. The true underlying cause may lie somewhere between or beyond these frameworks, necessitating further research.

Disease Management

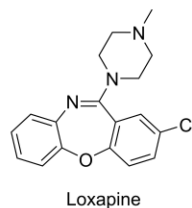
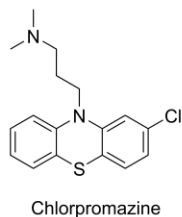
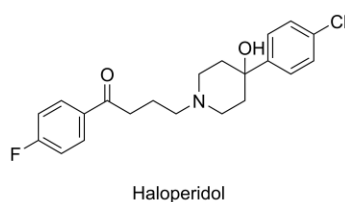
The primary approach to SCH treatment relies on antipsychotic medications, which have undergone considerable evolution over the past several decades, resulting in three generations of antipsychotics. First-generation antipsychotics (FGAs), also known as typical antipsychotics, primarily function as D2R antagonists, like haloperidol, chlorpromazine, and loxapine (Figure 24). While these drugs are effective in treating positive symptoms, they have limited efficacy against negative and cognitive symptoms and may even worsen them. Studies have found that D2R occupancy must remain within a therapeutic window of 60-78% to ensure efficacy while minimising the risk of adverse effects [200]. Exceeding this threshold increases the likelihood of side effects significantly. A major drawback of FGAs is their high risk of extrapyramidal symptoms (EPS), which result from non-selective D2R blockade. Since DARs are not targeted regiospecifically, D2R antagonism in the nigrostriatal pathway leads to severe motor dysfunctions, including tremor, akathisia, and dystonia, while blockade in the

tuberoinfundibular pathway can cause hyperprolactinemia. Furthermore, a significant number of patients do not respond adequately to FGA treatment [201]. Due to the limitations associated with FGAs, the development of second-generation antipsychotics (SGAs) led to the introduction of drugs such as risperidone, olanzapine, and quetiapine (Figure 24). Unlike FGAs, SGAs target a broader range of receptors, including dopaminergic (D1R-D4R), serotonergic, adrenergic, histaminergic, and muscarinic receptors. A key characteristic of SGAs is their ability to block both D2R and 5-HT_{2A} receptors. This broader receptor profile not only effectively addresses positive symptoms of psychosis but also reduces the risk of EPS [202]. Among SGAs, clozapine is considered the gold standard in SCH pharmacotherapy. Unlike most antipsychotics, it exhibits high efficacy against negative symptoms and even reduces suicidality [17,202]. It is also the treatment of choice for refractory and therapy-resistant SCH [203]. Interestingly, clozapine has a higher affinity for D4R than for D2R, which may contribute to its unique therapeutic effects. However, clozapine carries a significant risk of agranulocytosis, a potentially fatal condition. Close monitoring of patients is required to ensure management of this adverse effect [204].

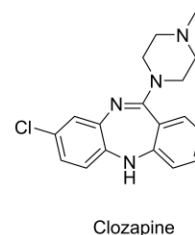
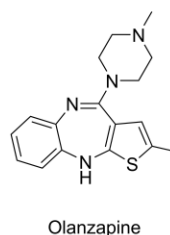
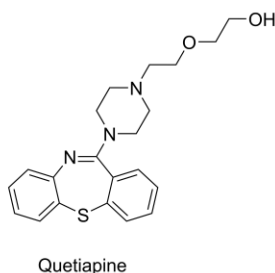
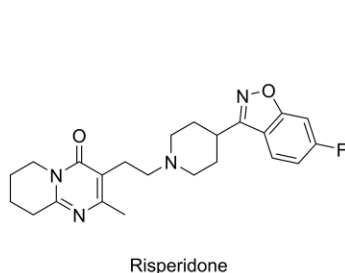
While SGAs were initially regarded as superior to FGAs, recent meta-analyses have questioned this assumption, particularly regarding efficacy [205], and treatment of negative and cognitive symptoms [206,207]. Moreover, due to their broader receptor affinity, SGAs remain associated with significant side effects, including metabolic side effects like weight gain and diabetes, sedation, and cardiovascular risks [208–210]. To overcome these limitations, researchers have developed a new class of antipsychotics: D2R/D3R partial agonists, often referred to as third-generation antipsychotics (TGAs). This category currently includes aripiprazole, brexpiprazole, and cariprazine (Figure 24), all of which act as partial agonists, modulating DA activity based on regional neurotransmitter levels. In hyperdopaminergic areas (e.g., mesolimbic pathway), they reduce excessive DA transmission, while in hypodopaminergic regions (e.g., mesocortical pathway), they enhance DA signalling [202]. Their additional 5-HT_{1A} partial agonism and 5-HT_{2A} antagonism contribute to their antipsychotic efficacy while reducing the risk of extrapyramidal symptoms [211]. All three TGA are approved for the treatment of SCH. In addition, aripiprazole and cariprazine are also indicated for bipolar mania, while aripiprazole and brexpiprazole are approved as adjunct treatments for major depressive disorder [212]. The development of second- and third-generation antipsychotics exemplifies the potential of multitargeted drug design in neuropsychiatric disorders. They are yet another example of how “selectively non-selective drugs” can be advantageous for multifactorial diseases [17].

1.6 Schizophrenia

First-generation antipsychotics



Second-generation antipsychotics



Third-generation (atypical) antipsychotics

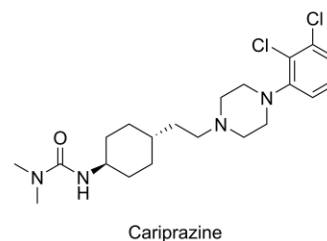
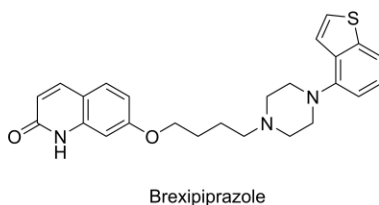
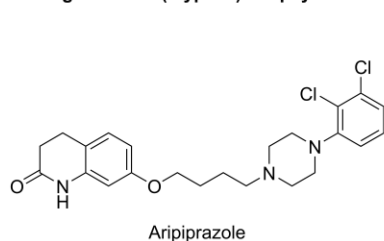


Figure 24: Typical first-, second-, and third-generation antipsychotics.

Among the three approved TGAs, cariprazine demonstrates superior efficacy in treating predominant negative symptoms and cognitive impairment, and shows lower incidents of side effects [213]. Cariprazine has a sub-nanomolar in vitro affinity for D3R, 10-fold higher than for the other two TGAs (Table 2). It has approximately 6-fold selectivity for D3R over the D2R, making it the only high-affinity D3R-preferring antipsychotic currently available [211]. Given that D3R is highly expressed in brain regions implicated in cognition and mood regulation, this receptor selectivity is hypothesised to underlie cariprazine's unique therapeutic benefits. Preclinical studies have shown that cariprazine reverses cognitive impairment and anhedonia-like symptoms in wild-type mice but fails to do so in D3R knockout models, strongly suggesting a D3R-dependent mechanism [214]. Clinical trials further support cariprazine's superior efficacy in reducing negative symptoms. It has demonstrated significantly greater improvement compared to placebo, aripiprazole [215], and risperidone [216]. The precise mechanism of action is only partially clear. One proposed explanation involves differences in receptor binding kinetics. Unlike aripiprazole, which exhibits slow, monophasic dissociation from the D3R, cariprazine displays a fast, biphasic dissociation process [217]. Furthermore, it functions as a bitopic ligand,

interacting not only with the OBS but also with a secondary allosteric site, which may influence receptor conformation and downstream signalling. This rapid receptor dissociation may contribute to cariprazine's ability to adapt to fluctuating DA levels. Another key difference between aripiprazole and cariprazine is the in vivo receptor occupancy. Despite exhibiting notable D3R affinity in vitro, aripiprazole and most antipsychotics either lack significant in vivo occupancy or display only low levels of it [218]. Ultimately, due to its unique pharmacological profile, cariprazine represents a promising lead compound for further drug development.

Table 2: Binding affinities (K_i values [nM]) of Cariprazine, Aripiprazole, and Brexipiprazole for relevant DA and 5-HT receptors. Modified table and data from [219].

Receptor	Cariprazine	Aripiprazole	Brexipiprazole	Therapeutic effects
D2R	0.49	0.34	0.30	Antipsychotic
D3R	0.085	0.8	1.1	Antipsychotic (including negative symptoms), antidepressant
5-HT _{1A}	2.6	1.7	0.12	Antidepressant, anxiolytic
5-HT _{2A}	18.8	3.4	0.47	Effective against extrapyramidal symptoms
5-HT _{2C}	134	15	34	Antidepressant

1.6 Schizophrenia

2 SCOPE AND OBJECTIVES

The development of effective therapeutics for neuropsychiatric and neurodegenerative disorders remains challenging due to their multifaceted nature. Traditional, single-target approaches often fail to address the complexity of conditions such as depression, AD, ASD, and SCH. MTDLs, ligands that simultaneously modulate key receptors and pathways, have emerged as a promising strategy to enhance therapeutic efficacy and reduce side effects.

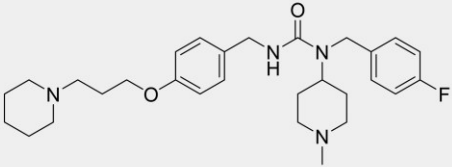
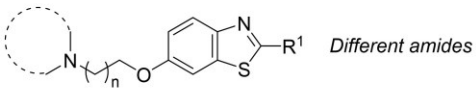
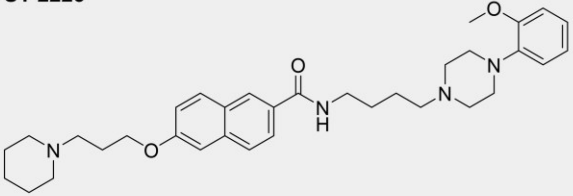
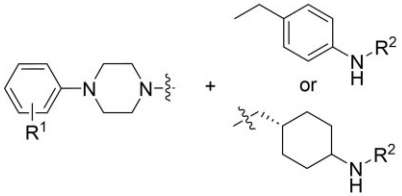
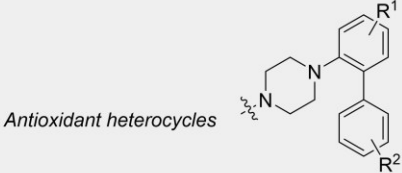
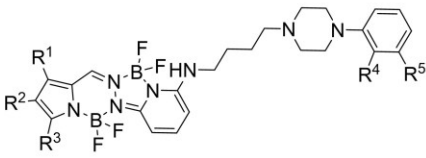
This cumulative dissertation focuses on the pharmacological characterisation of novel aminergic MTDLs targeting histaminergic (H3R) and/or dopaminergic (D2R/D3R) receptors, which are critically involved in the regulation of neurotransmission, cognition, motivation, and mood. These neurotransmitter systems are linked to the pathophysiology of multiple CNS disorders. By incorporating additional serotonergic (e.g., 5-HT_{2A}) or enzymatic (e.g. AChE) activity, the novel aminergic ligands are intended to act at various neurochemical levels.

The primary objective of this cumulative work is to explore the pharmacological profile of novel aminergic ligands for histaminergic and dopaminergic receptors. Various molecular scaffolds are explored, including phenoxypropyl piperidine, arylpiperazines, benzothiazole derivatives, which are combined in a multitargeting approach to interact with different drug targets. The compounds were evaluated through in vitro radioligand binding and enzyme inhibition assays to assess receptor affinity, selectivity, and activity profiles. Complementary in vivo experiments were conducted to investigate antidepressant-, anxiolytic-like effects and neuroprotective properties in established mouse models. The presented publications investigate structure-activity relationships to identify molecular motifs enhancing receptor binding, as well as functional and behavioural outcomes. Furthermore, this thesis includes the development of fluorescently labelled D2R/D3R ligands for a potential use in bioimaging or biophysical assays.

By addressing these objectives, this cumulative dissertation contributes to the field of multitarget drug design by identifying novel MTDL scaffolds, offering new insights for therapeutics with improved efficacy, selectivity and reduced side effects for CNS disorders.

2 Scope and Objectives

Table 3: Overview of the MTDLs pharmacologically evaluated in this cumulative dissertation: The illustrated compounds represent rationally designed ligands targeting H3R, D2R/D3R, and other selected targets. It shows the key structural scaffolds and their corresponding multitarget activity profiles across the six publications incorporated in this dissertation.

	Structure	Multi-Targets
ST-2300		
Publ. 1		H3R/5-HT _{2A/2C}
Publ. 2	 Different amides Different amines	H3R/MAO/ChE
ST-2223		
Publ. 3		H3R/D2R/D3R
Publ. 4		D3R/5-HT _{2A}
Publ. 5	 Antioxidant heterocycles Biphenyl-like systems	D2R/5-HT _{1A/2A} /neuroprotection
Publ. 6	 Fluorescent properties	D2R/D3R/bioimaging

3 PUBLICATIONS

3.1 PUBLICATION 1

The Novel Pimavanserin Derivative ST-2300 with Histamine H₃ Receptor Affinity Shows Reduced 5-HT_{2A} Binding, but Maintains Antidepressant- and Anxiolytic-like Properties in Mice

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Contribution: Shared first author. M.D. designed and performed the H₃R, H₁R, D₃R and D₁R binding studies. M.D. evaluated the pharmacological data, wrote parts of the introduction, major parts of the in vitro characterisation, parts of the in vivo assessment, and major parts of results and discussion. She was involved in the proofreading and review process.

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Article

The Novel Pimavanserin Derivative ST-2300 with Histamine H₃ Receptor Affinity Shows Reduced 5-HT_{2A} Binding, but Maintains Antidepressant- and Anxiolytic-like Properties in Mice

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Abstract: The therapy of depression is challenging and still unsatisfactory despite the presence of many antidepressant drugs on the market. Consequently, there is a continuous need to search for new, safer, and more effective antidepressant therapeutics. Previous studies have suggested a potential association of brain histaminergic/serotonergic signaling and antidepressant- and anxiolytic-like effects. Here, we evaluated the in vivo antidepressant- and anxiolytic-like effects of the newly developed multiple-active ligand ST-2300. ST-2300 was developed from 5-HT_{2A/2C} inverse agonist pimavanserin (PIM, ACP-103) and incorporates a histamine H₃ receptor (H₃R) antagonist pharmacophore. Despite its parent compound, ST-2300 showed only moderate serotonin 5-HT_{2A} antagonist/inverse agonist affinity (K_i value of 1302 nM), but excellent H₃R affinity (K_i value of 14 nM). In vivo effects were examined using forced swim test (FST), tail suspension test (TST), and the open field test (OFT) in C57BL/6 mice. Unlike PIM, ST-2300 significantly increased the anxiolytic-like effects in OFT without altering general motor activity. In FST and TST, ST-2300 was able to reduce immobility time similar to fluoxetine (FLX), a recognized antidepressant drug. Importantly, pretreatment with the CNS-penetrant H₃R agonist (*R*)- α -methylhistamine reversed the antidepressant-like effects of ST-2300 in FST and TST, but failed to reverse the ST-2300-provided anxiolytic effects in OFT. Present findings reveal critical structural features that are useful in a rational multiple-pharmacological approach to target H₃R/5-HT_{2A}/5-HT_{2C}.

Keywords: histamine H₃ receptor; 5-HT_{2A} receptor; pimavanserin; ACP-103; fluoxetine; antidepressant; anxiolytic; forced swim test; tail suspension test; open field test

1. Introduction

Nearly four decades since its discovery, the histamine H₃ receptor (H₃R) has become a widely accepted drug target for multiple central nervous disorders. H₃R is mainly localized in the central nervous system. The function as an autoreceptor is based on a blockage of Ca²⁺ current, which is part of histaminergic neurons' pacemaker function, controlling firing, histamine synthesis, and release [1]. As heteroreceptors, H₃R controls the release of several other neurotransmitters from GABAergic, glutamatergic, serotonergic, noradrenergic, cholinergic, and dopaminergic neurons in the mammalian brain [2,3].

To date, the only marketed selective H₃R antagonist/inverse agonist is Pitolisant (Wakix®), which demonstrated a pronounced wake-promoting effect and is currently used in the EU and the U.S. for the therapeutic management of narcolepsy with or without cataplexy. Additionally, in a recent trial to treat obstructive sleep apnoea, pitolisant has shown excellent clinical outcomes in ameliorating the excessive daytime sleepiness leading to EMA approval (Ozawade®) [4].

Many H₃R ligands have demonstrated attractive benefits in various preclinical models for neuropsychiatric and neurodegenerative diseases, including cognitive deficits [5,6], attention deficit hyperactivity disorder [7–9], autistic behavior [10–12], epilepsy [13,14], and depression [15–17]. Though the clinical significance is still unclear, high-level expression of H₃R in the prefrontal cortex of schizophrenia patients suggested an involvement of the H₃R in schizophrenia [18]. However, no breakthrough has been achieved by H₃R ligands so far in the field of depression and schizophrenia.

Pimavanserin (PIM, ACP-103, Nuplazid®) is an atypical antipsychotic drug approved by the FDA in 2016 for the treatment of hallucinations and delusions associated with Parkinson's disease psychosis. PIM acts as an inverse agonist at 5-HT_{2A} and 5-HT_{2C}, where the affinity for 5-HT_{2A} is higher than that for 5-HT_{2C}. No striking off-target binding to a series of other G-protein coupled receptors (GPCRs), including H₃R, ion channels, or transporters was reported [19]. Moreover, in a Phase II clinical trial, PIM has shown potential efficacy in treating psychosis associated with Alzheimer's disease [20]. In a further Phase II clinical trial (CLARITY study, ClinicalTrials.gov Identifier: NCT03018340), PIM has shown robust effects in major depression disorder (MDD) patients who did not properly respond to first- and second-generation antidepressants [21]. Additionally, an improved condition of insomnia and daytime sleepiness has been observed [22]. PIM has a generally good safety and tolerability profile. Only a low rate of typical side effects of antidepressants and antipsychotics, such as daytime sleepiness, weight gain, sexual dysfunction, extrapyramidal symptoms, and metabolic disorders have been observed.

PIM may have further advantages in treating MDD. Evidence suggests that the 5-HT_{2A} and 5-HT_{2C} receptors may be associated with suicidal behavior in depressive patients [23,24]. However, subjects with previous suicide attempts were excluded from the CLARITY study. The benefit regarding suicidal ideation could not be determined in this subpopulation. However, further studies are still required to gain a firm conclusion [25]. Therefore, simultaneous antagonism of H₃R and 5-HT_{2A/2C} may provide synergistic effects in the treatment of depression and psychiatric disorders with additional benefits, such as suicidal ideation reduction, wake-promoting, and pro-cognitive properties.

Following the concept of developing multiple-acting ligands with presumably more efficient therapeutic effects than selective agents, the multiple-active ligand ST-2300 was designed, comprising a modified structure of PIM and the H₃R antagonist pharmacophore *N*-(3-phenoxypropyl)piperidine (Figure 1) (for review see references [26–31]). In this work, we present the synthesis of ST-2300, its *in vitro* characterization at various histaminergic, dopaminergic, and serotonergic receptors, as well as the *in vivo* evaluation, in preclinical animal models, of depression- and anxiety-like behaviors, namely the forced swim test (FST), tail suspension test (TST), and open field test (OFT).

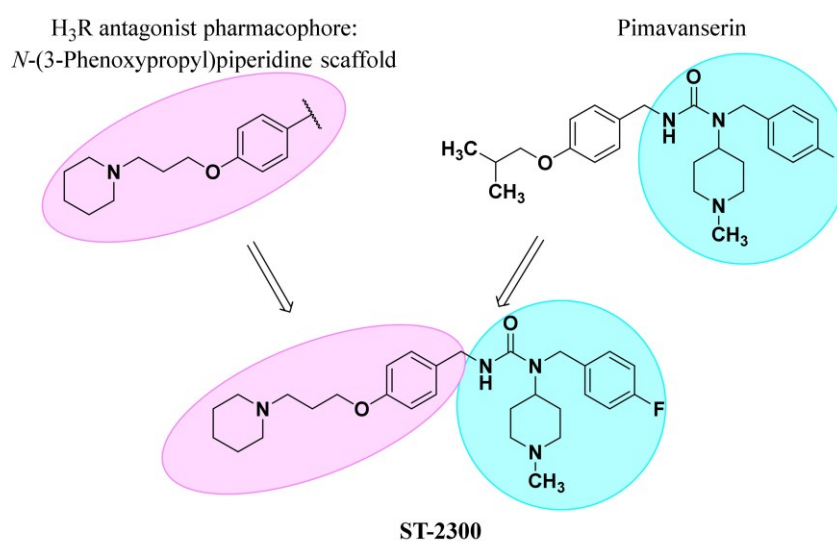


Figure 1. Design of multiple-active compound ST-2300.

2. Materials and Methods

2.1. Chemistry

Compound ST-2300 was synthesized according to the procedure described for PIM in a patent of ACADIA [32], from isocyanate **4** and secondary amine **5**. Instead of the gaseous phosgene applied in the patent, liquid diphosgene was used to prepare the isocyanate derivative **4** from the benzylamine derivative **3**, which was obtained from a series of nucleophilic substitutions and hydrogenation reactions (Figure 2).

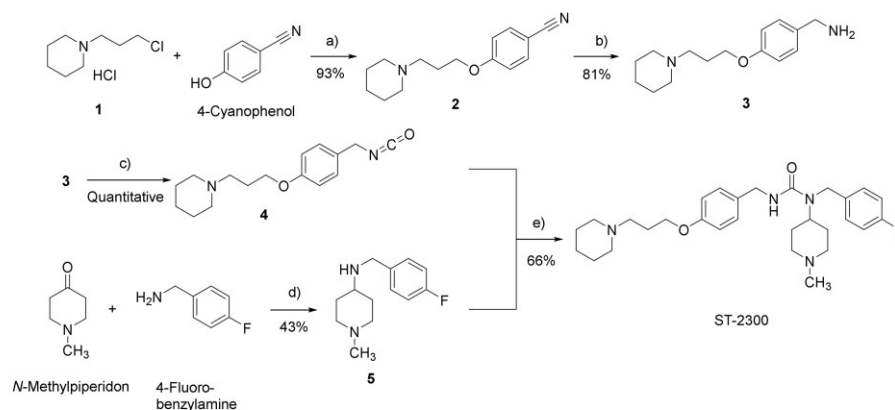


Figure 2. The synthesis of compound ST-2300. (a): K₂CO₃, KI (catalytic amount), acetone, reflux, 18 h; (b): Raney-Nickel, NH₃ (saturated in MeOH), H₂, 5 bar, 40 °C, 18 h; (c): Diphosgene, triethylamine, dichloromethane, 0 °C—room temperature, 2 h; (d): CH₃COOH, NaBH(CH₃COO)₃, 1,2-dichloroethane, room temperature 18 h; (e): Tetrahydrofuran, room temperature, 18 h.

2.1.1. General Notes

Nuclear magnetic resonance (NMR) spectroscopy: Avance III 75, 300 MHz (Bruker, Reinstädten, Germany). Data for proton NMR are reported in the following order: multi-

plicity (br, broad; d, doublet; m, multiplet; p, quintet; s, singlet; and t, triplet; q, quartet; p, quintet), approximate coupling constants J expressed in Hertz (Hz), and the number of protons. Mass spectrometry: atmospheric pressure chemical ionization (APCI+ / −) on Advion Expression L CMS (Advion, Ithaca, NY, USA). Liquid chromatography coupled with mass spectrometry (LC-MS): LC system: Elute SP (HPG 700) (Bruker Daltronics) equipped with a vacuum degasser, autosampler, column heater; column: Intensity Solo 2 C18 RP column (100 mm × 2.1 mm); MS-system: amaZon speed ETD ion trap LC/MSn system (Bruker Daltronics), equipped with electrospray; nebulizer: nitrogen, 15 Psi; dry gas: nitrogen, 8 L/min, 200 °C; mass range mode: Ultrascan. Alternating ion-polarity has been turned on, scan range: m/z : 80–1200. Sample preparation: the stock solutions in MeOH (approximately 1 mg/mL) have been diluted with MeOH (LC-MS grade), and the concentrations of approximately 0.1–0.2 mg/mL were obtained. The volume of injection was 2 μ L. The column temperature has been conditioned at 50 °C. The mobile phase consisted of gradients from acetonitrile (LC-MS grade, Sigma Aldrich, St. Louis, MO, USA) and H₂O (LC-MS grade, Sigma Aldrich, St. Louis, MO, USA) with 0.1% of formic acid (FA) (v/v) (Merck KGaA, Darmstadt, Germany). The flow rate was 0.2 mL/min, the measurements were only analyzed in positive mode, and the relative purity of the compounds was determined. Eluent mixture: acetonitrile:H₂O (v/v): 0–4 min. 95:5, 4–5 min. gradient to 90:10, 5–12 min gradient to 80:20, 12–13 min. 80:20, 13–15 min. gradient to 5:95, reconditioning: 15–17 min. gradient to 95:5.

2.1.2. Syntheses and Analyses

4-[3-(Piperidin-1-yl)propoxy]benzonitrile (2) [33].

The preparation of compound **2** was prepared according to the following process: 4-Cyanophenol (2.62 g, 22 mmol), alkyl chloride **1**, whose synthesis was described previously [34] (3.96 g, 20 mmol), K₂CO₃ (16 g, 119 mmol), and KI (0.70 g, 4.2 mmol) were suspended in acetone 100 mL. The reaction mixture was heated at reflux for 18 h. Upon the completion of the reaction monitored via thin-layer chromatography (TLC), the inorganic residue was removed by filtration. The solution was concentrated and diluted with ethyl acetate 100 mL and washed with 1 M NaOH 60 mL. The wash process was repeated until all phenolic derivative was removed from the organic layer (monitored via TLC). The organic layer was washed with one portion of 50 mL H₂O and one portion of 50 mL brine was subsequently dried with anhydrous MgSO₄, and evaporated in a rotary evaporator. The product was obtained in a yield of 93% as a transparent brownish viscous liquid and used without further purification in the next steps.

¹H NMR (300 MHz, DMSO-*d*₆) δ 7.74 (d, J = 8.9 Hz, 2H, Ph-2,6-*H*), 7.09 (d, J = 8.9 Hz, 2H, Ph-3,5-*H*), 4.07 (t, J = 6.4 Hz, 2H, -O-CH₂-), 2.41–2.24 (m, 6H, overlap: Piperidine-CH₂, Piperi-2,6-*H*₂), 1.97–1.78 (m, 2H, -O-CH₂-CH₂-), 1.53–1.42 (m, 4H, Piperidine-3,5-*H*₂), 1.41–1.31 (m, 2H, Piperidine-4-*H*₂). Molecular weight calculated for chemical formula: C₁₅H₂₀N₂O: 244.3 g/mol. APCI-MS (+): m/z : 245.2 [M + H]⁺.

{4-[3-(Piperidin-1-yl)propoxy]phenyl}methanamine (3) [35].

Preparation of Raney-Nickel: 1.0 g of nickel–aluminium alloy was treated with 80 mL of 10% NaOH solution (aq. m/V), the mixture was heated at 90 °C for 60 min, and the colorless solution was decanted. The black-colored nickel particles were washed with H₂O. The wash process was repeated until the pH value of the wash water was neutral. The nickel particles were subsequently washed three times with 20 mL of MeOH to remove the rest of the water and transferred cautiously to an autoclave tube.

Hydrogenation with Raney-Nickel: the cyano-derivative **2** (4.5 g, 18.5 mmol) was dissolved in 100 mL MeOH (NH₃), and this solution was given to the activated nickel particles. The reaction was stirred under 5 bar of hydrogen and heated at 40 °C. After 18 h, the pressure of the gas was released cautiously, the suspension of the reaction was filtered through kieselgur. The filter cake was rinsed three times with 20 mL of MeOH. The filtrate was collected and evaporated to dryness to obtain compound **3** in a yield of 81% as

a yellowish transparent viscous liquid. The product was sufficiently pure to use in the next steps without further purification.

^1H NMR (300 MHz, CDCl_3) δ 7.20 (d, J = 8.1 Hz, 2H, Ph-2,6-*H*), 6.86 (d, J = 8.0 Hz, 2H, Ph-3,5-*H*), 3.99 (t, J = 6.4 Hz, 2H, -O- CH_2 -), 3.82 (s, 2H, Ph- CH_2 -), 2.55–2.32 (m, 6H, overlap: Piperi- CH_2 -, Piperi-2,6- H_2), 1.97 (p, J = 6.6 Hz, 2H, -O- CH_2 - CH_2 -), 1.78 (br, 2H, - NH_2), 1.60 (p, J = 5.6 Hz, 4H, Piperi-3,5- H_2), 1.50–1.37 (m, 2H, Piperi-4- H_2). Molecular weight calculated for chemical formula: $\text{C}_{15}\text{H}_{24}\text{N}_2\text{O}$: 248.4 g/mol. APCI-MS (+): m/z : 249.2 $[\text{M} + \text{H}^+]^+$.

1-[3-[4-(Isocyanatomethyl)phenoxy]propyl]piperidine hydrochloride (**4**).

The isocyanate derivative **4** was synthesized via the following method: a solution of the benzylamine derivative **3** (0.28 g, 1.12 mmol) and triethylamine (0.56 mL, 4 mmol) in 5 mL of dichloromethane was given to a solution of diphosgene (0.12 mL, 1 mmol) in 5 mL of dichloromethane at 0 °C. The mixture was allowed to reach r.t. and further stirred for 1.5 h. The solvent was subsequently removed in the rotary evaporator. The crude product was yielded quantitatively as a white solid and used in the next step without further purification.

^1H NMR (300 MHz, CDCl_3) δ 7.19 (d, J = 8.6 Hz, 2H, Ph-2,6-*H*), 6.83 (d, J = 8.7 Hz, 2H, Ph-3,5-*H*), 4.33 (d, J = 5.9 Hz, 2H, Ph- CH_2 -), 4.01 (t, J = 5.9 Hz, 2H, -O- CH_2 -), 2.89–2.71 (m, 6H, overlap, Piperi- CH_2 -, Piperi-2,6- H_2), 2.29–2.14 (m, 2H, -O- CH_2 - CH_2 -), 1.86 (p, J = 5.7 Hz, 4H, Piperi-3,5- H_2), 1.63–1.51 (m, 2H, Piperi-4- H_2). Molecular weight calculated for chemical formula: $\text{C}_{16}\text{H}_{22}\text{N}_2\text{O}_2$: 274.4 g/mol. APCI-MS (+): m/z : 275.2 $[\text{M} + \text{H}^+]^+$.

N-(4-Fluorobenzyl)-1-methylpiperidin-4-amine (**5**) [32].

Compound **5** was synthesized via Borch reductive amination: 4-fluorobenzylamine (0.50 g, 4 mmol), 1-methylpiperidin-4-one (0.45 g, 4 mmol), and glacial acetic acid (0.34 mL, 6 mmol) were dissolved in 20 mL of dichloroethane. The reaction mixture was stirred in r.t. for 1 h. $\text{NaBH}(\text{CH}_3\text{COO})_3$ (1.00 g, 4.7 mmol) was added to the aforementioned solution, and the reaction was further stirred at r.t. for 18 h. Upon the completion of the reaction, monitored via TLC, 50 mL of NaHCO_3 solution was given to the mixture. The resulting mixture was extracted with three portions of 50 mL of DCM. The combined organic layer was dried with anhydrous MgSO_4 and concentrated in a rotary evaporator. The residue was purified with column chromatography, where the solvent mixtures of DCM and MeOH (NH_3) in ratios of 98:2 and 95:5, respectively, were used as eluents. The product was obtained in a yield of 43% as a yellow-colored liquid.

^1H NMR (300 MHz, CDCl_3) δ 7.34–7.22 (m, 2H, Ph-2,6-*H*), 7.07–6.93 (m, 2H, Ph-3,5-*H*), 3.78 (s, 2H, Ph- CH_2 -), 2.87–2.74 (m, 2H, Piperidine-2,6- H_{eq}), 2.58–2.40 (m, 1H, Piperidine-4-*H*), 2.26 (s, 3H, - CH_3), 2.06–1.82 (m, 4H, Piperidine-2,6- H_{ax} , Piperi-3,5- H_{eq}), 1.53–1.34 (m, 2H, Piperidine-3,5- H_{ax}). Molecular weight calculated for chemical formula: $\text{C}_{13}\text{H}_{19}\text{FN}_2$: 222.3 g/mol. APCI-MS (+): m/z : 223.0 $[\text{M} + \text{H}^+]^+$.

1-(4-Fluorobenzyl)-1-(1-methylpiperidin-4-yl)-3-[4-[3-(piperidin-1-yl)propoxy]benzyl] urea (**ST-2300**).

The PIM analog **ST-2300** was synthesized in analogy to the methods patented previously by ACADIA [32]. A solution of the isocyanate derivative **4** (0.31 g, 1.12 mmol) in 10 mL of tetrahydrofuran was given to a solution of compound **5** (0.32 g, 1.44 mmol) in 10 mL tetrahydrofuran. The reaction mixture was stirred at r.t. for 18 h. The solvent was subsequently reduced in the rotary evaporator. The residue was purified with column chromatography, where the solvent mixture of DCM and MeOH in a ratio of 90:10 and the mixture of DCM and MeOH (NH_3) in a ratio of 90:10 were used as eluents. **ST-2300** was obtained in a yield of 66% as a white to beige solid with a melting point of 114.0 °C.

^1H NMR (300 MHz, CDCl_3) δ 7.21–7.10 (m, 2H, 4-F-Ph-2,6-*H*), 7.05–6.89 (m, 4H, 4-O-Ph-2,6-*H*, 4-F-Ph-3,5-*H*), 6.76 (d, J = 8.7 Hz, 2H, 4-O-Ph-3,5-*H*), 4.47 (t, J = 5.4 Hz, 1H, -CO-NH-), 4.36–4.27 (m, 3H, overlap: 4-F-Ph- CH_2 -, 1-Me-Piperidine-4-*CH*-), 4.25 (d, J = 5.4 Hz, 2H, 4-O-Ph- CH_2 -), 3.94 (t, J = 6.4 Hz, 2H, -O- CH_2 -), 2.90–2.77 (m, 2H, 1-Me-Piperidine-2,6- H_{eq}), 2.49–2.33 (m, 6H, overlap: Piperidine- CH_2 -, Piperi-2,6- H_2), 2.23 (s, 3H, - CH_3), 2.04 (td, 3J = 11.6, 3.2 Hz, 2H, 1-Me-Piperidine-2,6- H_{ax}), 1.99–1.88 (m, 2H, -O- CH_2 - CH_2 -), 1.77–1.62

(m, 4H, 1-Me-Piperidine-3,5- H_2), 1.57 (p, $J = 5.2$ Hz, 4H, Piperidine-3,5- H_2), 1.47–1.36 (m, 2H, Piperidine-4- H_2). ^{13}C NMR (75 MHz, CDCl_3) δ 162.06, 158.29, 158.15, 134.20, 131.38, 128.71, 127.75, 115.79, 114.59, 66.60, 56.06, 55.35, 54.71, 52.31, 46.17, 45.18, 44.49, 30.26, 26.88, 26.03, 24.49. Molecular weight calculated for chemical formula: $\text{C}_{29}\text{H}_{41}\text{FN}_4\text{O}_2$: 496.7 g/mol. Purity determined via LC-MS: 96.18%, m/z : 249.08 $[(M + 2H^+)/2]^+$ and 497.33 $[M + H^+]^+$.

2.2. In Vitro Characterization

2.2.1. In Vitro Radioligand Displacement Assays at Different GPCRs

Histamine H_3 Receptor

Radioligand displacement assays were performed as described previously [31,36]. HEK293 cells stably expressing the human histamine H_3 receptor [37] were grown in Dulbecco's modified Eagle's medium, supplemented with 2 mM glutamine, 10 mM HEPES, 10% fetal bovine serum, and 10 $\mu\text{L}/\text{mL}$ penicillin/streptomycin in an atmosphere of 5% CO_2 at 37 °C. Cells were grown to confluence. The medium was removed, and cells were collected in 15 mL ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , pH 7.4). The cell suspension was centrifuged two times at $3000 \times g$ for 10 min with subsequent aspiration in 20 mL of PBS buffer at 4 °C. After the last centrifugation step, cells were disrupted and homogenized using an ULTRA-TURRAX® T25 digital (IKA, Staufen, Germany) in ice-cold binding buffer (12.5 mM MgCl_2 , 100 mM NaCl, and 75 mM Tris/HCl, pH 7.4). The cell membrane homogenate was centrifuged two times at $20,000 \times g$ for 20 min at 4 °C and homogenized again by sonication at 4 °C, and kept in ice-cold binding buffer. The membranes were stored at -80 °C until use.

Competition binding experiments were carried out as follows: membranes (20 $\mu\text{g}/\text{well}$ in a final volume of 0.2 mL binding buffer) were incubated with $[^3\text{H}]\text{-N}^{\alpha}$ -methylhistamine (2 nM), purchased from PerkinElmer (Waltham, MA, USA), and different concentrations of the respective test ligand. Assays were run at least in duplicates with eleven appropriate concentrations between 0.01 nM and 100 μM of the test compound. The preparation of competitor concentrations was carried out by serial dilution of 10 and 3 mM stock solution using a Freedom EVO pipetting instrument (TECAN®, Männersdorf, Switzerland). Incubations were performed for 90 min at r.t. under shaking. Non-specific binding was determined in the presence of the selectively acting histamine H_3 receptor inverse agonist/antagonist pitolisant (10 μM). The bound radioligand was separated from free radioligand by filtration through GF/B filters pretreated with 0.3% (m/v) polyethylenimine using an Inotech cell harvester (Dottikon, Switzerland). Unbound radioligand was removed by three washing steps with approximately 0.25 mL/well of cold deionized water. 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. The competition binding data were analyzed by the software GraphPad Prism™ (2016, version 7.01, San Diego, CA, USA) using a non-linear regression fit "one-site competition". The inhibitory constant (K_i) was calculated from the IC_{50} values according to the Cheng-Prusoff equation [38], expressed as the means from at least three independent experiments in duplicate with 95% confidence intervals.

Histamine H_1 Receptor

To determine the human $H_1\text{R}$ affinity, compounds and reference substances were tested in a $[^3\text{H}]\text{-pyrilamine}$ displacement assay. The assay was conducted with membrane preparations of CHO-K1 cells stably expressing h $H_1\text{R}$. Transfection, cell culture, and membrane preparation were described in detail by Smit and colleagues [39]. For the competition binding assay, membrane fractions (40 $\mu\text{g}/\text{well}$ in a final volume of 0.2 mL binding buffer) were incubated with $[^3\text{H}]\text{-pyrilamine}$ (1 nM) and the test compounds in different concentrations for 120 min at r.t. under shaking. The experimental assays were carried out in triplicate with at least six appropriate concentrations between 0.1 nM and 100 μM for the test compounds. Nonspecific binding was evaluated in the presence of the

standard H₁R antagonist chlorpheniramine hydrogen maleate at a concentration of 10 μ M. The following steps were performed as described before.

Histamine H₄ Receptor

To determine the human H₄R affinity, compounds and reference structures were examined in a [³H]-histamine dihydrochloride competition binding assay. Cell culture and membrane preparation of Sf9 cells co-expressing hH₄R-G α_{i2} fusion protein with G $\beta_1\gamma_2$ was prepared as described by Schneider and co-workers [40].

The competition binding assay has been performed as described previously by Sander, Kottke, and Schwed from the Stark Lab [41,42]. Cell membranes were sedimented by 3 min centrifugation at 25 °C and 130 rpm and were resuspended in binding buffer. Competition binding experiments were carried out by incubating the membranes (40 μ g/0.2 mL binding buffer) and [³H]-histamine dihydrochloride (10 nM) with test compounds for 60 min at r.t. Assays were run in triplicates with four to seven appropriate concentrations between 0.1 nM and 100 μ M for the test compound. Nonspecific binding was determined in the presence of 100 μ M JNJ7777120. The following steps were performed as described before.

Dopamine D₃ and D₁ Receptor

To determine the human D₃R affinity, compounds and reference substances were tested in a [³H]-spiperone competition binding assay as described by Bautista-Aguilera et al. [43]. The assay was conducted with membrane preparations of CHO-K1 cells stably expressing hD₃R. Membrane fractions (20 μ g/well in a final volume of 0.2 mL binding buffer) were incubated with [³H]-spiperone (0.2 nM) and the test compounds for 120 min at r.t. under shaking. Assays were run in triplicates with at least six appropriate concentrations between 0.1 nM and 100 μ M for the test compound. Nonspecific binding was determined in the presence of 10 μ M haloperidol.

hD₁R affinity was determined according to the procedure of hD₃R [43]. Membrane preparations of HEK293 cells stably expressing the hD₁R were used with 20 μ g/well. [³H]-SCH23390 was used as radioligand in a concentration of 0.3 nM. Nonspecific binding was determined in the presence of 100 μ M fluphenazine. The following steps were performed as described before.

Dopamine D₂ and Serotonin (5-HT_{1A}, 5-HT_{2A}, 5-HT₆, and 5-HT₇) Receptor

HEK293 cells with a stable expression of human 5-HT_{1A}, 5-HT₆, 5-HT_{7b}, and D_{2L} (prepared with the use of Lipofectamine 2000), or CHO-K1 cells with plasmids containing the sequence coding for the human serotonin 5-HT_{2A} receptor (PerkinElmer), were maintained at 37 °C in a humidified atmosphere with 5% CO₂ and grown in Dulbecco's Modified Eagle Medium containing 10% dialyzed fetal bovine serum and 500 μ g/mL G418 sulfate. For membrane preparation, cells were subcultured in 150 cm² flasks, grown to 90% confluence, washed twice with prewarmed to 37 °C phosphate buffered saline (PBS) and pelleted by centrifugation (200 $\times$ g) in PBS containing 0.1 mM EDTA and 1 mM dithiothreitol. Prior to membrane preparation, the pellets were stored at −80 °C.

Cell pellets were thawed and homogenized in 10 volumes of assay buffer using an Ultra Turrax tissue homogenizer, and were then centrifuged twice at 35,000 $\times$ g for 15 min at 4 °C, with incubations for 15 min at 37 °C in between. The composition of the assay buffers was as follows: for 5-HT_{1A}: 50 mM Tris HCl, 0.1 mM EDTA, 4 mM MgCl₂, 10 μ M pargyline, and 0.1% ascorbate; for 5-HT_{2A}: 50 mM Tris HCl, 0.1 mM EDTA, 4 mM MgCl₂, and 0.1% ascorbate; for 5-HT₆: 50 mM Tris HCl, 0.5 mM EDTA, and 4 mM MgCl₂; for 5-HT_{7b}: 50 mM Tris HCl, 4 mM MgCl₂, 10 μ M pargyline, and 0.1% ascorbate; for D₂R: 50 mM Tris HCl, 1 mM EDTA, 4 mM MgCl₂, 120 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, and 0.1% ascorbate. All assays were incubated in a total volume of 200 μ L in 96-well microtiter plates for 1 h at 37 °C, except for 5-HT_{1A} and 5-HT_{2A}, which were incubated at room temperature and 27 °C, respectively. The process of equilibration was terminated by rapid filtration through Unifilter plates with a 96-well cell harvester and the radioactivity retained

on the filters was quantified on a Microbeta plate reader (PerkinElmer, Waltham, MA, USA). For displacement studies, the assay samples contained as radioligands (PerkinElmer, Waltham, MA, USA) were: 2.5 nM [^3H]-8-OH-DPAT (135.2 Ci/mmol) for 5-HT_{1A}; 1 nM [^3H]-ketanserin (53.4 Ci/mmol) for 5-HT_{2A}; 2 nM [^3H]-LSD (83.6 Ci/mmol) for 5-HT₆; 0.8 nM [^3H]-5-CT (39.2 Ci/mmol) for 5-HT₇ or or 2.5 nM [^3H]-raclopride (76.0 Ci/mmol) for D_{2L}R. Non-specific binding was defined with 10 μM of 5-HT in 5-HT_{1A} and 5-HT₇ binding experiments, whereas 20 μM of mianserin, 10 μM of methiothepine, or 10 μM of haloperidol were used in 5-HT_{2A}, 5-HT₆, and D_{2L} assays, respectively. Each compound was tested in triplicate at 7 concentrations (10^{-10} – 10^{-4} M). The inhibition constants (K_i) were calculated from the Cheng-Prusoff equation [38]. Results are expressed as means of two separate experiments.

2.3. In Vivo Assessment

2.3.1. Animals

Inbred adult male C57BL/6 mice (aged 10–12 weeks and weighing 20–25 g) were obtained from the animal facility in the College of Medicine and Health Sciences, United Arab Emirates University. The animals were maintained in a separate air-conditioned room with controlled temperature and humidity (24 ± 2 °C and $55 \pm 15\%$, respectively), with a 12 h light/dark cycle, and ad libitum to food and water. All the experiments were performed between 9.00 am and 3.00 pm. The procedures were approved by the Institutional Animal Ethics Committee of College of Medicine and Health Sciences/United Arab Emirates University (Approval No. ERA-2019-6010). To reduce the suffering of the animals, a minimum number of animals were used in this study, whereas the objectives were not compromised.

2.3.2. Drugs

The multiple-active test compound 1-(4-fluorobenzyl)-1-(1-methylpiperidin-4-yl)-3-[4-[3-(piperidin-1-yl)propoxy]benzyl]urea (ST-2300, 5, 10 and 15 mg/kg, i.p.) and the reference drug PIM (5, 10, and 15 mg/kg, i.p.) were provided by the Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Germany. Diazepam (DZP, 1 mg/kg, i.p.), manufactured by Gulf Pharmaceutical Industries (Ras Al Khaimah, United Arab Emirates), was obtained from Dr. Essam Emam (Department of Medicine, Tawam Hospital, Al Ain, United Arab Emirates) and fluoxetine (Prozac®, 10 mg/kg) was obtained from Eli Lilly (Indianapolis, IN, USA). The brain-penetrant H₃R agonist (*R*)- α -methylhistamine (RAM, 10 mg/kg, i.p.) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3.3. Behavioral Studies

These experiments provide the first behavioral assessment of the ST-2300 in anxiety-like and depression-like behaviors in mice. All doses are expressed in terms of the free base of all test compounds. The drugs used in the current study were dissolved in saline and injected i.p. at a volume of 1 mL/kg. All experimental procedures were conducted in a blinded fashion in which the experimenter was uninformed about the specific treatment groups to which an animal group belonged. Saline, ST-2300 (5, 10, and 15 mg/kg), PIM (5, 10, and 15 mg/kg), FLX (10 mg/kg), RAM (10 mg/kg), and DZP (1 mg/kg) were injected i.p. 30–45 min before the beginning of the behavioral experiments.

Forced Swim Test

The FST test was performed according to previously described protocols [16,44,45]. Mice were individually placed into glass cylinders containing 15 cm of water at ~ 25 °C. The mice were left in the cylinders for 6 min and immobility was observed and analyzed. The mice were then removed from the container and left to dry in a heated enclosure before being returned to their home cages. The mice were considered to be immobile when they ceased struggling and remained floating motionless in the water (without any vertical or

horizontal movements), making only the movements necessary to keep their heads above the water level as described previously [16,45].

Tail Suspension Test

This experimental protocol was followed as previously described, however with slight modifications [16]. Each mouse was suspended on the edge of a rod 50 cm above a table top using adhesive Scotch tape placed approximately 1 cm from the tip of the tail. Tail climbing was prevented by passing the mouse's tail through a small plastic cylinder prior to suspension, as described by Can et al. [46]. The duration of immobility was manually scored for a 6 min observation period. Mice were considered immobile only when they hung down passively and were completely motionless. The parameter recorded was the number of seconds spent immobile.

Open Field Test

To analyze the effect of ST-2300 on locomotion and anxiety behaviors in animals, OFT was carried out. In addition to locomotor activity, the OFT is usually used to measure anxiety-like behaviors in experimental rodents [10,16,47,48]. The test provides a unique opportunity to systematically assess novel environment exploration, general locomotor activity, and provide an initial screen for anxiety-related behavior in experimental rodents. The OF box consisted of a square box (45 × 45 × 30 cm). A 23 × 23 cm area in the center region was defined as the central arena, and the remaining area was defined as periphery area. The mice with a higher degree of anxiety prefer to stay closer to the walls of the box and spend less time in the center, whereas increased time spent in the central area indicates low anxiety level and high exploratory behaviors. The first 5 min of the experiment were considered as habituation, followed by 10 min test session. Total distance moved in the whole arena, time spent in the center, and in the periphery were recorded for 10 min using a charge-coupled, camera-assisted, motion-tracking apparatus and software (EthoVision 3.1, Noldus Information Technology, Wageningen, The Netherlands). After each mouse completed the test, the OF chamber was cleaned thoroughly with 70% (volume/volume; vol/vol) alcohol.

2.3.4. Statistics

For behavioral assessments, data are expressed as means ± SEM. The data were analyzed for normality by assessing the sample distribution or skewness (−1.5 to +1.5 was considered as normally distributed). After the results had passed the tests for normality, the effects of drug treatments were analyzed by a two-way analysis of variance (ANOVA) and post hoc comparisons were performed with the Tukey's test in case of a significant main effect. For statistical comparisons, the software package SPSS 25.0 (IBM Middle East, Dubai, United Arab Emirates) was used. The *p* values of less than 0.05 were considered as statistically significant.

3. Results

For the pharmacological in vitro evaluation of ST-2300, competitive radioligand binding assays at various GPCRs were performed (Table 1). Our study confirmed the high receptor binding affinity of PIM to the 5-HT_{2A} receptor with a *K_i* value in the low nanomolar range. It should be noted that a *K_i* value in the picomolar concentration range, reported from Vanover et al. [19], could not be reproduced under our assay conditions. Furthermore, PIM demonstrated a good binding affinity at the 5-HT₆ receptor and weaker binding at the dopamine D₂ receptor. It was previously reported [19] that PIM does not show appreciable activity at dopaminergic/histaminergic GPCRs and lacks D₂R antagonistic activity [49], which may explain the reduced side effect profile of PIM. Under our assay conditions, a good 5-HT₆ affinity and a weak D₂R affinity were observed.

Table 1. Binding affinity or percentage inhibition of ST-2300 or PIM at different GPCRs.

Receptor Subtype	ST-2300 $K_i \pm SD$ (n) or K_i [CI 95%] (n)	Pimavanserin $K_i \pm SD$ (n)
H ₃ R	14.4 nM [2.98 nM; 70.0 nM] (4) ^b	inhibition at 10 μ M: <50% ^a
5-HT _{2A}	1302 $\pm$ 331 nM (2) ^c	0.7 $\pm$ 0.4 nM (2) ^c
5-HT _{1A}	>10,000 (2) ^d	>10,000 (2) ^d
5-HT ₆	>10,000 (2) ^d	59 $\pm$ 11 nM (2) ^d
5-HT ₇	>10,000 (2) ^d	>10,000 (2) ^d
D ₁ R	>10,000 (3) ^b	inhibition at 10 μ M: <50% ^a
D ₂ R	>10,000 (2) ^e	2417 $\pm$ 667 nM (2) ^e
D ₃ R	>10,000 (3) ^f	inhibition at 10 μ M: 60% ^a
H ₁ R	>5000 (2) ^f	inhibition at 10 μ M: <50% ^a
H ₄ R	>1000 (2) ^g	n.d. ^h

^a Determined by Vanover et al. [19]. ^b Displacement assay carried out using HEK293 cells, stably expressing the human histamine H₃R or dopamine D₁R against [³H]-N^α-methylhistamine or [³H]-SCH23390 as radioligands, respectively. ^c Displacement assay carried out using CHO-K1 cells expressing the human serotonin 5-HT_{2A} receptor against [³H]-ketanserin as a radioligand. ^d Displacement assay carried out using HEK293 cell lines stably expressing the serotonin h5-HT_{1A}, h5-HT₆, or h5-HT₇ against [³H]-8-OH-DPAT, [³H]-LSD, or [³H]-5-CT as radioligands, respectively. ^e Displacement assay carried out using HEK293 cells stably expressing the human dopamine D₂R against [³H]-raclopride as a radioligand. ^f Displacement assay carried out using CHO-K1 cells stably expressing the human histamine H₁R or dopamine D₃R against [³H]-pyrilamin or [³H]-spiperone as radioligands, respectively. ^g [³H]-Histamine displacement assay performed with cell membrane preparation of Sf9 cells transiently expressing the human histamine H₄R, co-expressed with Gα_{i2} and Gβ_{1γ2} subunits. ^h n.d., not determined.

ST-2300 has shown excellent binding affinity at H₃R (Table 1). However, despite the high structural similarity to PIM, ST-2300 displayed only a moderate affinity at 5-HT_{2A}. Screenings performed on other monoamine receptors subtypes revealed no notable off-target affinities for ST-2300.

In vivo antidepressant- and anxiolytic-like effects of ST-2300 were evaluated using FST, TST, and OFT in adult male C57BL/6 mice. FST and TST were used to determine the antidepressant-like effects, while OFT was used to determine anxiolytic-like effects. Mice were pretreated with ST-2300 (0–15 mg/kg, intraperitoneal, i.p.) or PIM (0–15 mg/kg, i.p.). Fluoxetine (FLX, 10 mg/kg, i.p.) and diazepam (DZP, 1 mg/kg, i.p.) were used as reference compounds in the depressive-like and anxiety-like animal model, respectively. To determine whether the observed effects were associated with antagonism of H₃Rs, (R)-α-methyl histamine (RAM, 10 mg/kg, i.p.) was co-administrated with ST-2300. To exclude any confounding effects of the CNS-penetrant H₃R agonist RAM, RAM was administered to the control mice.

3.1. Forced Swim Test (FST)

The results observed in FST revealed a significant and dose-dependent decrease of immobility time for mice pretreated with ST-2300 or PIM (Figure 3). Indeed, the decrease of immobility time for mice treated with ST-2300 (10 or 15 mg/kg) was not significantly different from the decrease of immobility time for mice treated with the reference drug PIM (10 or 15 mg/kg). The co-administration of the CNS-penetrant H₃R agonist RAM with ST-2300 led to a significant increase of immobility time (Figure 4), demonstrating the reversal of the ST-2300-provided effects on immobility time with the co-administration of the H₃R agonist. The latter conclusion shed light on the involvement of histaminergic neurotransmission mediated through H₃Rs.

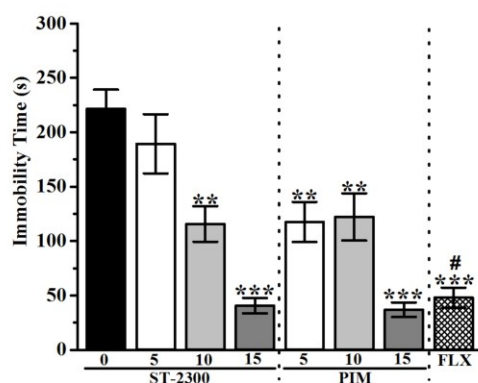


Figure 3. Effects of acute ST-2300 pre-treatment on depression-like behavior in FST. Acute ST-2300 dose-dependently decreased the immobility time in the FST. ** Denotes significant differences between drug doses and saline controls ($p < 0.01$); *** Denotes significant differences between drug doses and controls ($p < 0.001$). # Denotes significant differences between FLX and ST-2300 (5 and 10 mg/kg, i.p.) as well as PIM (5 and 10 mg/kg, i.p.) ($p < 0.05$). Vehicle ($n = 9$); ST-2300 (5 mg/kg, i.p.) ($n = 7$); ST-2300 (10 mg/kg, i.p.) ($n = 6$); ST-2300 (15 mg/kg, i.p.) ($n = 7$); PIM (5 mg/kg, i.p.) ($n = 6$); PIM (10 mg/kg, i.p.) ($n = 7$); PIM (15 mg/kg, i.p.) ($n = 6$); FLX (10 mg/kg, i.p.) ($n = 9$). Data are shown as the mean $\pm$ standard error of the mean. Abbreviations: PIM, pimavanserin; FLX, fluoxetine; FST, forced swim test.

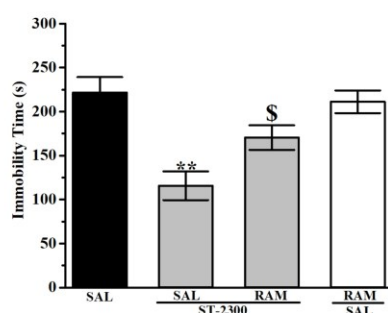


Figure 4. The H_3R agonist RAM reversed the ST-2300-provided antidepressant-like effects in FST. Acute ST-2300 (10 mg/kg, i.p.) significantly decreased the immobility time in the FST. ** Denotes significant differences between ST-2300 (10 mg/kg, i.p.) and saline-treated control mice group ($p < 0.01$); \$ Denotes significant difference when compared to ST-2300 (10 mg/kg, i.p.)-treated mice ($p < 0.05$). Vehicle ($n = 9$); ST-2300 (10 mg/kg) ($n = 6$); ST-2300 (10 mg/kg) co-administered with RAM (10 mg/kg) ($n = 6$); RAM (10 mg/kg) co-administered with vehicle ($n = 9$). Data are shown as the mean $\pm$ standard error of the mean. Abbreviations: RAM, (R)- α -methyl histamine; FST, forced swim test; SAL, saline.

3.2. Tail Suspension Test (TST)

The results obtained in the TST show the effects of ST-2300 on depression-like behavior. Acute systemic pretreatment with ST-2300 (10 or 15 mg/kg, i.p.) significantly reduced the immobility time of tested mice as compared to control mice injected with saline (Figure 5). Interestingly, there is a remarkable difference between treatment with ST-2300 (10 or 15 mg/kg) or PIM (5–15 mg/kg). Accordingly, pretreatment with PIM failed to affect the overall immobility time when compared to control mice. Similar to the results observed for

the FST, the effect of the ST-2300-induced reduction of immobility time was reversed by systemic co-administration with the CNS-penetrant H₃R agonist RAM (Figure 6).

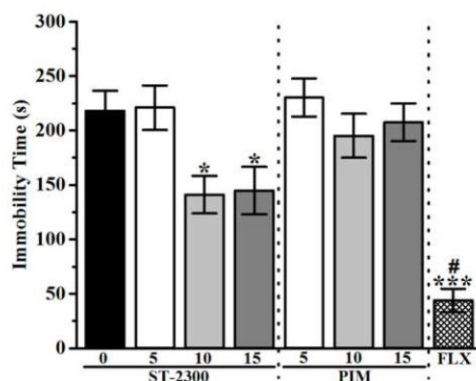


Figure 5. Effects of acute ST-2300 pre-treatment on depression-like behavior in TST. Acute ST-2300 (15 mg/kg, i.p.) decreased the immobility time in the tail suspension test (TST). * Denotes significant differences between ST-2300 doses and controls ($p < 0.05$); *** Denotes significant differences between FLX and saline controls ($p < 0.001$). # Denotes significant differences between FLX and administered drug doses of ST-2223 and PIM ($p < 0.05$). Vehicle (n = 9); ST-2300 (5 mg/kg, i.p.) (n = 7); ST-2300 (10 mg/kg, i.p.) (n = 6); ST-2300 (15 mg/kg, i.p.) (n = 6); PIM (5 mg/kg, i.p.) (n = 6); PIM (10 mg/kg, i.p.) (n = 7); PIM (15 mg/kg, i.p.) (n = 6); FLX (10 mg/kg, i.p.) (n = 9). Data are shown as the mean $\pm$ standard error of the mean. Abbreviations: PIM, pimavanserin; FLX, fluoxetine; TST, tail suspension test.

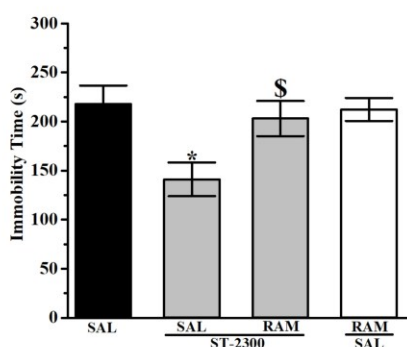


Figure 6. Effects of the H₃R agonist RAM abrogated ST-2300-provided antidepressant-like effects in TST. Acute ST-2300 (10 mg/kg, i.p.) significantly decreased the immobility time in TST. * Denotes significant difference between ST-2300 (10 mg/kg, i.p.) and saline-treated control mice group ($p < 0.05$); \$ Denotes significant difference when compared to ST-2300 (10 mg)-treated mice ($p < 0.05$). Vehicle (n = 9); ST-2300 (10 mg/kg, i.p.) (n = 6); ST-2300 (10 mg/kg, i.p.) co-administered with RAM (10 mg/kg, i.p.) (n = 6); RAM (10 mg/kg, i.p.) co-administered with vehicle (n = 9). Data are shown as the mean $\pm$ standard error of the mean. Abbreviations: RAM, (R)- α methyl histamine; TST, tail suspension test; SAL, saline.

3.3. Open Field Test

To test the effect of ST-2300 on anxiety-like behavior, an open field test (OFT) was performed. Pretreatment with ST-2300 or PIM did not alter the number of total line crossings (Figure 7A), excluding any effects of ST-2300 on locomotor activity. However, systemic ST-

2300 pretreatment led to a dose-dependent increase of time spent in the center of the arena (Figure 7B), indicating the low anxiety level of mice when pretreated with ST-2300 (10 or 15 mg/kg, i.p.). On the contrary, the reference drug PIM (5–15 mg/kg, i.p.) failed to provide any appreciable effects on the anxiety levels in the assessed mice. Notably, the significant increase of time spent in the center following pretreatment with ST-2300 could not be reversed by co-administration with RAM, suggesting that histaminergic neurotransmission by H₃Rs is not involved in the ST-2300-provided anxiolytic effects (Figure 7C).

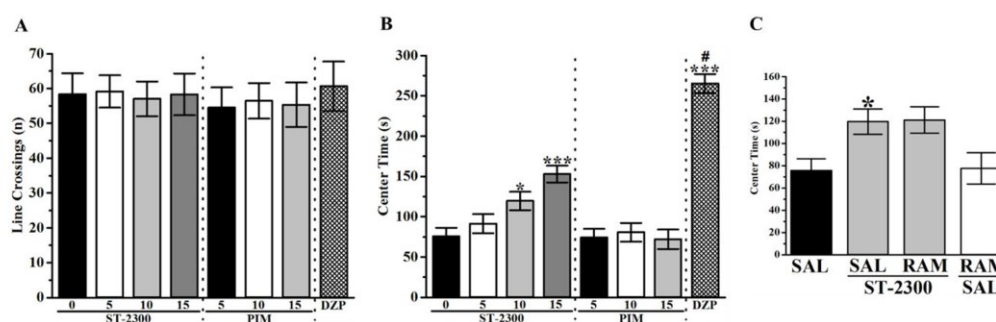


Figure 7. Effects of acute ST-2300 pre-treatment on anxiety-like behavior and locomotor activity in OFT. In OFT, ST-2300 had no effect on total line crossing (A) but dose-dependently increased the time spent in the center of the arena (B). Pre-treatment with the H₃R agonist RAM did not reverse the anxiolytic effect of ST-2300 (C). * Denotes significant difference between ST-2300 (10 mg/kg, i.p.) and controls ($p < 0.05$); *** Denotes significant difference between ST-2300 (10 mg/kg, i.p.) or DZP (1 mg/kg, i.p.) and controls ($p < 0.001$); # Denotes significant differences between DZP and drug doses ($p < 0.05$). Vehicle ($n = 9$); ST-2300 (5 mg/kg, i.p.) ($n = 7$); ST-2300 (10 mg/kg, i.p.) ($n = 6$); ST-2300 (15 mg/kg, i.p.) ($n = 7$); PIM (5 mg/kg, i.p.) ($n = 6$); PIM (10 mg/kg, i.p.) ($n = 7$); PIM (15 mg/kg, i.p.) ($n = 6$); DZP (1 mg/kg, i.p.) ($n = 9$). ST-2300 (10 mg/kg, i.p.) co-administered with RAM (10 mg/kg, i.p.) ($n = 6$); RAM (10 mg/kg, i.p.) co-administered with vehicle ($n = 9$). Data are shown as the mean $\pm$ standard error of the mean. Abbreviations: PIM, pimavanserin; DZP, diazepam; RAM, (R)- α -methyl histamine; OFT, open field test; SAL, saline.

4. Discussion

With the novel designed multiple-active ligand ST-2300, we present a selective and simultaneously targeting H₃R/5-HT_{2A} ligand with an innovative option for treating depressive and psychotic disorders. The linkage of PIM and the histamine H₃R pharmacophore is a promising attempt to combine the antipsychotic properties of 5-HT_{2A/2C} antagonists/inverse agonists with the pro-cognitive effects of H₃R antagonists/inverse agonists. The presented synthesis route is easily adaptable for further lead optimization. As a prototype of its class, ST-2300 already showed high receptor binding affinity at H₃R and decent activity at 5-HT_{2A}. It is known for PIM, which displays selective affinity at 5-HT_{2A} over 5-HT_{2C} [19]. For further investigation of ST-2300, the 5-HT_{2C} affinity should be determined. ST-2300 was lacking any appreciable off-target affinity, which was also previously reported for the reference drug PIM [19]. Though, with our assay conditions, PIM displayed a good affinity at 5-HT₆ and a weak affinity at D₂R. Since 5-HT₆ antagonists are already under investigation for cognitive improvements in patients diagnosed with Alzheimer's disease (AD) [50], this might contribute to a potential use of PIM and its derivatives for AD.

Interestingly, the observed in vivo results showed that ST-2300 was superior or comparable to the reference drug PIM, using depressive-like and anxiety-like animal models. Accordingly, ST-2300 provided antidepressant-like effects in the FST as well as in the TST. In contrast to PIM, ST-2300 was capable of increasing the time spent in the center of the arena in the OFT, without altering the overall locomotor activity of the tested mice

(overall line-crossing events were not influenced by the doses used). Importantly, the CNS-penetrant H₃R agonist RAM reversed the antidepressant-like effects provided by ST-2300 in both paradigms assessed in FST and TST, indicating that the antagonism of H₃Rs was responsible for the provided effects of ST-2300 in tested animals. However, RAM failed to reverse the anxiolytic-like effects provided by ST-2300 in OFT, indicating that neurotransmitters other than histamine are involved in the ST-2300-provided effects on anxiety levels of assessed mice in the OFT. Notably, previous reports in experimental rodents showed antidepressant- or anxiolytic-like effects of several H₃R antagonists/inverse agonists (e.g., ST-1283, pK_i 9.62) [15–17]. However, the exact mechanisms of H₃R antagonists regarding their anxiolytic and antidepressant effects remain to be fully elucidated. Further experimental efforts are necessary to fully understand the mechanisms of histaminergic and/or other monoaminergic action involved in anxiety and depression.

In order to comprehend the therapeutic potential of the concept leading to ST-2300 applications, further approaches should focus on multiple-active ligands with a balanced H₃R/5-HT_{2A} affinity. Moreover, the potential antipsychotic activity of ST-2300 still needs to be assessed in a battery of animal models to be able to generalize the current conclusions.

5. Conclusions

Taken together, the novel multiple-active ligand ST-2300 with H₃R and 5-HT_{2A} antagonist/inverse agonist affinities showed promising H₃R-mediated in vivo antidepressant as well as anxiolytic-like effects in tested animals. Therefore, multiple-active ligands with H₃R affinity might address the need for safe and effective drugs with potential to be used in the treatment of depression- and anxiety-related neuropsychiatric disorders.

Author Contributions: Conceptualization, H.S.; methodology, B.S. and H.S.; synthesis, S.Z. and H.S.; receptor screenings, M.D., G.S. and H.S.; in vivo screening, K.V. and B.S.; formal analysis, S.Z., M.D., G.S. and K.V.; resources, H.S. and B.S.; writing—B.S. and H.S.; writing—review and editing, all authors; supervision, B.S. and H.S.; project administration, H.S.; funding acquisition, H.S. and B.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The procedures were approved by the Institutional Animal Ethics Committee of College of Medicine and Health Sciences/United Arab Emirates University (Approval No. ERA-2019-6010). To reduce the suffering of animals, minimum number of animals were used in this study, whereas the objectives were not compromised.

Data Availability Statement: The datasets generated and analysed are available from the corresponding authors on reasonable request.

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3.2 PUBLICATION 2

Novel benzothiazole derivatives as multitargeted-directed ligands for the treatment of Alzheimer's disease

Donia E. Hafez^a, Mariam Dubiel^b, Gabriella La Spada^c, Marco Catto^c, David Reiner-Link^b, Yu-Ting Syu^d, Mohammad Abdel-Halim^a, Tsong-Long Hwang^{d,e,f,g}, Holger Stark^b and Ashraf H. Abadi^a

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Contribution: Co-authorship. M.D. prepared, planned, and conducted H3R binding studies and selectivity screenings at H1R and H4R, and performed the corresponding data analysis. She wrote major parts of the biological assay section and carried out extensive proofreading and corrections of the manuscript.

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Novel benzothiazole derivatives as multitargeted-directed ligands for the treatment of Alzheimer's disease

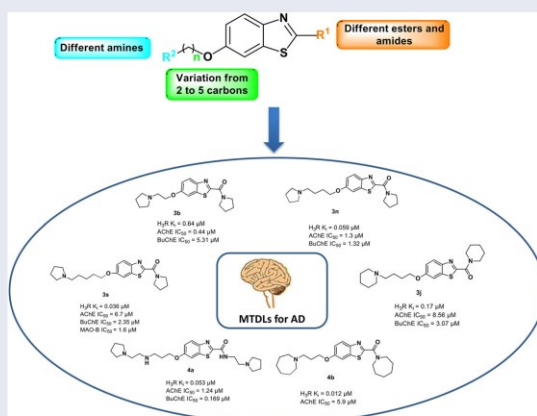
Donia E. Hafez^a, Mariam Dubiel^b, Gabriella La Spada^c, Marco Catto^c, David Reiner-Link^b, Yu-Ting Syu^d, Mohammad Abdel-Halim^a, Tsong-Long Hwang^{d,e,f,g} , Holger Stark^b and Ashraf H. Abadi^a

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ABSTRACT

Neurodegenerative diseases such as Alzheimer's disease (AD) are multifactorial with several different pathologic mechanisms. Therefore, it is assumed that multitargeted-directed ligands (MTDLs) which interact with different biological targets relevant to the diseases, might offer an improved therapeutic alternative than using the traditional "one-target, one-molecule" approach. Herein, we describe new benzothiazole-based derivatives as a privileged scaffold for histamine H₃ receptor ligands (H₃R). The most affine compound, the 3-(azepan-1-yl)propoxy-linked benzothiazole derivative **4b**, displayed a *K_i* value of 0.012 μM. The multitargeting potential of these H₃R ligands towards AChE, BuChE and MAO-B enzymes was evaluated to yield compound **3s** (pyrrolidin-1-yl-(6-((5-(pyrrolidin-1-yl)pentyl)oxy)benzo[d]thiazol-2-yl)methanone) as the most promising MTDL with a *K_i* value of 0.036 μM at H₃R and IC₅₀ values of 6.7 μM, 2.35 μM, and 1.6 μM towards AChE, BuChE, and MAO-B, respectively. These findings suggest that compound **3s** can be a lead structure for developing new multi-targeting anti-AD agents.

GRAPHICAL ABSTRACT



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Introduction

AD is a progressive irreversible neurodegenerative brain disorder and it is the most common form of dementia.^{1,2} The histopathological changes observed in AD patients include deficiency in cholinergic neurotransmission, accumulation of amyloid- β ($A\beta$) peptide in the form of amyloid plaques in the brain, intracellular neurofibrillary tangles and progression of inflammation and damage caused by oxidative stress.³ The drugs approved for treating AD only offer a symptomatic treatment, but fail to stop the neurodegenerative process. Owing to the complex pathology of the disease, a single ligand for a certain target is not sufficient to produce a relevant therapeutic effect.^{4,5} Therefore, the new pharmacological approach is to design single molecules that modulate multiple targets simultaneously, so-called multitarget-directed ligands (MTDLs). MTDLs are synthesised mainly as potential therapeutics for the treatment of multifactorial diseases such as neurodegenerative diseases.^{6,7} MTDLs are compounds that affect more than one pathophysiological pathway since they can be rationally designed as structures with pharmacophore moieties that bind to different receptors and enzymes.⁸ The pathogenesis of most neurodegenerative diseases (especially AD) is linked to different pharmacological targets. Among these targets, neurotransmitters such as ACh, histamine, dopamine and glutamate play major roles in the progressive deterioration of cognitive functions. The combination of H_3 R antagonist pharmacophore and acetylcholinesterase pharmacophore is one of the most interesting and promising combinations among MTDLs for the treatment of AD.^{9,10}

Histamine H_3 receptors (H_3 Rs) belong to the family of G protein-coupled receptors (GPCR). H_3 R was first identified on pre-synaptic histaminergic neurons acting as autoreceptors. Besides its inhibitory activity as an autoreceptor, modulating the release of other neurotransmitters such as noradrenaline, serotonin, dopamine and acetylcholine has been observed by H_3 hetero receptor located on non-histaminergic neurons.¹¹ Thus the precognitive use of H_3 R antagonists/inverse agonists for the treatment of CNS-related disorders, such as depression, schizophrenia, PD, and AD is being investigated.¹² Pitolisant, is the first H_3 R antagonist/inverse agonist which received the approval in Europe and United States for the treatment of narcolepsy with or without cataplexy.¹³ In the early stage of its discovery, pitolisant showed selectivity towards H_3 R and induced central histaminergic transmissions in animal models increasing wakefulness and resulted in increased release of dopamine and ACh in the prefrontal cortex of rats. New classes of H_3 R antagonists with additional pharmacological properties have been described.¹⁴ These classes may have synergistic effect leading to the increase of their therapeutic efficacy in the treatment of disorders related to neurotransmitter deficits, such as AD. Accordingly, the development of multitarget compounds including H_3 receptor-blocking properties has been of great interest.^{9,11}

Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are enzymes responsible for the hydrolysis of acetylcholine and the increase in their activity leads to cognitive disorders.¹⁵ Moreover, the inhibition of AChE may not only result in increased levels of ACh but may also affect the $A\beta$ aggregation.¹⁶ So far, the anti-Alzheimer's drugs include acetylcholinesterase inhibitors (AChEI), such as donepezil, rivastigmine and galantamine and the N -methyl-D-aspartate (NMDA) receptor antagonist memantine.³ Unfortunately, these drugs can only slow down the progression of the disease and relief the symptoms but they do not provide effective treatment for AD. In AD, the cognitive decline is caused by neuronal degeneration of brain regions where the cholinergic activity is predominant. The loss of cholinergic transmission can be counteracted by AChE inhibitors, whose efficacy indeed declines during progression of the disease, while at the same time

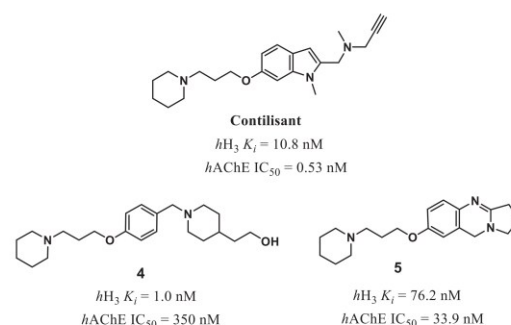


Figure 1. Compounds having dual activities on AChE and H_3 R.

BuChE remains unaltered or increased. Hence, the inhibition of BuChE may provide additional cognitive benefits in the late stages of disease.¹⁷ Hence, cholinergic neurotransmission can be enhanced by cholinesterase inhibitors (ChEIs) which counteract such depletion of ACh neurotransmitter.¹⁸

Several studies proposed structures similar to those designed in this work to have dual activity on AChE and H_3 R such as contilisant, compound 4 and 5 as shown in Figure 1.¹¹ Therefore, the combination of both H_3 R inhibition and cholinesterase enzymes inhibition in a single drug might lead to significant synergistic effects on cognitive function and memory. Using these hybrid compounds can lead to huge progress in the treatment of cognitive disorders.^{9,19}

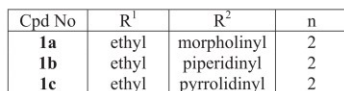
Herein, novel benzothiazole derivatives as H_3 R ligands were developed. Benzothiazole is a privileged heterocyclic scaffold used worldwide for a variety of therapeutic applications.²⁰ Several novel benzothiazoles have been developed and exhibited significant antitumour, antimicrobial, antidiabetic, anti-inflammatory, antiviral, antioxidant, antimalarial, antiasthmatic, anthelmintic, and other activities.^{21–27} New benzothiazole molecules are currently under development and are being evaluated as therapeutic drug candidates for the treatment of neurodegenerative diseases such as Alzheimer's.²⁸ For instance, riluzole (2-amino-6-trifluoromethoxy) benzothiazole is used to treat amyotrophic lateral sclerosis due to its neuroprotective effect acting as voltage-gated sodium channel blockers, non-competitive inhibition of NMDA receptors and inhibition of glutamate release. Recently, evaluation of riluzole in a clinical trial for AD was reported.²⁹

Inspired by the similarity in the pharmacophoric features of the H_3 R antagonists and AChE inhibitors, the activity of the synthesised compounds on AChE enzyme to reach multitarget inhibition was tested.¹⁴ The dual-acting compounds (**3b**, **3h**, **3j**, **3n**, **3s**, **3t**, **4a**, and **4b**) were also tested against BuChE as well as MAO-B enzyme to extend the multitarget range against AD. MAO-B enzyme inhibition was assessed since some literature stated that MAO-B activity is increased in the brain of patients with neurodegenerative diseases and suggested that MAO-B regulates β -amyloid production via γ -secretase.^{30,31} Moreover, molecular modelling studies have revealed possible interactions between active compounds and both AChE and BuChE as well as H_3 R.

Results and discussion

Compounds' design

The presented compounds were designed to match the general structure suggested for histamine H_3 R antagonists/inverse agonists (Figure 2).³² This pharmacophore blueprint contains a basic



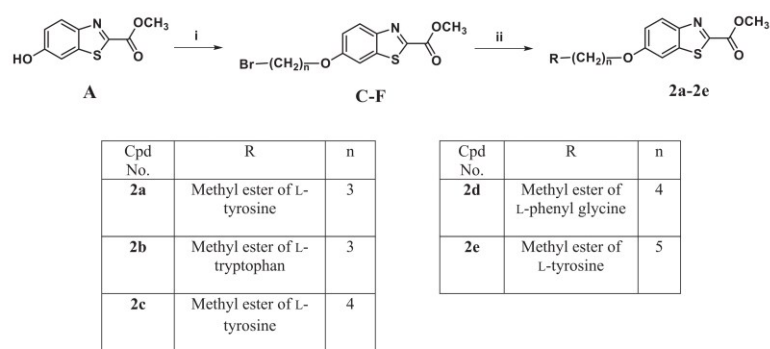
Cpd No	R ¹	R ²	n
1d	methyl	morpholinyl	2
1e	methyl	piperidinyl	2

moiety (mostly a tertiary amine), linked by a spacer to a central core, which is connected to further affinity-enhancing elements in the eastern arbitrary region which can be another basic moiety or hydrophilic/lipophilic groups as shown in Figure 2.^{33–35} The following structural modification strategies were applied to design potent H₃R antagonists: (1) employing benzothiazole ring as a central core;^{36–38} (2) several amines were examined as a basic head including alicyclic amines, cycloalkyl amines and amino acids; (3) the spacer connecting the central benzothiazole core and the basic head was varied from two to five carbons; (4) different esters and amides were added to the eastern arbitrary region of the compound.

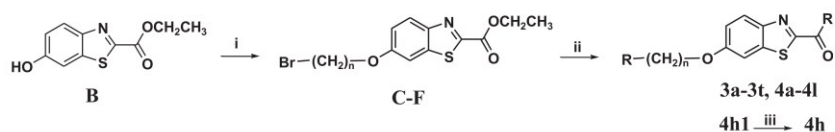
The adopted synthetic pathways for the intended compounds are depicted in **Schemes 1–4**. The synthesis of methyl/ethyl 6-hydroxybenzothiazole-2-carboxylate core was accomplished through a

three-step synthesis (Scheme 1). First, 1,4-benzoquinone was reacted with cysteine methyl/ethyl ester hydrochloride through Michael addition to afford the hydroquinone. Then, the hydroquinone intermediate underwent an oxidation reaction by potassium ferricyanide to give the benzothiazine derivative, which underwent contraction to benzothiazole ring in the presence of an acidic medium to yield compounds **A** and **B**. The mechanism of ring contraction was proposed to take place via benzothiazine ring hydrolysis to give a mercaptoaldehyde derivative, intramolecular attack to form the contracted ring, followed by oxidation and decarboxylation of the aldehyde group.³⁹ Compounds **A** and **B** were refluxed with different chloroalkyl amines in presence of potassium carbonate to yield compounds **1a–1e**. In Scheme 2, the 6-hydroxybenzothiazole-2-carboxylate methyl ester (**A**) reacted with the appropriate alpha, omega-dibromoalkane to give an ether in presence of potassium carbonate and potassium iodide to produce compounds **C–F**, which were then refluxed with the methyl ester of different aromatic amino acids to yield compounds **2a–2e**. In Scheme 3, the alkyl bromides (**C–F**) were

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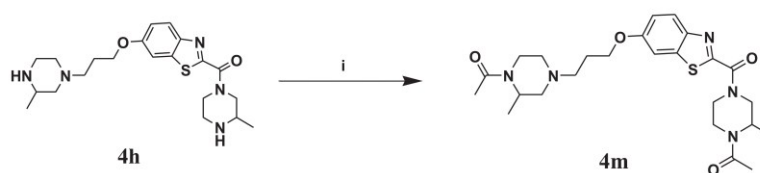


Scheme 2. Reagents and conditions: (i) 3 equiv. of α,ω -dibromoalkane, 4 equiv. of K_2CO_3 , KI, acetone, reflux, overnight, (ii) 3 equiv. of the methyl ester of the aromatic amino acid, MeOH, reflux, overnight.



Cpd No.	R	n	Cpd No.	R	n	Cpd No.	R	n
3a	piperidinyl	2	4d	phenethyl amino	3	3j	piperidinyl	4
3b	pyrrolidinyl	2	4e	tetrahydroisoquinolinyl	3	3k	cycloheptyl amino	4
3c	cyclohexyl methylamino	2	4f	4-ethyl piperazinyl	3	3l	cyclohexyl methyl amino	4
3d	piperidinyl	3	4g	4-methyl piperazinyl	3	3m	cyclopropyl amino	4
3e	cycloheptyl amino	3	4h1	4-Boc-3-methyl piperazinyl		3n	pyrrolidinyl	4
3f	cyclohexyl methylamino	3	4h	3-methyl piperazinyl	3	3o	piperidinyl	5
3g	cyclopropyl amino	3	4i	4-(2-hydroxyethyl) piperazinyl	3	3p	cycloheptyl amino	5
3h	pyrrolidinyl	3	4j	benzyl amino	3	3q	cyclohexyl methyl amino	5
4a	2-pyrrolidin-1-yl-ethylamino	3	4k	3-methyl piperidinyl	3	3r	cyclopropyl amino	5
4b	azepanyl	3	4l	4-methyl piperidinyl	3	3s	pyrrolidinyl	5
4c	3,5-dimethyl piperidinyl	3	3i	cyclopentyl amino	3	3t	cyclopentyl amino	5

Scheme 3. Reagents and conditions: (i) 3 equiv. of α,ω -dibromoalkane, 4 equiv. of K_2CO_3 , KI, acetone, reflux, overnight, (ii) 3 equiv. of appropriate amine, MeOH, reflux, overnight, (iii) TFA, DCM, 0 °C, RT, 2 h for Boc-protected derivative.



Scheme 4. Reagents and conditions: (i) 4 equiv. of glacial acetic acid, 4 equiv. of DMAP, 4 equiv. of EDC in DCM and DMF in ratio 1:1, RT, overnight.

refluxed with corresponding amines (added in excess) to give compounds **3a–3t** and **4a–4l**. Compound **4m** was synthesised by direct coupling of acetic acid with compound **4h** using EDC in the presence of DMAP that acts as a base (Scheme 4).⁴⁰

Biological evaluation

Initial screening was done to all compounds shown in Tables 1–3 in which the spacer length was varied against hH₃R (10 μ M), hH₄R (10 μ M) and hH₃R (1 μ M) in a 1-point radioligand displacement assays. K_i values were determined for the most promising compounds. Additionally, the compounds were screened against AChE at 10 μ M, compounds that showed percent inhibition above 50 as threshold were selected for IC₅₀ value determination. Compounds from the 2nd stage in which the 3-methylene spacer was only adopted (**4a–4m**) were screened against H₁R (1 μ M), H₃R and AChE as shown in Table 4.

Human histamine hH₃R affinity

We tried attaching an alkyl ether ending with diverse basic heads to the benzothiazole core and an ester or amide in the eastern arbitrary region in the presented series (Figure 2).

An alicyclic amine as the basic head, a 2-carbon spacer, and short alkyl esters at the eastern arbitrary region were used in the first cluster of compounds. However, these analogues (**1a–1e**, Table 1) showed weak ligand displacement at H₃R at 1 μ M of the tested compound (% inhibition 25–63%).

Next, in the 2nd cluster of compounds we tried aromatic L-amino acid esters at the basic head (L-Tyr, L-Trp and L-PhGly), while varying the spacer length between 3–5 carbons and methyl ester at the eastern part of the molecule. Like the first cluster of compounds, none of the adopted changes led to potent compounds at H₃R (Table 2).

In the 3rd group of compounds, we adopted a synthetic strategy in which the same terminal basic group in the western part of the molecule, was also used in the eastern arbitrary region but incorporated via an amide linkage to the benzothiazole core (Table 3). This cluster includes several derivatives with variable spacer length ($n = 2–5$).

Effect of the spacer length on H₃R affinity

Generally, we found that compounds with 3- and 5-carbon spacers between the basic amine nitrogen and the ether oxygen provide higher affinity to H₃R compared to compounds with 2- and 4-carbon spacers. For instance, in piperidine and pyrrolidine derivatives, the alkyl spacer with 3 or 5 methylene groups (compounds **3d**, **3o**, **3h**, **3s**) was statistically at the same affinity ($K_i = 0.074$, 0.068 , 0.038 , and $0.036 \mu\text{M}$, respectively) than those with 4 methylene groups (compounds **3j**, **3n**) with K_i values 0.17 and $0.059 \mu\text{M}$, respectively. The 2 methylene spacer (**3a**, **3b**) showed the lowest affinity with K_i values of $>1 \mu\text{M}$ for **3a** and $0.64 \mu\text{M}$ for **3b**. Based on this, in our last stage of synthesis, we made more diversification in the basic head focussed only on compounds having a 3-carbon spacer, since it provides the so far best balance in the gain of affinity and lipophilicity (Table 4).

The basic amine head. Generally, the 3rd alicyclic amines like pyrrolidine and piperidine demonstrated higher affinity to H₃R than the 2nd cycloalkyl amines, which is in accordance with previous investigations.³⁴ It can be protonated for ionic interactions at physiological pH values (cf. Figure 3).

Pyrrolidine analogues. The use of pyrrolidine afforded the ligands with the highest affinity to H₃R in the present series. Compound **3s** (5C spacer) showed the highest affinity with K_i value $0.036 \mu\text{M}$. Decreasing the spacer length to 3 and 4 methylene groups led to a close inhibitory activity with K_i values $0.038 \mu\text{M}$ for **3h** and $0.059 \mu\text{M}$ for **3n**. While compound **3b** with two methylene groups showed a 10-fold decrease in affinity ($K_i = 0.64 \mu\text{M}$) (Table 3). In compound **4a** having the 1-(2-aminoethyl) pyrrolidine group as the basic part, it only slightly reduced the activity ($K_i = 0.053 \mu\text{M}$, Table 4) compared to the pyrrolidine analogue (**3h**) indicating that having two basic nitrogen atoms can be tolerated in this part of the molecule.

Piperidine analogues. Piperidine ring was also tried as a basic amine. Compounds **3d**, **3j**, **3o** with 3-, 4- and 5-carbon spacers showed good inhibitory activity with $K_i = 0.074$, 0.17 , $0.068 \mu\text{M}$, respectively (Table 3). Obeying the general trend, compound **3a** with a 2-carbon spacer showed the lowest affinity to H₃R with $K_i > 1 \mu\text{M}$. Introduction of a mono methyl substituent at position 3 of the piperidine moiety (**4k**) seemed to be more tolerated than at position 4 (**4l**). Compound **4k** showed the highest affinity among all compounds bearing the substituted piperidine moiety with K_i value of $0.064 \mu\text{M}$ (Table 4). Also, fusing a benzene ring to the piperidine ring as in the tetrahydro-isoquinoline derivative **4e** led to a clear reduction in the affinity with K_i value of $0.34 \mu\text{M}$ (Table 4).

Piperazine analogues. The piperazine ring was tried as a basic head to see the influence of having two basic nitrogen atoms at the western part of the compound. First, the 1-ethyl and 1-methyl piperazine derivatives were used in compounds **4f** and **4g**. Compound **4f** showed moderate inhibitory activity with K_i value of $0.43 \mu\text{M}$ while compound **4g** showed weak inhibitory activity with K_i value of $>1 \mu\text{M}$ (Table 4). Also, we tried 3-methyl piperazine and 1-(2-hydroxyethyl) piperazine to yield compounds **4h** and **4i** respectively. The inhibition by **4h** was close to **4f** with K_i value of $0.47 \mu\text{M}$ while **4i** showed a 4-fold boost in the affinity with K_i value of $0.12 \mu\text{M}$. Moreover, acetylation of **4h** was done to give **4m**, which showed K_i value of $0.4 \mu\text{M}$, similar to the values of **4f** and **4h** (Table 4).

Azepane ring. In compound **4b**, the azepane ring was tried as the basic part (with a 3-carbon spacer). This compound showed the highest affinity towards H₃R in the present series with a K_i value of $0.012 \mu\text{M}$. The increased lipophilicity may contribute to this effect.

Secondary amines. The effect of replacing the 3rd alicyclic amine as a basic centre with different 2nd amines was also examined. Cyclopentyl amino derivatives showed low potency, e.g. **3t**, which has a 5-carbon spacer, only showed a marginal K_i value of $>1 \mu\text{M}$ (Table 3). The same applies for the cyclohexyl methyl amino derivative **3q** (also having 5-carbon spacer) with a K_i value $>3 \mu\text{M}$. Ring expansion to give cycloheptyl amino (**3e**, **3k**, **3p**) and ring contraction to cyclopropyl amino (**3g**, **3m**, **3r**) did not recover the activity towards H₃R regardless of the spacer length. The use of aralkyl amines like the phenyl ethyl amino **4d** and benzyl amino **4j** led to ligands with K_i values >1 and $0.64 \mu\text{M}$ respectively (Table 4).

Selectivity

Within the group of histamine receptors, subtype H₃R shows the highest sequence homology to H₂R in the histamine H₄ receptor (H₄R). It

Table 1. Screening at H₁R, H₃R, H₄R, and AChE by the first cluster of compounds (1a–1e).

1a–1e

Cpd No.	R ¹	R ²	n	hH ₁ R ^a	hH ₃ R ^b	hH ₄ R ^c	AChE ^d	
				Inhibition at 10 μM [%] ±SD (n)	Inhibition at 1 μM [%] ±SD (n)	Inhibition at 10 μM [%] (n)	Inhibition at 10 μM [%]	IC ₅₀ (μM)
1a	–Et		2	NI (2)	63.3 ± 4.2% (2)	39.2 ± 34.7% (4)	7.64 ± 7.33	>10
1b	–Et		2	NI (2)	35.2 ± 14.4% (2)	61.7 ± 35.4% (4)	9.25 ± 7.88	>10
1c	–Et		2	1.0 ± 9.7 (2)	50.2 ± 10.9% (2)	54.0 ± 12.1% (4)	57.19 ± 5.41	7.63 ± 1.6
1d	–Me		2	11.2 ± 10.5 (2)	25.3 ± 5.4% (2)	51.3 ± 18.2% (4)	5.52 ± 6.39	>10
1e	–Me		2	16.3 ± 41.7 ^e (3)	54.2 ± 28.4% (2)	ND	84.05 ± 4.25	2.92 ± 0.37

Values are mean values of *n* experiments; ND: not determined; NI: no inhibition. % inhibition at H₁R, H₃R and H₄R is calculated relative to radioligand binding (100%). ^aChlorpheniramine maleate was used as a positive control with *K_i* value of 0.011 μM. ^bPitolisat was used as a positive control with *K_i* value of 0.010 μM. ^cJNJ777120 was used as a positive control with *K_i* value of 0.024 μM. ^dDonepezil was used as a positive control with an IC₅₀ value of 36.45 ± 8.31 nM.

was therefore essential to test newly developed histamine H₃R ligands for their potential interaction with this most closely related subtype of the histamine receptor family (testing was performed at 10 μM), since modulation of H₃Rs is more specific towards neurodegenerative diseases while H₄Rs mainly have role in inflammation and immune response. Based on the obtained data, it was shown that the active compounds at H₃R did not possess remarkable affinities at the H₄R. Additionally, to exclude side effects of first-generation sedating H₁ antagonists which readily cross the blood–brain barrier, leading to drowsiness, fatigue, and psychomotor disturbances, the affinity at the H₁R was screened mostly at a concentration of 10 μM and was, except for one compound (**3o**), considered negligible (Tables 1–3).

Cholinesterase inhibitory activity

AChE inhibition. To achieve our aim to find MTDLs for Alzheimer's disease, all compounds were tested against human AChE as shown in Tables 1–4. The compounds were tested at 10 μM screening concentration then IC₅₀ values were determined for compounds showing higher than 50% inhibition at 10 μM. Similar to the activity towards H₃R, pyrrolidine derivatives were among the most potent compounds against AChE regardless of the spacer length. All compounds having pyrrolidine showed % inhibition of more than 50% at 10 μM, however, the strongest inhibitor was found to be compound **3b** bearing the 2 methylene groups with an IC₅₀ value of 0.44 μM. Interestingly, and unlike what was observed towards H₃R, the 2nd best pyrrolidine derivative is compound **3n** with 4-carbon spacer (IC₅₀ = 1.3 μM), indicating that spacer length of two and four methylene groups (Table 2 and Figure 2) was generally superior for AChE inhibition.

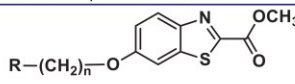
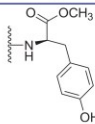
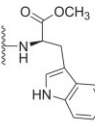
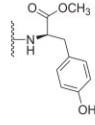
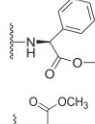
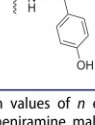
Compound **4b**, the most affine compound to H₃R, having azepanyl head and 3-carbon spacer showed the ability to inhibit AChE with IC₅₀ = 5.91 μM. This compound showed higher potency towards AChE than the respective pyrrolidine analogue **3h**. Piperidine derivatives showed potency only with the 4-carbon spacer in compound **3j** with IC₅₀ = 8.56 μM. A remarkable drop in inhibitory activity towards AChE occurred in all the secondary amines except cyclopentyl amino at spacer 3 and 5 (compounds **3i** and **3t**) which showed IC₅₀ values of 7.31 and 3.55 μM, respectively. Compounds **1c** (pyrrolidine derivative) and **1e** (piperidine derivative) which have simple ethyl and methyl esters respectively (2 carbon spacer) showed AChE inhibitory activity (IC₅₀ = 7.63 μM, 2.92 μM respectively). This confirms the superiority of the 2-carbon spacer as a favourable requirement for the AChE inhibitory activity.

BuChE inhibition. As for BuChE, only compounds with dual activity towards H₃R and AChE were tested for their inhibitory activity towards this enzyme in addition to three compounds (**3d**, **3o**, and **4k**) with very good activity towards H₃R with *K_i* values 0.074, 0.068, and 0.064 μM, respectively (Table 5). Most of the tested compounds showed inhibitory activity towards BuChE in the micromolar range with IC₅₀ values below 5.5 μM. Interestingly, compound **4a** having 1-(2-amino ethyl) pyrrolidine ring and three carbon spacer showed the highest inhibitory activity with IC₅₀ of 0.169 μM.

MAO-A and B inhibition

The activity of MAO-B enzyme is increased in the cortical areas and in the hippocampus of the brains of patients with AD. This overexpression of MAO-B leads to stimulation of free radical formation and thus may feasibly advance the neurodegenerative

Table 2. Inhibition of H₁R, H₃R, H₄R and AChE by the 2nd cluster of compounds (2a–2e).

 2a-2e							
Cpd No.	R	n	hH ₁ R ^a	hH ₃ R ^b	hH ₄ R ^c	AChE ^d	
			Inhibition at 10 μM [%] (n)	Inhibition at 1 μM [%] ±SD (n)	Inhibition at 10 μM [%] (n)	Inhibition at 10 μM [%]	IC ₅₀ (μM)
2a		3	NI (2)	54.8 ± 21.2% (2)	35.5 ± 32.1% (3)	NI	>10
2b		3	9.2 ± 19.1 (2)	33 ± 13.8% (2)	48.0 ± 30.0% (3)	NI	>10
2c		4	NI (2)	39.5 ± 20.8% (2)	69.5 ± 28.0% (2)	1.8 ± 5.0	>10
2d		4	NI (2)	64.5 ± 10.3% (2)	58.5 ± 11.6% (3)	NI	>10
2e		5	NI (2)	26.0 ± 6.3% (2)	62.8 ± 14.7% (3)	NI	>10

Values are mean values of *n* experiments; ND: not determined, NI: no inhibition. % inhibition at H₁R, H₃R and H₄R is calculated relative to radioligand binding (100%). ^aChlorpheniramine maleate was used as a positive control with *K_i* value of 0.011 μM. ^bPitolisant was used as a positive control with *K_i* value of 0.010 μM. ^cJNJ777120 was used as a positive control with *K_i* value of 0.024 μM. ^dDonepezil was used as a positive control with an IC₅₀ value of 36.45 ± 8.31 nM.

mechanisms occurring in AD. Therefore, many new MAO-B inhibitors are designed and developed as potential disease-modifying AD drugs.⁴¹ On the other hand MAO-A was found to increase in the hypothalamus and frontal pole. MAO-A activity appears to be lower in the locus ceruleus in patients with AD, and is accompanied by an approximately 80% decrease in the number of neurons, revealing that activated MAO-A in neurons is involved in the pathology of this disease as a predisposing factor.⁴² Consequently, the same set of compounds selected for testing against BuChE was further tested against MAO-A and -B enzymes (Table 5). Only compounds having piperidine and pyrrolidine as a basic head with 5-carbon spacer (compounds **3o** and **3s**) displayed significant and selective MAO-B inhibitory activity (>60% inhibition at 10 μM) with IC₅₀ values 3.04 and 1.6 μM, respectively.

Molecular modeling

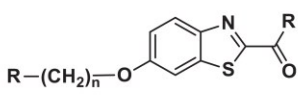
Docking studies on H₃R

Among the four known histamine receptor subtypes, crystal structure only for the histamine H₁ receptor was known up to date

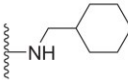
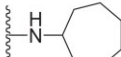
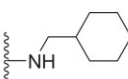
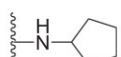
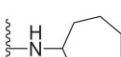
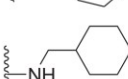
(PDB ID: 3RZE).⁴³ Therefore, due to the lack of crystal structures, homology modelling methods are used to search for new H₃R ligands and to explain SAR. Using the prepared homology model,⁴⁴ we made an attempt to demonstrate how the tested compounds interacted with the H₃R active site. We chose one of the most active H₃R ligands **3s** (*K_i* = 0.036 μM), to present the observed interactions. The homology model was kindly provided by Marek Bajda group.⁴⁴ Compound **3s** interacts with the homology model through different types of interactions (Figure 3) described as follows: (1) The protonated pyrrolidine nitrogen interacts with Glu176 through ionic interaction, (2) One of the methylene groups in the spacer interacts with Phe163 through CH–π interaction, (3) The CH groups in the pyrrolidine ring formed two hydrogen bonds involving Glu176 (CH...O interaction) and Cys88 (CH...S interaction) and one CH–π interaction involving Trp341, 4) The carbonyl group in the eastern arbitrary region interacts with Arg351 through hydrogen bond interaction.

The overall binding mode apparently resembles that described for the known H₃R inverse agonists such as ABT-239, JNJ5207852, and others.⁴⁴ These inverse agonists contain in their structure

Table 3. Inhibition of H_1R , H_3R , H_4R and AChE by 3rd cluster of compounds (3a–3t).



3a–3t

Cpd No.	R	n	H_1R^a	H_3R^b	H_4R^c	$AChE^d$	
			Inhibition at 10 μM [%] (n)	Inhibition at 1 μM [%] $\pm$ SD (n) or K_i [μM] (n)	Inhibition at 10 μM [%] (n)	Inhibition at 10 μM [%]	IC_{50} (μM)
3a		2	NI (2)	68.0 $\pm$ 9.1% (2)	30.3 $\pm$ 24.9% (4)	35.5 $\pm$ 13.44	>10
3b		2	NI (2)	0.64 μM (3)	44.9 $\pm$ 18.1% (4)	95.3 $\pm$ 0.8	0.44 $\pm$ 0.10
3c		2	5.3 $\pm$ 14.2 (2)	31.5 $\pm$ 11.4% (2)	52.8 $\pm$ 11.8% (4)	NI	>10
3d		3	65.3 $\pm$ 9.2 (2)	0.074 μM (3)	63.1 $\pm$ 11.2% (4)	33.6 $\pm$ 10.82	>10
3e		3	12.1 $\pm$ 18.3 (2)	35.7 $\pm$ 11.5% (2)	53.5 $\pm$ 15.2% (4)	7.3 $\pm$ 2.31	>10
3f		3	47.6 $\pm$ 12.9 (2)	46.3 $\pm$ 14.7% (2)	60.8 $\pm$ 30.4% (4)	3.2 $\pm$ 3.69	>10
3g		3	13.6 $\pm$ 10.7 (2)	27.7 $\pm$ 12.8% (2)	30.2 $\pm$ 14.3% (3)	15.4 $\pm$ 11.25	>10
3h		3	39.1 $\pm$ 13.0 (2)	0.038 μM (3)	54.3 $\pm$ 13.0% (4)	56.8 $\pm$ 7.14	7.21 $\pm$ 2.30
3i		3	35.5 $\pm$ 9.8 (2)	51.2 $\pm$ 8.7% (2)	50.5 $\pm$ 13.0% (4)	59 $\pm$ 6.16	7.31 $\pm$ 1.59
3j		4	42.8 $\pm$ 7.7 (2)	0.17 μM (3)	53.1 $\pm$ 11.7% (4)	54.8 $\pm$ 7.01	8.56 $\pm$ 2.83
3k		4	14.2 $\pm$ 11.7 (2)	39.0 $\pm$ 8.9% (1)	61.5 $\pm$ 8.9% (4)	9.7 $\pm$ 4.91	>10
3l		4	14.7 $\pm$ 8.5 (2)	57.3 $\pm$ 12.7% (1)	49.7 $\pm$ 41.0% (4)	16.7 $\pm$ 7.79	>10
3m		4	27.0 $\pm$ 10.6 (2)	67.8 $\pm$ 2.5% (2)	53.6 $\pm$ 11.1% (4)	13.2 $\pm$ 8.07	>10

(continued)

Table 3. Continued.

Cpd No.	R	n	hH ₁ R ^a	hH ₃ R ^b	hH ₄ R ^c	AChE ^d	
			Inhibition at 10 μ M [%] (n)	Inhibition at 1 μ M [%] $\pm$ SD (n) or K_i [μ M] (n)	Inhibition at 10 μ M [%] (n)	Inhibition at 10 μ M [%]	IC ₅₀ (μ M)
3n		4	37.5 $\pm$ 7.5 (2)	0.059 μ M (3)	57.9 $\pm$ 13.3% (4)	83 $\pm$ 3.27	1.30 $\pm$ 0.10
3o		5	91.2 $\pm$ 8.2 (6)	0.068 μ M (4)	38.8 $\pm$ 28.0% (4)	27.8 $\pm$ 5.91	>10
3p		5	17.5 $\pm$ 8.4 (2)	34.0 $\pm$ 7.5% (2)	58.4 $\pm$ 8.3% (4)	9.5 $\pm$ 3.58	>10
3q		5	40.6 $\pm$ 8.2 (2)	>3 μ M (2)	49.1 $\pm$ 22.8% (4)	22.2 $\pm$ 5.5	>10
3r		5	23.7 $\pm$ 12.7 (2)	38.8 $\pm$ 9.7% (2)	51.6 $\pm$ 15.8% (4)	14.8 $\pm$ 6.73	>10
3s		5	66.2 $\pm$ 7.3 (2)	0.036 μ M (3)	63.7 $\pm$ 13.0% (4)	58.6 $\pm$ 3.73	6.70 $\pm$ 1.13
3t		5	47.3 $\pm$ 8.914 (2)	>1 μ M (2)	41.8 $\pm$ 31.7% (4)	75.5 $\pm$ 4.73	3.55 $\pm$ 0.54

Values are mean values of *n* experiments; ND: not determined, NI: no inhibition. %inhibition in H₁R, H₃R and H₄R is calculated relative to radioligand binding (100%). ^aChlorpheniramine maleate was used as a positive control with K_i value of 0.011 μ M. ^bPitolisant was used as a positive control with K_i value of 0.010 μ M. ^cJNJ777120 was used as a positive control with K_i value of 0.024 μ M. ^dDonepezil was used as a positive control with an IC₅₀ value of 36.45 $\pm$ 8.31 nM.

nitrogen atom capable of protonation at physiological pH values. The docking results suggested that the ionic interaction with Glu176 in the active site is essential for efficient binding of non-imidazole H₃ antagonists. Compound **3s** like the other antagonists showed this type of interaction through the protonated nitrogen atom in the pyrrolidine ring. The overall interactions including ionic, hydrogen bond and hydrophobic interactions with their appropriate distances led to the high affinity of this compound at H₃R.

Upon comparing the docking results of the most potent pyrrolidine derivative **3s** (K_i = 0.036 μ M) with the least potent **3b** (K_i = 0.64 μ M), it is clear that the number of interactions is lower in **3b** than **3s**. In **3b**, only two amino acids (Glu176 and Arg351) were shown to interact with **3b**. In addition, the distance between the amino acids and the compound is larger in case of **3b** since the distance of the ionic interaction in **3b** was 3.95 Å while in **3s** it was shorter (3.15 Å). Also, the distance of the hydrogen bonds formed with Glu176 was greater in case of **3b** (Figure 4).

Docking studies on AChE enzyme

In an attempt to get a better insight into the binding mode of our most potent inhibitor against human AChE **3b** (IC₅₀ = 0.44 μ M), molecular docking was performed at AChE binding pocket (PDB code: 4EY5) using MOE. As depicted in Figure 5, **3b**

binds to AChE binding site in a way analogous to the cocrystallized ligand (Huperzine A) with a score −43.3102 and rmsd 1.4070. Compound **3b** was extended along the active gorge. The pyrrolidine ring was located close to the amino acid residues from the anionic site and the catalytic triad.

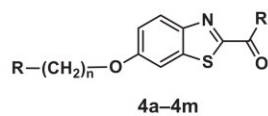
Docking studies on BuChE enzyme

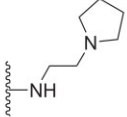
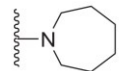
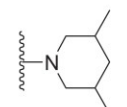
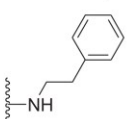
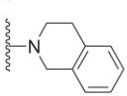
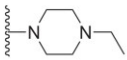
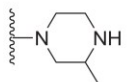
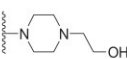
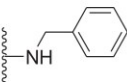
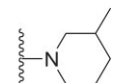
Compound **4a** was docked to the active site of human BuChE. The binding mode of the active compound **4a**, which revealed significant hBuChE inhibitory activity with IC₅₀ 0.169 μ M is shown in Figure 6.

In-silico evaluation of drug-like properties

Since the major indication of these compounds is the treatment of AD, it seemed interesting to assess the ability of the most potent and selective novel analogues to pass BBB. Different physicochemical properties associated with a high probability of CNS penetration have been previously reported in the literature.^{45,46} CNS-active drug candidates are assumed to have definite attributes exemplified in: molecular weight <450, solubility >60 μ g/ml, logP <5, HBA <7 and HBD <3. The molecule topological polar surface area (TPSA) is one of the concepts used to assess drug-likeness and ability to cross BBB where its ideal value is less than 70 Å². However, the upper limit of PSA for a molecule to

Table 4. Inhibition of H₃R, and AChE (compounds 4a–4m).



Cpd No.	R	n	hH ₃ R ^a Inhibition at 1 μM [%] (n)	hH ₃ R ^a Inhibition at 1 μM [%] ±SD (n) or K _i [μM] (n)	AChE ^b	
					Inhibition at 10 μM [%]	IC ₅₀ (μM)
4a		3	28.1 ± 39.1% (3)	0.053 μM (3)	86.9 ± 1.51	1.24 ± 0.07
4b		3	41.9 ± 33.8% (7)	0.012 μM (3)	62.8 ± 3.35	5.91 ± 0.67
4c		3	26.8 ± 31.0% (3)	0.34 μM (3)	6.9 ± 1.67	>10
4d		3	10.9 ± 37.1% (3)	>1 μM (3)	5.2 ± 2.36	>10
4e		3	9.7 ± 23.0% (3)	0.34 μM (3)	6.2 ± 3.15	>10
4f		3	3.0 ± 36.0% (3)	0.43 μM (3)	8.2 ± 1.64	>10
4g		3	4.8 ± 37.7% (3)	>1 μM (3)	7.7 ± 1.87	>10
4h		3	18.4 ± 27.0% (3)	0.47 μM (5)	8.3 ± 1.02	>10
4i		3	22.5 ± 27.4% (3)	0.12 μM (4)	7.3 ± 2.77	>10
4j		3	27.0 ± 39.4% (3)	0.64 μM (3)	20.3 ± 3.55	>10
4k		3	42.9 ± 28.3% (7)	0.064 μM (3)	34.4 ± 2.15	>10

(continued)

3 Publications

Table 4. Continued.

Cpd No.	R	n	hH ₃ R ^a	hH ₃ R ^a	AChE ^b	
			Inhibition at 1 μ M [%] (n)	Inhibition at 1 μ M [%] $\pm$ SD (n) or K_i [μ M] (n)	Inhibition at 10 μ M [%]	IC ₅₀ (μ M)
4l		3	26.8 $\pm$ 26.3% (3)	0.43 μ M (3)	8.4 $\pm$ 0.51	>10
4m		3	27.0 $\pm$ 27.6% (3)	0.40 μ M (4)	2.4 $\pm$ 2.06	>10

Values are mean values of *n* experiments; ND: not determined, NI: no inhibition. %inhibition in H₃R is calculated relative to radioligand binding (100%). ^aPitolisant was used as a positive control with K_i value of 0.010 μ M. ^bDonepezil was used as a positive control with an IC₅₀ value of 36.45 $\pm$ 8.31 nM.

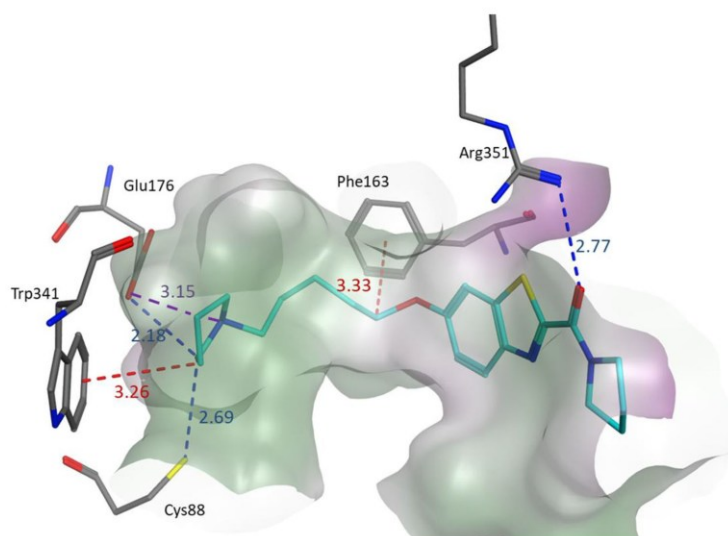


Figure 3. Binding mode of compound **3s** within the homology model of H₃R. **3s** was docked into the active site using MOE. In the binding model, compound **3s** formed three hydrogen bond interactions with Glu176, Cys88, and Arg351, a salt bridge with Glu176 and two CH- π interactions involving Trp341 and Phe163. Interactions are indicated by dashed lines and distances between the heavy atoms are given in Å.

penetrate CNS was around 90 Å².⁴⁵ Therefore, different physico-chemical parameters of several potent inhibitors with dual activity from the current series were calculated using MOE software as shown in Table 6 to estimate their drug-likeness. The calculated parameters for most of our potent compounds were within the ideal ranges for brain penetration as reported in the literature. In particular, compounds **3b**, **3h**, and **3n** have reduced logP, logD, and low molecular weight. These values would likely allow BBB penetration. Also, those compounds showed an advantageous enhancement of solubility. Altogether, a high possibility for brain penetration for the highly potent and selective compounds in the current work was anticipated.

Conclusion

In this work, we present benzothiazole derivatives as multi-target ligands to efficiently treat AD. The presented derivatives have a

benzothiazole-based central core, which is connected to piperidine, pyrrolidine, azepane and other rings via an alkoxy linker. H₃R affinity was determined by radioligand displacements assay against [³H] N-methylhistamine. Several of the presented compounds reached K_i values in the nanomolar range. The most affine compound, the azepane-linked benzothiazole derivative displayed a K_i value of 0.012 μ M. The linker length had a slight influence on H₃R affinity in that the ethoxy linker was the least favourite linker. Additionally, affinity at the H₄ receptor was tested against [³H]-histamine, but no notable receptor inhibition at 10 μ M was detected. To exclude side effects, the affinity at the H₁ receptor was determined against [³H]-pyrilamine and was considered negligible. Upon examining the inhibitory activity of all our compounds towards AChE, some of them showed good potency towards the inhibition of these enzymes with IC₅₀ values in the low or sub-micromolar range. Moreover, some of the compounds were tested towards BuChE and showed inhibitory activity towards the

Table 5. Inhibition of selected compounds at BuChE and MAO-A and B enzymes.

Cpd No.	R	n	MAO-A		MAO-B		hBuChE	
			% Inhibition at 10 μM^a		% Inhibition at 10 μM^a	IC_{50} (μM)	% Inhibition at 10 μM^a	IC_{50} (μM)
3b		2	58 $\pm$ 2		47 $\pm$ 3	ND	70 $\pm$ 1	5.31 $\pm$ 0.01
3d		3	33 $\pm$ 3		28 $\pm$ 5	ND	31 $\pm$ 1	ND
3h		3	22 $\pm$ 4		24 $\pm$ 4	ND	13 $\pm$ 2	ND
3j		4	29 $\pm$ 5		48 $\pm$ 5	ND	70 $\pm$ 2	3.07 $\pm$ 0.24
3n		4	31 $\pm$ 4		49 $\pm$ 4	ND	80 $\pm$ 1	1.32 $\pm$ 0.12
3o		5	37 $\pm$ 4		70 $\pm$ 4	3.04 $\pm$ 0.46	81 $\pm$ 1	2.38 $\pm$ 0.32
3s		5	45 $\pm$ 3		75 $\pm$ 3	1.60 $\pm$ 0.16	69 $\pm$ 1	2.35 $\pm$ 0.23
3t		5	39 $\pm$ 4		47 $\pm$ 5	ND	84 $\pm$ 1	1.75 $\pm$ 0.06
4a		3	29 $\pm$ 4		51 $\pm$ 1	ND	97 $\pm$ 1	0.169 $\pm$ 0.019
4b		3	29 $\pm$ 2		46 $\pm$ 5	ND	51 $\pm$ 2	ND
4k		3	32 $\pm$ 2		48 $\pm$ 5	ND	30 $\pm$ 5	ND

^aValues are mean values of three experiments; standard deviation <20%.

enzyme in the micromolar range with IC_{50} values below 5.5 μM . Among the 42 synthesised compounds, we have 6 compounds (**3b**, **3j**, **3n**, **3s**, **3t**, and **4a**) that can be described as MTDL with affinities for hH₃R and inhibitory activity for AChE and BuChE. These structures using the benzothiazole moiety as a privileged scaffold serve as a starting point for further structural optimizations to improve MTDL pharmacological properties and to yield potential drugs for the treatment of neurodegenerative diseases.

Experimental section

Chemistry

Solvents and reagents were purchased from commercial suppliers and used as received. ^1H NMR and ^{13}C NMR spectra were recorded by a Bruker DRX 500 spectrometer, Bruker Fourier 300 as

well as Varian 400 spectrometer. ^1H shifts are referenced to the residual protonated solvent signal (δ 2.50 for DMSO- d_6), (δ 7.24 for CDCl_3), (δ 4.87 for CD_3OD) and ^{13}C shifts are referenced to the deuterated solvent signal (δ 39.5 for DMSO- d_6), (δ 77.0 for CDCl_3), (δ 49.5 for CD_3OD). Chemical shifts are given in parts per million (ppm), and all coupling constants (J) are given in Hz. All final compounds have a percentage purity of at least 95%, and this was verified utilising HPLC coupled with mass spectrometry. Mass spectrometric analysis (UHPLC-ESI-MS) was done utilising Waters ACQUITY Xevo TQD framework, which consisted of an ACQUITY UPLC H-Class system and XevoTM TQD triple-quadrupole tandem mass spectrometer with an electrospray ionisation (ESI) interface (Waters Corp., Milford, MA, USA). Acquity BEH C18 50 mm $\times$ 2.1 mm column (particle size, 1.7 μm) was utilised to separate analytes (Waters, Ireland). Two LC methods were used. Method I: The percentage of B started at an initial of 5% and maintained for

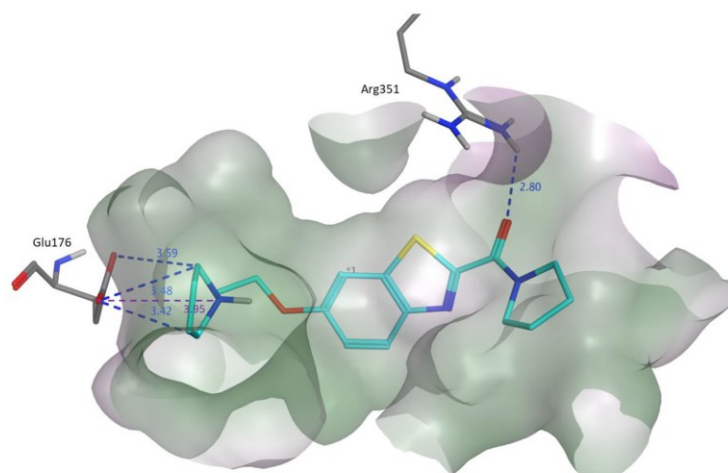


Figure 4. Binding mode of compound 3b within the homology model of H₃R. 3b was docked into the active site using MOE. In the binding model, compound 3b formed three hydrogen bond interactions with Glu176, a salt bridge with Glu176 and one hydrogen bond with Arg351. Dashed lines indicated the interactions and distances between the heavy atoms are given in Å.

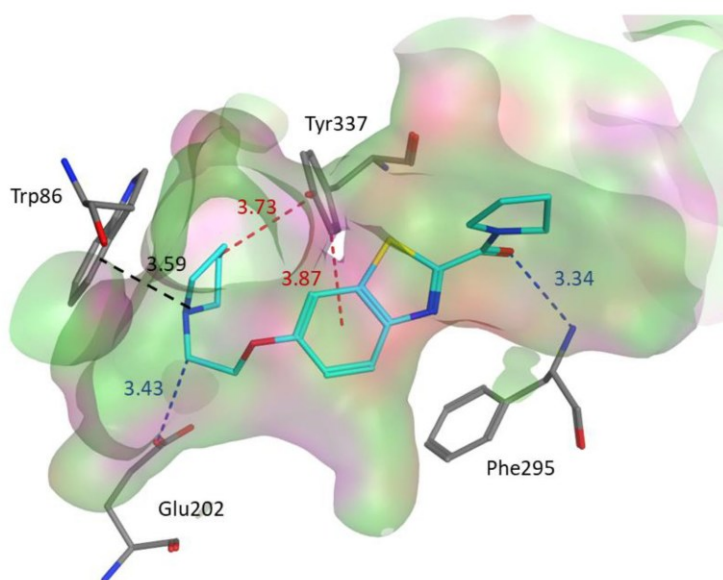


Figure 5. Binding mode of compound 3b within the AChE active site (PDB code: 4EY5). 3b was docked into the active site of AChE using MOE. In the binding model, compound 3b is anchored between amino acids Phe295, Tyr337, Glu202 and Trp86. It formed cation- π interaction with Trp86. In addition, we found two CH- π interactions involving Tyr337 and two hydrogen bond interactions with Glu202 and Phe295. Dashed lines indicate the interactions and distances between the heavy atoms are given in Å.

1 min, then increased up to 100% during 10 min, kept at 100% for 2 min, and flushed back to 5% in 3 min then kept at 5% for 1 min. Method II: The solvent system consisted of water containing 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). HPLC was used for purity determination, HPLC-technique: flow rate

400 μ L/min. The percentage of B started at an initial of 5% and maintained for 0.5 min, then increased up to 100% for 4 min, kept at 100% for 2 min, and flushed back to 5% in 1 min. The MS scan was carried out at the accompanying conditions: capillary voltage 3.5 kV, cone voltage 20 V, radio frequency (RF) lens voltage 2.5 V,

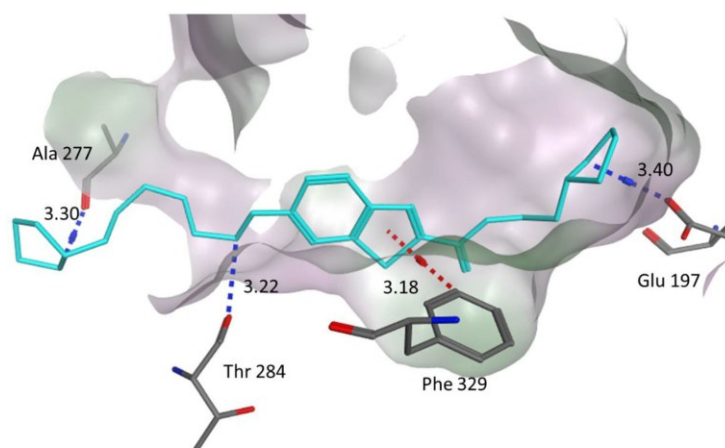


Figure 6. Binding mode of compound 4a within the BuChE active site (PDB code: 1POI). 4a was docked into the active site of BuChE using MOE. In the binding model, compound 4a is anchored between amino acids Phe329, Thr284, Ala277 and Glu197. It formed three hydrogen bond interactions involving Glu197, Ala277 and Thr284. In addition one CH- π interaction with Phe329. Dashed lines indicate the interactions and distances between the heavy atoms are given in Å.

Table 6. Calculated physicochemical properties of compounds (3b, 3h, 3j, 3n, 3s, 3t, 4a, 4b)*.

Cpd. No.	MW [gm/mol]	logS	logP	logD	TPSA [Å ²]	HBD	HBA	BBB crossing prediction
3b	346.475	-2.97	2.630	0.838	46.87	0	3	+
3h	360.502	-3.17	3.072	0.559	46.87	0	3	+
3j	402.583	-3.78	4.398	0.977	46.87	0	3	+
3n	374.529	-3.37	3.514	0.532	46.87	0	3	+
3s	388.556	-3.58	3.956	0.718	46.87	0	3	+
3t	416.61	-4.36	5.501	0.490	67.83	1	3	+
4a	416.61	-3.98	2.361	-2.088	46.87	2	4	+
4b	416.61	-3.98	4.840	1.435	46.87	0	3	+

*Ideal ranges: MW: <450 g/mol, solubility >60 μ g/ml, logP: <5; logD <3 (at pH = 7.4), TPSA <90 Å², HBD: <3; HBA <7.

source temperature 150 °C and desolvation gas temperature 500 °C. Nitrogen was used as the desolvation and cone gas at a flow rate of 1000 and 20 L/h, respectively. System operation and data acquisition were controlled using Mass Lynx 4.1 software (Waters). Full-scan HRMS experiments were done with a dual electrospray interface (ESI) and a quadrupole time-of-flight mass spectrometer (Q-TOF, Agilent 6530 Series, Agilent Technologies, Cernusco s. N., Italy). Spectra were recorded in the mass/charge (m/z) range 50–3000 Da. Melting points were obtained using a Buchi B-540 melting point apparatus and are uncorrected.

General synthetic methods and experimental details

General procedure for the synthesis of 6-hydroxybenzothiazole-2-carboxylic acid ethyl and methyl ester (A, B). A solution of 1, 4-benzoquinone (5 mmol) in MeOH (20 ml) was added dropwise to a solution of L-cysteine ethyl or methyl ester hydrochloride (5 mmol) in MeOH (20 ml) with stirring at room temperature overnight. The reaction was monitored by TLC (petroleum ether/EtOAc, 6/4) and upon the reaction completion, the solvent was evaporated. The residue was dissolved in isopropanol (50 ml) then an aqueous solution of 1 M K₃Fe(CN)₆ (20 ml) and 4 M NaOH (2.5 ml) were added, respectively. The mixture was stirred at room temperature overnight. At the end of the reaction (known by TLC monitoring), the solid was removed by filtration and the solution was extracted with ethyl acetate. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. To a solution of the

obtained benzothiazine in EtOH (20 ml), HCl (1 M, 12 ml) was added. After stirring for 24 h at room temperature, H₂O was added and the mixture was extracted with CH₂Cl₂. The organic layer was dried over anhydrous Na₂SO₄ and evaporated under reduced pressure, and the resulting residue was purified by column chromatography (CC) using (EtOAc/petroleum ether, 4:6) as a solvent system to give compounds **A** and **B**.

6-Hydroxybenzothiazole-2-carboxylic acid methyl ester (a). Synthesised according to the method outlined above using L-cysteine methyl ester hydrochloride; brown solid; yield: 50.87%; mp 90.2–90.8 °C; MS (ESI): m/z = 210 (M + H)⁺.³⁹

6-Hydroxybenzothiazole-2-carboxylic acid ethyl ester (B). Synthesised according to the method outlined above using L-cysteine ethyl ester hydrochloride. The product was purified by CC (EtOAc: Petroleum ether, 4:6); yellow solid; yield: 60%; mp 90.6–90.9 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 8.00 (d, J = 8.9 Hz, 1H), 7.46 (d, J = 2.4 Hz, 1H), 7.09 (dd, J = 8.9, 2.4 Hz, 1H), 4.39 (q, J = 7.1 Hz, 2H), 1.33 (t, J = 7.1 Hz, 3H), 1.20 (s, 1H); ¹³C NMR (101 MHz, DMSO-d₆) δ 160.63, 158.19, 154.42, 146.82, 138.60, 126.21, 118.20, 107.04, 62.77, 14.48; MS (ESI): m/z = 224.03 (M + H)⁺.

General procedure for the synthesis of compounds (1a–1e). Different derivatives of chloro ethyl amine hydrochloride (4 mmol) were added to a solution of 6-hydroxybenzothiazole ester

(0.446 g, 2 mmol) in acetone (20 ml). Then (1.1 g, 8 mmol) potassium carbonate and a few specs of potassium iodide were added to the mixture and heated to reflux for 6 h. Afterward, the mixture was concentrated *in vacuo*. The residue was partitioned between ethyl acetate and distilled water, the aqueous layer was re-extracted by ethyl acetate. The organic layers were collected and the volume was reduced under reduced pressure. Afterward, the product was purified by CC to yield compounds (**1a–1e**).

Ethyl 6-(2-morpholinoethoxy)benzo[d]thiazole-2-carboxylate (1a). The title compound was synthesised according to the procedure in Section "General procedure for the synthesis of compounds (1a–1e)", using 4-(2-chloroethyl) morpholine hydrochloride. The product was purified by CC (DCM:MeOH:TEA, 100:1:1); light brown solid; yield: 46.3%; mp 128.4–128.8 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.10 (d, *J* = 9.1 Hz, 1H), 7.82 (d, *J* = 2.4 Hz, 1H), 7.26 (dd, *J* = 9.1, 2.5 Hz, 1H), 4.43 (q, *J* = 7.1 Hz, 2H), 4.22 (t, *J* = 5.7 Hz, 2H), 3.64–3.56 (m, 4H), 2.77 (t, *J* = 5.6 Hz, 2H), 2.58–2.47 (m, 4H), 1.37 (t, *J* = 7.1 Hz, 3H); ¹³C NMR (75 MHz, DMSO-*d*₆) δ 160.56, 158.83, 155.65, 147.69, 138.56, 126.08, 118.50, 105.67, 66.56, 66.44, 62.94, 57.25, 54.00, 14.51; MS (ESI): *m/z* = 337.11 (M + H)⁺.

Ethyl 6-(2-(piperidin-1-yl)ethoxy)benzo[d]thiazole-2-carboxylate (1b). The title compound was synthesised according to the procedure in Section "General procedure for the synthesis of compounds (1a–1e)", using 1-(2-chloroethyl) piperidine hydrochloride. The product was purified by CC (DCM:MeOH:TEA, 100:3:1); yellow semisolid; yield: 35.2%; ¹H NMR (400 MHz, CDCl₃) δ 8.09 (d, *J* = 9.1 Hz, 1H), 7.36 (d, *J* = 2.3 Hz, 1H), 7.15 (dd, *J* = 9.1, 2.4 Hz, 1H), 4.52 (q, *J* = 7.1 Hz, 2H), 4.30 (t, *J* = 5.3 Hz, 2H), 3.01 (s, 2H), 2.74 (s, 4H), 1.77–1.70 (m, 4H), 1.51 (d, *J* = 4.7 Hz, 2H), 1.46 (t, *J* = 7.1 Hz, 3H); ¹³C NMR (101 MHz, CDCl₃) δ 160.67, 158.23, 155.95, 147.99, 138.61, 126.19, 117.80, 104.35, 65.74, 62.92, 57.34, 54.84, 24.96, 23.44, 14.29; MS (ESI): *m/z* = 335.14 (M + H)⁺.

Ethyl 6-(2-(pyrrolidin-1-yl)ethoxy)benzo[d]thiazole-2-carboxylate (1c). The title compound was synthesised according to the procedure in Section "General procedure for the synthesis of compounds (1a–1e)", using 1-(2-chloroethyl) pyrrolidine hydrochloride. The product was purified by CC (DCM:MeOH:TEA, 100:4:1); yellow semisolid; yield: 23.5%; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.59 (d, *J* = 8.9 Hz, 1H), 7.27 (d, *J* = 2.4 Hz, 1H), 6.87 (dd, *J* = 8.9, 2.5 Hz, 1H), 4.47–4.40 (m, 2H), 4.29 (t, *J* = 4.5 Hz, 2H), 3.59 (s, 4H), 3.52–3.41 (m, 4H), 2.17–1.92 (m, 5H); ¹³C NMR (75 MHz, DMSO-*d*₆) δ 168.34, 163.00, 156.18, 148.22, 138.15, 124.47, 115.96, 105.09, 64.71, 54.51, 54.18, 53.35, 23.10, 14.20; MS (ESI): *m/z* = 335.14 (M + H)⁺.

Methyl 6-(2-morpholinoethoxy)benzo[d]thiazole-2-carboxylate (1d). The title compound was synthesised according to the procedure in Section "General procedure for the synthesis of compounds (1a–1e)", using 4-(2-chloroethyl) morpholine hydrochloride. The product was purified by CC (DCM:MeOH:TEA, 100:3:1); brown solid; yield: 44.8%; mp 139.2–139.5 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.09 (d, *J* = 9.1 Hz, 1H), 7.81 (d, *J* = 2.5 Hz, 1H), 7.26 (dd, *J* = 9.1, 2.5 Hz, 1H), 4.21 (t, *J* = 5.7 Hz, 2H), 3.97 (s, 3H), 3.63–3.56 (m, 4H), 2.77 (t, *J* = 5.6 Hz, 2H), 2.51 (t, *J* = 3.7 Hz, 4H); ¹³C NMR (75 MHz, DMSO-*d*₆) δ 160.94, 158.86, 155.23, 147.65, 138.54, 126.07, 118.54, 105.65, 66.57, 66.47, 57.25, 54.00, 53.81; MS (ESI): *m/z* = 323.1 (M + H)⁺.

Methyl 6-(2-(piperidin-1-yl)ethoxy)benzo[d]thiazole-2-carboxylate (1e). The title compound was synthesised according to the procedure in Section "General procedure for the synthesis of compounds

(1a–1e)", using 1-(2-chloroethyl) piperidine hydrochloride. The product was purified by CC (DCM:MeOH:TEA, 100:3:1); yellowish white solid; yield: 51.6%; mp 121–121.5 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.07 (d, *J* = 9.0 Hz, 1H), 7.80 (d, *J* = 2.0 Hz, 1H), 7.23 (dd, *J* = 8.9, 2.3 Hz, 1H), 4.17 (t, *J* = 5.2 Hz, 2H), 3.94 (s, 3H), 2.72 (s, 2H), 2.47–2.44 (m, 4H), 1.49 (s, *J* = 50.0 Hz, 4H), 1.36 (s, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 160.91, 158.88, 155.19, 147.60, 138.52, 126.03, 118.53, 105.66, 66.68, 57.50, 54.73, 53.77, 25.87, 24.23; MS (ESI): *m/z* = 321.1 (M + H)⁺.

General procedure for preparing compounds (2a–2e). Preparation of the amino acid methyl ester. A 250 ml round bottom flask containing methanol (30 ml), was cooled in an ice bath, then the solid amino acid (10 mmol) was added in one portion. The solution was stirred for 5 min, then acetyl chloride (4 ml) was added dropwise using a dropping funnel over 15 min, the solution was then heated to reflux for 5 h, allowed to cool to room temperature and the solvent was removed under reduced pressure to give the methyl ester hydrochloride of the amino acid. The free base was obtained by adding dil. NH₄OH and extraction with CH₂Cl₂. The organic layer was dried over anhydrous MgSO₄, evaporated under reduced pressure and was used in the next step without further purification.

Preparation of compounds (C–F). A suspension of methyl 6-hydroxybenzothiazole-2-carboxylate (compound **A**) (2 mmol), appropriate alpha, omega-dibromoalkanes (6 mmol), anhydrous K₂CO₃ (8 mmol) and few specs of potassium iodide in acetone (30 ml) was stirred at 80 °C for 6 h. The suspension was partitioned between ethyl acetate and distilled water. The combined organic phase was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography over silica gel using methylene chloride as eluent to give the product a solid (**C–F**). The LC-MS confirmed the purity and the masses of these compounds and they were used in the next step without further elucidation.

Preparation of compounds (2a–2e). To a solution of the compounds (**C–F**) dissolved in methanol (20 ml), the methyl ester of the amino acid (3 equiv.) was added and the reaction mixture was left to reflux overnight. The solvent was evaporated *in vacuo* and extraction was done using methylene chloride and water. The organic layer was dried over anhydrous Na₂SO₄, concentrated *in vacuo* and the residue was purified by CC.

Methyl (S)-6-(3-((3-(4-hydroxyphenyl)-1-methoxy-1-oxopropan-2-yl)amino)propoxy)benzo[d]thiazole-2-carboxylate (2a). The title compound was synthesised according to the procedure in Section "General Procedure for preparing compounds (2a–2e)", using methyl ester of L-tyrosine amino acid and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:1); yellow semisolid; yield: 47.9%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.19 (s, 1H), 8.08 (d, *J* = 9.0 Hz, 1H), 7.73 (d, *J* = 2.5 Hz, 1H), 7.20 (dd, *J* = 8.5, 2.1 Hz, 1H), 6.94 (d, *J* = 7.8 Hz, 2H), 6.62 (d, *J* = 8.5 Hz, 2H), 5.76 (s, 1H), 4.08 (t, *J* = 6.3 Hz, 2H), 3.96 (s, 3H), 3.53 (s, 3H), 3.37 (t, *J* = 6.7 Hz, 1H), 2.78–2.65 (m, 3H), 2.55 (dd, *J* = 12.4, 5.8 Hz, 1H), 1.84 (dd, *J* = 13.0, 6.5 Hz, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 174.53, 160.48, 158.58, 155.78, 154.66, 147.08, 138.09, 129.97, 127.67, 125.55, 118.01, 114.90, 105.01, 66.51, 62.89, 53.30, 51.13, 43.80, 37.93, 28.95; MS (ESI): *m/z* = 445.12 (M + H)⁺.

Methyl (S)-6-(3-((3-(1H-indol-2-yl)-1-methoxy-1-oxopropan-2-yl)amino)propoxy)benzo[d]thiazole-2-carboxylate (2b). The title compound was synthesised according to the procedure in Section "General

Procedure for preparing compounds (**2a–2e**)¹⁸, using methyl ester of L-tryptophan amino acid and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:1.5); orange semisolid; yield: 8.3%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.80 (s, 1H), 8.05 (d, *J* = 9.0 Hz, 1H), 7.68 (d, *J* = 2.2 Hz, 1H), 7.45 (d, *J* = 7.9 Hz, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.18–7.15 (m, 1H), 7.09 (d, *J* = 1.5 Hz, 1H), 7.02 (d, *J* = 7.6 Hz, 1H), 6.93 (t, *J* = 7.3 Hz, 1H), 4.08 (t, *J* = 6.2 Hz, 2H), 3.95 (s, 3H), 2.98 (d, *J* = 6.6 Hz, 3H), 2.72–2.65 (m, 1H), 2.61–2.54 (m, 1H), 1.88–1.81 (m, 3H), 1.22 (s, 3H); MS (ESI): *m/z* = 468.12 (M + H)⁺.

Methyl 2-(6-(3-(((R)-2-methoxy-2-oxo-1-phenylethyl)amino)propoxy)-benzo[d]thiazole-2-carboxamido)-2-phenylacetate (2c). The title compound was synthesised according to the procedure in Section “General Procedure for preparing compounds (**2a–2e**)¹⁸”, using methyl ester of L-tyrosine amino acid and 1,4-dibromobutane. The product was purified by CC (DCM:MeOH, 100:1); yellow semisolid; yield: 74.8%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.18 (s, 1H), 8.08 (d, *J* = 9.1 Hz, 1H), 7.77 (d, *J* = 2.5 Hz, 1H), 7.23 (dd, *J* = 9.0, 2.6 Hz, 1H), 6.95–6.93 (m, 2H), 6.64–6.62 (m, 2H), 4.05 (t, *J* = 6.5 Hz, 2H), 3.96 (s, 3H), 3.53 (s, 3H), 3.38 (dd, *J* = 9.1, 4.8 Hz, 1H), 2.75–2.70 (m, 2H), 2.55 (dt, *J* = 11.5, 7.1 Hz, 1H), 2.48–2.42 (m, 1H), 1.78–1.72 (m, 2H), 1.55–1.49 (m, 2H), 1.23 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 169.66, 160.47, 158.61, 155.78, 154.64, 147.07, 138.11, 129.95, 127.62, 125.57, 118.05, 114.92, 105.01, 68.16, 62.87, 53.30, 51.14, 46.73, 37.97, 26.19, 25.85; MS (ESI): *m/z* = 459.14 (M + H)⁺.

Methyl (R)-6-(4-((3-(4-hydroxyphenyl)-1-methoxy-1-oxopropan-2-yl)amino)butoxy)benzo[d]thiazole-2-carboxylate (2d). The title compound was synthesised according to the procedure in Section “General Procedure for preparing compounds (**2a–2e**)¹⁸”, using methyl ester of L-phenyl glycine amino acid and 1,4-dibromobutane. The product was purified by CC (DCM:MeOH, 100:2); brown semisolid; yield: 13.5%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.07 (dd, *J* = 9.1, 2.8 Hz, 1H), 7.74 (d, *J* = 2.4 Hz, 1H), 7.59 (d, *J* = 7.3 Hz, 1H), 7.40–7.25 (m, 5H), 4.39 (dd, *J* = 13.3, 6.1 Hz, 1H), 4.05 (t, *J* = 6.3 Hz, 2H), 3.94 (s, 3H), 3.68 (s, 1H), 3.58 (s, 2H), 2.01–1.94 (m, 1H), 1.60–1.54 (m, 1H), 1.35 (t, *J* = 7.1 Hz, 1H), 1.22 (s, 4H); MS (ESI): *m/z* = 429.12 (M + H)⁺.

Methyl (R)-6-(4-((2-methoxy-2-oxo-1-phenylethyl)amino)butoxy)benzo[d]thiazole-2-carboxylate (2e). The title compound was synthesised according to the procedure in Section “General Procedure for preparing compounds (**2a–2e**)¹⁸”, using methyl ester of L-tyrosine amino acid and 1,5-dibromopentane. The product was purified by CC (DCM:MeOH, 100:2); yellow semisolid; yield: 21%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.18 (s, 1H), 8.08 (d, *J* = 9.0 Hz, 1H), 7.77 (d, *J* = 2.5 Hz, 1H), 7.23 (dd, *J* = 9.1, 2.5 Hz, 1H), 6.95–6.93 (m, 2H), 6.66–6.62 (m, 2H), 4.05 (t, *J* = 6.5 Hz, 2H), 3.96 (s, 3H), 3.53 (s, 3H), 3.36 (t, *J* = 5.9 Hz, 2H), 2.76–2.69 (m, 2H), 2.43–2.37 (m, 1H), 1.76–1.70 (m, 2H), 1.45–1.39 (m, 4H), 1.23 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 174.58, 160.48, 158.64, 155.78, 154.64, 147.07, 138.11, 129.94, 127.65, 125.57, 118.06, 114.91, 104.99, 68.24, 62.90, 53.30, 51.11, 47.04, 37.99, 29.05, 28.31, 23.13; MS (ESI): *m/z* = 473.15 (M + H)⁺.

General procedure for preparing compounds (3a–3t, 4a–4l). A suspension of 6-hydroxy benzothiazole-2-carboxylic acid ester **B** (0.446 g, 2 mmol), 1, 2-dibromoalkanes (6 mmol), anhydrous K₂CO₃ (1.1 g, 8 mmol) and few specs of KI in acetone (30 ml) was stirred at 80 °C for 6 h. The suspension was partitioned between ethyl

acetate and distilled water. The combined organic phase was dried over anhydrous Na₂SO₄, and concentrated *in vacuo*. The residue was purified by column chromatography over silica gel using methylene chloride as an eluent to give the product as a solid. To a solution of this product dissolved in methanol (20 ml), the appropriate amine (3 equiv.) was added and the reaction mixture was left to reflux overnight. The solvent was evaporated *in vacuo* and extraction was done using methylene chloride and water. The organic layer was dried over anhydrous Na₂SO₄, concentrated *in vacuo* and the residue was purified by CC.

Piperidin-1-yl-(6-(2-(piperidin-1-yl)ethoxy)benzo[d]thiazol-2-yl)methanone (3a). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (**3a–3t, 4a–4l**)¹⁸”, using piperidine and 1,2-dibromoethane. The product was purified by CC (DCM:MeOH, 100:7); white semisolid; yield: 15.7%; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.97 (d, *J* = 9.0 Hz, 1H), 7.73 (d, *J* = 2.5 Hz, 1H), 7.16 (dd, *J* = 9.0, 2.6 Hz, 1H), 4.20 (s, 2H), 4.14 (t, *J* = 5.9, 3.4 Hz, 2H), 3.63 (s, 2H), 2.67 (dd, *J* = 10.6, 4.7 Hz, 2H), 2.45–2.40 (m, 4H), 1.59 (d, *J* = 4.4 Hz, 4H), 1.48 (m, 5H), 1.40–1.35 (m, 2H), 1.21 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 160.48, 158.58, 155.78, 129.97, 125.55, 118.01, 114.90, 105.01, 66.51, 62.89, 53.30, 51.13, 43.80, 37.93, 28.95; MS (ESI): *m/z* = 374.2 (M + H)⁺.

Pyrrolidin-1-yl-(6-(2-(pyrrolidin-1-yl)ethoxy)benzo[d]thiazol-2-yl)methanone (3b). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (**3a–3t, 4a–4l**)¹⁸”, using pyrrolidine and 1,2-dibromoethane. The product was purified by CC (DCM:MeOH, 100:7); white solid; yield: 21.4%; mp 79.1–79.7 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.00 (d, *J* = 9.0 Hz, 1H), 7.75 (d, *J* = 2.5 Hz, 1H), 7.19 (dd, *J* = 9.0, 2.6 Hz, 1H), 4.19 (t, *J* = 5.8 Hz, 2H), 4.09 (t, *J* = 6.8 Hz, 2H), 3.56 (t, *J* = 6.9 Hz, 2H), 2.89 (m, 2H), 2.60 (m, 4H), 2.00–1.93 (m, 2H), 1.90–1.83 (m, 2H), 1.71 (m, 4H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 163.04, 158.27, 157.65, 147.54, 137.28, 125.11, 117.10, 105.03, 54.05, 53.94, 48.53, 47.50, 26.02, 23.08; MS (ESI): *m/z* = 346.2 (M + H)⁺; HRMS (ESI): calculated for (C₁₈H₂₃N₃O₂S + Na)⁺ 368.1404, found 368.1403 (M + Na)⁺.

N-(Cyclohexylmethyl)-6-(2-(cyclohexylmethyl)amino)ethoxybenzo[d]thiazole-2-carboxamide (3c). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (**3a–3t, 4a–4l**)¹⁸”, using cyclohexyl methyl amine and 1,2-dibromoethane. The product was purified by CC (DCM:MeOH, 100:6); yellow semisolid; yield: 10.4%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.00 (t, *J* = 6.2 Hz, 1H), 7.99 (d, *J* = 9.0 Hz, 1H), 7.76 (d, *J* = 2.5 Hz, 1H), 7.21 (dd, *J* = 9.0, 2.6 Hz, 1H), 4.12 (t, *J* = 5.7 Hz, 2H), 3.13 (t, *J* = 6.6 Hz, 2H), 2.92 (t, *J* = 5.7 Hz, 2H), 2.44 (d, *J* = 6.7 Hz, 2H), 1.76–1.57 (m, 11H), 1.43–1.35 (m, 1H), 1.24–1.10 (m, 7H), 0.96–0.82 (m, 4H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 162.02, 159.52, 157.70, 147.08, 137.85, 124.49, 117.36, 105.47, 67.96, 55.90, 48.18, 45.26, 37.40, 30.94, 30.39, 26.25, 26.01, 25.57, 25.32; MS (ESI): *m/z* = 430.24 (M + H)⁺.

Piperidin-1-yl-(6-(3-(piperidin-1-yl)propoxy)benzo[d]thiazol-2-yl)methanone (3d). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (**3a–3t, 4a–4l**)¹⁸”, using piperidine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH:TEA, 100:5:1); brown solid; yield: 52.4%; mp 90.1–90.6 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.98 (d, *J* = 9.0 Hz, 1H), 7.73 (d, *J* = 2.5 Hz, 1H), 7.17 (dd, *J* = 9.0,

2.6 Hz, 1H), 4.23 (t, $J = 6.2$ Hz, 2H), 4.11 (t, $J = 6.3$ Hz, 2H), 3.66 (t, $J = 5.3$ Hz, 2H), 2.64–2.53 (m, 4H), 2.02–1.91 (m, 2H), 1.69–1.51 (m, 11H), 1.42 (d, $J = 5.1$ Hz, 3H); ^{13}C NMR (75 MHz, DMSO- d_6) δ 162.80, 159.17, 158.23, 147.34, 137.63, 125.40, 117.57, 105.38, 66.93, 55.18, 54.18, 44.51, 26.83, 25.44, 24.42, 24.02; MS (ESI): $m/z = 388.2$ (M + H) $^{+}$.

N-Cycloheptyl-6-(3-(cycloheptylamino)propoxy)benzo[d]thiazole-2-carboxamide (3e). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using cycloheptyl amine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:6); brown solid; yield: 33%; mp 177.1–177.7 °C; ^1H NMR (300 MHz, DMSO- d_6) δ 8.87 (d, $J = 8.4$ Hz, 1H), 8.02 (d, $J = 9.0$ Hz, 1H), 7.79 (d, $J = 2.5$ Hz, 1H), 7.22 (dd, $J = 9.0$, 2.5 Hz, 1H), 4.19 (t, $J = 5.9$ Hz, 2H), 4.02–3.92 (m, 1H), 3.27–3.18 (m, 1H), 3.15–3.06 (m, 2H), 2.21–2.00 (m, 4H), 1.85 (dd, $J = 12.6$, 5.5 Hz, 2H), 1.61 (m, 21H); ^{13}C NMR (75 MHz, DMSO- d_6) δ 162.89, 158.71, 157.87, 147.60, 138.29, 125.07, 117.82, 106.04, 65.91, 58.68, 51.31, 34.51, 30.65, 28.10, 27.85, 24.39, 23.80; MS (ESI): $m/z = 444.4$ (M + H) $^{+}$.

N-(Cyclohexylmethyl)-6-(3-(cyclohexylmethylamino)propoxy)benzo[d]thiazole-2-carboxamide (3f). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using cyclohexyl methyl amine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:7); white solid; yield: 26.2%; mp 227.2–228.2 °C; ^1H NMR (300 MHz, DMSO- d_6) δ 9.03 (t, $J = 6.2$ Hz, 1H), 8.00 (d, $J = 9.0$ Hz, 1H), 7.77 (d, $J = 2.5$ Hz, 1H), 7.20 (dd, $J = 9.0$, 2.5 Hz, 1H), 4.16 (t, $J = 6.1$ Hz, 2H), 3.14 (t, $J = 6.5$ Hz, 2H), 2.86 (t, $J = 7.1$ Hz, 2H), 2.56 (d, $J = 6.8$ Hz, 2H), 2.07–1.96 (m, 2H), 1.80–1.45 (m, 13H), 1.28–1.07 (m, 6H), 0.93 (dd, $J = 23.3$, 15.3 Hz, 4H); ^{13}C NMR (75 MHz, DMSO- d_6) δ 162.54, 159.99, 158.08, 147.57, 138.35, 125.01, 117.80, 105.90, 66.53, 55.11, 46.00, 45.76, 37.82, 36.57, 31.03, 27.75, 26.50, 26.43, 25.81; MS (ESI): $m/z = 444.4$ (M + H) $^{+}$.

N-Cyclopropyl-6-(3-(cyclopropylamino)propoxy)benzo[d]thiazole-2-carboxamide (3g). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using cyclopropyl amine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:6); brown semisolid; yield: 10.2%; ^1H NMR (300 MHz, DMSO- d_6) δ 8.89 (d, $J = 4.8$ Hz, 1H), 7.73 (d, $J = 9.0$ Hz, 1H), 7.53 (d, $J = 2.5$ Hz, 1H), 6.97 (dd, $J = 9.0$, 2.6 Hz, 1H), 3.92 (t, $J = 6.1$ Hz, 2H), 3.16 (d, $J = 33.9$ Hz, 2H), 2.84–2.63 (m, 2H), 1.77 (m, 2H), 0.49–0.36 (m, 8H), 0.13 (d, $J = 41.7$ Hz, 1H); ^{13}C NMR (75 MHz, DMSO- d_6) δ 162.35, 161.18, 158.00, 147.64, 138.30, 125.02, 117.82, 105.97, 66.24, 45.53, 30.24, 26.93, 23.61, 6.13, 4.44; MS (ESI): $m/z = 332.12$ (M + H) $^{+}$.

Pyrrolidin-1-yl-6-(3-(pyrrolidin-1-yl)propoxy)benzo[d]thiazol-2-yl)methanone (3h). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using pyrrolidine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:6); brown solid; yield: 22.6%; mp 190.2–190.9 °C; ^1H NMR (300 MHz, DMSO- d_6) δ 8.03 (d, $J = 9.0$ Hz, 1H), 7.77 (d, $J = 2.5$ Hz, 1H), 7.20 (dd, $J = 9.0$, 2.5 Hz, 1H), 4.19 (t, $J = 6.0$ Hz, 2H), 4.09 (t, $J = 6.7$ Hz, 2H), 3.57 (t, $J = 6.8$ Hz, 2H), 3.19–3.05 (m, 2H), 2.51 (dd, $J = 3.6$, 1.8 Hz, 2H), 2.21 (dd, $J = 15.2$, 6.0 Hz, 2H), 2.02–1.82 (m, 10H); ^{13}C NMR (75 MHz, DMSO- d_6) δ 163.71, 158.71, 157.91, 148.17, 137.73, 125.66, 117.55, 105.64, 65.99, 53.61, 51.72, 49.04, 48.02, 26.52,

25.67, 23.66, 23.16; MS (ESI): $m/z = 360.2$ (M + H) $^{+}$; HRMS (ESI): calculated for (C₁₉H₂₅N₃O₂S + Na) $^{+}$ 382.1560, found 382.1591 (M + Na) $^{+}$.

N-(2-(Pyrrolidin-1-yl)ethyl)-6-(3-(2-(pyrrolidin-1-yl)ethyl)amino)propoxy)benzo[d]thiazole-2-carboxamide (4a). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using 1-(2-aminoethyl) pyrrolidine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH:TEA, 100:0.5:0.5); white solid; yield: 21.2%; mp 120.5–120.8 °C; ^1H NMR (400 MHz, CDCl₃) δ 8.42 (t, $J = 5.7$ Hz, 1H), 8.15 (d, $J = 7.6$ Hz, 1H), 7.33 (d, $J = 2.4$ Hz, 1H), 7.13 (dd, $J = 9.0$, 2.5 Hz, 1H), 3.95 (q, $J = 6.1$ Hz, 2H), 3.57 (q, $J = 7.3$ Hz, 4H), 3.42 (m, 6H), 3.22 (d, $J = 4.6$ Hz, 5H), 2.10 (dd, $J = 8.1$, 5.3 Hz, 5H), 1.43 (dd, $J = 9.2$, 5.2 Hz, 7H), 1.22 (s, 2H); ^{13}C NMR (101 MHz, CDCl₃) δ 158.03, 147.58, 138.55, 125.51, 118.15, 117.56, 106.71, 104.86, 69.36, 63.98, 54.48, 53.01, 46.00, 40.15, 23.30, 8.62, 7.99; MS (ESI): $m/z = 446.2$ (M + H) $^{+}$; HRMS (ESI): calculated for (C₂₃H₃₅N₅O₂S + H) $^{+}$ 446.2582, found 446.2572 (M + H) $^{+}$.

Azepan-1-yl-6-(3-(azepan-1-yl)propoxy)benzo[d]thiazol-2-yl)methanone (4b). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using azepane and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:5); white solid; yield: 57.5%; mp 130.6–131.1 °C; ^1H NMR (400 MHz, CDCl₃) δ 7.92 (d, $J = 9.0$ Hz, 1H), 7.32 (d, $J = 2.3$ Hz, 1H), 7.06 (dd, $J = 9.0$, 2.4 Hz, 1H), 4.21–4.16 (m, 2H), 4.14 (t, $J = 5.2$ Hz, 2H), 3.81–3.65 (m, 4H), 3.29–3.23 (m, 2H), 2.46 (d, $J = 3.9$ Hz, 2H), 2.10–1.59 (m, 18H); ^{13}C NMR (101 MHz, CDCl₃) δ 163.16, 160.95, 157.24, 148.22, 137.86, 125.38, 116.27, 104.44, 65.52, 64.15, 55.1, 54.66, 48.96, 48.43, 29.42, 27.1, 26.99, 26.90, 26.72, 24.44, 23.39; MS (ESI): $m/z = 416.23$ (M + H) $^{+}$; HRMS (ESI): calculated for (C₂₃H₃₃N₃O₂S + Na) $^{+}$ 438.2184, found 438.2209 (M + Na) $^{+}$.

(3,5-Dimethylpiperidin-1-yl)-6-(3-(3,5-dimethylpiperidin-1-yl)propoxy)benzo[d]thiazol-2-yl)methanone (4c). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using 3,5-dimethyl piperidine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:5); white solid; yield: 42%; mp 182.3–183.1 °C; ^1H NMR (400 MHz, CDCl₃) δ 7.97–7.93 (d, $J = 2.6$ Hz, 1H), 7.34 (d, $J = 2.4$ Hz, 1H), 7.07 (dd, $J = 9.0$, 2.5 Hz, 1H), 4.15 (t, $J = 5.6$ Hz, 2H), 3.75 (dd, $J = 11.2$, 3.6 Hz, 1H), 3.68 (dd, $J = 11.2$, 5.6 Hz, 1H), 3.41 (d, $J = 9.7$ Hz, 2H), 3.15 (s, 2H), 2.95 (s, 2H), 2.87 (s, 2H), 2.45 (s, 2H), 2.30 (dd, $J = 26.7$, 14.4 Hz, 3H), 2.14 (s, 1H), 1.90 (dd, $J = 12.0$, 10.4 Hz, 2H), 1.76 (d, $J = 4.0$ Hz, 2H), 0.94 (dd, $J = 14.9$, 6.6 Hz, 12H); ^{13}C NMR (101 MHz, CDCl₃) δ 162.90, 159.54, 157.32, 147.89, 137.82, 125.33, 116.35, 104.42, 65.67, 64.21, 53.35, 50.72, 42.43, 32.41, 31.32, 19.16, 18.79; MS (ESI): $m/z = 444.26$ (M + H) $^{+}$.

N-Phenethyl-6-(3-(phenethylamino)propoxy)benzo[d]thiazole-2-carboxamide (4d). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using phenethyl amine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:1); grey solid; yield: 63.2%; mp 90.5–91 °C; ^1H NMR (400 MHz, DMSO- d_6) δ 8.90 (t, $J = 5.0$ Hz, 1H), 7.77 (d, $J = 9.0$ Hz, 1H), 7.52 (s, 1H), 7.11–6.95 (m, 11H), 3.91 (t, $J = 5.6$ Hz, 2H), 3.36–3.30 (m, 2H), 3.08 (dd, $J = 10.6$, 5.6 Hz, 3H), 2.68 (t, $J = 7.2$ Hz, 2H), 2.30 (s, 3H), 1.67 (dd, $J = 13.5$, 7.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ

162.16, 159.90, 158.26, 147.42, 140.88, 139.60, 138.32, 129.05, 128.80, 126.22, 117.83, 105.81, 72.93, 63.52, 51.52, 46.17, 36.30, 35.29, 29.43; MS (ESI): $m/z = 460.2$ (M + H)⁺.

(3,4-Dihydroisoquinolin-2(1H)-yl)(6-(3-(3,4-dihydroisoquinolin-2(1H)-yl)propoxy)benzo[d]thiazol-2-yl)methanone (4e). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using tetrahydroisoquinoline and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:1); white solid; yield: 76.6%; mp 134.2–134.7 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 8.02 (d, $J = 8.4$ Hz, 1H), 7.74 (s, 1H), 7.22 (d, $J = 22.2$ Hz, 5H), 7.05 (d, $J = 14.3$ Hz, 4H), 5.46 (s, 1H), 4.82 (s, 1H), 4.47 (s, 1H), 4.14 (t, $J = 5.6$ Hz, 2H), 3.88 (s, 1H), 3.56 (s, 2H), 2.94 (d, $J = 17.3$ Hz, 2H), 2.79 (s, 2H), 2.69–2.58 (m, 4H), 2.04–1.99 (m, 2H); ¹³C NMR (101 MHz, DMSO-d₆) δ 159.89, 158.44, 147.38, 137.72, 135.33, 134.88, 134.61, 133.14, 130.17, 128.94, 128.81, 127.05, 126.83, 126.73, 126.35, 125.86, 125.58, 125.51, 117.71, 105.37, 67.08, 56.05, 54.60, 50.96, 45.85, 44.22, 29.43, 29.16, 26.77; MS (ESI): $m/z = 484.2$ (M + H)⁺.

(4-Ethylpiperazin-1-yl)(6-(3-(4-ethylpiperazin-1-yl)propoxy)benzo[d]thiazol-2-yl)methanone (4f). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using 1-ethyl piperazine and 1,3-dibromopropane. The product was purified by adding a small amount of DCM followed by the addition of petroleum ether; beige solid; yield: 48.1%; mp 114.6–114.9 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 7.97 (d, $J = 9.0$ Hz, 1H), 7.71 (d, $J = 2.5$ Hz, 1H), 7.15 (dd, $J = 9.0, 2.5$ Hz, 1H), 4.29 (s, 4H), 4.08 (t, $J = 6.4$ Hz, 2H), 3.67 (s, 2H), 2.47–2.33 (m, 16H), 1.94–1.86 (m, 2H), 1.00 (td, $J = 7.2, 4.3$ Hz, 6H); ¹³C NMR (101 MHz, DMSO-d₆) δ 162.47, 159.14, 158.34, 147.29, 137.66, 125.43, 117.66, 105.34, 72.92, 66.98, 63.52, 54.59, 53.20, 52.44, 51.84, 26.45, 12.28; MS (ESI): $m/z = 446.25$ (M + H)⁺.

(4-Methylpiperazin-1-yl)(6-(3-(4-methylpiperazin-1-yl)propoxy)benzo[d]thiazol-2-yl)methanone (4g). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using 1-methyl piperazine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH:TEA, 100:5:0.5); beige solid; yield: 28.1%; mp 99.2–99.7 °C; ¹H NMR (400 MHz, CDCl₃) δ 7.95–7.91 (d, $J = 8.0$ Hz, 1H), 7.37 (d, $J = 11.1$ Hz, 1H), 7.10 (d, $J = 8.8$ Hz, 1H), 4.48 (s, 2H), 4.08 (s, 2H), 3.85 (s, 2H), 2.67–2.50 (m, 14H), 2.38 (s, 3H), 2.34 (s, 3H), 2.02 (d, $J = 5.4$ Hz, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 162.12, 159.66, 158.23, 147.55, 137.90, 125.19, 117.09, 104.04, 66.64, 55.49, 54.66, 52.41, 45.96, 43.46, 29.66, 26.50; MS (ESI): $m/z = 418.2$ (M + H)⁺.

(3-Methylpiperazin-1-yl)(6-(3-(3-methylpiperazin-1-yl)propoxy)benzo[d]thiazol-2-yl)methanone (4h). The title compound was synthesised according to procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using 1-boc-2-methyl piperazine and 1,3-dibromopropane to give compound 4h1, which was deprotected using TFA in DCM. The product was purified by CC (DCM:MeOH:TEA, 100:5:0.5); white solid; yield: 27.6%; mp 114.3–114.8 °C; ¹H NMR (400 MHz, CD₃OD) δ 8.02 (d, $J = 9.0$ Hz, 1H), 7.64 (d, $J = 2.4$ Hz, 1H), 7.25 (dd, $J = 9.1, 2.4$ Hz, 1H), 4.24 (t, $J = 5.9$ Hz, 2H), 3.72–3.42 (m, 9H), 3.35–3.31 (m, 7H), 2.39 (s, 1H), 2.31–2.24 (m, 2H), 1.43 (dt, $J = 13.6, 6.6$ Hz, 7H); ¹³C NMR (101 MHz, CD₃OD) δ 160.89, 159.77, 158.50, 147.41, 137.95, 125.05, 117.34, 104.07, 65.60, 54.59, 54.22, 52.19, 51.13, 50.09, 48.29, 46.48, 41.67, 24.63, 14.79, 7.79, 6.22; MS (ESI): $m/z = 418.2$ (M + H)⁺.

(4-(2-Hydroxyethyl)piperazin-1-yl)(6-(3-(4-(2-hydroxyethyl)piperazin-1-yl)propoxy)benzo[d]thiazol-2-yl)methanone (4i). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using 1-(2-hydroxyethyl) piperazine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH:TEA, 100:5:0.5); red solid; yield: 22.5%; mp 173.5–174 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 7.97 (d, $J = 9.3$ Hz, 1H), 7.70 (s, 1H), 7.15 (d, $J = 9.5$ Hz, 1H), 4.27 (s, 2H), 4.07 (s, 2H), 3.65 (s, 1H), 3.48 (d, $J = 21.4$ Hz, 3H), 2.43–2.32 (m, 18H), 1.87 (s, 4H), 1.20 (s, 2H); ¹³C NMR (101 MHz, DMSO-d₆) δ 162.40, 159.17, 158.35, 147.25, 137.64, 125.42, 117.67, 105.31, 72.89, 63.49, 60.33, 58.87, 53.63, 53.23, 26.51; MS (ESI): $m/z = 477.24$ (M + H)⁺.

N-Benzyl-6-(3-(benzylamino)propoxy)benzo[d]thiazole-2-carboxamide (4j). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using benzyl amine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:6); white solid; yield: 57.4%; mp 90.1–90.7 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 9.59 (t, $J = 6.4$ Hz, 1H), 7.97 (d, $J = 9.0$ Hz, 1H), 7.74 (d, $J = 2.5$ Hz, 1H), 7.34–7.17 (m, 11H), 4.46 (d, $J = 6.3$ Hz, 2H), 4.13 (t, $J = 6.4$ Hz, 2H), 3.71 (s, 2H), 2.67 (t, $J = 6.8$ Hz, 2H), 1.94–1.87 (m, 2H), 1.29–1.20 (m, 1H); ¹³C NMR (101 MHz, DMSO-d₆) δ 162.00, 160.06, 158.31, 147.44, 139.40, 138.38, 128.74, 128.52, 128.43, 127.90, 127.35, 127.03, 124.99, 117.89, 105.84, 67.02, 53.30, 45.63, 43.12, 29.28; MS (ESI): $m/z = 431.17$ (M + H)⁺.

(3-Methylpiperidin-1-yl)(6-(3-(3-methylpiperidin-1-yl)propoxy)benzo[d]thiazol-2-yl)methanone (4k). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using 3-methyl piperidine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:6); white solid; yield: 76.4%; mp 161.1–161.6 °C; ¹H NMR (400 MHz, CDCl₃) δ 7.93 (d, $J = 8.8$ Hz, 1H), 7.34 (s, 1H), 7.08 (d, $J = 8.9$ Hz, 1H), 4.51 (d, $J = 12.4$ Hz, 1H), 4.11 (t, 2H), 3.20 (dd, $J = 23.8, 9.7$ Hz, 2H), 2.88 (s, 2H), 2.25 (s, 3H), 1.88 (ddd, $J = 41.8, 24.6, 11.8$ Hz, 9H), 1.67–1.58 (m, 1H), 1.25 (d, $J = 11.0$ Hz, 2H), 0.94 (dd, $J = 18.5, 4.9$ Hz, 8H); ¹³C NMR (101 MHz, CDCl₃) δ 162.67, 159.72, 157.69, 147.70, 137.79, 125.18, 116.62, 104.30, 66.25, 60.62, 55.43, 53.57, 50.99, 47.01, 33.08, 31.79, 29.77, 26.16, 25.23, 23.84, 19.29; MS (ESI): $m/z = 415.23$ (M + H)⁺.

(4-Methylpiperidin-1-yl)(6-(3-(4-methylpiperidin-1-yl)propoxy)benzo[d]thiazol-2-yl)methanone (4l). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using 4-methyl piperidine and 1,3-dibromopropane. The product was purified by CC (DCM:MeOH, 100:6); white solid; yield: 80.2%; mp 162.2–162.5 °C; ¹H NMR (400 MHz, CDCl₃) δ 7.93 (d, $J = 8.9$ Hz, 1H), 7.34 (s, 1H), 7.08 (d, $J = 9.0$ Hz, 1H), 4.66 (d, $J = 12.6$ Hz, 1H), 4.12 (s, 2H), 3.32 (d, $J = 9.8$ Hz, 2H), 3.20 (t, $J = 12.7$ Hz, 1H), 2.87 (dd, $J = 31.4, 18.9$ Hz, 3H), 2.45 (s, 2H), 2.31 (s, 2H), 1.70 (dd, $J = 62.5, 34.6$ Hz, 9H), 1.34–1.23 (m, 2H), 0.99 (t, $J = 5.7$ Hz, 6H); ¹³C NMR (101 MHz, CDCl₃) δ 162.71, 159.75, 157.60, 147.75, 137.80, 125.19, 116.55, 104.34, 66.15, 64.19, 53.39, 46.79, 44.15, 34.90, 33.92, 31.10, 21.65; MS (ESI): $m/z = 415.23$ (M + H)⁺.

N-Cyclopentyl-6-(3-(cyclopentylamino)propoxy)benzo[d]thiazole-2-carboxamide (3i). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using cyclopentyl amine and 1,3-

dibromopropane. The product was purified by CC (DCM:MeOH:TEA, 100:3:1); grey solid; yield: 15.6%; mp 169.4–171.3 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.96 (d, *J* = 7.9 Hz, 1H), 8.02 (d, *J* = 9.0 Hz, 1H), 7.79 (d, *J* = 2.5 Hz, 1H), 7.22 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.22 (dt, *J* = 11.6, 6.6 Hz, 2H), 3.53–3.45 (m, 1H), 3.35 (s, 1H), 3.12–3.04 (m, 1H), 2.16–2.07 (m, 1H), 2.03–1.83 (m, 4H), 1.78–1.47 (m, 14H), 1.24 (s, 1H); ¹³C NMR (75 MHz, DMSO-*d*₆) δ 162.85, 159.78, 157.74, 147.96, 138.57, 125.22, 117.89, 106.36, 65.87, 58.91, 51.49, 43.71, 32.28, 29.79, 24.12, 24.06; MS (ESI): *m/z* = 388.2 (M + H)⁺.

Piperidin-1-yl-6-(4-(piperidin-1-yl)butoxy)benzo[d]thiazol-2-yl)methanone (3j). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using piperidine and 1,4-dibromobutane. The product was purified by CC (DCM:MeOH, 100:9); yellowish white solid; yield: 44.2%; mp 169.7–171.5 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.00 (d, *J* = 9.0 Hz, 1H), 7.73 (d, *J* = 2.5 Hz, 1H), 7.17 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.22 (t, *J* = 5.1 Hz, 2H), 4.09 (t, *J* = 5.8 Hz, 2H), 3.65 (t, *J* = 5.2 Hz, 2H), 3.34 (s, 1H), 2.72 (s, 5H), 1.77 (d, *J* = 2.9 Hz, 4H), 1.64 (m, 10H), 1.45 (m, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 162.28, 158.69, 157.74, 146.85, 137.13, 124.92, 117.09, 104.92, 67.78, 52.81, 46.72, 44.01, 26.33, 26.11, 25.47, 24.49, 23.92; MS (ESI): *m/z* = 402.21 (M + H)⁺; HRMS (ESI): calculated for (C₂₂H₃₁N₃O₂S + Na)⁺ 424.2038, found 424.2040 (M + Na)⁺.

N-Cycloheptyl-6-(4-(cycloheptylamino)butoxy)benzo[d]thiazole-2-carboxamide (3k). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using cycloheptyl amine and 1,4-dibromobutane. The product was purified by CC (DCM:MeOH, 100:6); white solid; yield: 22%; mp 193.3–194.1 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.83 (d, *J* = 8.4 Hz, 1H), 8.00 (d, *J* = 9.0 Hz, 1H), 7.77 (d, *J* = 2.5 Hz, 1H), 7.19 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.11 (t, *J* = 6.0 Hz, 2H), 3.99–3.91 (m, 1H), 3.20–3.12 (m, 1H), 3.01–2.95 (m, 2H), 2.05–1.97 (m, 2H), 1.87–1.74 (m, 6H), 1.72–1.63 (m, 6H), 1.60–1.37 (m, 15H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 162.25, 158.23, 157.58, 147.07, 137.86, 124.56, 117.26, 105.42, 67.52, 58.14, 50.82, 43.89, 34.02, 30.25, 27.61, 27.34, 23.90, 23.31; MS (ESI): *m/z* = 458.4 (M + H)⁺.

N-(Cyclohexylmethyl)-6-(4-(cyclohexylmethyl)amino)butoxy)benzo[d]thiazole-2-carboxamide (3l). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using cyclohexyl methyl amine and 1,4-dibromobutane. The product was purified by CC (DCM:MeOH, 100:7); white solid; yield: 22%; mp 234.8–235.6 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.02 (t, *J* = 6.1 Hz, 1H), 8.00 (d, *J* = 9.0 Hz, 1H), 7.77 (d, *J* = 2.4 Hz, 1H), 7.20 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.11 (t, *J* = 5.5 Hz, 2H), 3.13 (t, *J* = 6.5 Hz, 2H), 2.99–2.92 (t, *J* = 5.9 Hz, 2H), 2.75 (t, *J* = 6.9 Hz, 2H), 1.68 (m, *J* = 35.4, 23.9, 15.6 Hz, 17H), 1.26–1.09 (m, 6H), 0.93 (d, *J* = 7.9 Hz, 4H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 162.01, 159.49, 157.58, 147.09, 137.87, 124.56, 117.29, 105.45, 67.56, 52.81, 47.19, 45.27, 37.32, 30.39, 29.97, 26.00, 25.56, 25.32, 24.98; MS (ESI): *m/z* = 458.28 (M + H)⁺.

N-Cyclopropyl-6-(4-(cyclopropylamino)butoxy)benzo[d]thiazole-2-carboxamide (3m). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using cyclopropyl amine and 1,4-dibromobutane. The product was purified by CC (DCM:MeOH, 100:9);

reddish semisolid; yield: 20.5%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.11 (d, *J* = 4.9 Hz, 1H), 7.96 (d, *J* = 9.0 Hz, 1H), 7.75 (d, *J* = 2.5 Hz, 1H), 7.18 (dd, *J* = 9.0, 2.6 Hz, 1H), 4.08 (t, *J* = 6.4 Hz, 2H), 3.58 (t, *J* = 6.5 Hz, 1H), 2.91 (m, 1H), 2.81 (t, *J* = 6.8 Hz, 1H), 2.63–2.56 (m, 1H), 2.16 (d, *J* = 6.6 Hz, 2H), 1.82–1.79 (m, 1H), 1.68–1.63 (m, 1H), 0.72–0.69 (m, 3H), 0.58–0.56 (m, 2H), 0.52–0.50 (m, 1H), 0.48 (m, 1H), 0.37–0.34 (m, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 171.72, 161.72, 157.73, 147.04, 137.86, 124.50, 117.34, 105.37, 67.92, 57.56, 29.85, 26.12, 23.11, 22.15, 5.57, 4.70; MS (ESI): *m/z* = 346.1 (M + H)⁺.

Pyrrolidin-1-yl-6-(4-(pyrrolidin-1-yl)butoxy)benzo[d]thiazol-2-yl)methanone (3n). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using pyrrolidine and 1,4-dibromobutane. The product was purified by CC (DCM:MeOH, 100:9); white solid; yield: 37.8%; mp 234.8–235.1 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.01 (d, *J* = 9.0 Hz, 1H), 7.74 (d, *J* = 2.5 Hz, 1H), 7.19 (dd, *J* = 9.0, 2.6 Hz, 1H), 4.09 (dt, *J* = 10.4, 6.4 Hz, 4H), 3.56 (t, *J* = 6.9 Hz, 2H), 3.10–2.98 (m, 4H), 2.00 (m, 14H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 163.05, 158.25, 157.73, 147.55, 137.28, 125.12, 117.09, 105.04, 67.57, 53.53, 52.80, 48.53, 47.51, 26.02, 23.16, 22.69; MS (ESI): *m/z* = 374.2 (M + H)⁺; HRMS (ESI): calculated for (C₂₀H₂₇N₃O₂S + H)⁺ 374.1896, found 374.1900 (M + H)⁺.

Piperidin-1-yl-6-((5-(piperidin-1-yl)pentyl)oxy)benzo[d]thiazol-2-yl)methanone (3o). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using piperidine and 1,5-dibromopentane. The product was purified by CC (DCM:MeOH:TEA, 100:8.5:0.5); yellowish white solid; yield: 52%; mp 76.2–77.1 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.00 (d, *J* = 9.0 Hz, 1H), 7.73 (d, *J* = 2.5 Hz, 1H), 7.17 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.23 (s, 2H), 4.07 (t, *J* = 6.4 Hz, 2H), 3.66 (s, 2H), 3.46 (d, *J* = 12.0 Hz, 2H), 2.62 (s, 4H), 1.79 (dd, *J* = 14.0, 7.0 Hz, 2H), 1.60 (dd, *J* = 16.2, 11.4 Hz, 12H), 1.49–1.37 (m, 4H); ¹³C NMR (75 MHz, DMSO-*d*₆) δ 162.73, 159.17, 158.32, 147.30, 137.65, 125.40, 117.57, 105.32, 68.50, 57.87, 53.69, 47.22, 44.51, 28.77, 26.83, 25.96, 24.42, 23.69; MS (ESI): *m/z* = 416.23 (M + H)⁺.

N-Cycloheptyl-6-((5-(cycloheptylamino)pentyl)oxy)benzo[d]thiazole-2-carboxamide (3p). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using cycloheptyl amine and 1,5-dibromopentane. The product was purified by CC (DCM:MeOH, 100:6); yellowish white solid; yield: 42.6%; mp 80.2–80.4 °C; ¹H NMR (400 MHz, CDCl₃) δ 7.88 (d, *J* = 9.0 Hz, 1H), 7.34 (d, *J* = 2.4 Hz, 1H), 7.28 (d, *J* = 8.5 Hz, 1H), 7.10 (dd, *J* = 9.0, 2.4 Hz, 1H), 4.14 (m, 1H), 4.00 (t, *J* = 6.2 Hz, 2H), 3.21–3.13 (m, 1H), 2.99–2.93 (m, 2H), 2.24–2.17 (m, 1H), 2.08–1.99 (m, 3H), 1.86–1.78 (m, 4H), 1.69–1.43 (m, 18H), 1.29–1.18 (m, 4H), 0.87–0.81 (m, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 161.80, 158.80, 158.05, 147.33, 138.71, 124.66, 117.33, 104.65, 68.03, 59.74, 50.96, 44.96, 34.98, 31.34, 28.56, 28.05, 27.54, 26.17, 24.07, 23.61; MS (ESI): *m/z* = 472.2 (M + H)⁺.

N-(Cyclohexylmethyl)-6-((5-(cyclohexylmethyl)amino)pentyl)oxy)benzo[d]thiazole-2-carboxamide (3q). The title compound was synthesised according to the procedure in Section “General procedure for preparing compounds (3a–3t, 4a–4l)”, using cyclohexyl methyl amine and 1,5-dibromopentane. The product was purified by CC (DCM:MeOH, 100:8); white solid; yield: 32.8%; mp 149–149.4 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.03 (t, *J* = 6.1 Hz, 1H), 8.00 (d,

$J = 9.0$ Hz, 1H), 7.78 (d, $J = 2.5$ Hz, 1H), 7.20 (dd, $J = 9.0$, 2.5 Hz, 1H), 4.09 (t, $J = 6.2$ Hz, 2H), 3.14 (t, $J = 6.5$ Hz, 2H), 2.93–2.85 (m, 2H), 2.73 (d, $J = 6.8$ Hz, 2H), 1.87–1.43 (m, 18H), 1.29–0.86 (m, 11H); ^{13}C NMR (75 MHz, DMSO- d_6) δ 162.49, 160.00, 158.19, 147.52, 138.37, 125.02, 117.81, 105.85, 68.36, 53.30, 47.86, 45.75, 37.82, 35.02, 30.88, 30.51, 28.52, 26.50, 26.07, 25.82, 25.50, 23.17; MS (ESI): $m/z = 472.29$ (M + H) $^{+}$.

N-Cyclopropyl-6-((5-(cyclopropylamino)pentyl)oxy)benzo[d]thiazole-2-carboxamide (3r). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using cyclopropyl amine and 1,5-dibromopentane. The product was purified by CC (DCM:MeOH, 100:6); brown solid; yield: 70%; mp 141.8–142.5 °C; ^1H NMR (400 MHz, CDCl_3) δ 7.85 (d, $J = 8.7$ Hz, 1H), 7.39 (s, 1H), 7.32 (s, 1H), 7.09 (d, $J = 8.9$ Hz, 1H), 4.00 (s, 2H), 2.96–2.86 (m, 3H), 2.32 (d, $J = 2.3$ Hz, 1H), 1.81 (dd, $J = 17.9$, 7.3 Hz, 4H), 1.55 (d, $J = 6.1$ Hz, 2H), 1.24 (s, 1H), 0.89 (d, $J = 6.5$ Hz, 2H), 0.73 (d, $J = 21.6$ Hz, 4H), 0.61 (d, $J = 5.8$ Hz, 2H); ^{13}C NMR (101 MHz, CDCl_3) δ 161.28, 161.00, 158.24, 147.22, 138.76, 124.67, 117.45, 104.52, 68.24, 48.98, 30.30, 28.79, 27.70, 23.65, 22.77, 6.65, 4.98; MS (ESI): $m/z = 360.17$ (M + H) $^{+}$.

Pyrrolidin-1-yl-6-((5-(pyrrolidin-1-yl)pentyl)oxy)benzo[d]thiazol-2-yl)methanone (3s). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using pyrrolidine and 1,5-dibromopentane. The product was purified by CC (DCM:MeOH, 100:7); light brown solid; yield: 24%; mp 151.2–151.8 °C; ^1H NMR (300 MHz, DMSO- d_6) δ 8.01 (d, $J = 9.0$ Hz, 1H), 7.75 (d, $J = 2.5$ Hz, 1H), 7.18 (dd, $J = 9.0$, 2.5 Hz, 1H), 4.11–4.07 (m, 4H), 3.56 (t, $J = 6.8$ Hz, 2H), 3.20–3.12 (m, 4H), 2.05–1.65 (m, 14H), 1.55–1.44 (m, 2H); ^{13}C NMR (75 MHz, DMSO- d_6) δ 163.49, 158.73, 158.30, 147.98, 137.79, 125.62, 117.57, 105.43, 68.35, 54.30, 53.69, 49.03, 48.01, 28.45, 26.52, 25.43, 23.66, 23.04; MS (ESI): $m/z = 388.2$ (M + H) $^{+}$; HRMS (ESI): calculated for $(\text{C}_{21}\text{H}_{29}\text{N}_3\text{O}_2\text{S} + \text{Na}^+)$ 410.1872, found 410.1894 (M + Na) $^{+}$.

N-Cyclopentyl-6-((5-(cyclopentylamino)pentyl)oxy)benzo[d]thiazole-2-carboxamide (3t). The title compound was synthesised according to the procedure in Section "General procedure for preparing compounds (3a–3t, 4a–4l)", using cyclopentyl amine 1,5-dibromopentane. The product was purified by CC (DCM:MeOH:TEA, 100:8.5:0.5); grey semisolid; yield: 34%; ^1H NMR (300 MHz, DMSO- d_6) δ 8.93 (d, $J = 7.9$ Hz, 1H), 7.99 (dd, $J = 9.0$, 3.8 Hz, 1H), 7.76 (d, $J = 2.5$ Hz, 1H), 7.19 (dd, $J = 9.0$, 2.5 Hz, 1H), 4.23 (m, 1H), 4.07 (t, $J = 6.2$ Hz, 2H), 3.35 (m, 1H), 2.88–2.74 (m, 2H), 1.95–1.83 (m, 4H), 1.81–1.56 (m, 13H), 1.50 (dd, $J = 11.6$, 5.9 Hz, 6H); ^{13}C NMR (75 MHz, DMSO- d_6) δ 162.52, 159.58, 158.20, 147.51, 138.36, 124.98, 117.78, 105.79, 68.42, 58.79, 51.48, 46.34, 32.29, 29.88, 28.59, 26.32, 24.05, 23.32; MS (ESI): $m/z = 416.23$ (M + H) $^{+}$.

Procedure for preparing compound (4m). Compound (4h) (1 mmol, 1 equiv.) was dissolved in a mixture of 10 ml DCM and 10 ml DMF, then glacial acetic acid (4 equiv.), DMAP (4 equiv.) and EDC (4 equiv.) were added. The reaction was stirred at room temperature overnight. After the completion of the reaction, the solvent was evaporated under vacuum then the compound was extracted using ethyl acetate and distilled water. The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography over silica gel.

1-(4-(6-(3-(4-Acetyl-3-methylpiperazin-1-yl)propoxy)benzo[d]thiazole-2-carbonyl)-2-methylpiperazin-1-yl)ethan-1-one (4m). The title compound was synthesised according to the procedure in Section "Procedure for preparing compound (4m)", using compound (4h). The product was purified by CC (DCM:MeOH, 100:8); white semisolid; yield: 34%; ^1H NMR (400 MHz, CDCl_3) δ 7.93 (s, 1H), 7.37 (s, 1H), 7.12 (d, $J = 8.7$ Hz, 1H), 4.53 (s, 1H), 4.17–3.95 (m, 3H), 3.57 (dd, $J = 107.2$, 45.2 Hz, 3H), 3.17–2.30 (m, 6H), 2.14 (dd, $J = 30.7$, 21.1 Hz, 10H), 1.65 (s, 2H), 1.35–1.16 (m, 7H); ^{13}C NMR (101 MHz, CDCl_3) δ 169.26, 150.73, 138.05, 134.27, 132.92, 125.51, 117.11, 106.48, 104.06, 63.33, 53.43, 50.00, 47.65, 46.21, 43.71, 35.90, 33.63, 29.65, 24.18, 21.70, 21.22, 16.43, 16.21; MS (ESI): $m/z = 502.2$ (M + H) $^{+}$.

Biological assays

Cell culture and membrane preparations

HEK-293 cells stably expressing the human H_3R were cultured in DMEM (10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1.1% HEPES buffer (1M)). CHO-K1 cells stably expressing the human H_1R were cultivated in DMEM (10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 1% NEAA). For human H_4R assays, Sf-9 insect cells were cultured in Spodopan (5% FBS, 1% penicillin/streptomycin) and co-infected with baculoviruses containing G-protein $\text{G}_{\alpha i2}$, $\text{G}_{\beta 1;2}$ and human H_4R . Cell membrane preparations were obtained according to Bautista et al. and stored at -80°C until use.¹⁴

Radioligand displacement assay

Human H_3R . The displacement assay for H_3R was performed according to the protocol reported by Bautista et al.¹⁴ Briefly, membrane fractions (20 μg /200 μL binding buffer) were co-incubated with [^3H]- N^2 -methylhistamine (2 nM) and test ligand for 90 min at room temperature while shaking. Assays for K_i value determination were run in duplicates with appropriate concentrations between 100 μM and 0.01 nM of the tested compound. Assays for one-point inhibition were performed in triplicates at a concentration of 1 μM test ligand, calculating percent inhibition relative to total radioligand binding. Non-specific binding was determined in the presence of 10 μM pitolisant. Data were analysed with GraphPad Prism 6.1 (San Diego, CA, USA) using non-linear regression fit (one-site competition with a logarithmic scale). The K_i values were calculated from the IC_{50} values using the Cheng-Prusoff equation.⁴⁷ Statistical calculations were performed on $-\log(K_i)$.

Human H_1R . The displacement assay for H_1R was performed according to the protocol reported by Bautista et al.¹⁴ Briefly, membrane fractions (40 μg /200 μL binding buffer) were co-incubated with [^3H]histamine (1 nM) and test ligand for 120 min at room temperature while shaking. Assays for one-point inhibition were performed in triplicates at a concentration of 10 μM test ligand, calculating percent inhibition relative to total radioligand binding. Non-specific binding was determined in the presence of 10 μM chlorpheniramine. Data were analysed with GraphPad Prism 6.1 (San Diego, CA, USA).

Human H_4R . The displacement assay for H_4R was performed according to the protocol reported by Bautista et al.¹⁴ Briefly, membrane fractions (40 μg /200 μL binding buffer) were co-incubated with [^3H]histamine (10 nM) and test ligand for 60 min at

room temperature while shaking. Assays for one-point inhibition were performed in triplicates at a concentration of 1 μ M test ligand, calculating percent inhibition relative to total radioligand binding. Non-specific binding was determined in the presence of 100 μ M JNJ7777120. Data were analysed with GraphPad Prism 6.1 (San Diego, CA, USA).

Inhibition of AChE

The AChE assay was performed using an AChE inhibitor screening kit (BioVision, #K197-100, San Francisco, USA). According to the manufacturer's protocol, tested compounds, donepezil, or DMSO (1%, as solvent control) were incubated with AChE for 15 min in dark at 25 °C, and then the probe and substrate were added for 30 min. The activity of AChE was obtained using a spectrophotometer at 412 nm. The assays were performed in triplicates.

Inhibition of BuChE

The hBuChE assay was performed using the Ellman's spectrophotometric method adapted to a 96-well plate procedure as previously described. Briefly, BuChE from human serum (50 U/mg), substrate butyrylthiocholine iodide and chromophoric reagent 5,5'-dithiobis(2-nitrobenzoic acid) (all from Sigma Aldrich, Italy) were incubated with test compounds in clear flat-bottomed 96-well plates (Greiner Bio-One, Austria) in duplicate. Final concentrations where 0.09 U/mL (BuChE), 0.5 mM (butyrylthiocholine iodide), 0.33 mM (DTNB), while compounds were initially tested at 10 μ M concentration. Only for compounds showing >60% inhibition, IC50 values were measured in the range 30-0.01 μ M. The increase in absorbance was read at 412 nm for 5 min with a Tecan Infinite M1000 Pro multiplate reader (Tecan, Italy). Inhibition values were calculated with GraphPad Prism 5.0 software as the mean of three independent experiments.⁴⁸

Inhibition of monoamine oxidases

Inhibition of hMAOs was evaluated using human recombinant MAO-A and MAO-B (microsomes from baculovirus-infected insect cells) and kynuramine as non-selective MAO-A/B substrate (all from Sigma Aldrich Italy) through a previously described fluorescence-based method.⁴⁹ Experiments were performed in triplicates in black, round-bottomed polystyrene 96-well microtiter plates (Greiner). Fluorescence was measured at excitation/emission wavelengths of 310/400 nm in a 96-well microplate fluorescence reader (Tecan Infinite M1000 Pro) and inhibition values were calculated with GraphPad Prism 5.0 software as the mean of three independent experiments.

Molecular docking

All procedures were performed using the Molecular Operating Environment (MOE)[®] software package (version 2019, Chemical Computing Group). During the molecular modelling studies on H₃R a previously prepared homology model was used.⁴⁴ The ligands (**3b**, **3s**) were prepared by MOE (washing, calculating partial charges, adjusting tautomeric states and minimising the energy). The binding site was defined using Site Finder and the docking was performed using Amber10: EHT as a force field and the "triangle matcher" method (number of return poses set to 200), Induced fit as refinement and London dG as the scoring function. The number of returned poses was set to 20.

For the docking simulations on human AChE, PDB code 4EY5 (rhAChE cocrystallized with Huperzine A) was used. Molecular docking simulation with **3b** as a ligand was performed using the Amber10: EHT as a force field and the "triangle matcher" method (number of return poses set to 200), Induced fit as refinement and London dG as the scoring function. The number of returned poses was set to 20. Similarly, compound **4a** was docked in the active site of human BuChE using PDB code 1POL.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Supplementary Information

Novel benzothiazole derivatives as multitargeted-directed ligands for the treatment of Alzheimer's disease

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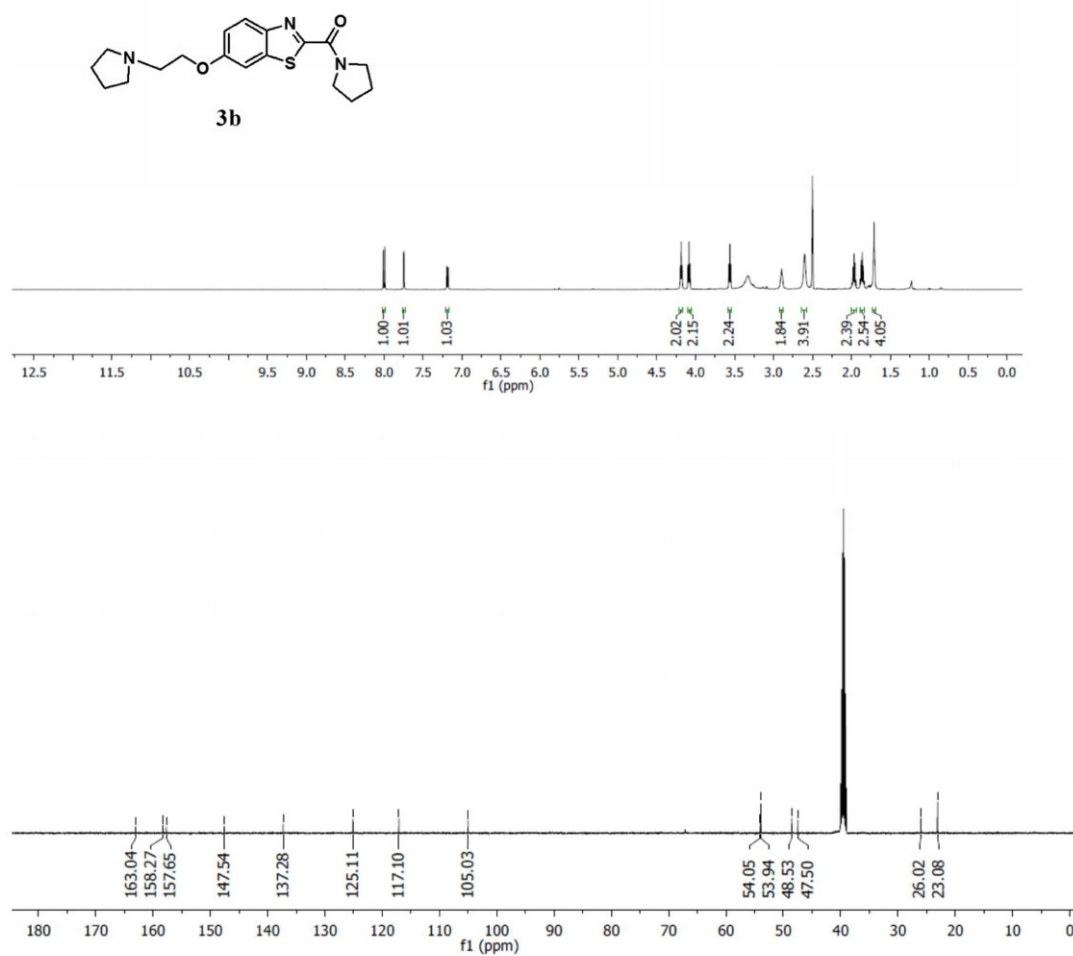
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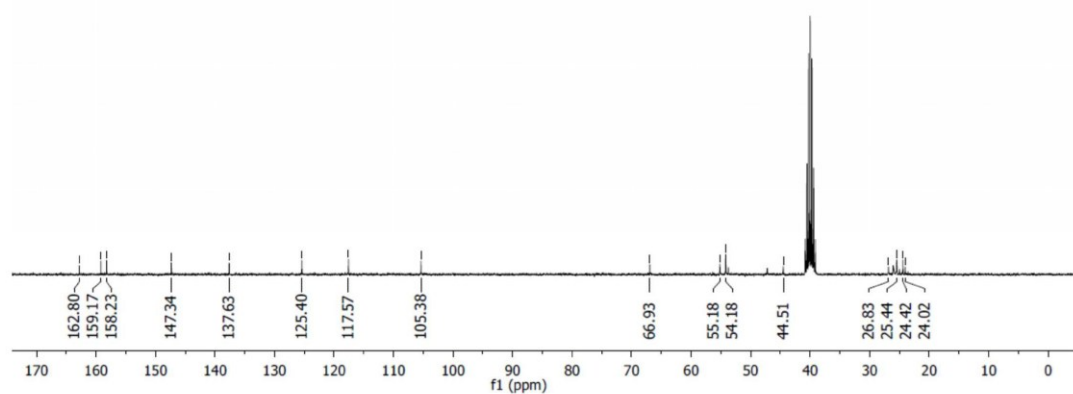
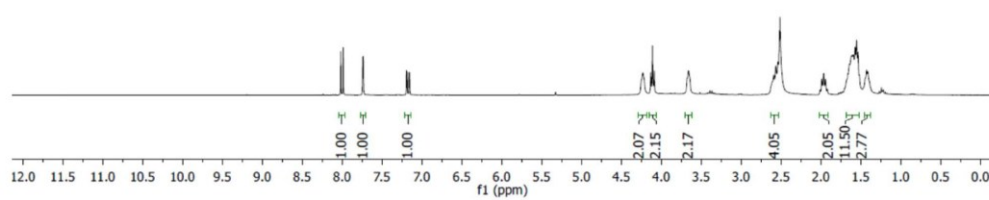
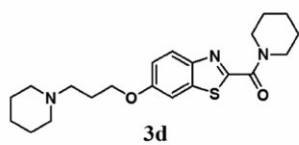
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Content

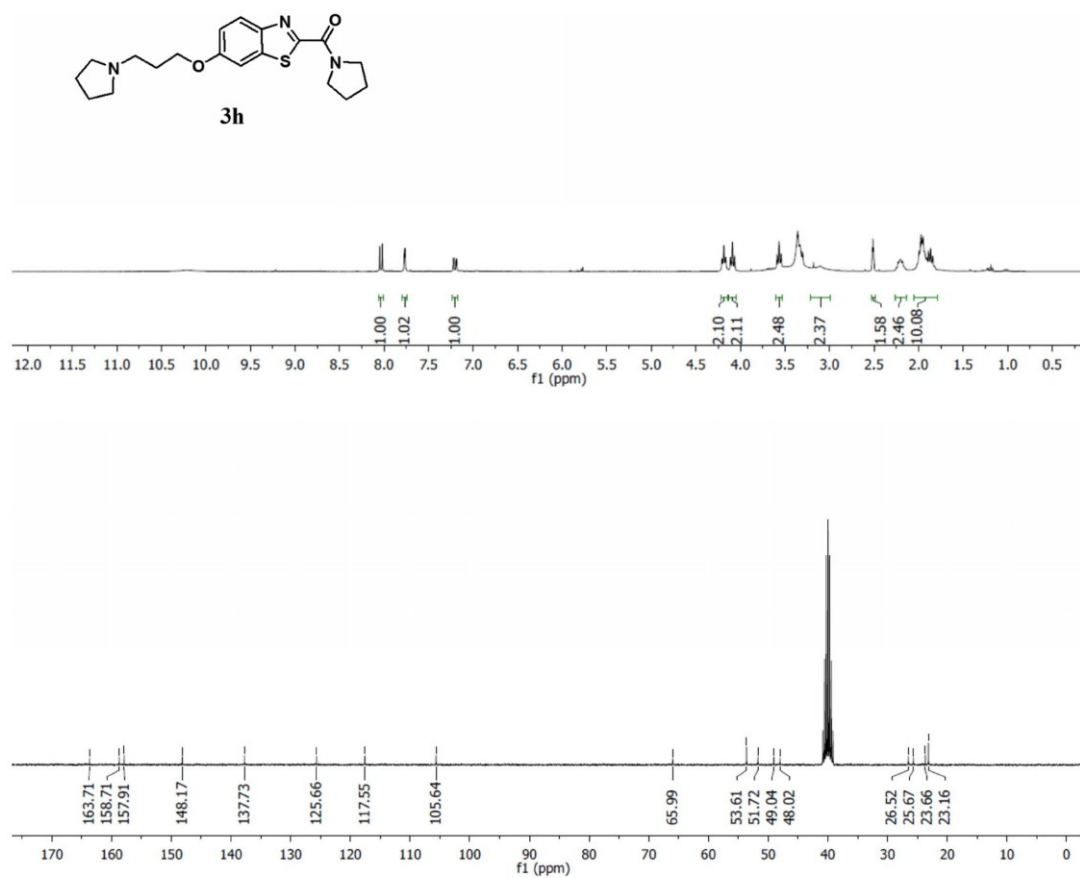
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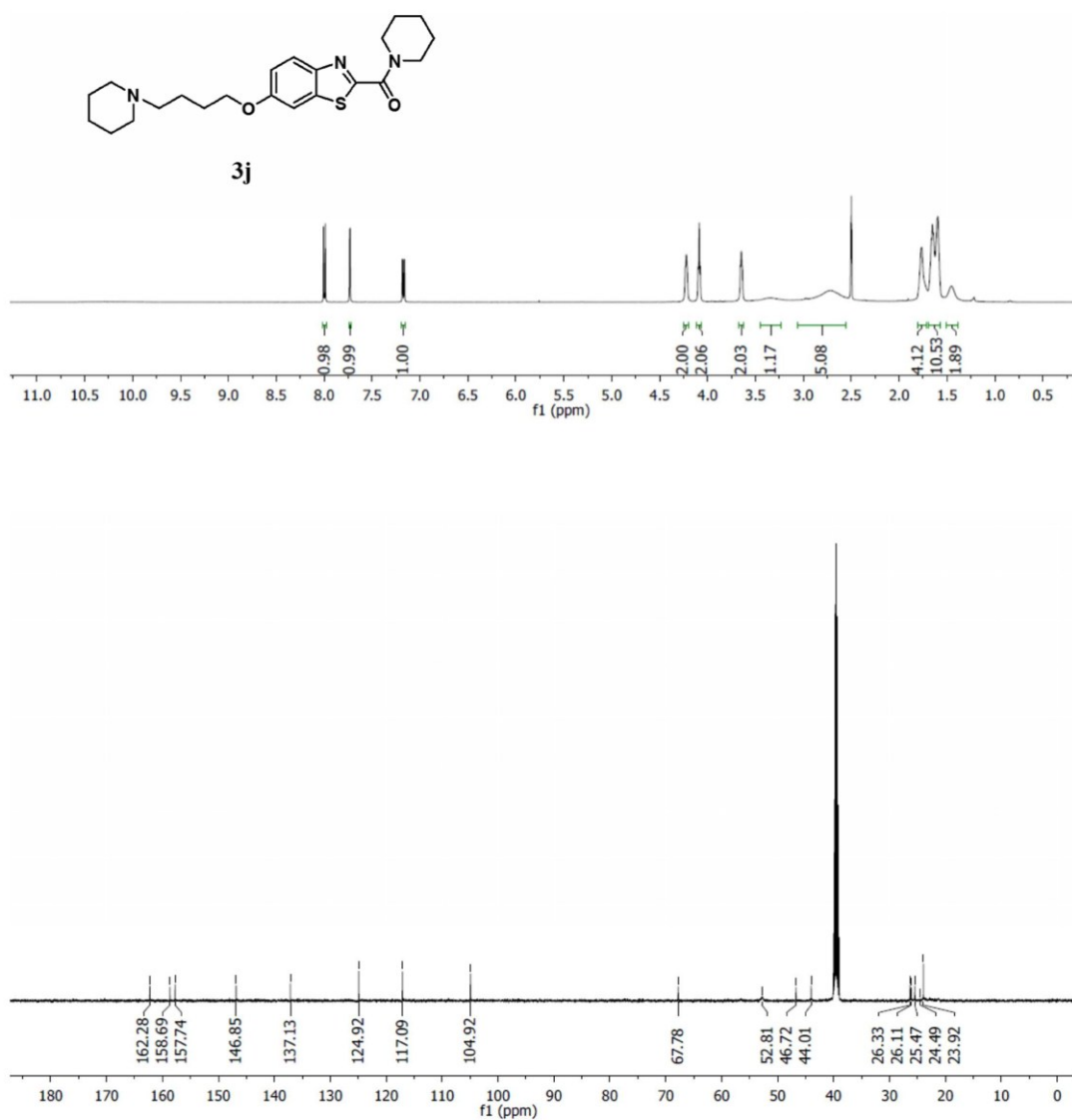
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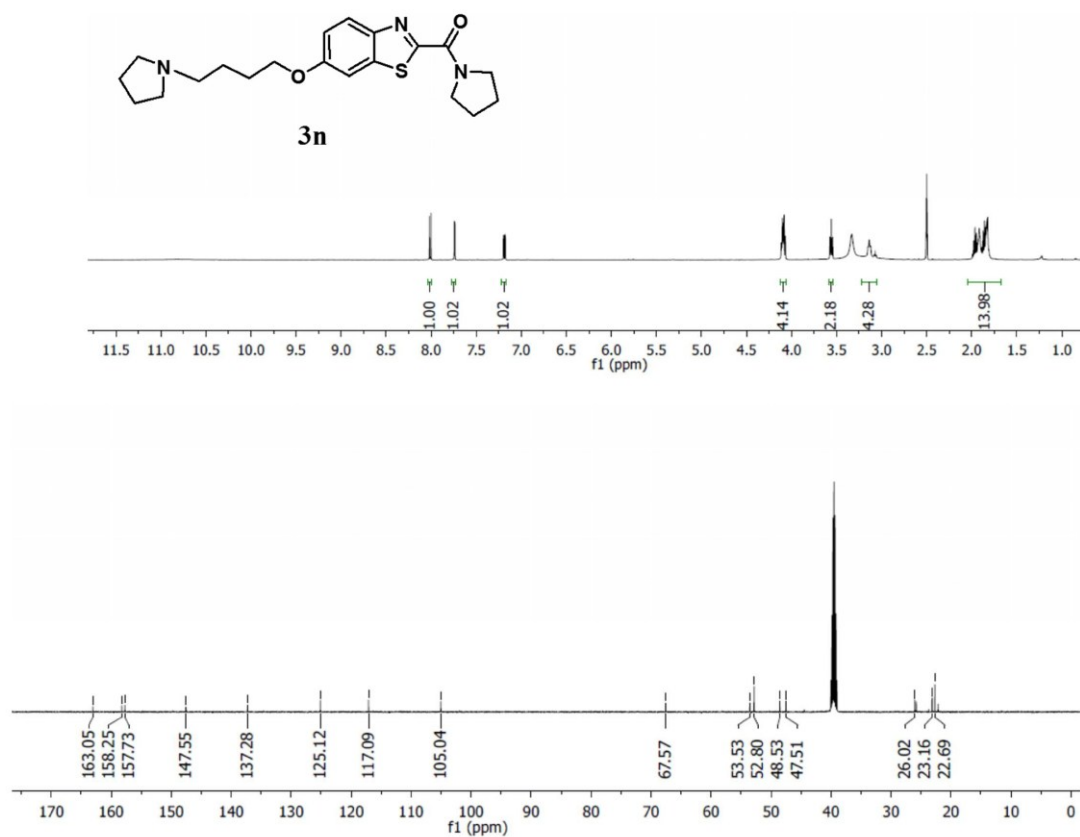


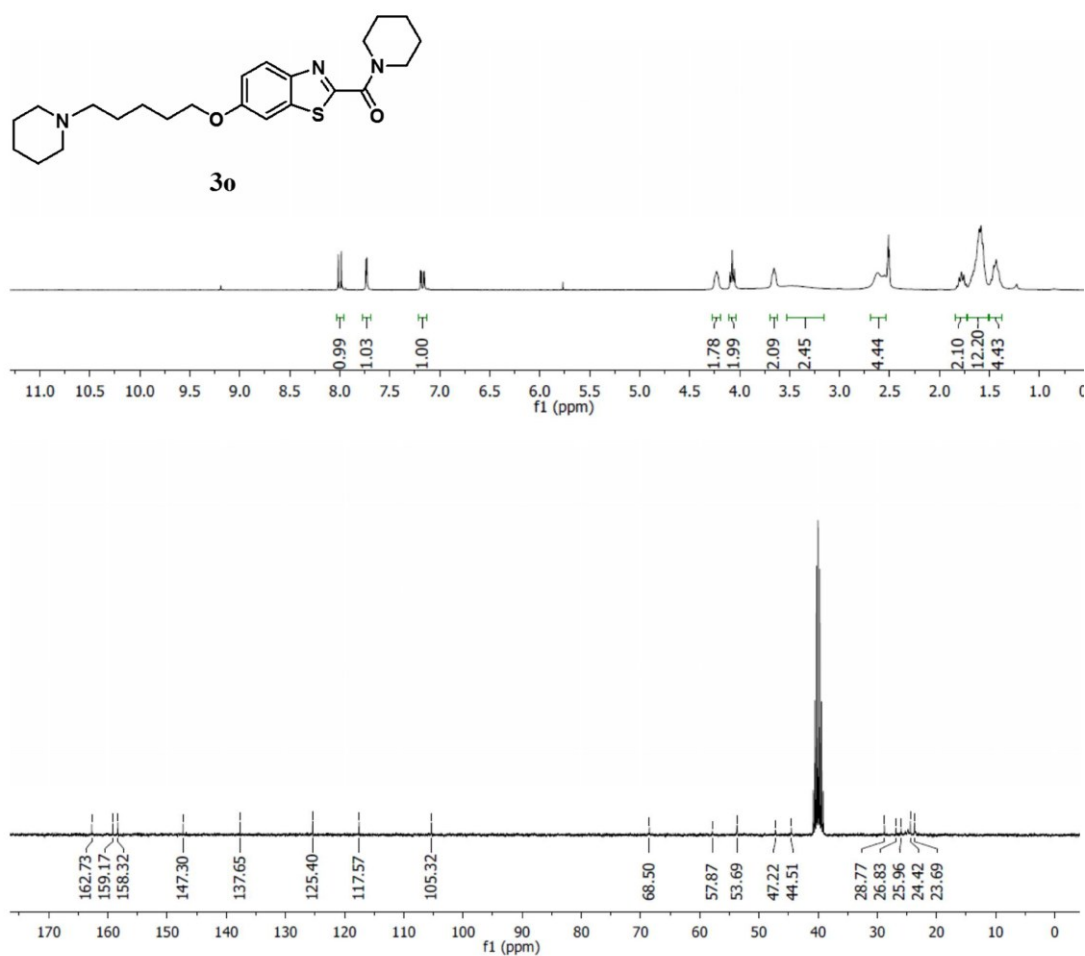
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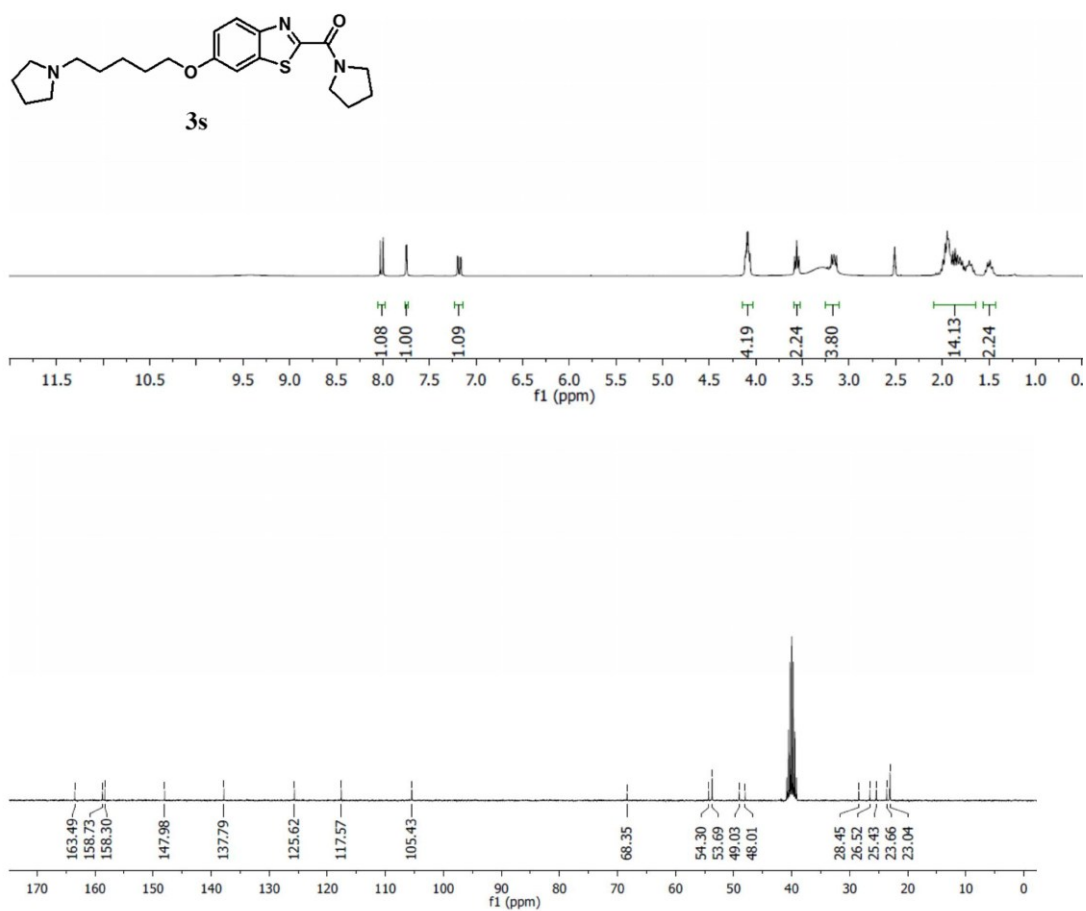


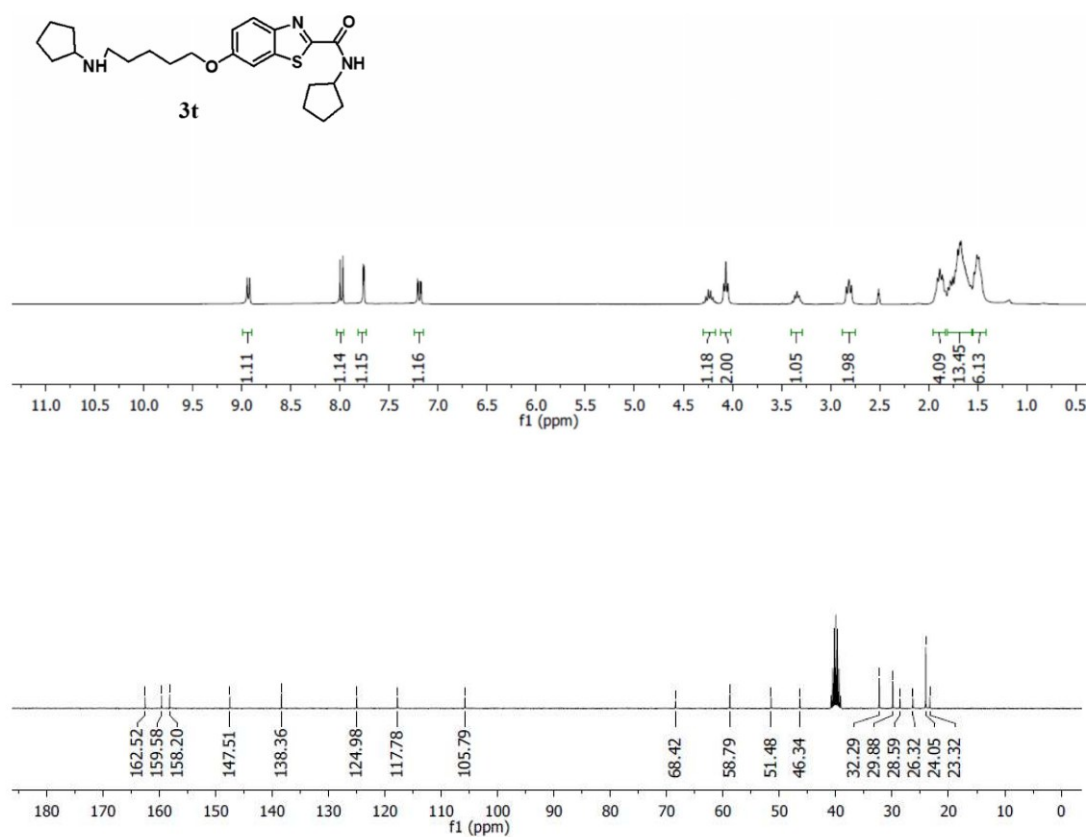
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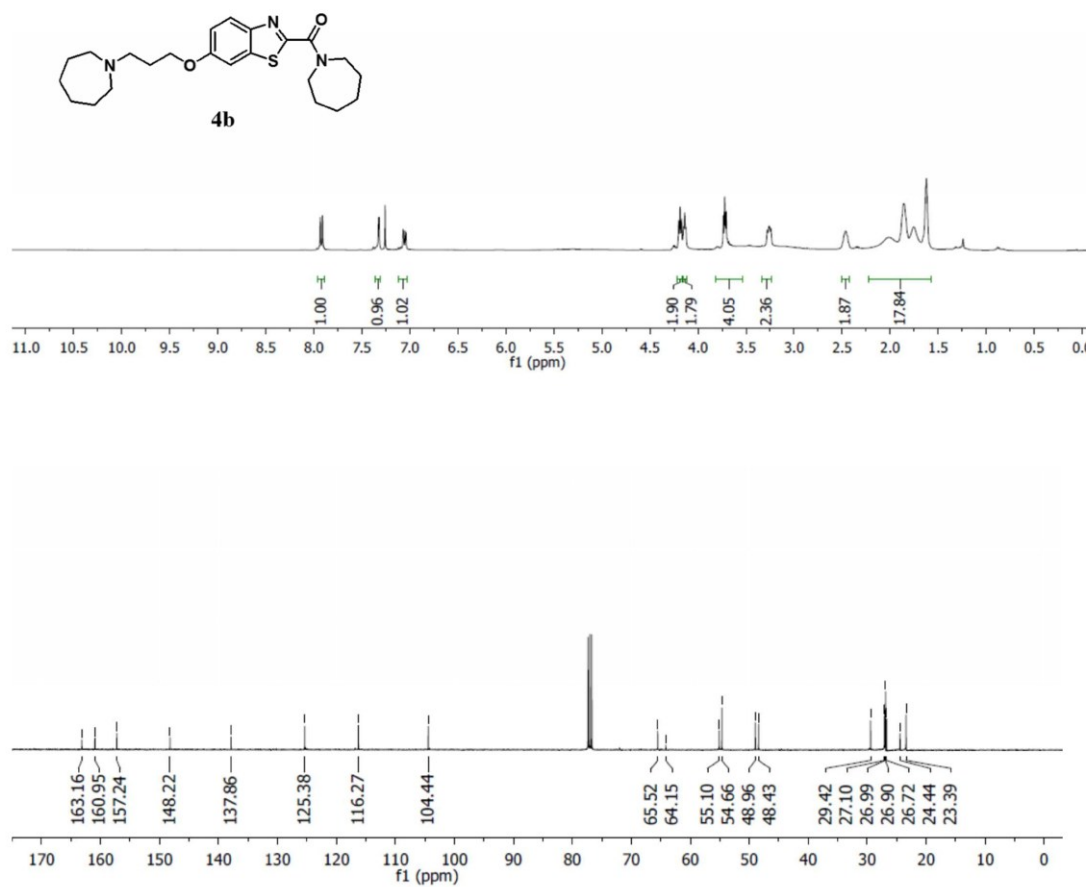


3 Publications





3 Publications



3.3 PUBLICATION 3

The Multi-Targeting Ligand ST-2223 with Histamine H₃ Receptor and Dopamine D₂/D₃ Receptor Antagonist Properties Mitigates Autism- Like Repetitive Behaviors and Brain Oxidative Stress in Mice

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Abstract: Autism spectrum disorder (ASD) is a complex heterogeneous neurodevelopmental disorder characterized by social and communicative impairments, as well as repetitive and restricted behaviors (RRBs). With the limited effectiveness of current pharmacotherapies in treating repetitive behaviors, the present study determined the effects of acute systemic treatment of the novel multi-targeting ligand ST-2223, with incorporated histamine H₃ receptor (H₃R) and dopamine D₂/D₃ receptor affinity properties, on ASD-related RRBs in a male Black and Tan Brachyury (BTBR) mouse model of ASD. ST-2223 (2.5, 5, and 10 mg/kg, i.p.) significantly mitigated the increase in marble burying and self-grooming, and improved reduced spontaneous alternation in BTBR mice (all $p < 0.05$). Similarly, reference drugs memantine (MEM, 5 mg/kg, i.p.) and aripiprazole (ARP, 1 mg/kg, i.p.), reversed abnormally high levels of several RRBs in BTBR ($p < 0.05$). Moreover, ST-2223 palliated the disturbed anxiety levels observed in an open field test (all $p < 0.05$), but did not restore the hyperactivity parameters, whereas MEM failed to restore mouse anxiety and hyperactivity. In addition, ST-2223 (5 mg/kg, i.p.) mitigated oxidative stress status by decreasing the elevated levels of malondialdehyde (MDA), and increasing the levels of decreased glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) in different brain parts of treated BTBR mice (all $p < 0.05$). These preliminary in vivo findings demonstrate the ameliorative effects of ST-2223 on RRBs in a mouse model of ASD, suggesting its pharmacological prospective to rescue core ASD-related behaviors. Further confirmatory investigations on its effects on various brain neurotransmitters, e.g., dopamine and histamine, in different brain regions are still warranted to corroborate and expand these initial data.

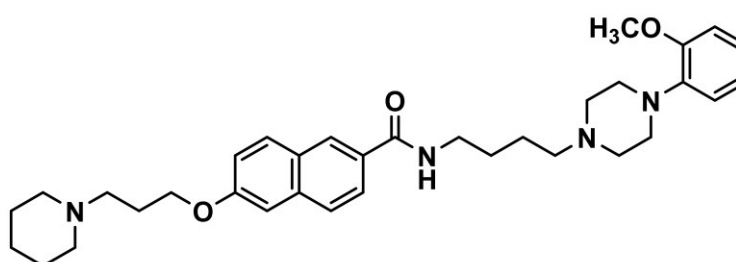
Keywords: autistic spectrum disorder; BTBR mice; histamine H₃ receptor antagonist; dopamine D₂/D₃R antagonist; repetitive and restricted behavior; anxiety; oxidative stress

1. Introduction

Autism spectrum disorder (ASD) is a highly heterogeneous neurodevelopmental disorder characterized by persistent social interaction deficits coupled with restricted, repetitive patterns of behaviors (RRBs), that are typically diagnosed during early developmental stages [1]. Behavioral observation is the primary diagnostic criteria for these core features

of ASD [2,3]. ASD individuals frequently display intense RRBs and thoughts that are reported to be a disturbing aspect for patients of ASD, while also being challenging for their families, influencing their daily functioning [4]. RRBs refer to a broad class of responses characterized by their repetition and rigidity, including motor stereotypies, compulsions, insistence on sameness, circumscribed interests and cognitive inflexibility [5–8]. Despite its clinical significance and the fact that a great deal of research has been pursued, it has proven difficult to understand the underlying mechanisms contributing to these behaviors, hence RRBs in ASD are still not remediable [9,10]. To date, the FDA has only approved risperidone and aripiprazole, for the management of irritability associated with ASD, but no medications have been approved to treat RRBs in ASD [11]. Hence, understanding the implicated neurochemical mechanisms in brain circuitry that contribute to RRBs, will be critical in developing potential pharmacotherapies [12]. Findings from several studies suggest that RRBs also appear in other CNS disorders, including obsessive-compulsive disorder, Tourette syndrome (TS), and schizophrenia (SCH), all of which are comorbid with ASD [13–18]. This overlap suggests that repetitive behavior likely arises from multiple etiologies or sources of CNS disruption. In addition to genetic and environmental factors, growing evidence suggests that a variety of neurotransmitters that have significant roles in initial brain development, such as acetylcholine (ACh), serotonin (5-HT), dopamine (DA), γ -aminobutyric acid (GABA), glutamate (Glu), and histamine (HA) are implicated in the onset and progression of ASD. Thus, substantiating these neurotransmitter systems as being a significant area in studying the etiology of RRBs in ASD [19]. Brain DA and HA have influences on behavior in brain disorders including Alzheimer's disease, SCH, anxiety, and narcolepsy, all of which show overlap with ASD [20–22]. The emergence of the widespread clinical use of antipsychotics that mainly target D_2 receptors (D_2 Rs), along with the pathophysiological role of dopaminergic system (DS) deficits in ASD, implicates the fundamental role of DA brain functioning [23,24]. Dysregulation of the DS has been reported to alter striatal dopaminergic neurotransmission and DA-dependent behaviors that are implicated in various neuropsychiatric disorders and recently in ASD [22]. ASD is strongly associated with a mutation in the dopamine transporter (DAT) gene *SLC6A3*, which codes for a protein that contributes to regulation of DA levels in the brain [25]. DAT knockout mice (hyperdopaminergic mutant mice) showed greater invariance in complex fixed action patterns, suggesting an association between abnormal DA levels and repetitive behaviors [26]. Furthermore, the siRNA-mediated inhibition of D_2 Rs in the dorsal striatum has been shown to replicate ASD-like phenotypes in D_2 R KO mice [27]. The brain histaminergic system (HS) has also been found to play a critical role in cognition, sleep and several neuropsychiatric disorders, including SCH and TS; comorbidities related to ASD [28]. TS, which is a condition characterized by stereotypies and tics, has been reported to be among the most prevalent comorbid neurodevelopmental disorders with ASD [29], sharing genetic risk factors [30,31]. In recent studies, it was reported that histaminergic signalling abnormalities may contribute to rare diseases such as TS. Moreover, TS has been associated with a premature termination codon (W317X) in the L-histidine decarboxylase (HDC) gene, hence, implicating the HS in the outcome of this syndrome and ASD [32–37]. Alteration of the brain's HA modulatory system has been identified as being a rare genetic cause of tic disorders; indeed, histidine decarboxylase knockout (*Hdc*-KO) mice have been generated, representing a promising model of this pathophysiology [38,39], implying that diminished histaminergic neurotransmission may be related to the exhibited repetitive and tic-like stereotypies [39]. Moreover, alteration in gene expression was found for the histamine-*N*-methyltransferase enzyme (HNMT, an enzyme responsible for the metabolism of central HA) and for histamine receptor (HR) subtypes (H_1 - H_3 R) in ASD [28]. Based on these preclinical experimental outcomes, the central H_3 Rs—as auto- and hetero-receptors that regulate the biosynthesis and release of HA and several neurotransmitters, including DA—are suggested to be attractive targets for developing novel H_3 R antagonists/inverse agonists, that may be potential therapeutics for the treatment of several neurological multi-neurotransmitter disorders, including cognitive impairments and RRBs. Additionally, the

implication of a variety of neurotransmitter systems, such as ACh, 5-HT, DA, GABA, Glu, and HA, in the onset and progression of ASD, has suggested that the recent advances in developing novel agents with multiple pharmacological effects as being a promising strategy for the treatment of multifactorial diseases, such as ASD [19]. Therefore, the present study aimed to investigate the effects of the newly developed multiple-targeting ligand ST-2223 [N-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)-6-(3-(piperidin-1-yl)propoxy)-2-naphthamide] with high in vitro H_3R affinity (hH_3R K_i = 4.8 nM) and potent hD_2/hD_3R affinity (hD_2R K_i = 19.8 nM, hD_3R K_i = 2.0 nM), with a preference for hD_3R (ratio K_i values hD_2R/hD_3R :10) (Figure 1) on RRBs in the Black and Tan BRachyury (BTBR) mouse model of ASD. ST-2223 combines the classical non-imidazole H_3R pharmacophore in an overlapping integration with the D_3R/D_2R ligand BP 897 [40–43]. Although a small reduction in affinities to DR subtypes is observed, this novel compound maintains affinities at the desired targets in a low nanomolar concentration range.



ST-2223

$$K_i (hH_1R)^b = 85.2 \text{ nM}$$

$$K_i (hH_3R)^a = 4.8 \text{ nM}$$

$$K_i (hD_2R)^c = 19.8 \text{ nM}$$

$$K_i (hD_3R)^c = 2.0 \text{ nM}$$

$$hD_2R/hD_3R \text{ ratio} = 10$$

$$K_i (hD_1R)^d = 564 \text{ nM}$$

$$K_i (hD_5R)^d = 5064 \text{ nM}$$

$$EeAChE^e \leq 60\% \text{ inhibition [1 } \mu\text{M]}$$

$$EqBuChE^f \leq 35\% \text{ inhibition [1 } \mu\text{M]}$$

Figure 1. Chemical structure of the multi-targeting ligand ST-2223 with affinities at hH_3R , hD_2R and hD_3R . ^a [³H] N^α -methylhistamine binding assay, performed with cell membrane preparation of Human embryonic kidney (HEK) cells stably expressing the human H_3R (n = 4) [44,45]. ^b [³H]pyrilamine binding assay, performed with cell membrane preparation of Chinese hamster ovary (CHO) cells stably expressing the hH_1R (n = 2). ^{c,d} Displacement assay was carried out as described previously, using membrane suspension of cell lines stably expressing the human dopamine hD_1Rs and hD_5Rs (HEK) against [³H]SCH23390 and hD_2SRs , hD_3Rs (CHO) using [³H]spiperone (n = 3) [44,45]. ^e AChE: Acetylcholine esterase; Ee; electric eel (preliminary data); ^f BuChE: Butyrylcholinesterase; Eq: equine (preliminary data) (n = 2) [46].

BTBR is an idiopathic model of ASD, that naturally displays deficits in social interactions, in addition to RRBs including cognitive inflexibility, elevated grooming behavior

and marble burying, when compared to B6 mice [47–49]. To understand whether ST-2223 treatment has a more general effect on motor behavior, the effect of ST-2223 on locomotor activity was also measured in both mice strains in order to simultaneously rule out possible intrinsic impairment of spontaneous locomotor activity that might mask anxiety parameters [50]. Moreover, since oxidative stress has been shown to be closely related with ASD [51,52], further assessment of the neuroprotective effects of ST-2223 on ASD-like RRBs in BTBR mice compared to B6 mice was carried out. This was due to the fact that BTBR mice have been reported to have elevated levels of oxidative stress with a deficient enzymatic anti-oxidant response that is suggested to be associated with the exaggerated repetitive behavior. To comprehend our observations, the ability of CNS-penetrant H₃R agonist (*R*)- α -methylhistamine (RAM) to counteract the ST-2223-provided H₃R antagonist effects was assessed in order to elucidate the possible involvement of brain HA and DA in the enhancements observed by ST-2223.

2. Results

2.1. Marble Burying Behavior

The effects of systemic injection of ST-2223 (2.5, 5, and 10 mg/kg, i.p.), memantine (MEM) (5 mg/kg, i.p.) and aripiprazole (ARP) (1 mg/kg, i.p.) on marble burying behavior in BTBR and B6 mice are shown in Figure 2. The results of a two-way ANOVA showed that there was a significant main effect for strain ($F_{(1,48)} = 3.08$, $p < 0.01$), treatment ($F_{(5,48)} = 4.52$, $p < 0.01$) and also for the strain $\times$ treatment interaction ($F_{(5,48)} = 3.16$, $p < 0.05$) (Figure 2). Post hoc tests revealed that vehicle-treated BTBR (63.00 ± 3.34 %) buried significantly more marbles compared to B6 mice (35.00 ± 2.45 %) with values of ($F_{(1,8)} = 7.36$, $p < 0.05$). ST-2223 (2.5, 5, and 10 mg/kg, i.p.) significantly decreased the percentage of marbles buried in BTBR mice with values of ($F_{(1,8)} = 7.38$, $p < 0.05$) ($F_{(1,8)} = 8.52$, $p < 0.05$), and ($F_{(1,8)} = 7.16$, $p < 0.05$), respectively. Moreover, no significant differences in the percentage of marbles buried was observed between the three doses of ST-2223, with values of ($F_{(1,8)} = 0.84$, $p = 0.39$) for 2.5 mg/kg versus 5 mg/kg, ($F_{(1,8)} = 1.85$, $p = 0.21$) for 5 mg/kg versus 10 mg/kg, and ($F_{(1,8)} = 4.07$, $p = 0.09$) for 2.5 mg/kg versus 10 mg/kg (Figure 2). Furthermore, the reference drugs MEM (5 mg/kg, i.p.) and ARP (1 mg/kg, i.p.), significantly reduced the percentage of marbles buried with values of ($F_{(1,8)} = 39.28$, $p < 0.01$) and ($F_{(1,8)} = 73.63$, $p < 0.01$), respectively. In B6 mice, ST-2223 did not alter the percentage of marbles buried (all p 's > 0.05). However, there was a trend for MEM to reduce marble burying, although systemic treatment with 5 mg/kg was not significant when compared to that of the vehicle treatment ($p > 0.05$). As depicted in Figure 2 and in the post hoc analyses observations, the ST-2223 (5 mg)-stimulated decrease in the percentage of buried marbles was reversed by co-administration with RAM (10 mg/kg, i.p.), with values of ($F_{(1,8)} = 5.88$; $p < 0.05$), as compared to the ST-2223 (5 mg)-treated BTBR mice (Figure 2). Interestingly, subchronic systemic pretreatment of BTBR mice with RAM (10 mg/kg, i.p.) did not alter the behaviors of tested mice in the marble burying task (MBT), with values of ($F_{(1,8)} = 0.12$; $p = 0.77$), as compared to the saline-treated BTBR mice (Figure 2).

2.2. Spontaneous Self-Grooming Behavior

The effects on spontaneous self-grooming in BTBR and B6 mice are shown in Figure 3. The results of the two-way ANOVA showed that there was a significant main effect for strain, treatment and strain $\times$ treatment interaction, with values of ($F_{(1,48)} = 97.54$, $p < 0.01$), ($F_{(5,48)} = 30.85$, $p < 0.01$), and ($F_{(5,48)} = 11.38$, $p < 0.01$), respectively. Post hoc tests revealed that vehicle-treated BTBR (161.20 ± 7.69 s) spent significantly more time grooming when compared to B6 mice (62.20 ± 5.32 s), with values of ($F_{(1,8)} = 81.69$, $p < 0.01$). ST-2223 (5 and 10 mg/kg, i.p.) significantly, and dose-dependently, decreased the time spent self-grooming in BTBR mice, with values of ($F_{(1,8)} = 29.43$, $p < 0.01$) and ($F_{(1,8)} = 29.56$, $p < 0.01$), respectively. However, ST-2223 (2.5 mg/kg, i.p.) failed to significantly mitigate the self-grooming behavior of treated BTBR mice ($p = 0.23$). Interestingly, the reducing effect observed with 10 mg/kg of ST-2223 on self-grooming behavior was significantly

higher than that witnessed with the lower dose (5 mg/kg) of the same compound, with values of ($F_{(1,8)} = 7.97$, $p < 0.05$). The reference drugs MEM (5 mg/kg, i.p.) and ARP (1 mg/kg, i.p.) significantly reduced the self-grooming time of BTBR mice with values of ($F_{(1,8)} = 186.96$, $p < 0.01$) and ($F_{(1,8)} = 20.89$, $p < 0.01$), respectively (Figure 3). In B6 mice, ST-2223 (2.5, 5, and 10 mg/kg, i.p.) and ARP (1 mg/kg, i.p.) did not alter the time spent self-grooming (all p 's > 0.05). However, MEM (5 mg/kg, i.p.) significantly reduced the self-grooming time of treated B6 mice with values of ($F_{(1,8)} = 19.50$, $p < 0.01$) (Figure 3). As depicted in Figure 3, and following the post hoc analyses of the observed results, the ST-2223 (5 mg)-induced decrease in the duration of time spent displaying self-grooming behaviors, was nullified by co-administration with RAM (10 mg/kg, i.p.), with values of ($F_{(1,8)} = 27.52$; $p < 0.001$), as compared to the ST-2223 (5 mg)-treated BTBR mice (Figure 3). Interestingly, systemic pretreatment of BTBR mice with RAM (10 mg/kg, i.p.) did not alter the behaviors of tested mice in the MBT, with values of ($F_{(1,8)} = 0.11$; $p = 0.72$) as compared to the vehicle-treated BTBR mice (Figure 3).

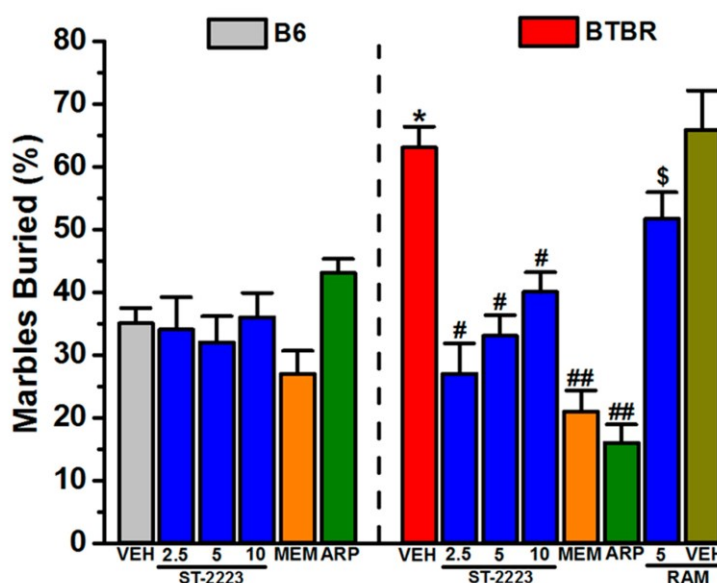


Figure 2. ST-2223 treatment attenuates marble burying in Black and Tan BRachyury (BTBR) mice. Percentage of marbles buried was assessed in B6 and BTBR mice. Each mouse received an i.p. injection of vehicle, ST-2223 (2.5, 5.0, or 10 mg/kg, i.p.), memantine (MEM, 5 mg/kg, i.p.), or aripiprazole (ARP, 1 mg/kg, i.p.) 30–45 min prior to marble exposure. BTBR mice buried significantly more marbles compared to B6 mice. Vehicle or ST-2223 treatment did not significantly affect marble burying in B6 mice. ST-2223 (2.5, 5, and 10 mg/kg, i.p.) and MEM significantly decreased marble burying in BTBR mice. The effects of systemic co-injection of (*R*)- α -methylhistamine (RAM, 10 mg/kg, i.p.) on the ST-2223 (5 mg)-induced decrease in stereotyped repetitive and compulsive behaviors of BTBR mice were also assessed. Data are expressed as the mean $\pm$ SEM ($n = 5$). * $p < 0.05$ vs. vehicle-treated B6 mice. # $p < 0.05$ vs. vehicle-treated BTBR mice. ## $p < 0.01$ vs. vehicle-treated BTBR mice. \$ $p < 0.05$ vs. ST-2223 (5 mg)-treated BTBR mice.

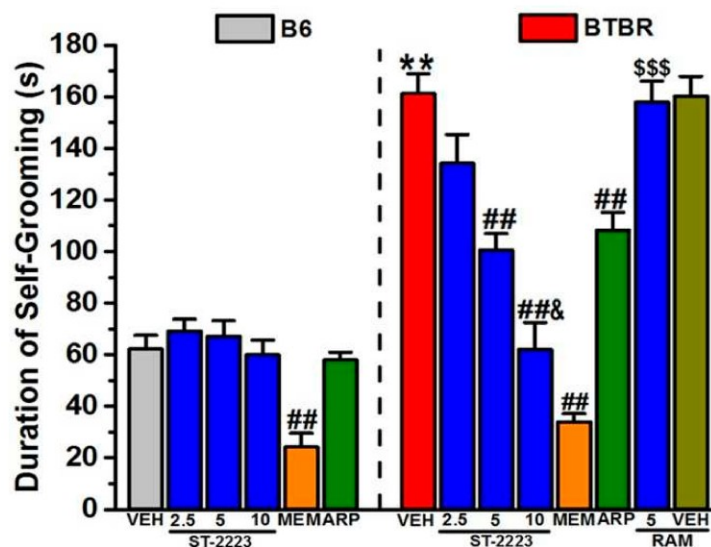


Figure 3. ST-2223 treatment mitigates spontaneous self-grooming behavior in BTBR mice. Self-grooming behavior was measured in B6 and BTBR mice. Each mouse received an i.p. injection of vehicle, ST-2223 (2.5, 5.0, or 10 mg/kg, i.p.), MEM (5 mg/kg, i.p.), or ARP (1 mg/kg, i.p.) 30–45 min prior to self-grooming behavior assessment. BTBR mice spent significantly more time grooming compared to that of B6 mice. Vehicle, ST-2223, MEM, or ARP treatment did not affect spontaneous self-grooming behavior in B6 mice. ST-2223 (5 and 10 mg/kg, i.p.), and MEM (5 mg/kg, i.p.) significantly decreased self-grooming behavior in BTBR mice. ARP (1 mg/kg, i.p.) failed to significantly alter the increased self-grooming behavior of BTBR mice. Effects systemic co-injection of (R)- α -methylhistamine (RAM, 10 mg/kg, i.p.) on the ST-2223 (5 mg)-induced decrease in self-grooming behavior of BTBR mice were also assessed. Data are expressed as the mean $\pm$ SEM time spent grooming all body regions ($n = 5$). ** $p < 0.01$ vs. vehicle-treated B6 mice. ## $p < 0.01$ vs. vehicle-treated BTBR mice. & $p < 0.05$ vs. ST-2223 (5 mg)-treated BTBR mice. \$\$\$ $p < 0.001$ vs. ST-2223(5 mg)-treated BTBR mice.

2.3. Spontaneous Alternation Behavior

Figure 4 shows the results observed for the spontaneous alternation of BTBR and B6 mice. The results of a two-way ANOVA showed that there was a significant main effect for strain, treatment and strain $\times$ treatment interaction, with values of ($F_{(1,48)} = 15.06$, $p < 0.01$), ($F_{(5,48)} = 16.04$, $p < 0.01$), and ($F_{(5,48)} = 5.22$, $p < 0.01$), respectively. Post hoc tests revealed that vehicle-treated BTBR displayed a significantly reduced spontaneous percentage of alternation when compared with vehicle-treated B6 mice, with values of ($F_{(1,8)} = 14.56$, $p < 0.01$). ST-2223 (2.5, 5 and 10 mg/kg, i.p.) and MEM (5 mg/kg, i.p.) significantly increased the percentage of spontaneous alternation in BTBR mice, with values of ($F_{(1,8)} = 11.23$, $p < 0.05$), ($F_{(1,8)} = 7.20$, $p < 0.05$), ($F_{(1,8)} = 11.29$, $p < 0.05$), and ($F_{(1,8)} = 8.47$, $p < 0.05$), respectively (Figure 4). However, ARP (1 mg/kg, i.p.) failed to significantly mitigate the decreased percentage of alternation in BTBR mice ($p = 0.05$) (Figure 4). Moreover, no significant differences in the percentage of spontaneous alternation were observed between the three doses of ST-2223 used, with values of ($F_{(1,8)} = 0.13$, $p = 0.72$) for 2.5 mg/kg versus 5 mg/kg, ($F_{(1,8)} = 0.07$, $p = 0.80$) for 5 mg/kg versus 10 mg/kg, and ($F_{(1,8)} = 0.01$, $p = 0.92$) for 2.5 mg/kg versus 10 mg/kg (Figure 4). In B6 mice, ST-2223 (2.5, 5, and 10 mg/kg, i.p.), MEM (5 mg/kg, i.p.) and ARP (1 mg/kg, i.p.) did not alter the percentage of spontaneous alternation (all p 's > 0.05) (Figure 4). As depicted in Figure 4, and following the post hoc analyses of detected results, the ST-2223 (5 mg)-induced enhancement in the alternation of

BTBR mice was entirely reversed by co-administration with RAM (10 mg/kg, i.p.), with values of ($F_{(1,8)} = 8.89$; $p < 0.05$) as compared to the ST-2223 (5 mg)-treated BTBR mice (Figure 4). Interestingly, systemic pretreatment of BTBR mice with RAM (10 mg/kg, i.p.) did not alter the behavior of tested mice in the MBT, with values of ($F_{(1,8)} = 0.09$; $p = 0.78$), as compared to the vehicle-treated BTBR mice (Figure 4).

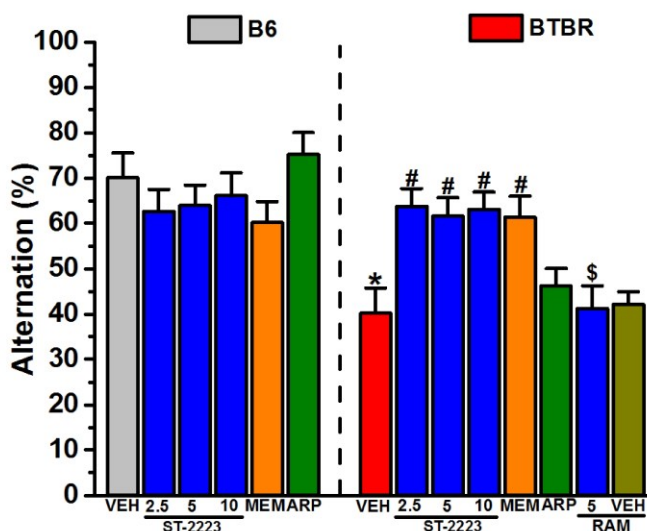


Figure 4. ST-2223 treatment enhances spontaneous alternation behavior in BTBR mice. Spontaneous alternation behavior was measured in B6 and BTBR mice. Each mouse received an i.p. injection of vehicle, ST-2223 (2.5, 5.0, or 10 mg/kg, i.p.), MEM (5 mg/kg, i.p.), or ARP (1 mg/kg, i.p.) 30–45 min prior to assessment of alternation behavior. BTBR mice demonstrated a significantly reduced percentage of spontaneous alternation behavior compared to that of B6 mice. Vehicle, ST-2223, MEM, or ARP treatment did not affect spontaneous alternation behavior in B6 mice. ST-2223 (2.5, 5 and 10 mg/kg, i.p.) and MEM (5 mg/kg, i.p.) significantly mitigated the decreased alternation behavior in BTBR mice. ARP (1 mg/kg, i.p.) failed to alter the decreased alternation behavior of BTBR mice. The effects of systemic co-injection of RAM (10 mg/kg, i.p.) on the ST-2223 (5 mg)-induced enhancement of the alternation behavior of BTBR mice were also assessed. Data are expressed as the mean $\pm$ SEM percentage of spontaneous alternation ($n = 5$). * $p < 0.05$ vs. vehicle-treated B6 mice. # $p < 0.05$ vs. vehicle-treated BTBR mice. \$ $p < 0.05$ vs. ST-2223 (5 mg)-treated BTBR mice.

2.4. Locomotor Activity and Anxiety Levels

The findings observed for locomotor activity in B6 and BTBR mice are shown in Figure 5. For the total distance travelled, there was a significant effect of strain ($F_{(1,48)} = 1059.37$, $p < 0.01$), but there was no significant effect for treatment or strain $\times$ treatment interaction (p 's > 0.05) (Figure 5A). Post hoc tests revealed that vehicle-treated BTBR showed a significant increase in the distance travelled when compared with vehicle-treated B6 mice, with values of ($F_{(1,8)} = 95.63$, $p < 0.01$). B6 and BTBR mice pretreated with ST-2223 (2.5, 5 and 10 mg/kg, i.p.), MEM (5 mg/kg, i.p.), or ARP (1 mg/kg, i.p.) did not show any alteration in the total distance travelled (all p 's > 0.05) (Figure 5A). The observed effects of systemic injection of the vehicle, ST-2223 (2.5, 5, or 10 mg/kg, i.p.), MEM or ARP on the time B6 and BTBR mice spent in the center (Figure 5B) and in the periphery (Figure 5C) in the open field test (OFT) are shown. For the time spent in the center, there was no significant effect of strain ($p > 0.05$), but there was significant effect for treatment and the strain $\times$ treatment interaction, with values of ($F_{(5,48)} = 8.01$, $p < 0.01$), and ($F_{(5,48)} = 3.56$, $p < 0.01$), respectively (Figure 5B). Post hoc tests revealed that vehicle-treated BTBR spent

significantly less time in the center of the arena when compared with vehicle-treated B6 mice, with values of ($F_{(1,8)} = 9.11, p < 0.05$). ST-2223 (2.5, 5 and 10 mg/kg, i.p.) and ARP (1 mg/kg, i.p.) significantly increased the amount of time BTBR mice spent in center, with values of ($F_{(1,8)} = 33.36, p < 0.01$), ($F_{(1,8)} = 46.76, p < 0.01$), ($F_{(1,8)} = 21.29, p < 0.01$), and ($F_{(1,8)} = 12.11, p < 0.01$), respectively (Figure 5B). However, MEM (5 mg/kg, i.p.) failed to significantly mitigate the reduced time spent in the center in BTBR mice ($p = 0.78$) (Figure 5B). In B6 mice, ST-2223 (2.5, 5, and 10 mg/kg, i.p.), MEM (5 mg/kg, i.p.) and ARP (1 mg/kg, i.p.) did not alter the amount of time spent in the central arena (all p 's > 0.05) (Figure 5B). The observed effects of systemic administration of the vehicle, ST-2223 (2.5, 5, or 10 mg/kg, i.p.), MEM or ARP on the amount of time B6 and BTBR mice spent in the periphery—tested in the OFT—are shown in Figure 5C. For the time spent in the periphery, there was no significant effect for strain, nor for treatment or strain $\times$ treatment interaction (all p 's > 0.05) (Figure 5C). Notably, post hoc analyses of the detected results revealed that the ST-2223 (5 mg)-induced increase in time spent in the center of the arena was not reversed by co-administration with RAM (10 mg/kg, i.p.), with values of ($F_{(1,8)} = 0.43; p = 0.53$), as compared to the ST-2223 (5 mg)-treated BTBR mice (Figure 5B). Interestingly, systemic pretreatment of BTBR mice with RAM (10 mg/kg, i.p.) alone, did not induce any alternation in the amount of time tested BTBR mice spent in the center, with values of ($F_{(1,8)} = 0.27; p = 0.62$), as compared to the saline-treated BTBR mice (Figure 5B).

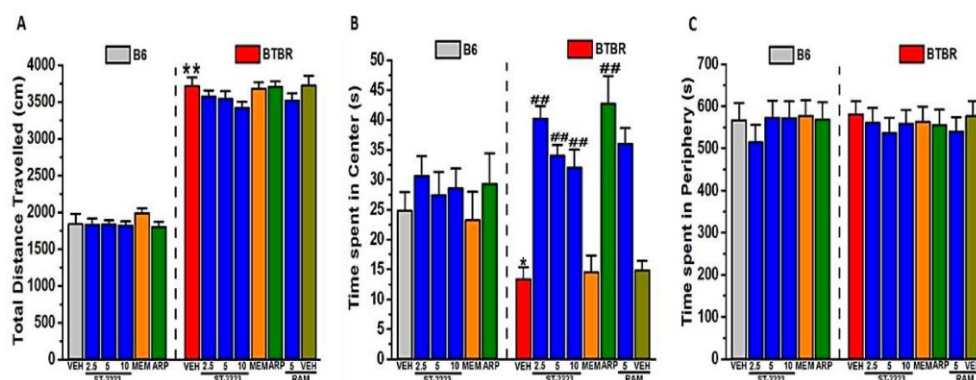


Figure 5. ST-2223 treatment did not affect locomotor activity in BTBR mice. Locomotor activity was assessed in B6 and BTBR mice. Each mouse received an i.p. injection of vehicle, ST-2223 (2.5, 5.0, or 10 mg/kg, i.p.), MEM (5 mg/kg, i.p.), or ARP (1 mg/kg, i.p.) 30–45 min prior to locomotion was measured. BTBR mice travelled significantly greater distances compared to those of B6 mice (A). Vehicle, ST-2223, MEM, or ARP treatment did not affect locomotor activity in B6 or BTBR mice (A,C). BTBR mice spent significantly less time in the center of the arena compared to that of B6 mice (B). ST-2223 (2.5, 5 and 10 mg/kg, i.p.) and ARP (1 mg/kg, i.p.) significantly mitigated the decreased amount of time spent by BTBR mice in the center. MEM (5 mg/kg, i.p.) failed to alter the decreased amount of time BTBR mice spent in the center. Data are expressed as the mean $\pm$ SEM ($n = 5$). * $p < 0.05$ vs. vehicle-treated B6 mice. ** $p < 0.01$ vs. vehicle-treated B6 mice. ## $p < 0.01$ vs. vehicle-treated BTBR mice.

2.5. Levels of Oxidative Stress Markers in Different Brain Parts of Treated BTBR Mice

Statistical analysis showed that MDA was significantly increased in the four assessed brain parts (all p values < 0.05), and GSH, SOD and CAT were significantly reduced in these brain parts (all p values < 0.05) of BTBR mice compared to B6 mice (Table 1). However, the cerebellum of BTBR mice that were pretreated with ST-2223 (5 mg/kg, i.p.) displayed a significant reduction in MDA in all regions assessed (all p values < 0.05), and this ST-2223-induced modulation of MDA was reversed when RAM (10 mg/kg, i.p.) was co-administered (all p values < 0.05). Moreover, the observed results showed that

systemic pretreatment with ST-2223 significantly increased the decreased levels of GSH in the cerebellum, prefrontal cortex and striatum (all p values < 0.05), without appreciable effects on MDA levels in the hippocampus. Interestingly, the enhancing effects observed for MDA were counteracted following co-administration with RAM but only in the prefrontal cortex and striatum (all p values < 0.05) (Table 1). Moreover, systemic administration of ST-2223 (5 mg/kg, i.p.) significantly increased the decreased level of SOD in the cerebellum (p < 0.05), without any appreciable effects on SOD in the hippocampus, prefrontal cortex or striatum. However, ST-2223 significantly increased the decreased levels of CAT in hippocampus, prefrontal cortex and striatum (all p values < 0.05), without any appreciable effects on CAT in the cerebellum. Similarly, the cerebellum of BTBR mice that were pretreated with ARP (1 mg/kg, i.p.) or MEM (5 mg/kg, i.p.) showed a significant reduction in MDA in all four brain parts (all p values < 0.05), a significant increase in GSH in the cerebellum, hippocampus and striatum, a significant increase in the cerebellum, prefrontal cortex and striatum, and a significant increase in CAT (only for ARP) in the cerebellum and striatum, without any appreciable effects for MEM on the assessed levels of CAT in all four brain regions (Table 1).

Table 1. ST-2223 mitigated levels of oxidative stress markers in different brain parts of treated BTBR mice.

		BTBR				
	B6 (VEH)	(VEH)	ST-2223 (5 mg/kg)	ST-2223 (5 mg/kg) + RAM (10 mg/kg)	ARP (1 mg/kg)	MEM (5 mg/kg)
MDA						
Cerebellum	29.9 ± 3.3	54.0 ± 6.4 ##	35.2 ± 1.8 **	45.0 ± 0.6 \$\$	36.8 ± 1.8 ***	26.7 ± 3.6 ***
Hippocampus	35.1 ± 3.0	53.8 ± 2.9 ##	38.1 ± 3.1 **	40.7 ± 3.3	32.9 ± 4.1 ***	32.8 ± 3.4 ***
Prefrontal Cortex	41.1 ± 7.0	58.3 ± 1.8 #	39.1 ± 3.2 ***	53.6 ± 1.2 \$\$	43.0 ± 2.5 **	40.0 ± 5.7 **
Striatum	30.8 ± 4.1	52.7 ± 3.4 ##	34.5 ± 5.5 *	51.4 ± 4.8 \$	41.2 ± 1.4 *	32.6 ± 4.1 **
GSH						
Cerebellum	1560 ± 103.5	827 ± 93.3 ##	1108 ± 41.1 *	1266 ± 70.2	1030 ± 36.2 *	877 ± 55.7
Hippocampus	2119 ± 425.1	1096 ± 16.7 #	916 ± 4.2	643.6 ± 49.9	611.5 ± 62.6 ***	642.6 ± 66.0 ***
Prefrontal Cortex	1776 ± 266.6	835 ± 97.4 #	1212 ± 302.6 *	1070.4 ± 64.8 \$	935 ± 98.0	719.3 ± 50.0
Striatum	1128 ± 199.5	583 ± 68.8 #	1007 ± 93.1 *	735.5 ± 59.9 \$	1139 ± 74.6 *	915.5 ± 97.3 *
SOD						
Cerebellum	60.6 ± 3.1	49.7 ± 1.3 #	64.3 ± 1.0 ***	49.2 ± 2.0 \$\$\$	56.4 ± 1.1 **	64.4 ± 3.1 **
Hippocampus	66.4 ± 5.4	49.5 ± 0.5 #	48.1 ± 4.2	39.5 ± 0.5 \$\$	51.8 ± 2.0	41.4 ± 3.4
Prefrontal Cortex	53.8 ± 2.2	45.1 ± 2.8 #	43.2 ± 3.4	42.9 ± 4.2	54.0 ± 5.2 **	44.9 ± 2.2
Striatum	68.5 ± 4.7	51.8 ± 1.2 #	51.2 ± 7.7	45.0 ± 1.0	65.7 ± 7.7 **	66.47 ± 4.4 **
CAT						
Cerebellum	156.7 ± 7.29	130.7 ± 3.3 #	120.6 ± 8.8	153.3 ± 5.4	159.2 ± 5.1 **	132.3 ± 4.5
Hippocampus	131.7 ± 3.4	111.4 ± 4.8 #	173.0 ± 1.9 *	100.6 ± 3.6 \$\$	118.3 ± 6.5	114.3 ± 11.2
Prefrontal Cortex	130.3 ± 9.4	80.1 ± 14.9 #	116.8 ± 5.3 *	70.9 ± 3.9 \$	96.2 ± 1.3	94.1 ± 4.8
Striatum	117.7 ± 2.5	104.6 ± 2.28 #	119.1 ± 3.7 *	76.0 ± 9.3 \$\$	120.5 ± 10.1 *	108.1 ± 3.4

Malondialdehyde (MDA), glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) were assessed in four different brain regions of treated BTBR mice, namely, the cerebellum, hippocampus, prefrontal cortex and striatum. BTBR mice showed a significant increase in MDA and significant decrease in GSH, SOD, and CAT compared to B6 mice. The effects of systemic administration of ST-2223 (5 mg/kg) were observed in B6 and BTBR mice. ST-2223 (5 mg/kg) significantly reduced the increased levels of MDA and significantly increased the reduced levels of GSH, SOD and CAT. Data are expressed as the mean ± SEM ($n = 3-6$). [#] p < 0.05 vs. B6 mice. ^{##} p < 0.01 vs. B6 mice. ^{*} p < 0.05 vs. BTBR mice. ^{**} p < 0.01 vs. BTBR mice. ^{***} p < 0.001 vs. BTBR mice. ^{\$} p < 0.05 vs. ST-2223 (5 mg)-treated BTBR mice. ^{\$\$} p < 0.01 vs. ST-2223 (5 mg)-treated BTBR mice. ^{\$\$\$} p < 0.001 vs. ST-2223 (5 mg)-treated BTBR mice. In biochemical assessments, 6 groups of 3-6 mice per group were used. The effects of ST-2223 were analyzed using two-way analysis of variance (ANOVA) with dose of drugs and animals (either BTBR or B6 mice) as the between-subjects factor, and post hoc comparisons were performed with Tukey's test in case of a significant effect. VEH: vehicle, RAM: (R)- α -methylhistamine, ARP: aripiprazole, MEM: memantine.

3. Discussion

3.1. In Vitro Affinities for *hH₁Rs*, *hH₃Rs*, *hD₁Rs*, *hD₂Rs*, *hD₃Rs* and *hD₅Rs*

The novel multiple-targeting test compound ST-2223 was evaluated for its in vitro *H₃R* affinity by [³H]N^α-methylhistamine displacement assays on membrane preparations of HEK-293 cells, stably expressing the *hH₃R*. The results show that ST-2223 had high in vitro affinity for the desired targets, i.e., *hH₃Rs* ($K_i = 4.8$ nM), *hD₂Rs* ($K_i = 19.8$ nM), and *hD₃Rs* ($K_i = 2.0$ nM), with a ratio of *hD₂Rs*/*hD₃Rs* of 10 (Figure 1). Moreover, the observed in vitro results showed that ST-2223 displayed low affinity for *hH₁Rs* (85.2 nM), *hD₁Rs* (564 nM), *hD₅Rs* (5064 nM), and neglectable inhibition of acetylcholine esterase enzyme (*eeAChE*, <60% at a concentration of 1000 nM of ST-2223, preliminary data) as well as butyrylcholine esterase enzyme (*eqBuChE*, <30% at a concentration of 1000 nM of ST-2223, preliminary data). The in vitro results for the selectivity profile of ST-2223 demonstrate that *hH₁Rs*, *hD₁Rs*, *hD₅Rs*, and *AChE* as well as *BuChE* enzymes are not involved in the observed in vivo behavioral enhancements.

3.2. In Vivo Ameliorative Effects of ST-2223 on Behaviors of Treated BTBR Mice

BTBR mice display a typical behavioral profile, characterized by robust social and communication deficits, together with increased RRBs [53]. Collectively, these traits have promoted the widespread use of this model in preclinical research to mimic the symptoms and generate novel hypotheses about the origin and components of neurodevelopmental disorders such as ASD, and to envisage prospective pharmacological modalities for the future therapeutic management of ASD. Therefore, the objective of this study was to assess the capability of the multi-targeting ligand ST-2223, incorporating *H₃R* and *D₂R*/*D₃R* antagonist properties, to ameliorate RRBs and the alternation behaviors observed in BTBR mice. Following numerous investigations of neurochemical pathways in the brain to better understand the pathophysiology of ASD, the dysfunction of several neurotransmitter systems has been shown to be implicated in ASD [54]. This evidence suggests that alterations in neurotransmission plays a crucial role in ASD, and could be useful for prospective pharmacological intervention in this disorder. As alterations in histaminergic and dopaminergic [53] neurotransmission are thought to be involved in the phenotypic outcomes of ASD-related behavioral features [25,28,39,53,55], the present experiments investigated whether the pharmacological modulation of brain HA and DA—using the novel multiple-targeting *H₃R* and *D₂R*/*D₃R* antagonist ST-2223—is capable of reducing RRBs in the BTBR mouse model of ASD. These conducted experiments allowed us to test the stereotyped repetitive behavior and behavioral rigidity through cognitively demanding insistence on sameness behaviors. Similarly to comparing the repetitive and cognitive inflexibility observed in ASD individuals vs. non-ASD individuals, BTBR mice displayed increased RRBs compared to B6 mice; B6 mice were used in the current study as normosocial comparators. Recent studies have suggested that histaminergic signaling abnormalities may contribute to a rare neurodevelopmental Tourette syndrome, characterized by stereotypies that are reported to be among the most prevalent comorbidities associated with ASD [29,32,56]. Moreover, a previous study revealed that activation of *H₃Rs* is implicated in triggering stereotypies in a mouse model of tic disorder with repetitive behavior-related pathologies [57]. Relative to BTBR mice, B6 mice displayed low levels of marble burying, and this observation was consistent with several previous preclinical studies (Figure 2) [47,58].

In the current study, acute systemic treatment of BTBR mice with ST-2223 (2.5, 5 or 10 mg/kg, i.p.) or with the reference drugs MEM (5 mg/kg, i.p.) or ARP (1 mg/kg, i.p.), resulted in comparable reductions in stereotyped repetitive behavior in the MBT test. The latter finding suggests that the functioning of auto and/or hetero *H₃Rs* in BTBR attenuated certain RRBs. Notably, patients diagnosed with ASD have been found to exhibit significant impairment in executive functioning, with some authors suggesting that deficits in cognitive flexibility and set-shifting are consistently associated with the presence of RRBs in ASD [59]. Additionally, mounting evidence indicates that, along with HA release,

the functioning of H₃Rs as hetero-receptors can also regulate the release of several other brain neurotransmitters, including DA, ACh, Glu, 5-HT, and GABA, in several brain regions [60,61]. Thus, central H₃R antagonism has been proposed to improve the cortical fast rhythms closely associated with cognitive behaviors [61]. Interestingly, a previous clinical study reported that neurocognitive deficit may selectively underlie the strong need for sameness in routine and in the environment that is exhibited by many individuals with ASD [8]. As a result, their findings related to the specific association of neurocognitive deficits to RRBs and the need for sameness, are supported by the results observed in the current study. Preclinically, numerous studies have indicated that H₃R antagonists in particular exhibited a unique feature in the form of their potential cognition-enhancing property [19,20,50,62–66], suggesting their potential therapeutic role in the treatment of RRBs. In accordance with this, a previous report showed that mice with valproic acid (VPA)-induced ASD displayed reduced repetitive behavior in the MBT following treatment with the H₃R antagonist ciproxifan [55]. Interestingly, the results observed for ST-2223 in the MBT are in agreement with a previous study, in which adult male Tuck-Ordinary mice with VPA-induced ASD features were sub chronically pretreated with the non-imidazole H₃R antagonist DL77, yielding a significant and dose-dependent (5, 10, 15 mg/kg, i.p.) reduction in the percentage of marbles buried [67]. These previous observations are in harmony with the current results of MBT which comprehend the capability of ST-2223 to modulate the neurotransmission of HA and DA, supporting their crucial role in repetitive/compulsive behaviors in mice. Notably, most of the previous studies focused on effects of H₃R antagonists on ASD, however, the current series of behavioral experiments are the first to assess the effects of a multiple-active H₃R and D₂R/D₃R antagonist on behavioral parameters altered in ASD. Comparable to observations with MBT, ST-2223 (5 and 10 mg/kg) significantly reduced the self-grooming duration in BTBR mice, with no effect of the highest dose of ST-2223 on locomotor activity (Figure 3). Similar results were observed with reference drug ARP (1 mg/kg), suggesting that the mechanism by which the self-grooming effect is improved, following administration of ST-2223, may involve its potent antagonistic interactions with D₂R/D₃R, which may modulate the levels of DA in several specific brain areas. Moreover, ARP is considered as a dopamine stabilizer, as when DA levels are high, it acts as a D₂R antagonist, and at low endogenous DA levels, it acts as a D₂R agonist [68]. The involvement of DA in self-grooming, as suggested by the present data, is supported by a recent study which demonstrated the attenuation of enhanced frequency of self-grooming induced by anorexigenic peptide neuromedin U following systemic pretreatment with ARP, as neuromedin U was reported to increase the *ex vivo* level of DA [68]. As previously reported by Rapanelli et al. (2017), the pathology of repetitive behaviors in mice derives from a deficiency of brain HA. Markedly elevated grooming was observed by specific ablation or chemogenetic silencing of histaminergic neurons in the tuberomammillary nucleus (TMN) of the hypothalamus, and the detection of elevated neuronal activity markers was observed in both the dorsal striatum and the medial prefrontal cortex [69]. However, their findings showed that direct infusion of HA into the striatum reversed this behavioral pathology [69]. In the current study, the addressed H₃R antagonistic effects of ST-2223 in alleviating self-grooming in BTBR mice are in line with those previously observed, namely its enhancement effect on the release of HA and other neurotransmitters in several brain regions.

Consequently, assessing the levels of different brain neurotransmitters, including HA and DA, in different brain areas of BTBR mice with ASD-like behaviors, as well as after pre-treatment with ST-2223, would further help to understand which neural circuits may be involved in this observed improvement in self grooming. In addition, our current observations demonstrate that the NMDAR antagonist MEM significantly rescued self-grooming in BTBR mice. These results are in accordance with a previous study in which suppression of elevated NMDAR function in a VPA mouse model of ASD was found to normalize repetitive behaviors [70]. However, in B6 mice, there was no appreciable trend for ST-2223 in altering the normal level of self-grooming, while MEM tended to

decrease self-grooming without altering the locomotor activity of B6 mice, as observed in the open field test (Figures 3 and 5). The latter observations may explain the specific involvement of NMDAR and glutamate transmission in self-grooming in dysregulated and normal conditions. Notably, self-grooming is an innate behavior and is the most frequently occurring awake behavior displayed by laboratory mice [71,72]. When the grooming patterns is not in a ritualistic manner and occurs sequentially and systematically, it resembles compulsive-like behaviors expressed in some ASD individuals and in other psychiatric disorders [71,72]. ST-2223 (5 and 10 mg/kg) decreased the elevated level of self-grooming of the whole body that is significantly elevated in BTBR mice reflecting RRB. On the other hand, all doses of ST-2223 showed no effect on the normal self-grooming displayed by B6 control mice, excluding any confounding effect of the test compound. Moreover, ST-2223 was unable to restore hyperactivity in BTBR mice, excluding any drug-induced depression like behaviors, or impaired motor function. Our observations are in agreement with the reported principal symptoms that characterize clinical depression seen in stressed animals, including decreased motor activity [73].

Neurocognitive deficit is clinically reported to be one of the selective mechanisms underlying the strong need for sameness in routine that is exhibited by many individuals with ASD [8]. Therefore, a Y maze assessing the spontaneous alternation is commonly used to evaluate cognition and working memory, as mice must recall the most recently visited arm. In line with this idea, the findings from Y maze behavioral assessments showed that ST-2223 and MEM enhanced cognitive performances by reversing the reduced percentage of spontaneous alternation behavior in BTBR mice, as decreases in the percentage of alternation can be attributed to same arm repeated visits reflecting cognition and attentional deficits (Figure 4). The B6 control animals showed high spontaneous alternations, suggesting a high randomness of their behaviors. Griebel et al. (2012) reported that SAR110894, a potent histamine H₃-receptor antagonist, reversed a deficit in working memory in the Y maze test, following an acute low dose of phencyclidine (PCP) in mice sensitized by repeated treatment with a high dose of PCP [74]. Taken together, the relationship between working memory and cognitive abilities suggests that the H₃R antagonist may be effective in treating RRBs in ASD, due to its therapeutic effects on certain aspects of cognition. Previous studies have reported that MEM rescued both social deficits and repetitive behaviors, such as self-grooming and jumping, in a VPA mouse model of ASD [70]. In agreement that suppression of elevated NMDAR functioning normalizes repetitive behaviors, here, we found that MEM significantly attenuated the reduced spontaneous alternation in BTBR mice, suggesting the observed reduction in sameness of the animal behavior. Accordingly, these collective observations reveal that the histaminergic and dopaminergic systems cross react or overlap in targeting the mechanistic RRBs of ASD; similarly effective as modulation of NMDA receptor functioning in BTBR mice.

The effects of ST-2223 on locomotor activity, as well as anxiety levels, were examined in order to exclude any false-positive effect in the behavioral tests. The results observed indicated that systemic treatment with ST-2223 in control B6 mice has no aversive effects in the open field test, indicating that ST-2223 does not change the baseline levels of motor, exploratory, or anxiety behaviors. However, ST-2223 significantly increased the amount of time spent by BTBR mice in the central arena, confirming the ability of ST-2223 to modulate anxiety-associated fear levels. In contrast, ST-2223 failed to restore hyperactivity, as no effect was exhibited with all doses on the total distance travelled (Figure 5). Consequently, and because ST-2223 treatment had no effect on locomotor activity in BTBR mice, the drug-induced reduction in marble burying and alternation behaviors cannot be explained by a more general reduction in activity. Instead, the results suggest that regulation of histaminergic and dopaminergic systems selectively modulated RRBs in BTBR mice. In contrast with a previous study [75], MEM failed to show significant anxiolytic effects in the open field test, a discrepancy that may be explained with the difference in dose regimen or anxiety level state in different mouse strains. Moreover, the failure of ST-2223 (at all doses), MEM and ARP to restore the hyperactivity observed in BTBR mice may have been

due to the excitatory (Glu) and inhibitory (GABA) neurotransmitter imbalance which is well known to exist, as such an imbalance was observed in several clinical trials in patients with ASD [76–79]. However, a previous randomized clinical trial showed that following 6 weeks of treatment of MEM, this may be considered as an alternative to methylphenidate in children with attention deficit hyperactivity disorder (ADHD), characterized by lack of attention and hyperactivity [80]. Taken together, the current collective observations suggest that chronic treatment with MEM may be effective with regard to reducing anxiety and hyperactivity.

The strong ASD-like behavioral profile of BTBR mice, i.e., abnormalities in neurotransmitter systems in addition to several physiological and neurological features resembles that observed in patients diagnosed with ASD [81]. The latter observation suggests that the promising results of our current study on RRBs in BTBR mice may be a new therapeutic approach in the treatment of the core symptoms in ASD patients.

3.3. *In Vivo* Mitigating Effects of ST-2223 on Oxidative Stress in Different Brain Areas of Treated BTBR Mice

Another major objective of the current study was to test the capability of the most promising dose of ST-2223 (5 mg/kg) to mitigate abnormal levels of oxidative stress in four different brain areas of treated BTBR mice, namely the cerebellum, hippocampus, prefrontal cortex, and striatum. In the current study, the results showed that BTBR mice with ASD-like behaviors displayed significant increases in MDA levels, connected with a decline in GSH, SOD, and CAT levels in different brain parts of tested BTBR mice and as compared to control mice B6. Moreover, systemic administration with the most promising dose of ST-2223 (5 mg/kg), ARP (1 mg/kg) or MEM (5 mg/kg) showed a significant reduction in MDA, as well as a significant elevation in GSH, SOD, and CAT in different areas of the brain. The results observed on the levels of oxidative stress markers are in agreement with several previous reports from our group and other research groups that investigated the effects of H₃R antagonists on oxidative stress in the brains of different rodent models [79,82–86]. Accordingly, previous reports showed that imidazole-based H₃R antagonists, including clobenpropit and ciproxifan, mitigated several oxidative stress markers (e.g., MDA and GSH) in amphetamine- or dizocilpine-augmented oxidative stress in a preclinical mouse model of schizophrenia, suggesting the protective effect of H₃R antagonists in such disease conditions [87–89]. Furthermore, systemic co-administration with RAM (10 mg) counteracted the ST-2223 (5 mg)-induced modulating effects on MDA, GSH, SOD, and CAT in BTBR mice, indicating that modulation of brain HA—provided by ST-2223—may have contributed to the correction of an unbalanced ratio of radical oxygen species through the generation of endogenous cellular antioxidant defensive mechanisms.

4. Materials and Methods

4.1. Animals

Male C57BL/6J (B6) and BTBR T+ tf/J (BTBR) mice (aged 8–10 weeks, weighing 25–35 g) (Jackson Laboratory, Bar Harbor, USA) from the central animal facility of the College of Medicine and Health Sciences, United Arab Emirates University [90] were utilized for all the *in vivo* assessments. All mice were housed in plastic cages in a temperature-controlled room (22–25 °C) on a standard 12 h light/dark cycle (lights on at 6 a.m.). The animals had free access to tap water and a standard rodent chow diet in their home cages. Experiments were performed during the light cycle. All experimental procedures described herein were performed according to the recommendations of the European Communities Council Directive of 24 November 1986 (86/609/EEC). All experiments were approved by the Institutional Animal Ethics Committee in the College of Medicine and Health Sciences/United Arab Emirates (Approval No. ERA-2017-5603).

4.2. Drugs

The drugs tested included ST-2223 [*N*-(4-(4-(2-methoxyphenyl)piperazin-1-yl)butyl)-6-(3-(piperidin-1-yl)propoxy)-2-naphthamide] that was designed and synthesized in the Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Germany, and in accordance with previously described methodologies [44,45,91] (Supplementary Materials). Aripiprazole (ARP) (1 mg/kg, i.p.) and memantine (MEM, 5 mg/kg, i.p.), as reference drugs, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The CNS-penetrant H₃R agonist (*R*)- α -methylhistamine (RAM, 10 mg/kg, i.p.) was purchased from Sigma-Aldrich, St. Louis, MO, USA. T. Protease and the phosphatase inhibitor cocktail were procured from Thermo Scientific, USA. The assay kit for reduced glutathione (GSH) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The lipid peroxidation assay kit for estimation of malondialdehyde (MDA) was purchased from North West Life Science (Vancouver, WA, USA). The assay kits for superoxide dismutase (SOD) and catalase (CAT) were purchased from Cayman Chemical (Ann Arbor, MI, USA). The drugs were prepared daily using 1% dimethyl sulfoxide (DMSO) in 0.9% saline, for intraperitoneal (i.p.) administration at a volume of 10 mL/kg of body weight. Vehicle treatment consisted of 1% DMSO in 0.9% normal saline. All drugs were i.p. administered 30 min before the behavioral test. The doses of these drugs were chosen based on previous reports. Neurobehavioral assessment was performed blindly with respect to experimental group and drug administration.

4.3. In Vitro Pharmacological Binding Assays for ST-2223

In accordance with previous experimental protocols, the binding affinity of ST-2223 for H₃Rs was assessed by applying the [³H]*N*^α-methylhistamine binding assay, performed with cell membrane preparation of HEK cells stably expressing the human H₃R (*n* = 4) [45,50,92–94]. In addition, and as previously described, the binding affinity of ST-2223 for human dopamine *h*D₁Rs and *h*D₅Rs (HEK) against [³H]SCH23390 and *h*D₂SRs, *h*D₃Rs (CHO) using [³H]spiperone (*n* = 3) was assessed (*n* = 3) [44,91]. Moreover, the inhibitory effects of the tested compound ST-2223 on acetylcholinesterase (AChE) and butyrylcholin esterase (BuChE) were determined using a modified Ellman's method [46].

4.4. In Vivo Behavioral Tests

4.4.1. Marble Burying Task (MBT)

The MBT is used for testing repetitive and compulsive behaviors as it accurately reflects repetitive digging behavior [95,96]. The test was performed as described previously, with slight modifications [96–98]. Briefly, cages (26 cm × 48 cm × 20 cm) were filled with fresh, unscented mouse bedding material to a depth of 5 cm, on top of which 20 marbles were placed in 4 rows of 5 marbles each (15 mm diameter). Before placing the marbles, the bedding surface was leveled by placing another cage of the same size onto the surface of the bedding and then each mouse was individually added for 10 min habituation. After habituation, the mouse was removed, and the marbles were carefully overlaid. The same mouse was then returned to its designated test cage and allowed to explore for 30 min. The total number of marbles buried (>50% marble covered by the bedding material) was determined at the end of the session [95].

4.4.2. Self-Grooming Paradigm (SGP)

In the SGP test, mice were scored for spontaneous grooming behaviors. Self-grooming may represent more of a repetitive motor pattern that does not have a predominant cognitive component as with reversal learning [99]. To measure SGP, each mouse was placed individually in a standard clear plastic cage (28 cm wide × 17 cm long × 12 cm high) for a total of 20 min. Mice were allowed to freely explore the cage for the entire test, the first 10 min served as a habituation period. After 10 min habituation, each mouse was scored with a stopwatch for 10 min for cumulative time spent grooming all body regions. Grooming behavior included head washing, body grooming, genital/tail grooming and

paw and leg licking. The observer sat approximately 1 m from the test cage during the second 10 min of testing [99].

4.4.3. Spontaneous Alternation Behavior (SAB)

The SAB test is based on the exploratory strategy of rodents to explore a new environment. In examining repetitive behaviors, the Y maze test took advantage of the rodents' natural tendency to spontaneously alternate or choose a different arm of the maze instead of the one they visited on their previous entry (i.e., least recently visited arm). A decrease in the percentage alternation can also be attributed to same arm repeated visits [100]. Spontaneous alternation behavior was measured on a Y maze apparatus, as previously described, with slight modifications. The Y maze composed of three arms (30 cm × 6 cm × 15 cm), where the arms were labeled with letters: A, B, or C. The test mouse was placed in the center of the maze and was allowed to freely explore the maze for 8 min. The number of entries into each arm and the total number of entries were recorded by the observer. A spontaneous alternation was defined as successive entry into three arms on an overlapping triplet set. Percentage spontaneous alternation was calculated as the number of spontaneous alternations (actual alternations) over the total number of entries (possible alternations). Two arm entries were subtracted from the denominator because an alternation required at least 3 entries. The apparatus was cleaned with 70% alcohol solution after each mouse was tested [101].

$$\text{Percentage spontaneous alternations} = \frac{\text{Total alternations}}{(\text{Total arm entries} - 2)} \times 100 \quad (1)$$

For example, the sequence C, B, A, B, C, B, A, C, B (starting in arm A) resulted in a percent spontaneous alternation of 5/7 = 71.4%.

4.4.4. Locomotor Activity

The open field test (OFT) systematically evaluates exploration behaviors executed in a novel open field (considered as a novel environment), as an independent control for the effects of the drugs on physical activity that could confound the interpretation of the results from the self-grooming, anxiety and spontaneous alternation. Mice were allowed to freely explore an open-field arena (45 × 45 × 30 cm), to assess general locomotor activity, and anxiety-related behaviors [82]. The center arena was defined as the central 23 × 23 cm area. Mice were given 5 min to habituate in the center area of the arena before the recording started for the actual behavioral assessments. Recording lasted for 10 min using charge-coupled device (CCD) camera-assisted motion tracking apparatus and software (EthoVision 3.1, Noldus Information Technology, The Netherlands), and the total distance moved in the whole arena, time spent in the center and periphery were recorded. Test chambers were cleaned with 70% ethanol after each trial and were allowed to dry until ethanol evaporation and odor dissipation had occurred. When interpreting the results observed in this assessment, less time spent in the center was considered as an indicator for high levels of anxiety-like behaviors and total distance travelled signified the overall locomotor activity of tested animals [98,102,103].

4.5. Brain Collection and Tissue Processing for Biochemical Analyses

At the end of the behavioral assessments, the animals were sacrificed following previously published protocols [67,84]. Animals were anesthetized with pentobarbital (40 mg/kg, body weight, i.p.), sacrificed, and perfused via intracardial infusion to wash out the blood, using 1 × PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) at pH 7.4. The blood removal was confirmed by the observation of whitish color liver, heart, and kidney, indicating that they were blood free. The brains were then quickly removed and placed on an ice plate. The cerebellum, hippocampus, prefrontal and striatum were excised from the brain and snap-frozen in liquid nitrogen for further use in biochemical assessments [67,84]. On the day of the biochemical tests, the tissues

were homogenized using 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) supplemented with protease and phosphatase inhibitors. Then, the homogenates were centrifuged at 14,000 rpm for 30 min (4 °C), and the supernatant was used for evaluation of lipid peroxidation, glutathione, and antioxidant enzymes using spectrophotometric measurements and enzyme-linked immunosorbent assays (ELISA). [90,104].

4.5.1. Oxidative Stress Marker Estimations

Malondialdehyde (MDA) Assay

This assay was performed to estimate the amount of lipid peroxidation in experimental animals using an MDA detection kit following the manufacturer's instructions, as previously described in our laboratories [84,105,106]. Briefly, 250 µL of sample or calibrator was incubated with thiobarbituric acid, followed by rigorous vortexing. After 1 h incubation at 60 °C, the mixture was centrifuged at 10,000 × *g* for 2–3 min and the reaction mixture was transferred to a cuvette. Spectra were measured at 532 nm and the results are expressed as µM MDA/mg protein.

Quantification of Glutathione (GSH)

Levels of GSH in tissue homogenates were carried out according to the manufacturer's instructions of the commercially available Sigma's glutathione assay kit (Sigma-Aldrich Chemie GmbH, Steinheim), and as previously described [84,105,106]. In brief, samples were first deproteinized with 5% 5-sulfosalicylic acid solution, centrifuged to remove the precipitated protein, and then the supernatant was used to estimate GSH. Ten microliter samples or standards were incubated with 150 µL of working mixture (assay buffer + 5,5'-dithiobis (2-nitrobenzoic acid) + GSH reductase) in 96-well plates for 5 min. Diluted NADPH solution (50 µL) was added into each well and mixed thoroughly. Absorbance was measured at 412 nm with the kinetics for 5 min by using the microplate reader. Results are expressed as µM GSH/mg protein.

4.5.2. Assay of Antioxidant Enzymes Activities

To assess the activity of antioxidant enzymes Superoxide dismutase (SOD) and Catalase (CAT), commercially available kits were used, following the manufacturer's instructions, and as previously reported [105,106]. Cayman assay kits (Cayman Chemicals Company, Ann Arbor, MI, USA) were used to assess antioxidant enzyme (superoxide dismutase (SOD) and catalase (CAT)) activities in experimental animals. Catalase assay: Twenty microliters of samples or standards and 30 µL of methanol was added to the assay buffer (100 µL) in 96-well plates. To this mixture, twenty microliter of hydrogen peroxide was added and incubated for 20 min at room temperature (RT) to initiate the reaction. Following incubation, 30 µL of potassium hydroxide was used to terminate the reaction, followed by subsequent addition of catalase purpald (30 µL) and catalase potassium periodate (10 µL). The plate was incubated for 5 min at room temperature in a shaker and the plate was read at 540 nm using a microplate reader. Catalase activity was expressed as nmol/min/mg protein. Superoxide dismutase assay: ten microliters of sample or standard was added in 96-well plates. Twenty microliters of xanthine oxidase were added to initiate the reaction. The reaction mixture was mixed for few seconds and incubated (covered) for 30 min at room temperature (RT). Absorbance was read at 450 nm using microplate reader. The activity of SOD was expressed as units/mg protein.

4.6. Statistics

Separate two-way analyses of variance ANOVAs (strain: B6, BTBR; treatment: vehicle, 2.5, 5, 10 mg/kg ST-2223) were conducted for biochemical assessments, marble burying, self-grooming, alternation behaviors, and locomotor activity. A significant interaction was followed by Tukey HSD post hoc tests to determine significant treatment differences in

both strains. For statistical comparisons, the software package SPSS 25.0 (IBM Middle East, Dubai, UAE) was used. *p* values less than 0.05 were considered statistically significant.

5. Conclusions

The novel multiple-active H₃R and D₂R/D₃R antagonist ST-2223 ameliorated ASD-like RRBs that are naturally exhibited in the BTBR mouse model of ASD. To the best of our knowledge, this is the first time the effects of the interplay between HA and DA on ASD-like RRBs have been explored directly. The *in vivo* demonstration of the effectiveness of a potent multiple-active test compound in palliating the RRBs observed in BTBR mice, provides evidence for the potential role of such compounds in treating ASD. Moreover, ST-2223 modulated the increase in the level of MDA and the decrease in levels of GSH, SOD and CAT in the cerebellum, hippocampus, as well as the prefrontal cortex, providing sufficient evidence that oxidative stress balance plays a crucial role in the severity of ASD-like RRBs in BTBR mice. Given the fact that there are no FDA approved effective treatments for the core symptoms of ASD, identification of novel treatments is necessary. As our treatment consisted of a single dose, further studies are still necessary to determine whether chronic treatment or repeated administration of ST-2223 is more effective than the acute dose, and whether this could possibly even extend the duration of the positive effects of the acute dose. The consistent autism-relevant behavioral phenotype of BTBR is an invaluable tool in deciphering ASD's complex pathophysiology and discovering valid treatment methods. Therefore, the results of our current study support the validity of the translational value for the possible clinical applicability of multiple-active H₃R and D₂R/D₃R antagonists, e.g., ST-2223, in the modulation of RRBs in ASD and several other neuropsychiatric diseases.

Supplementary Materials: The following are available online at <https://www.mdpi.com/1422-0067/22/4/1947/s1>, Figure S1: Synthesis and analytics of ST-2223., Figure S2: ¹H-NMR spectral results of ST-2223 in DMSO-d₆.

Author Contributions: B.S. was responsible for the study concept, design, and acquisition and analysis of animal data. N.E., K.V., and P.J. conducted the behavioral and biochemical experiments. M.F., A.F., D.R.-L., M.D. and H.S. were responsible for the generation, synthesis and pharmacological *in vitro* characterization of the test compound, ST-2223. N.E. and B.S. drafted the manuscript. M.F. and H.S. provided critical revision of the manuscript. All authors critically reviewed content and approved the final version for publication. All authors have read and agreed to the published version of the manuscript.

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Supplementary Materials

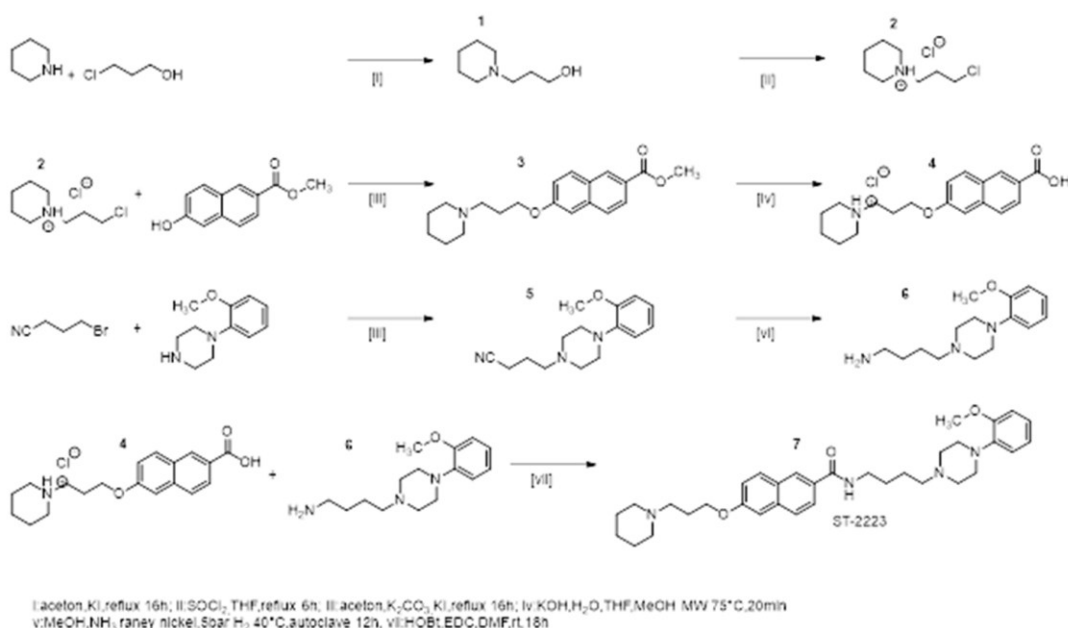


Figure S1. Synthesis and analytics of ST-2223.

All reagents and solvents were purchased by commercial suppliers. Mass spectra was determined with Advion CMS mass spectrometer using APCI. ¹H-NMR was measured by Bruker Avance III 300 MHz using DMSO-d₆ as solvent. Results were displayed as chemical shifts δ in parts per million with solvents as internal standard. Multiplicity described as s: singlet, d: doublet, t: triplet, m: multiplet and number of protons. Melting points were determined with Büchi M-564. LC-MS was performed on Bruker Elute SP with Bruker amazon speed ESI mass spectrometer using an Intensity Solo C18 RP 100x2.1mm column. As solvents acetonitrile and water with 0,1% formic acid in LCMS quality were used. Results given as retention time *t_R* in minutes, area fraction in % and mass to charge ratio *m/z*. Substances were accepted as pure with more than 95% area fraction. Column chromatography (CC) was performed with silica gel 60 (0.04-0.063) from Machery&Nagel with solvents as mentioned in technical quality. Flash chromatography was done with Biotage Isolera Spektra system with ACI and Assist. Biotage Sfär Silica D and Biotage Sfär Amino D were used as columns and solvents as mentioned in technical quality.

3-(Piperidin-1-yl)propan-1-ol 1¹

Compound 1 was synthesized from piperidine (5.0ml; 50mmol), 3-chloropropan-1-ol (4.2544g; 45mmol), potassium carbonate (13.8g; 100mmol) and potassium iodide (catalytic) in 150 ml acetone under reflux condition. Product was distilled at 20 mbar and 95°C. The oil was dissolved in isopropanol and precipitated with HCl in dioxane. Yield: 68%

APCI-MS: (+): *m/z*: 144.3/145.3 ¹H NMR (300 MHz, DMSO) δ 10.84 (s, 1H), 3.74 (t, *J* = 6.4 Hz, 2H), 3.38 (d, *J* = 12.1 Hz, 2H), 3.07 (dt, *J* = 12.3, 4.3 Hz, 2H), 2.93 – 2.75 (m, 2H), 2.29 – 2.15 (m, 2H), 1.93 – 1.62 (m, 5H), 1.37 (dtt, *J* = 17.5, 12.6, 6.7 Hz, 1H).

1-(3-Chloropropyl)piperidine hydrochloride 2¹

Compound 1 was dissolved in tetrahydrofuran, thionyl chloride was added and stirred 5 h under reflux conditions. The reaction mixture was cooled to room temperature and diluted with ether. The participate was filtered off and washed with ether. Yield: 98.7%

APCI-MS: (+): m/z: 161.1/163.1 (M+H)⁺ ¹H NMR (300 MHz, DMSO-*d*₆) δ 10.88 (s, 1H), 3.74 (t, *J* = 6.4 Hz, 2H), 3.43 – 3.35 (m, 2H), 3.12 – 3.01 (m, 2H), 2.84 (tdd, *J* = 12.3, 9.0, 3.6 Hz, 2H), 2.27 – 2.14 (m, 2H), 1.93 – 1.62 (m, 5H), 1.36 (qt, *J* = 12.8, 4.6 Hz, 1H).

Methyl 6-(3-(piperidin-1-yl)propoxy)-2-naphthoate ² 3

Compound 3 was synthesized via Williamson ether synthesis using methyl 6-hydroxy-2-naphthoate (2.997g; 14.8mmol), 1-(3-chloropropyl)piperidine (2.660g; 13.45mmol), potassium carbonate (9.20g; 67mmol) and potassium iodide (cat.) in acetone under reflux conditions for 16h. The solvent was filtered and evaporated. The residue was dissolved in DCM and washed three times with 1 N NaOH solution. The organic layer was dried with MgSO₄ and evaporated. Yield: 86.2%

APCI-MS: (+): m/z: 328.5/329.5 (M+H)⁺ ¹H NMR 8.50 (dd, *J* = 1.8, 0.8 Hz, 1H), 7.98 (dt, *J* = 8.7, 0.8 Hz, 1H), 7.86 (dd, *J* = 8.7, 1.8 Hz, 1H), 7.77 (d, *J* = 8.6 Hz, 1H), 7.24 – 7.13 (m, 2H), 3.80 (s, 3H), 2.32 (m, 6H), 1.85 (dt, *J* = 7.6, 6.5 Hz, 2H), 1.47 (p, *J* = 5.5 Hz, 4H), 1.36 (q, *J* = 6.0 Hz, 2H)

6-(3-(Piperidin-1-yl)propoxy)-2-naphthoic acid x HCl ² 4

Compound 4 (3.80g; 11.6mmol) and potassium hydroxide (2.8095g; 50mmol) were dissolved in tetrahydrofuran, methanol and water. The mixture was filled in a microwave vial and heated in a microwave to 70°C for 15 minutes. The organic solvents were evaporated and the water phase was neutralized with concentrated hydrochloric acid. At pH 2-3, the product precipitated as isoelectric structure. Dissolving in dioxane and adding HCl gave the protonated compound. Yield: 90.2%

APCI-MS:(+):m/z: 328.5/329.5 (-):m/z:326.5/327.5

¹H NMR (300 MHz, DMSO-*d*₆) δ 10.81 (s, 1H), 8.52 (d, *J* = 1.6 Hz, 1H), 8.03 (d, *J* = 9.0 Hz, 1H), 7.97 – 7.84 (m, 2H), 7.43 (d, *J* = 2.5 Hz, 1H), 7.24 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.22 (t, *J* = 6.1 Hz, 2H), 3.45 (d, *J* = 12.1 Hz, 2H), 3.26 – 3.13 (m, 2H), 2.87 (dh, *J* = 12.1, 3.7 Hz, 2H), 2.29 (dq, *J* = 11.6, 6.0 Hz, 2H), 1.94 – 1.66 (m, 5H), 1.39 (ddt, *J* = 12.6, 8.7, 4.2 Hz, 1H).

4-(4-(2-Methoxyphenyl)piperazin-1-yl)butanenitrile ³ 5

Compound 5 was synthesized via Williamson ether synthesis using 1-(2-methoxyphenyl)piperazine (1.75ml; 10mmol), 4-bromobutyronitrile (1.0ml; 10mmol), potassium carbonate (4.15g; 30mmol) and potassium iodide (cat.) in acetone under reflux conditions for 16h. The solvent was filtered and evaporated. The residue was dissolved in DCM and washed three times with 1 N NaOH solution. The organic layer was dried with MgSO₄ and evaporated. The product was purified using flash chromatography with silica gel and dichloromethane: methanol (97:3-93:7) as eluent. Yield: 94.6%

APCI-MS: (+): m/z: 260.4/261.4 ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.00 – 6.82 (m, 4H), 3.77 (s, 3H), 2.96 (s, 4H), 2.53 (d, *J* = 5.4 Hz, 2H), 2.51 (d, *J* = 1.9 Hz, 4H), 2.41 (t, *J* = 6.8 Hz, 2H), 1.76 (p, *J* = 7.0 Hz, 2H).

4-(4-(2-Methoxyphenyl)piperazin-1-yl)butan-1-amine ³ 6

Compound 5 (: 4.8321g; 8.6 mmol) was dissolved in methanol and some ammonia. Activated raney nickel was added and the mixture was stirred in an autoclave under 5bar hydrogen and 40°C for 12h. The

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mixture was filtered over Celite® and the solvent was evaporated. Yield: 89.4%

APCI-MS: (+): m/z : 264.5/265.5 ^1H NMR (300 MHz, DMSO- d_6) δ 6.97 – 6.81 (m, 4H), 3.76 (s, 3H), 2.94 (t, J = 4.7 Hz, 4H), 2.53 (dd, J = 10.1, 4.3 Hz, 2H), 2.48 (s, 4H), 2.29 (t, J = 7.2 Hz, 2H), 1.54 – 1.40 (m, 2H), 1.35 (dq, J = 8.7, 6.8 Hz, 2H).

N-(4-(2-Methoxyphenyl)piperazin-1-yl)butyl)-6-(3-(piperidin-1-yl)propoxy)-2-naphthamide 7 (ST-2223)

Compound **6** (2.1067g; 8mmol), acid **4** (2.8001g; 8mmol), HOBt (1.2241g; 8mmol) and EDC (1.40ml; 8mmol) were dissolved in DMF and stirred for 18h at room temperature. DMF was reduced and diluted with water and 1 N NaOH solution. The mixture was three times extracted with ethyl acetate. The combined organic layers were dried with MgSO_4 and evaporated. The products was recrystallized from acetone. Yield: 61.8%

APCI-MS: (+): m/z : 557.9/558.9 ($\text{M}+\text{H}^+$)

Melting point: 131.7°C-132.2°C

^1H NMR (300 MHz, CDCl_3) δ 8.21 (d, J = 1.7 Hz, 1H), 7.85 – 7.75 (m, 2H), 7.74 (d, J = 8.6 Hz, 1H), 7.24 – 7.13 (m, 2H), 7.01 (td, J = 7.6, 1.7 Hz, 1H), 6.95 – 6.78 (m, 4H), 4.16 (t, J = 6.4 Hz, 2H), 3.86 (s, 3H), 3.55 (q, J = 6.1 Hz, 2H), 3.06 (s, 4H), 2.66 (d, J = 5.5 Hz, 4H), 2.59 – 2.39 (m, 8H), 2.07 (dq, J = 8.9, 6.5 Hz, 2H), 1.98 (s, 1H), 1.81 – 1.56 (m, 7H), 1.48 (q, J = 5.9 Hz, 2H).

^{13}C NMR (75 MHz, CDCl_3) δ 168.03, 158.49, 152.36, 141.33, 136.31, 130.44, 130.09, 128.06, 127.24, 127.19, 124.41, 123.04, 121.07, 120.05, 118.30, 111.26, 106.57, 77.58, 77.36, 77.16, 76.74, 66.73, 58.21, 56.12, 55.46, 54.79, 53.60, 50.64, 40.19, 27.63, 26.85, 26.05, 24.67, 24.52.

LC-MS: t_r : 14.0 min Area Frak.: 100.0% m/z : 280.19 ($\text{M}+2\text{H}$) $^{2+}$

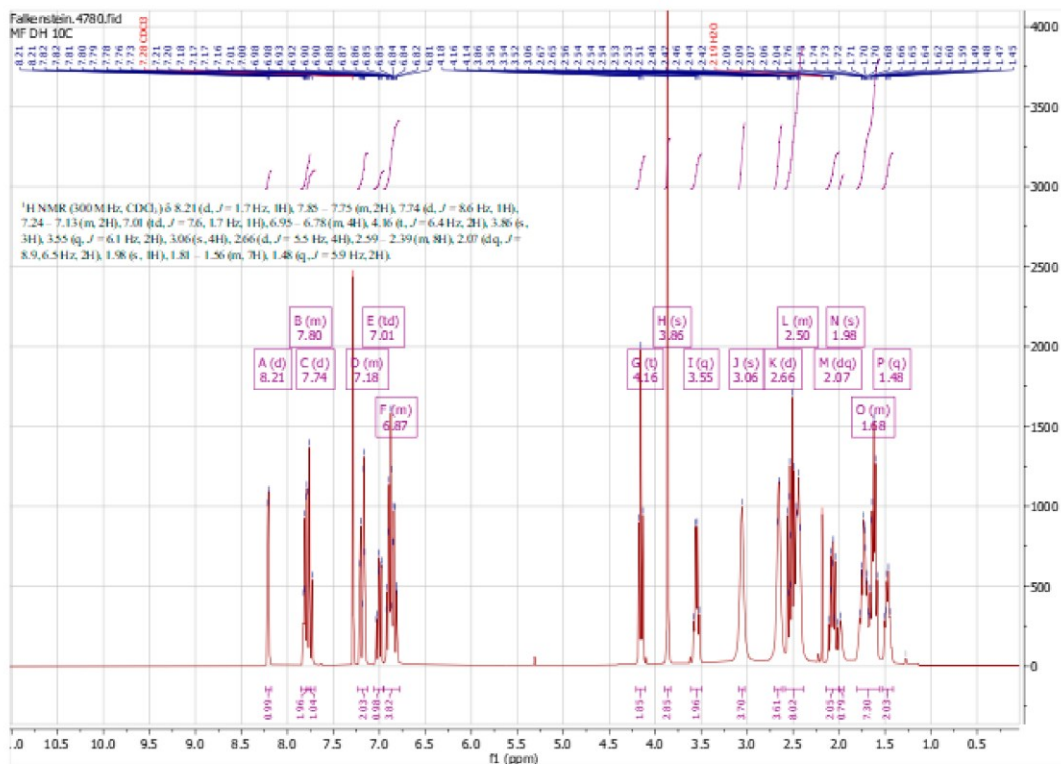


Figure S2. ^1H -NMR spectral results of ST-2223 in DMSO- d_6 .

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3.4 PUBLICATION 4

The impact of secondary site interactions on the binding specificity of bitopic dopamine D₂/D₃ receptor ligands

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The impact of secondary site interactions on the binding specificity of bitopic dopamine D₂/D₃ receptor ligands

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ABSTRACT

Dysfunction of dopamine receptors and interruption of normal dopaminergic signaling have been linked to severe neuropsychiatric diseases such as schizophrenia, Parkinson's disease and depression. Although these receptors are among the most drug-targeted families of GPCRs, designing compounds with subtype-specific pharmacological profile remains challenging due to the structural similarities among the subtypes of the family. Considering the structural moieties of cariprazine, a prototypic drug with D₃ receptor (D₃R) preference, we report a library of bitopic analogues to explore how the orthosteric and secondary binding motifs, coupled with different linkers, affect D₂R/D₃R selectivity and activity. Compounds with promising dopamine receptor selectivities and binding affinities were further characterized on a GPCR panel and in functional assays. Our efforts resulted in an advanced lead compound (**20b**) with improved D₃R and 5-HT_{2A}R, as well as decreased D₂R and 5-HT_{1A}R potencies (EC₅₀) compared to cariprazine. The cleaner receptor profile of this compound retained activities for the most important antidepressant mechanisms that were confirmed by reducing the depression-like behavior in mice.

1. Introduction

Dopamine receptors belong to Class A G-protein coupled receptors (GPCRs) characterized by seven transmembrane (TM) domains, and are widely distributed, albeit at different densities, in various areas of the brain [1]. They can be divided into two subfamilies based on their sequence homology, pharmacology and intracellular signalling [2]. The D₁ and D₅ subtypes belong to D1-like subfamily, while the D₂, D₃ and D₄ subtypes are classified as D2-like receptors. Within the latter group, the D₂ receptor shows 75 % sequence homology with the D₃ receptor and 53 % homology with D₄R, making the development of subtype selective ligands challenging [2–4].

Dysfunctions of the dopamine system have been linked to several important central nervous system (CNS) diseases such as schizophrenia [5], Parkinson's disease [6], depression [7] and substance use disorders [8]. D₂R is the most studied dopaminergic receptor due to its role in neuropsychiatric disorders [9]. Antipsychotics are the largest class of D₂R targeting drugs and include first-, second- and third-generation compounds. First-generation drugs, named typical antipsychotics, can be exemplified by haloperidol (**1**), a D₂R antagonist with the typical extrapyramidal symptoms (EPS) side effects (Fig. 1A) [10,11]. The second generation of compounds are classified as atypical antipsychotics. They include compounds such as risperidone (**2**, Fig. 1A), acting on both the D₂ and serotonin 5HT_{2A} receptors. They display lower risks

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of EPS, but a higher incidence of metabolic side effects, resulting in weight gain and diabetes [12–15]. While both first- and second-generation antipsychotics are effective against the positive symptoms of psychosis, treating the cognitive and negative symptoms has been more challenging [16–18].

Haloperidol (1) and risperidone (2) are bitopic compounds that bind both the orthosteric and the secondary binding pockets. The orthosteric pocket is used by the endogenous ligand, dopamine (Fig. 1F), while the

secondary pocket is in the extracellular vestibule of the receptor (Fig. 1B–E). The compounds are characterized by different pharmacological profiles, with haloperidol showing potent antagonism on dopaminergic receptors, mainly on the D₂R, and on α 1-AR, while having a moderate effect on the 5-HT_{2A} receptor. By contrast, risperidone (2) also binds with higher affinity to the 5-HT_{2A} and H₁ receptors. While both of them are effective against the positive symptoms, the side effect profile differs with haloperidol (1) strongly inducing extrapyramidal

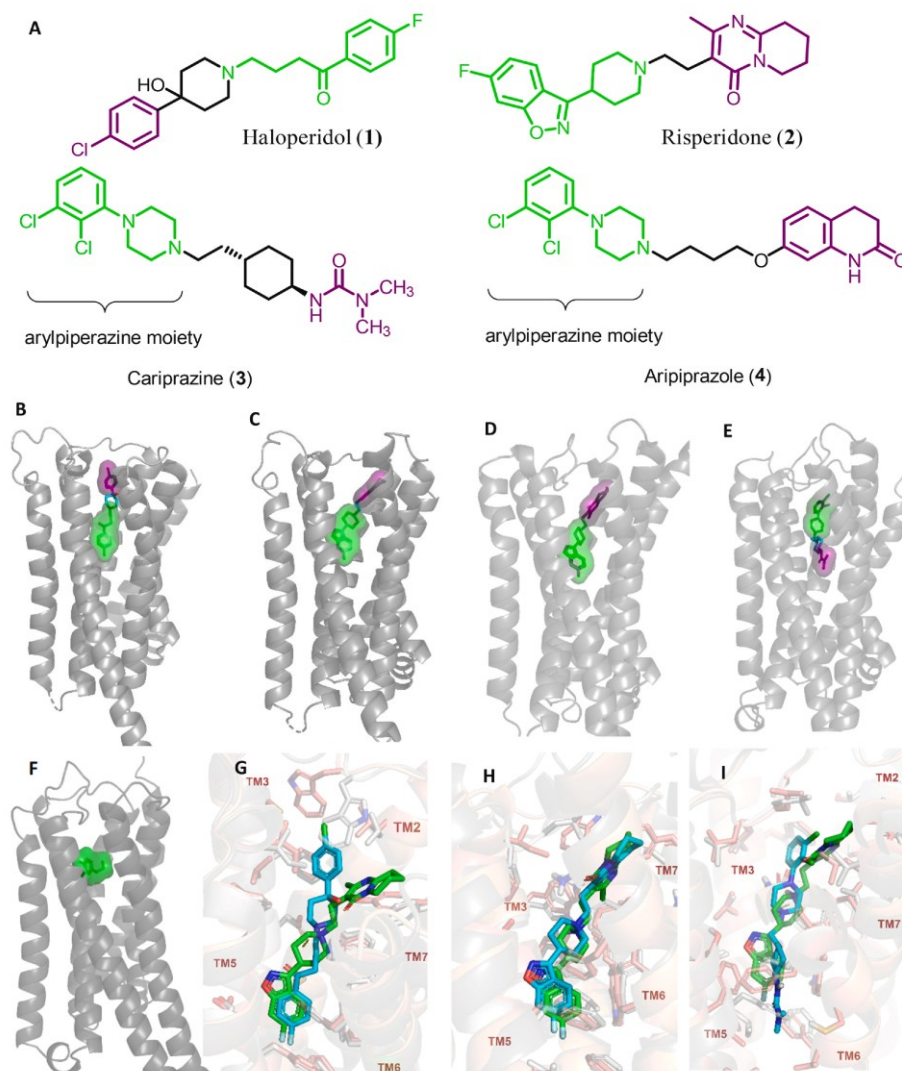


Fig. 1. A) The chemical structures of haloperidol (1), risperidone (2), cariprazine (3) and aripiprazole (4). The orthosteric binding pocket moiety (OBP) is shown in green and the secondary binding pocket moiety (SBP) in purple. B–E) Binding of first- and second-generation antipsychotics haloperidol (1) (PDB code: 6LUQ [19], B) and risperidone (2) (PDB code: 6CM4 [20], C) to D₂R. Both compounds are bitopic antagonists bound to the orthosteric (green) and secondary binding pockets (purple). Binding mode of risperidone (2) (PDB code: 6A93 [21], D) and third generation antipsychotic cariprazine (3) (PDB code: 7VOD [22], E) in the 5-HT_{2A} receptor. F) Dopamine in the D₂R (PDB code: 8TZQ [23]) representing the orthosteric binding pocket. (G–I) Closeup of the binding mode of haloperidol (1) (wheat cartoon, cyan ligand) and risperidone (grey cartoon, green ligand) in the D₂R (G), risperidone (2) in the D₂R (grey cartoon, green ligand) and 5-HT_{2A}R (wheat cartoon, cyan ligand) (H) and risperidone (2) (grey, green ligand) and cariprazine (3) in the 5-HT_{2A}R (I).

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symptoms, while risperidone is linked more to metabolic side effects like weight gain [24,25]. The ligands display markedly different binding modes in the D₂R. The arylpiperidine moiety is located in the orthosteric binding pocket (OBP) in the risperidone (2) structure and in the secondary binding pocket (SBP) in the haloperidol (1) structure. While the latter may be surprising, the symmetric nature of the ligand, regarding the aromatic rings and the centrally located basic nitrogen, may explain the unexpected binding mode. Both ligands form a hydrogen bond with the conserved Asp3.32 residue through their basic nitrogen and they similarly occupy the orthosteric pocket, with hydrophobic interactions with residues Phe6.51, Phe6.52 and Trp6.48 stabilizing the binding modes. The secondary moieties explore a different subpocket of the extracellular vestibule, with risperidone turning towards the top of TM2 and TM7, whereas haloperidol (1) towards the top of TM2, ECL1 and TM3 (Fig. 1G). Risperidone (2) conserved its binding mode also in the 5-HT_{2A} receptor (Fig. 1H).

Third-generation antipsychotics are classified as high-affinity, low intrinsic activity D₂R partial agonists, exemplified by two FDA-approved drugs, cariprazine (3) and aripiprazole (4), with cariprazine selected as a starting point for the present structure-activity study (Fig. 1A). Both compounds are used in the treatment of schizophrenia and mania, as they are effective against the positive symptoms. Furthermore, cariprazine can improve the negative symptoms [26] and was approved for treating full-spectrum bipolar disorders and as an adjunctive therapy to antidepressants for the treatment of major depressive disorder (MDD), a medical condition estimated to affect over 200 million people worldwide. Despite the formidable effort to treat these patients, there are still unmet medical needs; therefore, developing compounds with improved efficacy/side-effect profiles in treatment-resistant depression is paramount. D₃R, 5HT_{1A}R and 5HT_{2A}R were all suggested to underlie the antidepressant effect of third-generation antipsychotics. Combining the high affinity of cariprazine on these receptors and their established role as antidepressant targets [27–35] makes them an ideal subset to achieve antidepressant effect: this is the focus of our project.

Cariprazine and aripiprazole share the 2,3-dichlorophenylpiperazine moiety that is expected to bind to the orthosteric binding pocket of dopamine receptors, while the drugs show significant structural differences in the secondary binding motif (Fig. 1A). Table 1 shows the affinities of the compounds for different receptors. Cariprazine (3) has a higher affinity and higher preference (7.6-fold) towards the D₃R compared to D₂R, whereas the reverse is true for aripiprazole (4) that prefers D₂R (5-fold) [36]. Both compounds show comparable, low nanomolar affinities towards 5-HT_{1A}R, however, whereas aripiprazole has a similar affinity for 5-HT_{2A}R, cariprazine prefers the 5-HT_{1A}R. Nevertheless, cariprazine still shows a high affinity (18.7 nM K_i) for the 5-HT_{2A}R [36]. The compounds showed partial agonistic activity on both of the investigated dopaminergic [37] and serotonergic receptors [22].

The binding mode of cariprazine (3) has not been experimentally determined in the dopaminergic receptors yet, however, previous modelling studies in the D₃R propose a canonical binding mode with the N,N-dimethyl carbamate moiety exploring the extracellular vestibule, while the dichlorophenylpiperazine moiety occupies the same binding pocket as dopamine [38,39]. Interestingly, cariprazine (3) flipped to an

upside-down binding mode in the inactive 5-HT_{2A} crystal structure, compared to risperidone. Whereas the piperazine moiety binds to the conserved Asp3.32, the secondary urea motif penetrates towards the membrane between the central part of TM5 and TM6 (Fig. 1E and I).

In our previous study, we showed that receptor function can be modulated from the secondary binding pocket (SBP) [39], while the Newman group and others investigated the role of the orthosteric and secondary binding moieties and the linker on the selectivity, affinity and function of D₃ receptor ligands, focusing on drug abuse indication [40–45]. In the present study, we aim to investigate the influence of linker rigidity and extended amide- and sulfamoyl-derivative SBP-binding moieties on the binding affinity and selectivity of structural analogues of cariprazine, and identify compounds with promising multi-target profiles, focusing on the potential antidepressant effect. Compared to previous SAR studies, we are exploring one of the largest selections of secondary pharmacophores to map the influence of the secondary binding pocket, as they are key to achieving the desired pharmacological profile. Furthermore, we shift the focus from the original indication of schizophrenia to the antidepressant effect that was realized during the real-life clinical application of cariprazine and was added as a new indication in 2019.

2. Results

2.1. Chemistry

Cariprazine (3) is a bitopic compound that was used as the starting point for our work [46]. Previously, we have investigated three OBP binders (2,3-dichlorophenylpiperazine (5), 2-methoxyphenylpiperazine (6) and 3-cyano-5-trifluoromethylphenylpiperazine (7)) linked to two SBP moieties (dimethyl urea, *tert*-butyl carbamate) with a cyclohexyl linker [39]. We now explore how the D₃R activity and D₃R/D₂R selectivity changes with different SBP binders. Newman and coworkers suggested that the flexibility of the linker might have a significant effect on the D₃R/D₂R profile [40]. We aimed to investigate the 3D-likeness of the linker and therefore prepared two series of compounds, one with the cyclohexyl-ethyl linker of cariprazine (3) and another with its more planar phenyl-ethyl analogue. For compounds with the cyclohexyl linker, the key intermediates (16, 17) were prepared from N-(4-oxocyclohexyl)acetamide (8), which was suitable to form the desired urea (18a-c, 19a-c), carbamate (23a-b, 24a-b), sulfamoyl (20a-c, 21a-c), methanesulfonyl (22a-b) [47] or acetyl (14, 15) SBP groups in 1 step (Scheme 1) [48]. For the urea derivatives, the syntheses were realized in a one-pot manner. First, isocyanate was formed from the corresponding amine (16, 17) with triphosgene and then the desired products (18a-c, 19a-c) were obtained by adding the appropriate amine to the reaction mixture. For the sulfamide groups (20a-c, 21a-c) [49], the reactions were prepared with the corresponding sulfamoyl chloride derivatives (Scheme 1). For compounds 22a-b, methanesulfonic acid chloride was used in the last step. Carbamate derivatives (23a-b, 24a-b) were prepared by two different methods. The *tert*-butyl group was formed with di-*tert*-butyl carbonate, and the methyl group was formed with methanol after isocyanate formation with triphosgene, as described earlier (Scheme 2).

Compounds with a phenyl linker were synthesized in a similar way (Scheme 3). The amino group of 2-(4-aminophenyl)ethan-1-ol (26) was selectively protected [50] (27) and then the reaction with methanesulfonyl chloride [51] formed a good leaving group (28). The resulting 28 was reacted with the corresponding phenylpiperazine (29–31) and the protecting group was removed (32–34). The SBP-binding moieties were formed by the same methods described for the cyclohexyl derivatives to provide the corresponding analogues equipped with the aromatic linker (35–40 a-d, Scheme 3).

Table 1
Cariprazine (3) and aripiprazole (4) binding affinities measured by radioligand binding assay [36].

Receptor	Cariprazine (3) pK _i	Aripiprazole (4) pK _i
D _{2L}	9.31	9.68
D _{2S}	9.16	9.72
D ₃	10.07	9.03
5-HT _{1A}	8.59	8.97
5-HT _{2A}	7.73	8.57

[³H]spiperone radioligand was used for dopamine receptors, [³H]8-OH-DPAT for 5-HT_{1A}R and [³H]ketanserin for 5-HT_{2A}R. SDs were not presented.

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2.2. Structure-activity analysis

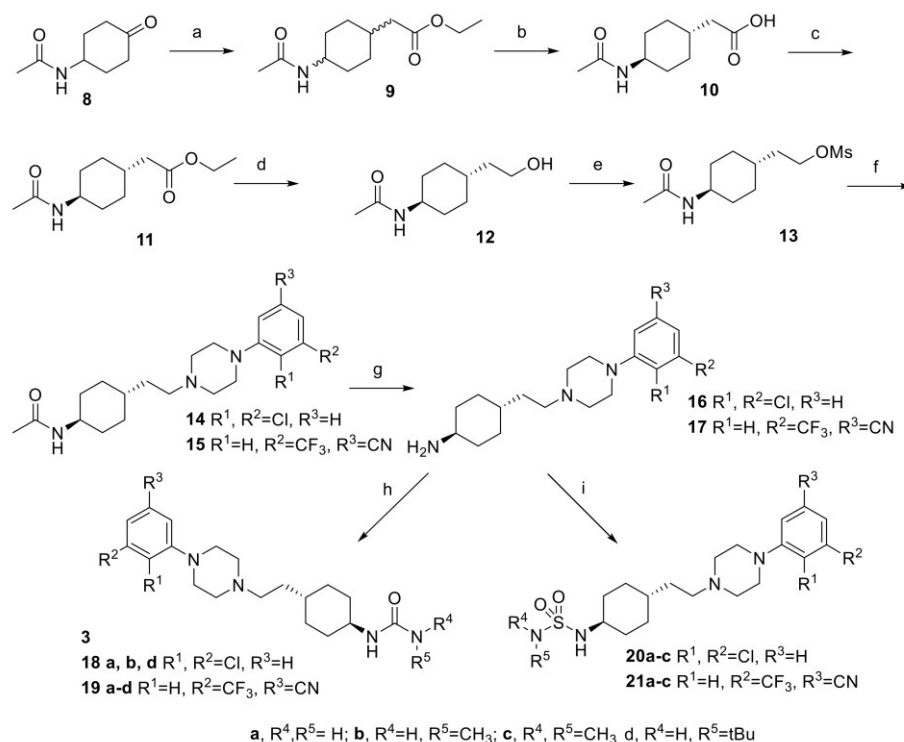
The synthesized compounds were first investigated against the D₂ and D₃ receptors in the corresponding [³H]-spiperone radioligand displacement assays. Results are summarized in Table 2.

Keeping the linker region and the 2,3-dichlorophenyl piperazine OBP-binding moiety of cariprazine (3), we first investigated the effect of the SBP-binder moiety (Table 2). Comparing these results to that of the OBP binding part (5), the attachment of the linker equipped with different SBP binders provided bitopic compounds with higher affinities for both the D₂ and D₃ receptors. We found that most of the analogues have high D₃R affinity in the subnanomolar or low nanomolar range, depending on the SBP functionality. Their D₂R affinity is typically weaker, however, in contrast to the OBP binder 5 and the bitopic cariprazine (3), compounds 23a and 25a have higher affinities to D₂R. This indicates that, in addition to the binding affinity, the SBP-binding region has a significant impact on D₃R/D₂R selectivity. Although in many cases the selectivity of the bitopic compounds is similar to that of the OBP binder, by selecting the appropriate SBP moiety, one can improve or even reverse the D₃R/D₂R selectivity. This effect was most remarkable for the sulfamide derivatives, as exemplified with compound 20b that showed the highest D₃R/D₂R selectivity (21.2), outperforming that of cariprazine (3).

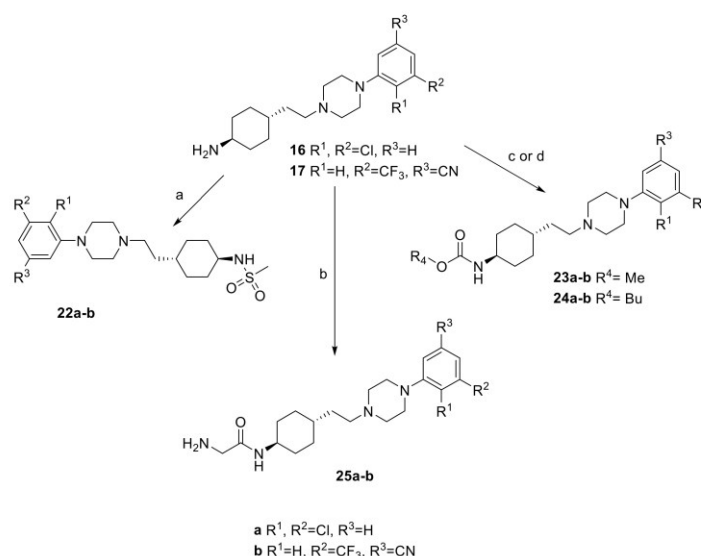
Next, we changed the cyclohexyl linker to the flat benzene ring and used the same set of SBP binding moieties while keeping the OBP unit of cariprazine (3) unchanged. This variation made both the D₃R and D₂R

affinities generally worse than that of the corresponding cyclohexyl-linked analogues. Like the cyclohexyl derivatives, some compounds showed preference for the D₂ receptors (35c and 35d), and some others showed improved D₃R/D₂R selectivity (38b, 38d, 41). Interestingly, however, SBP-binding moieties often induce different effects in the phenyl linker series compared to that of the cyclohexyl series. This suggests that the binding affinity of the bitopic compounds are influenced by both the linker and the SBP moiety.

The trends observed for the cyclohexyl and phenyl linkers were similar in both the 2,3-dichlorophenyl-piperazine and 3-cyano-5-trifluoromethylphenyl-piperazine (7) OBP series. The latter OBP binder is somewhat less potent on both receptors, with a slight D₃R preference. Bitopic compounds with the cyclohexyl linker showed low nanomolar affinities on D₃R, except for compounds 21a and 21c. These and other sulfamide analogues (21a-c, 22b) were less potent on D₂R, resulting in higher D₃R selectivity (up to 34.6-fold). Considering the similar D₃R/D₂R selectivity of the corresponding OBP binders (2,3-dichlorophenyl piperazine 5 and 3-cyano-5-trifluoromethylphenyl-piperazine 7) and the identical cyclohexyl linker used in both series, these data provide further support that both the linker and the SBP-binding moieties contribute to the overall affinity and selectivity of the compounds. Introducing the phenyl linker, we observed similar changes to that found for the 2,3-dichlorophenyl-piperazine analogues. D₃R and D₂R affinities were typically weaker than that of the cyclohexyl derivatives that resulted in lower selectivity, however, the beneficial effect of the sulfamides in the SBP is still reflected in the higher D₃R selectivity.



Scheme 1. The synthesis of cyclohexyl-containing compounds. a, 1. Horner-Wadsworth-Emmons (HWE) reaction with triethyl phosphonoacetate, t-BuOK, THF 2. H₂, 10 % Pd/C, EtOAc b, NaOH, H₂O, crystallization (EtOH) c, H₂SO₄, EtOH d, NaBH₄, MeOH, THF e, MsCl, DCM, TEA f, phenylpiperazine, K₂CO₃, CH₃CN g, 5 % HCl h, 1. triphosgene, TEA, DCM, 2. Amine I, CH₃CN, sulfamoyl chloride.



Scheme 2. Synthesis of mesylated and carbamate derivatives [47]. a, MsCl, DCM, TEA b,1, Boc-protected glycine, HATU, DIPEA, DCM c, Boc₂O, DCM d, 1. tri-phosgene, TEA, DCM 2. MeOH.

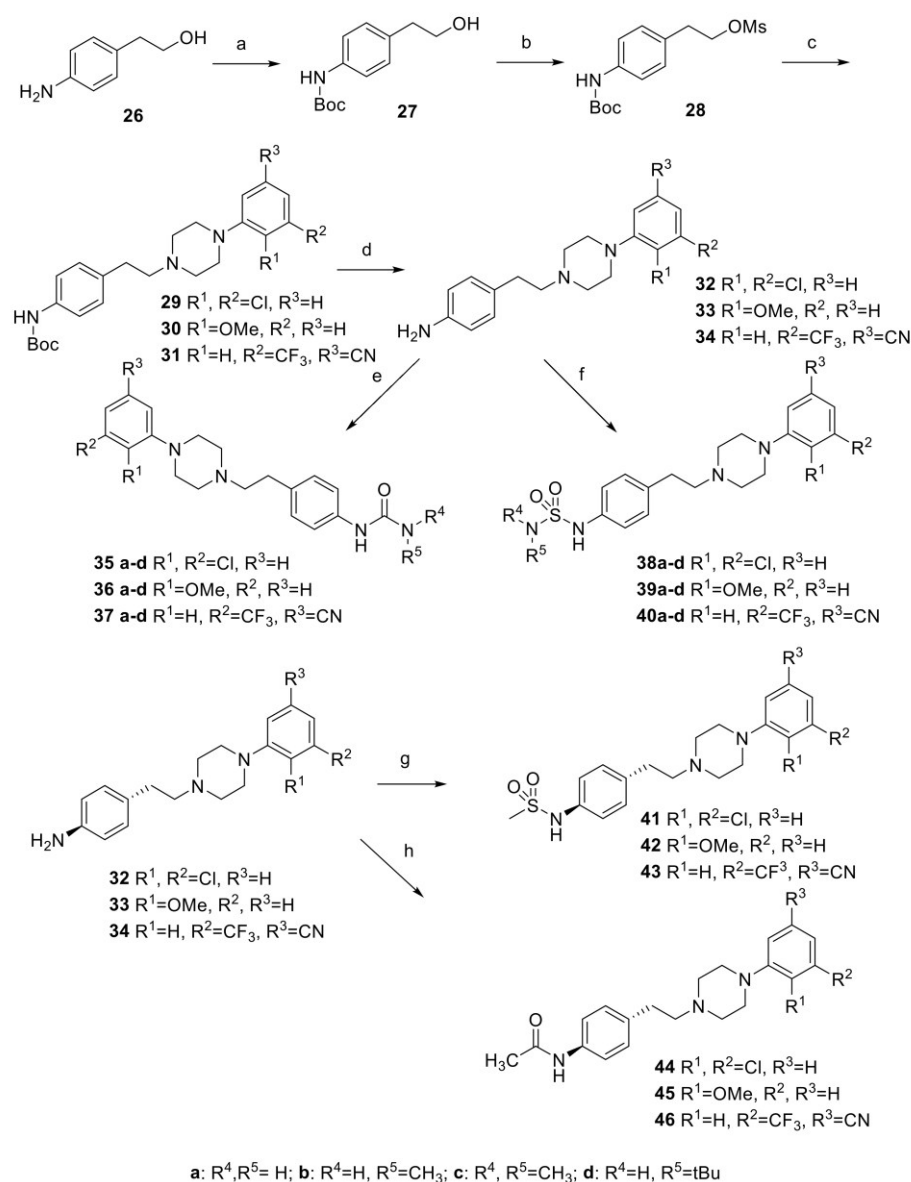
Finally, we tested a much weaker and D₂R-preferring OBP binder, 2-methoxyphenyl-piperazine (6), equipped with a phenyl linker. Attachment of the linker and the corresponding SBP-binding moiety improved the affinity of the compounds significantly. Interestingly, the D₂R preference of the OBP binder has been turned to D₃R selectivity by attaching most of the SBP binders through the phenyl linker. The D₃R preference of the sulfamide derivatives was further confirmed, with the most selective ligand (39c) in this series carrying a Me₂NSO₂ SBP group.

Based on the binding data on the dopamine receptors, we have selected the most selective compounds with high D₃R affinity and moderate D₂R affinity for further characterization on a panel of serotonin receptors. To initiate further understanding of the role of the OBPs, we have selected all compounds with the three investigated OBP binders (5–7), combined with the N-acetyl (MeCO) and its N-methylsulphon counterpart (MeSO₂) SBP moieties via both the phenyl and cyclohexyl linker (Table 2). For the investigated serotonin receptors (5-HT_{1A}R, 5-HT_{2A}R, 5-HT_{2B}R and 5-HT₇R), the major determinants of binding affinity came from the OBPs. Independently of the linker, compounds with the bulky 3-cyano-5-trifluoromethylphenylpiperazine (15, 21b, 22b, 43, 46) tend to show the least affinity for 5-HT_{1A}R and 5-HT_{2A}R, compared to the other compounds. Not much difference was observed between the phenyl vs. cyclohexyl linkers, nor between the 5 vs. 6 OBP binders on 5-HT_{1A}R. On 5-HT_{2A}R, larger differences were observed, with the best compound (41) having a binding affinity of 8 nM, and the worst (46) well above 30 μM. The two best 5HT_{2A}R-binding compounds (41, 22a) shared the same methylsulfamide SBP and the OBP binder 5. Interestingly, the more rigid compound with the phenyl linker showed the best affinity of 8 nM, while the analogue with the cyclohexyl linker has somewhat reduced affinity (43 nM). The OBP (6) was less tolerated by 5-HT_{2A}R, compared to 5-HT_{1A}R. Generally, all compounds showed reduced affinity for 5-HT₆R and 5-HT₇R, compared to 5HT_{1A}R, although some compounds (41, 22a) with the OBP binder (5) were showing good affinities on these receptors as well.

Based on the D₃R/D₂R selectivity data (Table 2) and their serotonin receptor profiles (Table 3), the most interesting compounds (20b, 21b, 22b) carry different sulfamide derivatives as SBP binders, combined

with 5 and 7 as OBP binders. These compounds were characterized on a GPCR panel (Table 4). All the compounds showed superior D₃R/D₂R selectivity, compared to cariprazine. Comparing the receptor binding affinity profile obtained with the radioligand binding assay of the compounds to that of cariprazine, 20b, that shares its OBP binder (5), preserved the D₃, 5-HT_{1A}, 5-HT_{2B} activities, however, showed better selectivity against 5-HT_{2A} and α_{1A} receptors. A somewhat improved binding on the 5-HT₇ and H₁ receptors was also observed. In contrast, 21b and 22b showed an almost clean receptor profile with affinities below 10 nM only for the D₃ and D₂ receptors.

Next, we carried out functional activity assays using BRET against D₃R (G_z), D₂R (G_{oB}), 5-HT_{1A}R (G_{oB}) and 5-HT_{2A}R (G_q) (Table 5), and selected pathways of other GPCRs activated by cariprazine (3) (Supplementary Table 1). The pathways were selected based on a systematic study investigating an extensive set of G-protein and β-arrestin pathways of several GPCRs, and the pathways that were best activated by cariprazine on each individual receptor have been chosen to assess our ligands. In the antagonist mode, we have tested the same pathways as we have selected for the agonist mode, as well as additional pathways if cariprazine showed more potent inhibition on them. These measurements revealed that 20b preserved the partial agonistic activity of cariprazine on D₂R and D₃R, while showed moderately increased efficacy on 5HT_{1A}R and 5HT_{2A}R. More importantly, although 20b and cariprazine have similar efficacies as partial agonists on D₃R, 20b was found to be an order of magnitude more potent than cariprazine. Furthermore, cariprazine (which showed high affinity on both D₂R and D₃R) was more potent on D₂R, whereas 20b (which displays lower D₂R affinity) showed similar potency on D₃R and D₂R in this functional assay. In terms of 5-HT_{1A}R and 5-HT_{2A}R, compound 20b showed the exact opposite profile of cariprazine. Cariprazine is a partial agonist with a higher potency on 5-HT_{1A}R (3.6 nM) than 5-HT_{2A}R (18.8 nM), whereas 20b is a slightly less potent partial agonist on 5-HT_{1A}R than 5-HT_{2A}R on the tested pathways (despite having a two orders of magnitude lower affinity towards 5HT_{2A}R). 21b and 22b did not preserve the partial agonistic activity on the tested D₃R pathway; instead they are antagonists with submicromolar potencies. Although these compounds were



Scheme 3. The synthesis of aromatic compounds a, EtOAc, Boc₂O b, MsCl, DCM, TEA c, phenylpiperazin, K₂CO₃, CH₃CN d, TFA DCM e, 1. triphosgene, TEA, DCM, 2. Amine f, CH₃CN, sulfamoyl chloride g, MsCl, TEA, DCM h, Ac₂O, AcOH.

found to be partial agonists at both the 5-HT_{1A} and 5-HT_{2A} receptors, their potency was much lower than that of **20b** and cariprazine (**3**). The compounds **20b**, **21b** and **22b** mostly showed weak activities on other receptors (5 H T₇R, 5HT_{1D}R, 5HT_{1B}R, M₂R activated by cariprazine (**3**), see [Supplementary Table 1](#)). Considering the potential of D₃R, 5-HT_{1A}R and 5-HT_{2A}R in the antidepressive effect, the improved potency on D₃R and 5-HT_{2A}R, together with D₃R preference over D₂R led us to

further investigate **20b** *in vivo*.

Finally, we investigated compound **20b** in comparison with cariprazine (**3**) in the tail suspension test (TST) as a depression-like behavior model ([Fig. 2](#)). Immobility time in the TST (which correlates with depression-like behavior) was significantly decreased by a 0.1 mg/kg p.o. dose of **20b** treatment ($p = 0,04$), compared to vehicle-treated controls. In contrast, cariprazine showed no effect in any tested dose

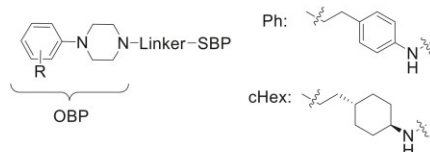
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Table 2

The measured D₂R and D₃R binding affinities obtained from radioligand displacement assay with [³H]spiperone. pK_i ± SEM values, D₃:D₂ ratio is quotient of K_i D₂R to K_i D₃R.



ID	OBP	linker	SBP	D ₂ R pK _i ±SEM	D ₃ R pK _i ±SEM	D ₃ R:D ₂ R ratio
5	diCl	H	–	6.80 (0.01) ^a [39]	7.07 (0.02) ^a [39]	1.86
6	OMe	H	–	6.19 (0.05) ^a [39]	5.88 (0.08) ^a [39]	0.49
7	CF ₃ CN	H	–	6.42 (0.01) ^a [39]	6.67 (0.09) ^a [39]	1.78
14	diCl	cHex	MeCO	9.12 ± 0.11	9.82 ± 0.20	5.07
15	CF ₃ CN	cHex	MeCO	7.81 ± 0.13	9.15 ± 0.13	21.5
16	diCl	cHex	H	8.10 ± 0.06	8.82 ± 0.07	5.3
17	CF ₃ CN	cHex	H	7.67 ± 0.14	6.44 ± 0.16	0.06
18a	diCl	cHex	NH ₂ CO	9.11 ± 0.08	9.70 ± 0.22	3.9
18b	diCl	cHex	MeNHCO	8.37 ± 0.11	8.70 ± 0.15	2.1
3	diCl	cHex	Me ₂ NCO	8.80 ± 0.07	9.24 ± 0.07	2.7
18d	diCl	cHex	tBuNHCO	8.17 ± 0.08	8.68 ± 0.16	3.2
19a	CF ₃ CN	cHex	NH ₂ CO	7.55 ± 0.14	8.70 ± 0.04	14.2
19b	CF ₃ CN	cHex	MeNHCO	7.76 ± 0.02	8.80 ± 0.12	11.0
19c	CF ₃ CN	cHex	Me ₂ NCO	7.56 ± 0.13	8.92 ± 0.26	22.8
19d	CF ₃ CN	cHex	tBuNHCO	7.90 ± 0.13	8.66 ± 0.05	5.8
20a	diCl	cHex	NH ₂ SO ₂	8.55 ± 0.18	8.84 ± 0.04	2.0
20b	diCl	cHex	MeNHSO ₂	7.67 ± 0.19	8.98 ± 0.26	20.3
20c	diCl	cHex	Me ₂ NSO ₂	7.82 ± 0.12	8.46 ± 0.18	4.4
21a	CF ₃ CN	cHex	NH ₂ SO ₂	6.27 ± 0.17	7.81 ± 0.21	34.6
21b	CF ₃ CN	cHex	MeNHSO ₂	6.86 ± 0.11	8.35 ± 0.18	30.7
21c	CF ₃ CN	cHex	Me ₂ NSO ₂	6.38 ± 0.23	7.62 ± 0.22	17.0
22a	diCl	cHex	MeSO ₂	8.43 ± 0.16	8.77 ± 0.07	2.1
22b	CF ₃ CN	cHex	MeSO ₂	7.25 ± 0.07	8.74 ± 0.15	31.2
23a	diCl	cHex	MeOCO	8.48 ± 0.04	8.21 ± 0.15	0.5
23b	CF ₃ CN	cHex	MeOCO	7.82 ± 0.14	8.37 ± 0.21	3.6
24a	diCl	cHex	Boc	7.92 ± 0.14	8.16 ± 0.12	1.7
24b	CF ₃ CN	cHex	Boc	7.22 ± 0.03	7.79 ± 0.13	3.7
25a	diCl	cHex	NH ₂ CH ₂ CO	8.59 ± 0.09	7.89 ± 0.07	0.2
25b	CF ₃ CN	cHex	NH ₂ CH ₂ CO	7.61 ± 0.04	8.07 ± 0.14	2.9
29	diCl	Ph	Boc	7.66 ± 0.17	7.53 ± 0.14	0.7
30	OMe	Ph	Boc	7.93 ± 0.17	7.79 ± 0.07	0.7
31	CF ₃ CN	Ph	Boc	6.94 ± 0.10	7.47 ± 0.08	3.4
32	diCl	Ph	H	7.42 ± 0.12	7.98 ± 0.10	3.6
33	OMe	Ph	H	7.34 ± 0.06	6.91 ± 0.10	0.36
34	CF ₃ CN	Ph	H	7.62 ± 0.03	7.49 ± 0.07	0.73
35a	diCl	Ph	NH ₂ CO	7.32 ± 0.05	8.01 ± 0.08	4.9
35b	diCl	Ph	MeNHCO	8.57 ± 0.05	8.71 ± 0.10	1.4
35c	diCl	Ph	Me ₂ NCO	8.34 ± 0.27	8.20 ± 0.09	0.7
35d	diCl	Ph	tBuNHCO	8.67 ± 0.06	8.34 ± 0.07	0.5
36a	OMe	Ph	NH ₂ CO	7.59 ± 0.12	7.73 ± 0.13	1.4
36b	OMe	Ph	MeNHCO	7.72 ± 0.06	8.02 ± 0.01	2.0
36c	OMe	Ph	Me ₂ NCO	7.71 ± 0.08	7.80 ± 0.11	1.2
36d	OMe	Ph	tBuNHCO	8.38 ± 0.07	7.82 ± 0.19	0.3
37a	CF ₃ CN	Ph	NH ₂ CO	7.21 ± 0.07	8.11 ± 0.08	8.0
37b	CF ₃ CN	Ph	MeNHCO	7.26 ± 0.07	7.90 ± 0.05	4.4
37c	CF ₃ CN	Ph	Me ₂ NCO	6.95 ± 0.06	7.52 ± 0.08	3.7
37d	CF ₃ CN	Ph	tBuNHCO	7.36 ± 0.06	7.54 ± 0.05	1.5
38a	diCl	Ph	NH ₂ SO ₂	7.80 ± 0.11	8.79 ± 0.13	9.9
38b	diCl	Ph	MeNHSO ₂	7.79 ± 0.10	8.88 ± 0.07	12.2
38c	diCl	Ph	Me ₂ NSO ₂	7.60 ± 0.02	8.17 ± 0.08	3.7
38d	diCl	Ph	tBuNHSO ₂	7.15 ± 0.14	8.34 ± 0.19	15.8
39a	OMe	Ph	NH ₂ SO ₂	7.38 ± 0.04	8.00 ± 0.10	4.2
39b	OMe	Ph	MeNHSO ₂	7.21 ± 0.03	7.74 ± 0.02	3.4
39c	OMe	Ph	Me ₂ NSO ₂	7.54 ± 0.09	8.66 ± 0.12	13.4
39d	OMe	Ph	tBuNHSO ₂	7.20 ± 0.07	7.63 ± 0.06	2.7
40a	CF ₃ CN	Ph	NH ₂ SO ₂	6.87 ± 0.07	7.80 ± 0.17	8.4
40b	CF ₃ CN	Ph	MeNHSO ₂	6.68 ± 0.16	7.96 ± 0.16	18.8
40c	CF ₃ CN	Ph	Me ₂ NSO ₂	6.47 ± 0.06	7.37 ± 0.01	8.1
40d	CF ₃ CN	Ph	tBuNHSO ₂	5.99 ± 0.01	6.58 ± 0.04	3.9
41	diCl	Ph	MeSO ₂	7.90 ± 0.13	8.95 ± 0.08	11.2
42	OMe	Ph	MeSO ₂	7.09 ± 0.12	7.64 ± 0.16	3.5
43	CF ₃ CN	Ph	MeSO ₂	6.45 ± 0.04	7.56 ± 0.02	13.0

(continued on next page)

Table 2 (continued)

ID	OBP	linker	SBP	D ₂ R pK _i ± SEM	D ₃ R pK _i ± SEM	D ₃ R:D ₂ R ratio
44	diCl	Ph	MeCO	8.23 ± 0.10	8.66 ± 0.06	2.7
45	OMe	Ph	MeCO	7.58 ± 0.04	8.28 ± 0.10	5.0
46	CF ₃ CN	Ph	MeCO	7.16 ± 0.05	8.24 ± 0.09	12.3

OBP (diCl: 2,3-dichlorophenyl-piperazine (5), CF₃CN: 3-cyano-5-trifluoromethylphenyl-piperazine (7), OMe: 2-methoxyphenyl-piperazine (6)) and SBP stand for the structural moieties bound to the OBP and SBP pockets, respectively. Linker (cHex: 1,4-trans-amino-cyclohexylethyl, Ph: para-aminophenethyl) is the connection element between the OBP and the SBP.

^a indicates pK_i and standard deviation (SD) from Egyed et al. [39].

Table 3

Receptor profile of selected compounds against 5-HT_{1A}, 5-HT_{2A}, 5-HT₆ and 5-HT₇ serotonin receptors obtained from radioligand binding assay.

Compd	pK _i ± SD			
	5-HT _{1A} R	5-HT _{2A} R	5-HT ₆ R	5-HT ₇ R
14	8.19 ± 0.14	6.45 ± 0.21	6.26 ± 0.05	6.54 ± 0.06
15	6.07 ± 0.08	4.70 ± 0.07	5.35 ± 0.14	5.43 ± 0.02
20b	8.59 ± 0.12 ^a	6.92 ± 0.09 ^a	NA	7.68 ± 0.14 ^a
21b	6.51 ± 0.05 ^a	5.64 ± 0.02 ^a	NA	6.31 ± 0.05 ^a
22a	7.98 ± 0.09	7.36 ± 0.19	6.29 ± 0.06	7.21 ± 0.02
22b	6.29 ± 0.13	5.18 ± 0.32	5.39 ± 0.07	6.00 ± 0.11
41	7.89 ± 0.14	8.07 ± 0.11	7.37 ± 0.02	7.03 ± 0.09
42	6.46 ± 0.02	5.78 ± 0.03	5.93 ± 0.08	5.77 ± 0.06
43	7.77 ± 0.04	6.21 ± 0.07	5.70 ± 0.05	6.37 ± 0.07
44	8.19 ± 0.05	6.83 ± 0.07	7.01 ± 0.02	6.37 ± 0.02
45	5.60 ± 0.13	4.50 ± 0.14	5.74 ± 0.20	5.15 ± 0.10
46	7.84 ± 0.11	5.20 ± 0.08	5.35 ± 0.04	5.57 ± 0.15

Radioligands: [³H]-8-OH-DPAT for 5-HT_{1A}R; [³H]-ketanserin for 5-HT_{2A}R; [³H]-LSD for 5-HT₆R; [³H]-5-CT for 5-HT₇R.

^a Results obtained as part of the GPCR panel from radioligand binding assay shown in Table 4. NA: not available; Data for reference compounds are available in Supplementary Table 2. Data is presented as pK_i ± SD as n = 2 (two independent in three replicates).

Table 4

Broader receptor profile of the selected compounds 20b, 21b, 22b, as compared to that of cariprazine (3) obtained from radioligand binding assay.

Receptors	pK _i ± SEM			
	3	20b	21b	22b
D ₂ R	8.80 ± 0.04	7.67 ± 0.05	6.99 ± 0.05	7.25 ± 0.05
D ₃ R	9.24 ± 0.06	8.98 ± 0.08	8.41 ± 0.07	8.74 ± 0.05
D ₂ R/D ₃ R ratio	2.7	20.3	26.3	31.2
D ₁ R	NA	15 ± 1.15 % ^a	5.75 ± 0.03	5.65 ± 0.03
5-HT _{1A} R	8.59 ± 0.11	8.59 ± 0.07	6.51 ± 0.03	6.52 ± 0.05
5-HT _{2A} R	7.73 ± 0.05	6.92 ± 0.05	5.64 ± 0.01	5.56 ± 0.02
5-HT _{2B} R	9.24 ± 0.08	9.30 ± 0.10	5.73 ± 0.04	5.64 ± 0.05
5-HT _{2C} R	6.87 ± 0.05	8.31 ± 0.09	3 ± 1.73 % ^a	1 ± 1.15 % ^a
5-HT ₇ R	6.95 ± 0.06	7.68 ± 0.08	6.31 ± 0.03	5.98 ± 0.03
α _{1A} R	6.81 ± 0.02	5.81 ± 0.06	5.33 ± 0.02	5.22 ± 0.01
M ₁ R	>1000 ±	25 ± 1.73 % ^a	6.25 ± 0.03	6.04 ± 0.04
H ₁ R	7.63 ± 0.10	8.03 ± 0.04	6.34 ± 0.02	6.26 ± 0.04

The following radioligands were used for the measurements: [³H]-spiperone for D₂R, D₃R; [³H]-SCH23390 for D₁R; [³H]-Pirenzepine for M₁R; [³H]-Pyrilamine for H₁R; [³H]-ketanserin for 5-HT_{2A}R; [³H]-LSD for 5-HT_{2B}R; [³H]-Mesulergine for 5-HT_{2C}R; [³H]-SB269970 for 5-HT₇R; [³H]-Prazosin for α_{1A}R. Data is presented as pK_i ± SEM, n = 3, D₃:D₂ ratio is quotient of K_i D₂R to K_i D₃R.

^a indicates % of inhibition at 10 μM. NA: not available.

Table 5

Functional activity of compounds 20b, 21b and 22b measured on selected aminergic pathways in agonist mode using BRET assays.

GPCR (pathway)	D ₂ R (Gz)		D ₂ R (GoB)		5HT _{1A} R (GoB)		5HT _{2A} R (Gq)	
	pEC ₅₀ ± SEM	E _{max} (%) ± SEM	pEC ₅₀ ± SEM	E _{max} (%) ± SEM	pEC ₅₀ ± SEM	E _{max} (%) ± SEM	pEC ₅₀ ± SEM	E _{max} (%) ± SEM
3	7.47 ± 0.31	61.74 ± 9.91	9.16 ± 0.10	79.07 ± 3.84	8.44 ± 0.22	61.52 ± 6.27	7.05 ± 0.05	80.42 ± 2.16
20b	8.75 ± 0.32 [*]	51.53 ± 7.46	8.63 ± 0.38	41.00 ± 7.07 [*]	7.29 ± 0.13 [*]	80.61 ± 5.72	7.49 ± 0.05 [*]	88.87 ± 2.38
21b	NA	NA	NA	NA	6.26 ± 0.11 [*]	82.01 ± 4.93	5.86 ± 0.10 [*]	43.77 ± 2.63 [*]
22b	NA	NA	NA	NA	6.96 ± 0.15 [*]	73.98 ± 5.69	5.51 ± 0.12 [*]	43.02 ± 3.31 [*]

NA: no activity, meaning that the efficacy was lower than 10 %. ^{*} Statistically significant based on one-way ANOVA. Statistical analysis was carried out with GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA), ^{*}p < 0.05 was considered as significant.

at the 1 h timepoint, likely due to its higher D₂R potency counteracting the effect. The concentration of compound 20b in the blood and the brain was checked by pharmacokinetic measurements and found to be sufficient to rationalize the measured *in vivo* effect (Supplementary Table 3).

2.3. Computational analysis

Completing the characterization of our selected three compounds and the reference (cariprazine), we have carried out docking studies to investigate the binding mode of the ligands in the active and inactive D₂R and D₃R structures (PDB IDs: 6CM4, 3PBL, 7JVR and 9F33) [52–55] vs. their functional activities.

All OBP binders formed stable, charge-assisted H-bonds with the conserved Asp114/110^{3,32} in both the D₂R and D₃R. An interesting feature of these OBP binders is that they can rotate 180° around the bond that connects the phenyl and piperazine rings. Docking has predicted binding modes with both OBP positions that were estimated to be energetically close, based on the similar docking scores. The secondary motifs of the molecules were all positioned fairly close to TM2 and TM7 residues, suggesting that in a dynamic environment, the distances may increase and some of the observed interactions in the SBP might be water-mediated, as we have seen in our previous MD simulations [38, 44] with cariprazine.

While our reference molecule (cariprazine) does not form a direct hydrogen bond in these active structures in the SBP, the novel compounds are able to gain further stabilization by interacting with secondary pocket residues through hydrogen bonds (Fig. 3). In line with their selectivity profiles, 21b and 22b form charged-assisted hydrogen bonds with Glu90^{2,65} of the D₃ receptor, and in the case of 21b, also with the backbone of Leu89^{2,64}. By contrast, they turn towards the non-charged Ser409^{7,36} residue in the inactive structure of the D₂R. Furthermore, the bulky headgroup positioning also led to suboptimal steric fitting of the cyclohexyl linker near Leu94^{2,64} in D₂R. Interestingly, in the active D₂R structure, the difference in the binding profile of 20b is smaller, in line with the decreased selectivity of 20b compared to 21b and 22b that display over 30-fold selectivity towards D₃R. The small inward shift of TM7 in the D₂R compared to the D₃R active structure pushes the ligand SBP upward, resulting in the loss of a second hydrogen bond to Glu95^{2,65} in D₂R.

In conclusion, while direct hydrogen bond formation with Glu95/90^{2,65} is not required to achieve high affinity on the target D₂R and D₃R receptors (based on the binding mode of our reference cariprazine and our previous MD studies [38,44]), the selectivity may be improved by designing headgroups that optimally form hydrogen bonds with D₃R,

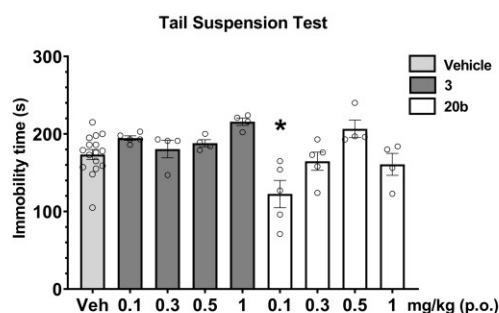


Fig. 2. Behavioral changes in vehicle-, cariprazine- and 20b-treated animals. Immobility times in the tail suspension test are represented as means $\pm$ SEM and analyzed with one-way ANOVA followed by Bonferroni's post-hoc test; * $p < 0.05$ shows statistically significant difference vs vehicle (Veh) treatment ($n = 4-16$).

while being sterically hindered to form these optimal hydrogen bonds with D₂R.

3. Discussion

As some levels of D₂R activity are beneficial for the antipsychotic effect, selective compounds with one-digit nanomolar D₃R affinity and reasonable D₂R affinity could have more potential in further development. We selected three compounds from our library for further characterization; **22b** and **21b** as the only compounds with >30-fold selectivity that displays D₂R and D₃R affinities below our set cut-off value (10 nM for D₃R and 150 nM for D₂R), and one further compound (**20b**) that share the same secondary pharmacophore as **21b** (a methyl sulfamide). The latter two share the cyclohexyl linker, with **20b** equipped with the 2,3-dichloro OBP (**5**), while **21b** belonging to the 3-cyano-5-trifluoromethyl OBP series (similarly to **22b**). First, we characterized their binding profile on nine aminergic GPCRs: D₁R, 5-HT_{1A}R, 5-HT_{2A}R, 5-HT_{2B}R, 5-HT_{2C}R, 5-HT₇R, α 1AR, M₁R and H₁R. Cariprazine (**3**) is considered a D₃R-preferring partial agonist with a 0.58 nM binding affinity on D₃R, however, it targets a broad range of other GPCRs as well, with sub- or low nanomolar affinities on D₂R, 5-HT_{1A}R, 5-HT_{2A}R, 5-HT_{2B}R and H₁R. On the 5-HT_{2C}R, 5-HT₇R and α 1AR receptors, the binding affinity still ranges between 100 and 200 nM, however there is no significant binding on M₁R. While this platform of targeted GPCRs surely reinforces the positive effects of cariprazine (**3**) in the treatment of schizophrenia (as well as its effect on negative symptoms), they likely also contribute to the observed side effects such as tremors, sleep issues, uncontrolled movements and weight gain [56]. The compounds with a bulkier orthosteric binding motif (**21b**, **22b**) show a much cleaner binding affinity profile with low nM affinity only on D₃R and 5-HT_{2C}R, and around 50–100 nM affinity on D₂R (nonetheless, they still maintain a few hundred nM affinity on the 5-HT_{1A}R that is also indicated in depression). The third compound (**20b**) that we have selected for characterization shares the OBP moiety with cariprazine, built up from the more three-dimensional cyclohexyl linker and a methylsulfamide headgroup. The OBP strongly influences the binding affinity of the ligands to the different GPCRs. In line with that, **20b** shows a closer profile to cariprazine (**3**) compared to **21b** and **22b**. Nevertheless, the secondary binding motif is crucial in tuning the affinity and selectivity and the change from the dimethylurea SBP to a bulkier sulfamide analogue results in a modified binding affinity profile. **20b** shows sub-nanomolar or single-digit nanomolar affinity on D₃R, 5-HT_{1A}R, 5-HT_{2B}R, 5-HT_{2C}R and H₁R, many of them linked to depression and mood regulation, while also showing a K_i around 20 nM on D₂R and

5-HT₇R. On 5-HT_{2A}R, the binding affinity was decreased to 119.8 nM, still reasonably expected to influence the overall pharmacological profile of the compound (**20b**), combined with the strong affinity on other depression targets like D₃R and 5-HT_{1A}R.

While binding affinity data is crucial in the characterization of the ligands, the functional activity of the receptors is key to assessing the benefits and risks of any compound. Therefore, we have measured the compounds in a functional BRET assay on selected pathways activated by cariprazine (**3**). As suggested by the binding data, the two compounds (**21b**, **22b**) sharing the 3-cyano-5-trifluoromethyl OBP (**7**) are more selective, showing activity on fewer pathways compared to cariprazine (**3**). Generally, the potency is lower for these compounds (**21b**, **22b**) on most investigated pathways, including the targets strongly linked to depression [7,32,35,57–59], as these compounds are weak partial agonists of 5-HT_{1A}R and 5-HT_{2A}R, and unfortunately they lost their agonistic effect on the D₃R G_s pathway as well. In our previous paper, we have confirmed that, in addition to affinity and selectivity, the functional profile can also be tuned from the secondary binding pocket; SBP moieties of bitopic compounds can change the functionality of the OBP binder [39]. For these two compounds (**21b**, **22b**), the ligands retained the functional activity of the OBP binder (**7**) and remained antagonists on the D₃R, although moderate partial agonism was also observed on D₂R.

In contrast, the compound (**20b**) sharing the OBP with cariprazine (**3**) displays an interesting polypharmacological profile. Overall, **20b** preserved the partial agonist nature of cariprazine (**3**), with an improved potency on D₃R, combined with similar efficacy. While cariprazine (**3**) preferred the D₂R G_{oB} pathway over D₂R G_s in our functional assay, **20b** showed the opposite preference, having lower potency on the D₂R. The dopaminergic receptor family is among the most targeted GPCRs and are linked to numerous CNS diseases like Parkinson's disease, schizophrenia, addiction and depression [7–9,35,37,60]. The signaling pathways contributing to the antidepressant effect of cariprazine have not been investigated in great detail. However, the drug-attenuated chronic unpredictable stress-induced anhedonic-like behavior in wild-type mice was not observed in D₃R-knockout animals [34], therefore partial agonism of D₃R could be favorable for cariprazine derivatives to exert their effect. Potent agonism of D₂R, however, could lead to extrapyramidal, motoric side effects, therefore the decreased potency on the D₂R G_{oB} pathway could aid in mitigating that.

On 5-HT_{1A}R, the potency was reduced compared to cariprazine (**3**) and the efficacy increased to 81 %, while compound **20b** retained comparable potency and displayed high efficacy also on other 5-HT₁ family receptors (5-HT_{1B}R, 5-HT_{1D}R, Table S1). More importantly, the 5-HT_{2A}R potency and efficacy on the G_i pathway increased, compared to cariprazine (**3**). Partial and full agonism of 5-HT_{1A}R has been known for a long time to contribute to the antidepressant effect, with marketed drugs exerting their effect via this mechanism. However, it was also linked to the delayed therapeutic action of common antidepressants [28, 61–63]. Agonists (and especially, biased partial agonists) of 5-HT_{2A}R recently gained a new wave of interest as antidepressants with more and more non-hallucinogenic compounds being developed recently [58, 64–66]. Vargas et al. proposed that these compounds promote neuroplasticity through intracellular 5-HT_{2A}R that could be linked to their therapeutic potential [59]. Recently, Gaitonde et al. showed that most atypical antipsychotics are partial agonists on 5-HT_{2A}R, among them cariprazine also activates several 5-HT_{2A}R-mediated pathways [67]. The increased potency and efficacy of the 5-HT_{2A}R-mediated pathway could contribute to the antidepressant effect of **20b**. The compound **20b** is an antagonist of the 5-HT_{2B}R G₁₁ pathway, similarly to cariprazine (**3**). Avoiding the activation of 5-HT_{2B}R is crucial, as it was linked with cardiac side effects [68].

The compound **20b** does not show relevant antagonist effect of the investigated muscarinic receptors (M₁R, M₂R, M₄R), neither H₁R, α 1AR (A and B), α 2AR, β 1AR and β 2AR (Table S1). Many of these pathways are associated with side effects observed for antipsychotics drugs like

cariprazine (**3**). Blocking the H₁R receptor by antihistamines has been linked to obesity [69], orthostatic hypotension was associated with alpha blockers [70], D₂R are coupled to motoric side effects [71,72], while antagonism on muscarinic receptors can lead to parasympatholytic effect [73,74].

Overall, **20b** is characterized by a cleaner multi-target profile with improved D₃R and 5-HT_{2A}R and decreased D₂R and 5-HT_{1A}R potencies, retaining activity for the most important pathways for antidepressant mechanisms, while reducing the chance of severe side effects [75,76]. The *in vivo* results confirmed our hypothesis for the ability to reduce depression-like behavior in mice. In the TST [77], the compound significantly reduced the immobility time in the 0.1 mg/kg dose. This effect was not observed in higher doses, presumably due to D₂R-related

actions. Meanwhile, cariprazine did not display antidepressant-like effect in this test in any doses 1 h after oral administration.

Our SAR study around cariprazine, was focused on improving the D₃R/D₂R selectivity profile of the compounds, while retaining activation of key signaling pathways. Mainly centered around the exploration of the role of the secondary pharmacophore, our efforts led to a novel derivative with a cleaner polypharmacological profile and *in vivo* antidepressant effect. The compound and the unfolded structure-activity relationship data on D₂R and D₃R could help to elucidate the clinically observed antidepressant effect of cariprazine (**3**), and offer a potential starting point for further, novel antidepressants with improved adverse effect profiles.

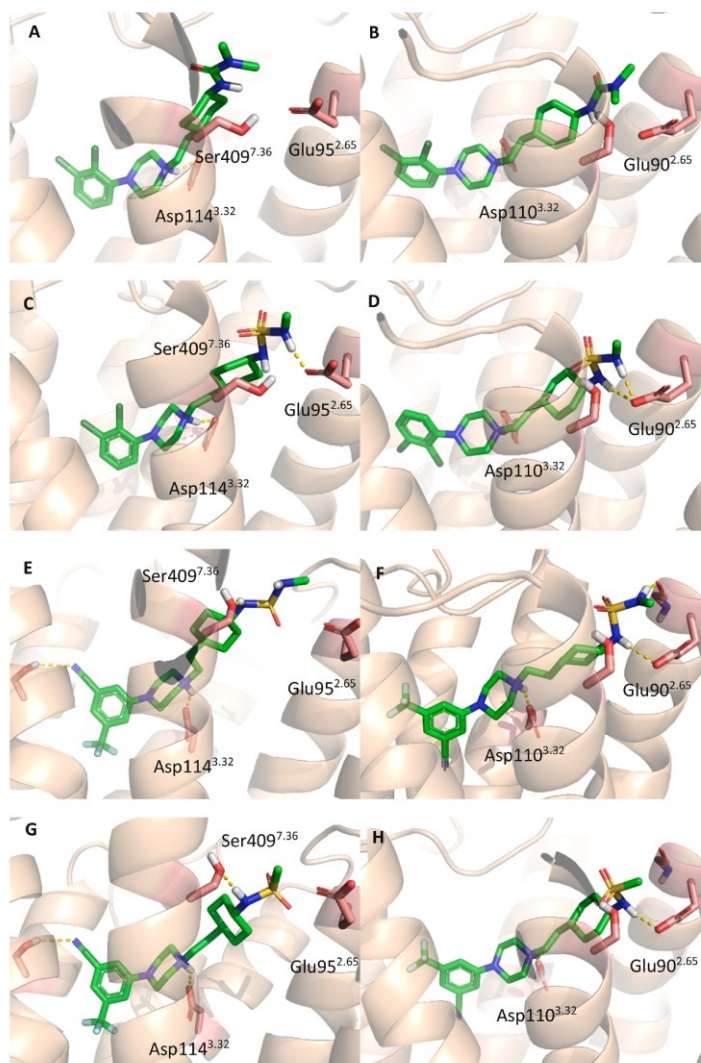


Fig. 3. Binding modes of cariprazine in the active A) D₂R and B) D₃R, **20b** in the active C) D₂R and D) D₃R, **21b** in the inactive E) D₂R and F) D₃R and **22b** in the inactive G) D₂R and H) D₃R obtained as the best-scoring pose from docking. Ligands were docked to structures 6CM4, 3PBL, 7JVR and 9F33 [52–55].

4. Conclusion

The market success of cariprazine can be linked to both its antipsychotic nature, beneficial in the treatment of the positive and negative symptoms of schizophrenia, as well as its antidepressant effect. While significant effort has been invested into clarifying the mechanism of action of antidepressants; in the case of cariprazine, a broad spectrum of receptor pathways makes these efforts harder. We have designed and synthesized over 60 derivatives of cariprazine by changing the orthosteric, linker and secondary moieties of the compounds to better understand their roles in the D₂R and D₃R binding affinity and selectivity, and characterize selected compounds with promising pharmacological profiles. The structure-activity relationship study confirmed the role of the SBP and the linker in achieving selectivity and increased binding affinity on dopamine receptors. Sulfamide derivatives positioned in the SBP emerged as promising binding motifs to enhance selectivity, while the phenyl linker ultimately decreased the affinity towards both D₂R and D₃R. Our functional BRET studies also showed that not only the OBP binder, but also the linker and the SBP binder play important roles in signaling and the functional outcome on the different receptor pathways. These observations, together with data collected previously by the Newman and Stark groups, represent a significant SAR knowledge, suggesting that the receptors are finely modulated by bitopic ligands [78–83]. Modification in any part (orthosteric, linker, secondary region) influences the rigidity and binding mode of the compounds and can consequently tune the selectivity, affinity and the functional profile, either directly through specific interactions, or indirectly through modulating the binding mode of the whole compound.

Depression is estimated to affect over 200 million people worldwide. This disorder is the result of complex neurochemical changes, such as monoamine deficit and neuroinflammation, involving several cytokines, as well as glutamate. The most well-known receptors affected in these processes are D₂, D₃, 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, NMDA and α_2 [84,85]. Therefore, we focused on the antidepressant effect of cariprazine and its derivatives, whose receptor-binding profiles are very complex, and include partial agonism on D₂ and D₃, as well as 5-HT_{1A} receptors and antagonism on 5-HT_{2A} and 5-HT_{2B} and α -receptors [86]. These characteristics make cariprazine suitable for the treatment of neuropsychiatric disorders, but also lead to a variety of side effects. Many extrapyramidal motoric side effects are likely to be linked to D₂R activation, therefore we selected compounds with strong D₃R binding affinities, combined with weaker D₂R affinities for further characterization, and looked for compounds with a cleaner functional profile. This might aid the understanding of the important signaling mechanisms behind the clinically observed antidepressant effect of cariprazine (3), while also benefitting the development of novel drugs with cleaner pharmacological profiles and fewer side effects. Although preclinical and clinical data refer to the antidepressant effect of cariprazine [87,88], it was not effective in the mouse TST. This finding might be the consequence of pharmacodynamic and pharmacokinetic properties of the drug, such as dose-dependent binding affinities to the receptors and metabolic changes. However, the TST experiments confirmed the antidepressant-like effect of one of our cariprazine derivatives built from the 2,3-dichlorophenylpiperazine OBP and *N*-methylsulfamoyl SBP connected with a cyclohexylethyl linker in mice (20b), which shows increased partial agonism on D₃R, but decreased potency on D₂R, compared to cariprazine. Furthermore, it shows some partial agonism on receptors previously linked to antidepressant effects, like 5-HT_{1A}R, 5-HT_{2A}R and 5-HT₇R, while it does not show relevant antagonist effects on off-target receptors linked to side effects, like muscarinic M₁R, M₂R and M₄R receptors, or H₁R, α_1 A (A and B), α_2 A, β_1 A and β_2 A. Together with the reasonable pharmacokinetics and brain penetration, this compound could be a good starting point for further investigations of novel antidepressants.

5. Experimental

5.1. Chemistry

All starting materials were purchased from commercial sources. NMR spectra were recorded at 30 °C, on a Varian Unity Inova 500 spectrometer (500 MHz for ¹H NMR and 125 MHz for ¹³C spectra) or Varian Unity Inova 300 spectrometer (300 MHz for ¹H NMR and 75 MHz for ¹³C spectra), with the deuterium signal of the solvent as the reference. Chemical shifts (δ) and coupling constants (*J*) are given in ppm and Hz, respectively. The solvent of the NMR measurement is indicated for each substance. HPLC-MS measurements were performed using a Shimadzu LCMS-2020 device equipped with a Reprospher 100C18 (5 μ m; 100 $\times$ 3 mm) column and positive-negative double ion source (DUIS) with a quadrupole MS analyser in a range of 50–1000 *m/z*. Sample was eluted with gradient elution using eluent A (0.1 % formic acid in water) and eluent B (0.1 % formic acid in acetonitrile). Flow rate was set to 1 ml/min. The initial condition was 0 % B eluent, followed by a linear gradient to 100 % B eluent by 2 min, from 2 to 3.75 min 100 % B eluent was retained; and from 3.75 to 4.5 min back to initial condition and retained to 5 min. The column temperature was kept at 30 °C and the injection volume was 1 μ l. High-resolution mass spectrometric measurements were performed using a Waters Q-TOF Premier mass spectrometer or Triple TOF 5600+ hybrid Quadrupole-TOF LC/MS/MS system in positive electrospray ionization mode. Here I only present the evaluation of the NMR and MS spectra, the spectra themselves can be viewed in Supplementary Information. For the yield percentage, I have indicated the quantity of products produced at the corresponding purity (>90 %, HPLC). In many cases, the conversion of the reactions was significantly better, but the solubility and other physical properties of the products strongly influenced the extent to which they could be isolated in good yields.

3, 18a, 18b, 20a, 21a, 21c, 22a, 22b, 24a, 24b, 32, and 33 are known compounds. Published descriptions were used for their synthesis [43,47,49,89–91]. The HPLC chromatograms required to verify their adequate purity are shown in the SI. Only the descriptions of new compounds are listed here.

5.2. 1-(*tert*-Butyl)-3-(4-(2-(4-(2,3-dichlorophenyl)piperazin-1-yl)ethyl)cyclohexyl)urea (18d)

Amine 16 (200 mg 0.56 mmol) and 15 ml DCM were measured into a flask. In the next step, triphosgene (166 mg 0.56 mmol) and TEA (311 μ l 2.24 mmol) were added to the suspension. The mixture was stirred at room temperature for 1 h and then *tert*-butylamine hydrochloride (155 mg 2.24 mmol) and a further 311 μ l TEA (2.24 mmol) were added. The suspension was allowed to stir overnight. At the end of the reaction, the mixture was filtered and extracted with 15 ml distilled water. The combined organic phases were dried on MgSO₄ and evaporated. Yield: 253 mg (99 %), HPLC purity: 93.4 %. HRMS [*M*+H]⁺ C₂₃H₃₇N₄OCl₂ expected: 455.2344 measured: 455.2333. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.40–7.31 (m, 2H), 7.21 (dd, *J* = 7.3, 2.3 Hz, 1H), 3.60 (d, *J* = 11.5 Hz, 2H), 3.44 (d, *J* = 12.1 Hz, 2H), 3.32–3.01 (m, 8H), 1.82 (q, *J* = 4.8 Hz, 2H), 1.77–1.69 (m, 2H), 1.60 (dt, *J* = 11.7, 6.8 Hz, 2H), 1.31–1.21 (m, 1H), 1.20 (d, *J* = 6.4 Hz, 9H), 1.01 (dq, *J* = 11.9, 5.5 Hz, 4H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 157.28, 149.93, 133.25, 129.06, 126.60, 125.79, 120.29, 54.35, 51.63, 51.57, 49.32, 48.29, 48.25, 42.25, 34.74, 33.51, 31.71, 30.40, 29.74.

5.3. 1-(4-(2-(4-(3-Cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)cyclohexyl)urea (19a)

Amine 17 (200 mg 0.48 mmol, 1 eq.), 93 mg (1.15 mmol, 2.4 eq.),

KOCN, 4 ml AcOH and 0.8 ml water were measured into a flask. The mixture was stirred at 120 °C for 24 h. The precipitate was filtered. The filtrate was extracted with DCM water and saturated Na₂CO₃, the combined organic phases were dried with MgSO₄, evaporated. The product was obtained as a yellow crystal, 30.4 mg (15 %). HPLC purity: 98.1 %. HRMS [M+H]⁺ C₂₁H₂₈N₅O₃ expected: 423.2366 measured: 423.2366. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.39–7.30 (m, 2H), 7.19 (dd, *J* = 7.6, 2.1 Hz, 1H), 3.58 (d, *J* = 12.0 Hz, 2H), 3.44 (d, *J* = 12.7 Hz, 2H), 3.31–3.13 (m, 5H), 3.02 (t, *J* = 12.2 Hz, 2H), 1.85–1.79 (m, 2H), 1.72 (d, *J* = 12.4 Hz, 2H), 1.56 (dt, *J* = 11.6, 6.9 Hz, 2H), 1.22 (d, *J* = 9.7 Hz, 1H), 1.13–1.05 (m, 2H), 1.00 (t, *J* = 12.1 Hz, 2H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 149.85, 133.23, 129.04, 126.58, 125.82, 120.32, 51.63, 48.37, 34.56, 33.01, 31.52, 30.45.

5.4. 1-(4-(2-(4-(3-Cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)cyclohexyl)-3-methylurea (**19b**)

Amine **17** 200 mg (0.48 mmol, 1 eq.), 10 ml DCM and 0.36 ml (2.54 mmol, 5.3 eq.) TEA were measured into a flask. After stirring for 10 min, 56.4 mg (0.19 mmol, 0.4 eq.) of triphosgene was added and stirred for 1 h at room temperature. 175 mg (2.59 mmol, 5.4 eq.) NH₂Me-HCl and 0.36 ml (2.54 mmol, 5.3 eq.) TEA were added to the solution. The mixture was stirred for 4 h at room temperature. The mixture was filtered. The filtrate was washed with DCM, extracted with DCM water, the combined organic phases were dried with MgSO₄, evaporated. The crude product was purified by flash chromatography: (DCM-MeOH). Yield: 71.3 mg (34 %) white powder. HPLC purity: 96.1 %. HRMS [M+H]⁺ C₂₂H₃₁N₅O₃ expected: 438.2475 measured: 438.2470. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.75 (d, *J* = 2.4 Hz, 1H), 7.59 (d, *J* = 3.4 Hz, 2H), 4.11 (d, *J* = 13.2 Hz, 2H), 3.58 (d, *J* = 11.3 Hz, 2H), 3.29 (tt, *J* = 11.2, 4.0 Hz, 1H), 3.21–3.06 (m, 6H), 2.53 (d, *J* = 0.9 Hz, 3H), 1.86–1.79 (m, 2H), 1.75–1.69 (m, 2H), 1.58 (dt, *J* = 11.7, 6.9 Hz, 2H), 1.28–1.20 (m, 2H), 1.12–0.98 (m, 4H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 150.45, 132.24, 131.98, 131.73, 131.47, 124.79, 122.62, 122.25, 118.94, 118.83, 118.80, 116.28, 116.25, 113.89, 54.37, 50.69, 48.83, 44.87, 34.72, 33.32, 31.64, 30.39, 26.65.

5.5. 3-(4-(2-(4-(3-Cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)cyclohexyl)-1,1-dimethylurea (**19c**)

Amine **17** 200 mg (0.48 mmol, 1 eq.), 10 ml DCM and 0.36 ml (2.54 mmol, 5.3 eq.) TEA were measured into a flask. After 10 min of stirring, 56.4 mg (0.19 mmol, 0.4 eq.) of triphosgene was added and stirred for 1 h at room temperature. Then 209 mg (2.56 mmol, 5.4 eq.) of NHMe₂-HCl and 0.36 ml (2.54 mmol, 5.3 eq.) of TEA were added to the solution. The mixture was stirred at room temperature for 4 h. The reaction mixture was extracted with DCM water, the combined organic phases were dried with MgSO₄, evaporated. The crude was purified by flash chromatography: (DCM-MeOH) yield: 24 mg, (11 %) slightly yellow powder. HPLC purity 96.9 %. HRMS C₂₃H₃₃N₅O₃ [M+H]⁺ expected: 452.2631 measured: 452.2626. ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.63 (t, *J* = 1.8 Hz, 1H), 7.50 (s, 1H), 7.47 (t, *J* = 2.0 Hz, 1H), 5.81 (d, *J* = 7.9 Hz, 1H), 2.75 (s, 6H), 2.37 (d, *J* = 9.3 Hz, 2H), 1.74 (td, *J* = 14.7, 3.6 Hz, 5H), 1.38 (dt, *J* = 14.9, 7.6 Hz, 2H), 1.27–1.13 (m, 3H), 0.96 (qd, *J* = 13.7, 3.9 Hz, 2H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 158.05, 151.73, 131.80, 124.93, 121.46, 118.53, 117.47, 115.21, 113.73, 56.07, 52.71, 49.91, 47.29, 36.35, 35.39, 33.41, 32.41.

5.6. 1-(*tert*-Butyl)-3-(4-(2-(4-(3-cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)cyclohexyl)urea (**19d**)

Amine **17** 200 mg (0.48 mmol, 1 eq.), 10 ml DCM and 0.36 ml (2.54 mmol, 5.3 eq.) TEA were measured into a flask, after stirring for 10 min, 56.4 mg (0.19 mmol, 0.4 eq.) of triphosgene was added to the mixture and stirred for 1 h at room temperature. 280 mg (2.56 mmol, 5.3 eq.) of *t*BuNH₂-HCl and 0.36 ml (2.54 mmol, 5.3 eq.) of TEA were added to the

solution. The mixture was stirred for 4 h at room temperature and was filtered. The reaction solution was extracted with DCM, water. The combined organic phases were dried with MgSO₄, evaporated. The crude product was purified by flash chromatography: (DCM-MeOH). Yield: 32.3 mg (14 %) slightly yellow powder. HPLC purity: 96.1 %. HRMS [M+H]⁺ C₂₅H₃₇N₅O₃ expected: 480.2944 measured: 480.2946. ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.64 (s, 1H), 7.50 (s, 1H), 7.47 (t, *J* = 2.0 Hz, 1H), 5.47–5.42 (m, 2H), 3.27–3.15 (m, 2H), 2.37 (s, 2H), 1.87–1.77 (m, 2H), 1.77–1.68 (m, 2H), 1.37 (q, *J* = 7.2 Hz, 2H), 1.25–1.23 (m, 1H), 1.20 (s, 9H), 1.04–0.89 (m, 4H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 157.26, 121.47, 118.52, 113.73, 56.08, 52.71, 49.36, 48.50, 47.25, 35.26, 33.87, 32.19, 29.82.

5.7. [(4-(2-[4-(2,3-dichlorophenyl)piperazin-1-yl]ethyl)cyclohexyl)sulfamoyl](methyl)amine (**20b**)

Amine **16** 35 mg (0.1 mmol, 1 eq.), 69 µl (0.5 mmol, 5 eq.) of TEA, 39 mg (0.3 mmol, 3 eq.) *N*-methylsulfamoyl chloride and 3 ml of DCM were measured into a flask. The mixture was stirred for 24 h at room temperature. The mixture was filtered and extracted with DCM water, the combined organic phases were dried with MgSO₄, evaporated. The crude product was purified by preparative HPLC (Gemini 5 µm NX-C18 column, water: ACN contain 0.1 % CHOOH, 0 % B eluent to 100 % in 25 min). Yield: 25 mg (56 %) white powder HPLC purity: 97.9 %. HRMS [M+H]⁺ C₁₉H₃₁N₄O₂SCl₂ expected: 449.1544 measured: 449.1537. ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.39–7.30 (m, 2H), 7.21 (dd, *J* = 7.4, 2.2 Hz, 1H), 3.60 (d, *J* = 11.9 Hz, 2H), 3.46 (d, *J* = 12.7 Hz, 2H), 3.20 (qd, *J* = 10.1, 3.9 Hz, 4H), 3.05 (t, *J* = 12.2 Hz, 2H), 2.91 (tt, *J* = 11.5, 4.0 Hz, 1H), 2.44 (s, 3H), 1.93 (dd, *J* = 13.3, 4.0 Hz, 2H), 1.78–1.71 (m, 2H), 1.57 (dt, *J* = 11.6, 6.8 Hz, 2H), 1.21 (ddd, *J* = 15.0, 10.6, 3.3 Hz, 3H), 1.04–0.92 (m, 2H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 149.31, 132.73, 128.44, 126.09, 125.26, 119.73, 53.80, 51.57, 51.11, 47.82, 33.94, 32.77, 31.13, 29.87, 28.25.

5.8. [(4-(2-[4-(2,3-Dichlorophenyl)piperazin-1-yl]ethyl)cyclohexyl)sulfamoyl]dimethylamine (**20c**)

Amine **16** 35 mg (0.1 mmol, 1 eq.), 69 µl (0.5 mmol, 5 eq.) of TEA, 42 mg (0.3 mmol, 3 eq.) *N,N*-dimethylsulfamoyl chloride and 3 ml of DCM were measured into a flask. The mixture was stirred for 24 h at room temperature. The mixture was filtered and extracted with DCM water, the combined organic phases were dried with MgSO₄, evaporated. The crude product was purified by preparative HPLC (Gemini 5 µm NX-C18 column, water: ACN contain 0.1 % CHOOH, 0 % B eluent to 100 % in 25 min). Yield: 20 mg (43 %) slightly yellow powder HPLC purity: 90.9 %. HRMS [M+H]⁺ C₂₀H₃₃N₄O₂SCl₂ expected: 463.1701 measured: 463.1695. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.39–7.31 (m, 2H), 7.20 (dd, *J* = 7.2, 2.4 Hz, 1H), 3.61 (d, *J* = 11.9 Hz, 2H), 3.45 (d, *J* = 12.6 Hz, 2H), 3.24–3.13 (m, 4H), 3.11–3.02 (m, 2H), 2.96 (tt, *J* = 11.4, 3.9 Hz, 1H), 2.64 (s, 6H), 1.92 (dd, *J* = 12.9, 4.3 Hz, 2H), 1.74 (d, *J* = 12.8 Hz, 2H), 1.57 (dt, *J* = 11.5, 6.8 Hz, 2H), 1.29–1.17 (m, 3H), 1.06–0.95 (m, 2H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 149.87, 133.27, 128.98, 126.64, 125.79, 120.25, 54.32, 52.95, 51.63, 48.34, 38.05, 38.04, 34.43, 33.53, 31.55, 30.33.

5.9. *N*-(4-(2-(4-(3-cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)cyclohexyl)-methanesulfonamide (**21b**)

Amine **17** 200 mg (0.48 mmol, 1 eq.), 380 mg (2.93 mmol, 6.1 eq.) of *N*-methylsulfamoyl chloride, 147 µl (1.06 mmol, 2.2 eq.) of TEA and 15 ml of DCM were measured into a flask. The mixture was stirred for 24 h at room temperature. The mixture was extracted with DCM water and saturated Na₂CO₃, the combined organic phases were dried with MgSO₄, evaporated. The crude product (0.14 g) was purified by flash chromatography (DCM:MeOH). Yield: 110 mg (48 %) white powder HPLC purity: 97.6 %. HRMS [M+H]⁺ C₂₁H₃₁N₅O₂SF₃ expected: 474.2150

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measured: 474.2127 ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.65 (s, 1H), 7.52 (s, 1H), 7.48 (s, 1H), 6.74 (d, J = 7.5 Hz, 1H), 6.55–6.48 (m, 1H), 3.38 (s, 4H), 2.93–2.86 (m, 1H), 2.62 (s, 4H), 2.44 (d, J = 5.2 Hz, 4H), 1.90 (d, J = 12.4 Hz, 2H), 1.75 (s, 2H), 1.39 (d, J = 8.1 Hz, 2H), 1.19 (dq, J = 24.4, 12.3 Hz, 4H), 0.94 (q, J = 12.8 Hz, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 221.64, 121.59, 118.50, 115.38, 113.76, 55.74, 52.43, 46.96, 46.22, 34.97, 33.63, 32.12, 28.99.

5.10. Methyl N-[4-(2-(4-(2,3-dichlorophenyl)piperazin-1-yl)ethyl)cyclohexyl]carbamate (23a)

Amine **16** (200 mg 0.56 mmol), triphosgene (166 mg 0.56 mmol) were added into a 20 mL reaction flask and were suspended in 15 mL DCM. Finally, TEA (311 µL, 2.24 mmol) was added to the reaction. The mixture was allowed to stir for 1 h at room temperature and methanol (119 µL, 2.24 mmol) was added dropwise. The suspension was then stirred overnight at room temperature. The mixture was extracted with water and with additional DCM. The combined organic phases were dried over MgSO₄ and evaporated. The crude product was purified by flash chromatography using a (DCM: MeOH) Yield: 138 mg (60 %) white powder. HPLC purity: 97.0 %. HRMS [M+H]⁺ C₂₀H₃₀N₃O₂Cl₂ expected: 414.1715 measured: 414.1709. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.38–7.30 (m, 2H), 7.20 (dd, J = 7.3, 2.3 Hz, 1H), 3.60 (d, J = 11.9 Hz, 2H), 3.50 (s, 3H), 3.45 (d, J = 12.8 Hz, 2H), 3.26–3.13 (m, 5H), 3.10–3.01 (m, 2H), 1.80 (dd, J = 12.8, 3.9 Hz, 2H), 1.76–1.70 (m, 2H), 1.57 (dt, J = 11.6, 6.8 Hz, 2H), 1.28–1.11 (m, 3H), 1.05–0.94 (m, 2H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 156.29, 149.87, 133.26, 129.00, 126.62, 125.79, 120.27, 54.33, 51.63, 51.34, 49.93, 48.34, 34.61, 32.64, 31.52, 30.43.

5.11. Methyl N-[4-(2-(4-[3-cyano-5-(trifluoromethyl)phenyl]piperazin-1-yl)ethyl)cyclohexyl]carbamate (23b)

Amine **17** 200 mg (0.48 mmol, 1 eq.), 15 mL DCM and 274 µL (1.97 mmol, 4.1 eq.) TEA were measured into a flask. After 10 min of stirring, 164 mg (0.55 mmol, 1.1 eq.) of triphosgene was added and the mixture was stirred for 1 h at room temperature. Then 50 µL (1.24 mmol, 2.6 eq.) of methanol was added dropwise. The mixture was stirred for 24 h at room temperature. Then the reaction mixture was extracted with DCM water, the combined organic phases were dried with MgSO₄ and evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 61 mg light brown crystalline (29 %) HPLC purity: 98.2 %. HRMS [M+H]⁺ C₂₂H₃₀N₅O₂F₃ expected: 437.2402 measured: 437.2410. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.76 (s, 1H), 7.60 (s, 2H), 4.11 (d, J = 12.4 Hz, 2H), 3.69–3.40 (m, 5H), 3.15 (h, J = 10.0 Hz, 7H), 1.86–1.68 (m, 4H), 1.59 (dd, J = 10.7, 5.8 Hz, 2H), 1.26–0.91 (m, 6H). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 156.29, 150.44, 132.49, 132.06, 131.62, 131.19, 125.52, 122.25, 118.80, 118.29, 116.27, 113.88, 54.27, 51.36, 50.63, 49.91, 44.84, 34.64, 32.65, 31.50, 30.33.

5.12. 2-Amino-N-(4-(2-(4-(2,3-dichlorophenyl)piperazin-1-yl)ethyl)cyclohexyl)acetamide (25a)

Step 1. Amine **16** (356 mg 1 mmol), 5 mL DCM, EDC-HCl (192 mg 1 mmol), TEA (209 µL 1.5 mmol), DMAP (13 mg 0.1 mmol) and 2-((*tert*-butoxycarbonyl)amino)acetic acid (175 mg 1 mmol) were added into a 20 mL flask. The mixture was stirred for 5 h at room temperature. After, the mixture was extracted with water and EtOAc. The combined organic phases were dried on MgSO₄, evaporated. We carried the raw product to the next step.

Step 2. Into a 100 mL flask was charged a mixture of previous crude product, 15 mL DCM and 5 mL trifluoroacetic acid. This mixture was stirred overnight and then 50 mL of DCM was added. The pH was set up to 10 with 25 % NH₄OH. The mixture was then extracted with water and DCM. The organic phase was dried on MgSO₄ and evaporated. Yield: 256 mg (62 %). HPLC purity: 95.2 %. HRMS [M+H]⁺

C₂₀H₃₁N₄O₃Cl₂ expected: 413.1874 measured: 413.1863. ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.58 (d, J = 8.1 Hz, 1H), 7.34–7.24 (m, 2H), 7.14 (dd, J = 7.1, 2.6 Hz, 1H), 3.49 (dt, J = 11.8, 7.8, 4.0 Hz, 1H), 3.04 (s, 2H), 2.97 (dd, J = 12.4, 7.3 Hz, 5H), 2.35 (t, J = 7.6 Hz, 2H), 1.81–1.71 (m, 5H), 1.36 (q, J = 7.1 Hz, 2H), 1.31–1.12 (m, 4H), 1.04–0.92 (m, 2H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 151.71, 149.82, 133.09, 128.88, 126.47, 124.75, 120.00, 56.16, 53.37, 51.45, 48.11, 45.08, 35.13, 33.90, 32.86, 32.09.

5.13. 2-Amino-N-[4-(2-(4-[3-cyano-5-(trifluoromethyl)phenyl]piperazin-1-yl)ethyl)cyclohexyl]acetamide (25b)

Step 1. Amine **17** 200 mg (0.48 mmol, 1 eq.), 94 mg (0.49 mmol, 1 eq.) EDC-HCl, 7.0 mg (0.06 mmol, 0.1 eq.) DMAP, 147 µL (1.06 mmol, 2.2 eq.) TEA, 2-((*tert*-butoxycarbonyl)amino)acetic acid (175 mg 1 mmol) and 10 mL DCM were added to a flask. The mixture was stirred for 24 h at room temperature. Then the mixture was filtered, the filtrate was extracted with DCM and water. The combined organic phases were dried with MgSO₄, evaporated. We carried the raw product to the next step.

Step 2. 75 mg (0.14 mmol, 1 eq.) crude product, 1 mL TFA and 3 mL DCM were added into a flask. The reaction mixture was stirred for 24 h at room temperature. Then, the pH was set up between pH = 7–8 with saturated Na₂CO₃, extracted with DCM water. The combined organic phases were dried with MgSO₄ and evaporated. Yield: 60.1 mg white powder (28 %). HPLC purity: 96.1 %. HRMS [M+H]⁺ C₂₂H₃₁N₅F₃ expected: 438.2480 measured: 438.2472. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.71 (t, J = 1.8 Hz, 1H), 7.56 (d, J = 2.5 Hz, 1H), 7.53 (s, 1H), 4.09 (d, J = 12.9 Hz, 2H), 3.60–3.46 (m, 5H), 3.22–3.06 (m, 6H), 1.86–1.74 (m, 4H), 1.58 (dt, J = 11.7, 6.8 Hz, 2H), 1.33–0.95 (m, 6H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 164.69, 150.11, 131.75, 131.49, 124.40, 122.23, 121.87, 117.87, 115.88, 113.60, 54.02, 50.39, 48.11, 44.56, 34.21, 31.91, 30.91, 29.95.

5.14. *tert*-Butyl 4-(2-(4-(2,3-dichlorophenyl)piperazin-1-yl)ethyl)phenylcarbamate (29)

First **28** (4.1 g, 13.0 mmol) was dissolved in ACN (75 mL). Then 1-(2,3-dichlorophenyl)piperazine hydrochloride (6.8 g, 4.6 mL 25.4 mmol) and K₂CO₃ (10.5 g, 76.1 mmol) were added to the solution. The reaction mixture was stirred at reflux temperature for 17 h. Then water (90 mL) was added to it and stirred for another 1.5 h. The appeared precipitation was filtered and washed with water until the pH reached 7. Then the crystals were suspended in the mixture of water (45 mL) and cc. HCl (0.75 mL). The reaction mixture was stirred for 2 h. After that the suspension was filtered and the precipitation was washed with water until it became neutral. Yield: 4.34 g (74 %) white powder. HPLC purity: 99.6 %. HRMS [M+H]⁺ C₂₃H₃₀N₃O₂Cl₂ expected: 450.1715 measured: 450.1708. ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.50 (s, 1H), 9.30 (s, 1H), 7.43 (d, J = 8.1 Hz, 2H), 7.41–7.33 (m, 2H), 7.20 (dd, J = 24.4, 7.6 Hz, 3H), 3.64 (d, J = 10.5 Hz, 2H), 3.45 (d, J = 10.9 Hz, 2H), 3.40–3.31 (m, 1H), 3.24 (dq, J = 21.3, 11.2 Hz, 4H), 3.13–2.99 (m, 2H), 1.48 (s, 9H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 153.26, 150.05, 138.74, 133.24, 130.88, 129.33, 129.12, 126.57, 125.76, 120.30, 118.88, 79.44, 56.78, 51.56, 48.20, 29.10, 28.61.

5.15. *tert*-Butyl 4-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)phenylcarbamate (30)

First, **28** (9.57 g, 30.4 mmol) was dissolved in ACN (115 mL). Then 1-(2-methoxyphenyl)piperazine (69) (11.7 g, 60.9 mmol) and K₂CO₃ (8.4 g, 60.9 mmol) were added to the solution. The reaction mixture was stirred at reflux temperature for 17 h. Then water (140 mL) was added to it and stirred for another 1.5 h. The reaction mixture was extracted with EtOAc. Then the combined organic phases were dried on Na₂SO₄ and

evaporated. The crude product was dissolved in the mixture of water (110 mL) and cc. HCl (1.8 mL) and the solution was stirred for 2 h at RT. The solution was extracted with DCM (250 mL). The mixed organic phases were dried over Na₂SO₄ and evaporated. This step was repeated once more. Yield: 9.13 g (73 %) white powder. HPLC purity: 98.5 %. HRMS [M+H]⁺ C₂₄H₃₄N₃O₃ expected: 412.2600 measured: 412.2599. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.46 (d, *J* = 8.1 Hz, 2H), 7.18 (d, *J* = 8.2 Hz, 2H), 7.06–6.87 (m, 4H), 3.80 (s, 3H), 3.64 (d, *J* = 12.0 Hz, 2H), 3.55 (d, *J* = 12.4 Hz, 2H), 3.41–3.34 (m, 2H), 3.26 (t, *J* = 12.2 Hz, 2H), 3.03–2.94 (m, 4H), 1.47 (s, 9H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 153.19, 152.37, 139.68, 138.77, 130.49, 129.29, 118.85, 114.51, 112.33, 112.21, 79.38, 56.91, 55.62, 51.78, 47.52, 29.30, 28.40.

5.16. tert-Butyl(4-(2-(4-(3-cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)phenyl)-carbamate (31)

28 (4.05 g, 12.8 mmol) was dissolved in ACN (75 mL). Then 3-(piperazin-1-yl)-5-(trifluoromethyl)benzonitrile (73) (6.4 g, 25.1 mmol) and K₂CO₃ (6.9 g, 50 mmol) were added to the solution. The reaction mixture was stirred at reflux temperature for 17 h. Then 90 mL of water was added to it and stirred for another 1.5 h. The reaction mixture was extracted with EtOAc. Then the combined organic phases were dried on Na₂SO₄ and evaporated. The crude product was purified with column chromatography (DCM-MeOH). Yield: 1.63 g (27 %) white powder HPLC purity: 99.5 %. HRMS [M+H]⁺ C₂₅H₃₀N₄O₂F₃ expected: 475.2315 measured: 475.2323. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.77 (s, 1H), 7.61 (d, *J* = 10.7 Hz, 2H), 7.44 (d, *J* = 8.1 Hz, 2H), 7.17 (d, *J* = 8.1 Hz, 2H), 4.14 (s, 2H), 3.65 (s, 2H), 3.45–3.10 (m, 7H), 3.01 (dd, *J* = 10.8, 6.2 Hz, 2H), 1.47 (s, 9H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 153.26, 153.18, 150.47, 138.82, 138.71, 132.00, 131.74, 130.66, 129.30, 127.01, 124.81, 122.64, 122.25, 118.90, 118.82, 118.30, 117.17, 116.25, 116.22, 114.86, 113.91, 79.42, 56.73, 50.68, 44.81, 29.21, 25.37.

5.17. 3-(4-(4-Aminophenethyl)piperazin-1-yl)-5-(trifluoromethyl)benzonitrile (34)

31 (11 g 23.2 mmol) was dissolved in the mixture of DCM (200 mL) and TFA (40 mL) and stirred at room temperature for 12 h. Then a mixture of DCM (160 mL) and water (100 mL) was added to the solution during stirring. The pH was set up to 10 with 25 % NH₃ aqueous solution. The mixture was extracted with DCM. The combined organic phases were dried over Na₂SO₄ and evaporated. Yield: 8.6 g (99 %) yellowish powder. HPLC purity: 94.8 %. HRMS[M+H]⁺ C₂₀H₂₂F₃N₄ expected: 375.1791 measured: 374.1822. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.77 (s, 1H), 7.67–7.55 (m, 3H), 7.43 (d, *J* = 8.2 Hz, 2H), 7.37 (d, *J* = 8.0 Hz, 2H), 4.12 (d, *J* = 26.8 Hz, 2H), 3.89–3.81 (m, 1H), 3.67 (s, 1H), 3.46–3.38 (m, 2H), 3.22 (s, 4H), 3.12–3.06 (m, 2H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 150.44, 137.73, 130.56, 124.01, 122.28, 118.27, 116.30, 113.90, 112.00, 56.28, 50.81, 44.86, 42.52, 29.31.

5.18. 1-(4-(2-(4-(2,3-Dichlorophenyl)piperazin-1-yl)ethyl)phenyl)urea (35a)

Amine **32** (200 mg, 0.57 mmol) was dissolved in DCM (17 mL) and TEA (0.285 mL) was added to the mixture. Then the triphosgene (61 mg, 0.21 mmol) dissolved in DCM (2 mL) was added to the solution dropwise. The reaction mixture was stirred for 1 h at rt. After that NH₃ gas bubbled through the system for 2 h at RT. Then, water was added to the solution. The mixture was extracted with water and the combined organic phase was dried over Na₂SO₄. The solvent was evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 24.1 mg (11 %) white powder HPLC purity: 99.2 %. HRMS [M+H]⁺ C₁₉H₂₃N₄OCl₂ expected: 393.1248 measured: 393.1238. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.37 (q, *J* = 7.9 Hz, 4H), 7.27–7.19

(m, 2H), 7.15 (d, *J* = 8.1 Hz, 2H), 3.69 (d, *J* = 11.9 Hz, 2H), 3.49 (d, *J* = 12.9 Hz, 2H), 3.41 (t, *J* = 8.6 Hz, 2H), 3.27 (d, *J* = 11.9 Hz, 3H), 3.08 (t, *J* = 12.3 Hz, 2H), 3.00–2.92 (m, 2H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 172.38, 149.90, 133.25, 129.43, 129.34, 129.09, 126.63, 125.86, 120.40, 118.52, 51.80, 48.39, 29.35, 21.44.

5.19. 1-(4-(2-(4-(2,3-Dichlorophenyl)piperazin-1-yl)ethyl)phenyl)-3-methylurea (35b)

Amine **32** (200 mg, 0.57 mmol) was dissolved in DCM (17 mL) and TEA (0.285 mL) was added to the mixture. Then the triphosgene (61 mg, 0.21 mmol) dissolved in DCM (2 mL) was added to the solution drop-by-drop. The reaction mixture was stirred for 1 h at rt. MeNH₂•HCl (150 mg, 2.22 mmol) and TEA (0.285 mL) were added to the solution and was stirred for 20 h at rt. Water was added to the solution and the two phases were separated. The organic phase was washed with water and dried over Na₂SO₄. The solvent was evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 25 mg (13 %) white powder. HPLC purity: 94.4 %. HRMS [M+H]⁺: C₂₀H₂₅N₄OCl₂ expected: 407.1405 measured: 407.1394. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.47 (d, *J* = 7.9 Hz, 1H), 7.37 (q, *J* = 8.5 Hz, 3H), 7.23 (dd, *J* = 12.5, 7.7 Hz, 2H), 7.14 (d, *J* = 8.1 Hz, 1H), 3.70 (t, *J* = 9.5 Hz, 2H), 3.53–3.38 (m, 4H), 3.29 (q, *J* = 10.4 Hz, 2H), 3.11 (d, *J* = 12.5 Hz, 2H), 2.97 (dt, *J* = 18.0, 8.3 Hz, 2H), 2.64 (s, 1H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 149.89, 133.26, 130.20, 129.51, 129.34, 129.06, 126.64, 125.84, 120.37, 118.84, 56.89, 51.78, 48.38, 29.36, 26.51.

5.20. 3-(4-(2-(4-(2,3-Dichlorophenyl)piperazin-1-yl)ethyl)phenyl)-1,1-dimethylurea (35c)

Amine **32** (200 mg, 0.57 mmol) was dissolved in DCM (17 mL) and TEA (0.285 mL) was added to the mixture. Then the triphosgene (61 mg, 0.21 mmol) dissolved in DCM (2 mL) was added to the solution drop-by-drop. The reaction mixture was stirred for 1 h at RT. Me₂NH•HCl (180 mg, 2.21 mmol) and TEA (0.285 mL) were added to the solution, and was stirred for 20 h at rt. Then, water was added to the solution and the two phases were separated. The organic phase was washed with water and dried over Na₂SO₄. The solvent was evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 39 mg (18 %) white powder HPLC purity: 99.3 %. HRMS [M+H]⁺: C₂₁H₂₇N₄OCl₂ expected: 421.1561 measured: 421.1547. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.45 (d, *J* = 8.2 Hz, 2H), 7.38–7.30 (m, 2H), 7.21 (dd, *J* = 7.2, 2.4 Hz, 1H), 7.15 (d, *J* = 8.3 Hz, 2H), 3.69 (d, *J* = 11.9 Hz, 2H), 3.48 (d, *J* = 12.8 Hz, 2H), 3.46–3.38 (m, 2H), 3.28 (td, *J* = 12.1, 3.0 Hz, 2H), 3.09 (t, *J* = 12.2 Hz, 2H), 3.00–2.93 (m, 2H), 2.91 (s, 6H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 156.19, 149.83, 139.94, 133.29, 129.92, 128.90, 126.67, 125.77, 120.41, 120.23, 56.88, 51.77, 48.32, 36.48, 29.32.

5.21. 1-(tert-Butyl)-3-(4-(2-(4-(2,3-dichlorophenyl)piperazin-1-yl)ethyl)phenyl)urea (35d)

Amine **32** (200 mg, 0.57 mmol) was dissolved in DCM (17 mL) and TEA (0.285 mL) was added to the mixture. Then the triphosgene (61 mg, 0.21 mmol) dissolved in DCM (2 mL) was added to the solution drop-by-drop. The reaction mixture was stirred for 1 h at rt. tBuNH₂•HCl (242 mg, 2.21 mmol) and TEA (0.285 mL) were added to the solution, and it was stirred for 20 h at RT. Then, water was added to the solution and the two phases were separated. The organic phase was washed with water and dried over Na₂SO₄. The solvent was evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 140 mg (50 %) white powder HPLC purity: 97.8 %. HRMS [M+H]⁺ C₂₃H₃₁N₄OCl₂ expected: 449.1874 measured: 449.1848. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.47 (d, *J* = 8.2 Hz, 1H), 7.42–7.28 (m, 3H), 7.23 (dt, *J* = 13.9, 5.5 Hz, 2H), 7.13 (d, *J* = 8.1 Hz, 1H), 3.70 (t, *J* = 9.5 Hz, 2H), 3.54–3.46 (m, 2H), 3.42 (td, *J* = 12.9, 7.8 Hz, 4H), 3.09 (dt, *J* =

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15.8, 7.8 Hz, 2H), 2.96 (dd, $J = 19.5, 9.8$ Hz, 2H), 1.29 (s, 9H). ^{13}C NMR (126 MHz, DMSO- d_6 , TFA- d_1) δ 149.90, 133.25, 129.51, 129.36, 129.08, 126.63, 125.85, 120.39, 118.93, 118.83, 118.09, 51.79, 49.78, 48.39, 29.45, 29.33.

5.22. 1-(4-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl)phenyl)urea (36a)

Amine **33** (200 mg, 0.64 mmol) was dissolved in the mixture of AcOH (2 mL) and water (1.28 mL) and KOCN (104 mg, 1.28 mmol) was added to the solution. Then the reaction mixture was stirred for 2 h at rt. The crude product was purified with flash chromatography (DCM: MeOH) Yield: 180.1 mg (80 %) yellowish-white powder HPLC purity: 98.9 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{20}\text{H}_{27}\text{N}_4\text{O}_2$ expected: 355.2134 measured: 355.2139. ^1H NMR (500 MHz, DMSO- d_6) δ 8.49 (s, 1H), 7.30 (d, $J = 8.1$ Hz, 2H), 7.07 (d, $J = 8.0$ Hz, 2H), 6.92 (d, $J = 7.4$ Hz, 2H), 6.87 (s, 2H), 5.79 (s, 2H), 3.76 (s, 3H), 2.96 (s, 3H), 2.67 (t, $J = 7.8$ Hz, 2H), 2.56 (s, 4H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 156.00, 151.90, 141.24, 138.37, 132.88, 128.59, 122.20, 120.76, 117.80, 117.78, 111.89, 59.98, 55.23, 52.90, 49.98, 32.02.

5.23. 1-(4-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl)phenyl)-3-methylurea (36b)

Amine **33** (200 mg, 0.64 mmol) was dissolved in DCM (15 mL) and TEA (0.260 mL) was added to the mixture. Then the triphosgene (68 mg, 0.23 mmol) dissolved in DCM (2 mL) was added to the solution drop-by-drop. The reaction mixture was stirred for 1 h at rt. MeNH $_2$ •HCl (150 mg, 2.22 mmol) and TEA (0.260 mL) were added to the solution and it was stirred for 20 h at rt. Then, water was added to the solution and the two phases were separated. The organic phase was washed with water and dried over Na $_2$ SO $_4$. The solvent was evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 26.5 mg (11 %) white powder. HPLC purity: 92.8 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{21}\text{H}_{29}\text{N}_4\text{O}_2$ expected: 369.2290 measured: 369.2295. ^1H NMR (500 MHz, DMSO- d_6) δ 7.46 (dd, $J = 15.0, 8.1$ Hz, 2H), 7.42–7.32 (m, 2H), 7.28–7.18 (m, 3H), 4.12 (q, $J = 7.1$ Hz, 2H), 3.69 (d, $J = 11.8$ Hz, 2H), 3.49 (d, $J = 12.9$ Hz, 2H), 3.46–3.38 (m, 2H), 3.28 (t, $J = 11.5$ Hz, 2H), 3.10 (t, $J = 12.2$ Hz, 2H), 2.99 (dt, $J = 8.4, 5.9$ Hz, 2H), 2.51 (p, $J = 1.9$ Hz, 3H), 1.25 (t, $J = 7.1$ Hz, 3H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 158.65, 153.95, 149.91, 138.43, 133.25, 130.86, 129.48, 129.09, 126.62, 125.85, 120.39, 118.86, 60.57, 51.74, 48.36, 29.32, 14.95.

5.24. 3-(4-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl)phenyl)-1,1-dimethylurea (36c)

Amine **33** (200 mg, 0.64 mmol) was dissolved in DCM (15 mL) and TEA (0.260 mL) was added to the mixture. Then the triphosgene (68 mg, 0.23 mmol) dissolved in DCM (2 mL) was added to the solution drop-by-drop. The reaction mixture was stirred for 1 h at RT. After that Me $_2$ NH•HCl (180 mg, 2.21 mmol) and TEA (0.260 mL) were added to the solution and it was stirred for 20 h at RT. Then, water was added to the solution and the two phases were separated. The organic phase was washed with water and dried over Na $_2$ SO $_4$. The solvent was evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 42 mg (18 %) white powder HPLC purity: 99.6 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{22}\text{H}_{31}\text{N}_4\text{O}_2$ expected: 383.2447 measured: 383.2434. ^1H NMR (500 MHz, DMSO- d_6 , TFA- d_1) δ 8.13 (s, 1H), 7.46 (d, $J = 8.0$ Hz, 2H), 7.21 (d, $J = 8.1$ Hz, 2H), 7.07–6.89 (m, 4H), 3.81 (s, 3H), 3.66 (d, $J = 11.5$ Hz, 2H), 3.56 (d, $J = 12.6$ Hz, 2H), 3.43–3.36 (m, 2H), 3.26 (s, 1H), 2.99 (td, $J = 10.0, 5.7$ Hz, 4H), 2.51 (p, $J = 1.8$ Hz, 6H). ^{13}C NMR (126 MHz, DMSO- d_6 , TFA- d_1) δ 163.24, 152.37, 139.67, 130.26, 129.49, 124.07, 121.30, 118.91, 118.84, 118.81, 112.03, 56.90, 55.75, 51.78, 47.55, 29.31.

5.25. 1-(tert-Butyl)-3-(4-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)phenyl)urea (36d)

Amine **33** (200 mg, 0.64 mmol) was dissolved in DCM (17 mL) and TEA (0.260 mL) was added to the mixture. Then the triphosgene (68 mg, 0.23 mmol) dissolved in DCM (2 mL) was added to the solution drop-by-drop. The reaction mixture was stirred for 1 h at RT. tBuNH $_2$ •HCl (250 mg, 2.28 mmol) and TEA (0.300 mL) were added to the solution, and it was stirred for 20 h at rt. Then, water was added to the solution and the two phases were separated. The organic phase was washed with water and dried over Na $_2$ SO $_4$. The solvent was evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 23.6 mg (9 %) white powder HPLC purity: 98.0 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{24}\text{H}_{35}\text{N}_4\text{O}_2$ expected: 411.2760 measured: 411.2765. ^1H NMR (500 MHz, CD $_3$ OD) δ 7.23 (d, $J = 8.1$ Hz, 2H), 7.13–7.08 (m, 2H), 7.03–6.86 (m, 4H), 3.84 (d, $J = 1.2$ Hz, 3H), 3.08 (s, 4H), 2.84–2.68 (m, 6H), 2.63 (ddd, $J = 11.0, 5.3, 1.9$ Hz, 2H), 1.35 (d, $J = 1.0$ Hz, 9H). ^{13}C NMR (126 MHz, CD $_3$ OD) δ 156.07, 152.51, 140.80, 137.91, 133.39, 128.58, 123.23, 120.80, 118.86, 118.05, 111.50, 60.23, 54.57, 52.87, 50.07, 49.65, 31.86, 28.27.

5.26. 1-(4-(2-(4-(3-Cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)phenyl)urea (37a)

Amine **34** (200 mg, 0.53 mmol) was dissolved in the mixture of AcOH (2 mL) and water (1 mL) and KOCN (86 mg, 1.06 mmol) was added to the solution. Then the reaction mixture was stirred for 2 h at rt. The crude product was purified with flash chromatography (DCM: MeOH) Yield: 120.0 mg (45 %) white powder. HPLC purity: 98.7 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{21}\text{H}_{23}\text{N}_5\text{O}_3$ expected: 418.1854 measured: 418.1830. ^1H NMR (500 MHz, DMSO- d_6) δ 8.43 (s, 1H), 7.63 (s, 1H), 7.47 (d, $J = 13.5$ Hz, 2H), 7.29 (d, $J = 8.0$ Hz, 2H), 7.07 (d, $J = 8.0$ Hz, 2H), 5.76 (s, 2H), 2.68 (t, $J = 7.8$ Hz, 2H), 2.55 (t, $J = 5.1$ Hz, 4H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ^{13}C NMR (126 MHz, DMSO- d_6) δ 156.51, 151.81, 138.93, 133.30, 131.79, 131.53, 129.16, 124.93, 121.44, 119.51, 118.53, 118.33, 117.38, 115.18, 115.16, 113.72, 60.17, 52.66, 47.40, 32.52.

5.27. 1-(4-(2-(4-(3-Cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)phenyl)-3-methylurea (37b)

Amine **34** (200 mg, 0.53 mmol) was dissolved in DCM (16 mL) and TEA (0.265 mL) was added to the mixture. Then the triphosgene (57 mg, 0.19 mmol) dissolved in DCM (2 mL) was added to the solution drop-by-drop. The reaction mixture was stirred for 1 h at RT. MeNH $_2$ •HCl (140 mg, 2.07 mmol) and TEA (0.265 mL) were added to the solution, and it was stirred for 20 h at RT. Then, water was added to the solution and the two phases were separated. The organic phase was washed with water and dried over Na $_2$ SO $_4$. The solvent was evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 35.9 mg (13 %) yellowish-white powder HPLC purity: 97.5 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{22}\text{H}_{25}\text{N}_5\text{O}_3$ expected: 432.2011 measured: 432.1991. ^1H NMR (500 MHz, DMSO- d_6) δ 9.09 (s, 1H), 7.63 (s, 1H), 7.46 (d, $J = 11.1$ Hz, 2H), 7.29 (d, $J = 8.2$ Hz, 2H), 7.04 (d, $J = 8.1$ Hz, 2H), 6.51 (q, $J = 4.6$ Hz, 1H), 3.32 (t, $J = 5.1$ Hz, 3H), 2.66 (t, $J = 7.8$ Hz, 1H), 2.60 (d, $J = 4.6$ Hz, 2H), 2.56 (s, 7H). ^{13}C NMR (126 MHz, DMSO- d_6) δ 156.68, 151.79, 139.30, 132.75, 131.77, 129.09, 121.45, 118.51, 117.92, 115.19, 113.70, 60.14, 52.63, 47.36, 45.74, 32.48, 26.42, 9.25.

5.28. 3-(4-(2-(4-(3-Cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)phenyl)-1,1-dimethylurea (37c)

Amine **34** (200 mg, 0.53 mmol) was dissolved in DCM (16 mL) and TEA (0.265 mL) was added to the mixture. Then the triphosgene (57 mg, 0.19 mmol) dissolved in DCM (2 mL) was added to the solution drop-by-drop. The reaction mixture was stirred for 1 h at RT. Me $_2$ NH•HCl (168

mg, 2.06 mmol) and TEA (0.205 mL) were added to the solution, and it was stirred for 20 h at rt. Then, water was added to the solution and the two phases were separated. The organic phase was washed with water and dried over Na_2SO_4 . The solvent evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 101 mg (35 %) white powder. HPLC purity: 99.9 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{23}\text{H}_{27}\text{N}_5\text{OF}_3$ expected: 446.2167 measured: 446.2145. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 9.50 (s, 1H), 8.16 (s, 1H), 7.65 (s, 1H), 7.49 (d, $J = 15.3$ Hz, 2H), 7.20–7.13 (m, 3H), 7.09 (d, $J = 8.1$ Hz, 2H), 3.34 (t, $J = 5.1$ Hz, 4H), 2.72 (dd, $J = 9.3, 6.4$ Hz, 2H), 2.60–2.52 (m, 8H), 2.46 (d, $J = 4.9$ Hz, 2H). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 151.80, 137.24, 134.98, 131.81, 131.54, 130.09, 129.57, 121.48, 119.12, 118.54, 117.43, 115.23, 113.73, 59.98, 52.62, 47.38, 32.40, 28.71.

5.29. 1-(tert-Butyl)-3-(4-(2-(4-(3-cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)phenyl)urea (37d)

Amine **34** (200 mg, 0.53 mmol) was dissolved in DCM (16 mL) and TEA (0.265 mL) was added to the mixture. Then the triphosgene (57 mg, 0.19 mmol) dissolved in DCM (2 mL) was added to the solution drop-by-drop. The reaction mixture was stirred for 1 h at RT. $\text{tBuNH}_2\cdot\text{HCl}$ (226 mg, 2.06 mmol) and TEA (0.265 mL) were added to the solution, and it was stirred for 20 h at RT. Then, water was added to the solution and the two phases were separated. The organic phase was washed with water and dried over Na_2SO_4 . The solvent evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 150 mg (60 %) white powder HPLC purity: 96.3 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{25}\text{H}_{31}\text{N}_5\text{OF}_3$ expected: 474.2480 measured: 474.2454. ^1H NMR (500 MHz, CD_3OD) δ 7.49–7.47 (m, 1H), 7.44–7.40 (m, 1H), 7.34 (s, 1H), 7.22 (dd, $J = 8.5, 2.0$ Hz, 2H), 7.13–7.08 (m, 2H), 3.30 (dq, $J = 3.3, 1.8$ Hz, 3H), 2.81–2.75 (m, 2H), 2.73–2.68 (m, 5H), 2.68–2.60 (m, 2H), 1.35 (d, $J = 1.9$ Hz, 9H). ^{13}C NMR (126 MHz, CD_3OD) δ 156.08, 151.73, 137.89, 133.40, 128.57, 120.74, 118.84, 117.56, 117.34, 114.91, 113.80, 60.00, 52.34, 49.64, 31.99, 28.24, 27.47.

5.30. N-(4-{2-[4-(2,3-Dichlorophenyl)piperazin-1-yl]ethyl}phenyl)-aminosulfonamide (38a)

Amine **32** (200 mg, 0.57 mmol) was dissolved in DCM (6 mL) and TEA (0.116 mL) and $\text{NH}_2\text{SO}_2\text{Cl}$ (97 mg, 0.84 mmol) were added to the mixture. Then it was stirred at rt for 24 h. The product was filtered and dried. Yield: 175 mg (7 %) white powder HPLC purity: 98.1 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{18}\text{H}_{23}\text{N}_4\text{O}_2\text{SCl}_2$ expected: 429.0918 measured: 429.0900. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 9.43 (s, 1H), 7.38–7.32 (m, 2H), 7.20–7.17 (m, 2H), 7.16–7.12 (m, 2H), 7.05 (s, 1H), 3.43–3.20 (m, 12H), 3.09–2.95 (m, 2H). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 149.50, 138.14, 132.67, 130.45, 128.96, 128.56, 125.99, 125.18, 119.71, 118.42, 56.34, 51.03, 47.68, 45.28, 28.56.

5.31. [(4-{2-[4-(2,3-Dichlorophenyl)piperazin-1-yl]ethyl}phenyl)sulfamoyl](methyl)amine (38b)

Amine **32** (212 mg, 0.64 mmol) was dissolved in DCM (5 mL) and TEA (0.278 mL) and methylsulfamoyl chloride (0.06 mL, 0.7 mmol) were added to the mixture. Then it was stirred at RT overnight. The solvent was evaporated and the crude product was purified with flash chromatography (DCM: MeOH). Yield: 83.3 mg (30 %) white powder HPLC purity: 96.3 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{19}\text{H}_{25}\text{N}_4\text{O}_2\text{SCl}_2$ expected: 443.1075 measured: 443.1053. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 9.48 (s, 1H), 7.32–7.26 (m, 2H), 7.15 (dt, $J = 6.9, 2.4$ Hz, 4H), 7.10–7.06 (m, 2H), 2.99 (t, $J = 4.8$ Hz, 4H), 2.70 (dd, $J = 9.3, 6.3$ Hz, 2H), 2.61 (s, 4H), 2.55 (t, $J = 7.8$ Hz, 1H), 2.45 (d, $J = 5.0$ Hz, 3H). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 151.12, 136.64, 134.52, 132.52, 129.00, 128.34, 125.91, 124.23, 119.48, 118.58, 59.57, 52.64, 50.83, 31.87, 28.15.

5.32. [(4-{2-[4-(2,3-Dichlorophenyl)piperazin-1-yl]ethyl}phenyl)sulfamoyl]dimethyl-amine (38c)

Amine **32** (212 mg, 0.64 mmol) was dissolved in DCM (5 mL) and TEA (0.278 mL) and dimethylsulfamoyl chloride (0.107 mL, 0.7 mmol) were added to the mixture. Then it was stirred at RT overnight. The solvent was evaporated, and the crude product was purified with flash chromatography (DCM: MeOH). Yield: 45 mg (15 %) white powder. HPLC purity: 98.1 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{21}\text{H}_{26}\text{N}_4\text{OCl}_2$ expected: 419.2116 measured: 419.2090. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 9.84 (s, 1H), 7.19 (q, $J = 8.3$ Hz, 3H), 7.00 (dt, $J = 18.5, 7.8$ Hz, 2H), 6.96–6.87 (m, 2H), 3.79 (s, 3H), 3.58 (s, 2H), 3.49 (s, 2H), 3.20 (s, 2H), 3.03 (d, $J = 8.9$ Hz, 2H), 2.69 (s, 4H). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 163.10, 151.77, 139.40, 129.22, 123.33, 120.77, 119.60, 118.18, 111.90, 55.30, 51.20, 46.97, 37.61.

5.33. tert-Butyl[(4-{2-[4-(2,3-dichlorophenyl)piperazin-1-yl]ethyl}phenyl)sulfamoyl]amine (38d)

Amine **32** (212 mg, 0.64 mmol) was dissolved in DCM (5 mL) and TEA (0.278 mL) and tert-butylsulfamoyl chloride (0.09 mL, 0.7 mmol) were added to the mixture. Then it was stirred at rt. overnight. The solvent was evaporated and the crude product was purified with flash chromatography (DCM:MeOH). Yield: 21.7 mg (7 %) yellowish powder HPLC purity: 90.1 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{22}\text{H}_{31}\text{N}_4\text{O}_2\text{SCl}_2$ expected: 485.1544 measured: 485.1516. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 9.41 (s, 1H), 7.30 (d, $J = 7.0$ Hz, 2H), 7.19–7.10 (m, 5H), 7.06 (d, $J = 8.1$ Hz, 2H), 3.00 (s, 4H), 2.71 (t, $J = 7.8$ Hz, 2H), 2.62 (s, 2H), 2.61–2.56 (m, 2H), 1.17 (s, 9H). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 151.67, 151.00, 137.83, 133.10, 129.37, 128.90, 124.81, 120.04, 117.81, 60.15, 53.18, 53.08, 51.36, 32.43, 29.82.

5.34. N-(4-{2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl}phenyl)aminosulfonamide (39a)

Amine **33** (200 mg, 0.64 mmol) was dissolved in DCM (6 mL) and TEA (0.134 mL) and $\text{NH}_2\text{SO}_2\text{Cl}$ (110 mg, 0.95 mmol) were added to the mixture. Then it was stirred at rt. for 24 h. During the reaction precipitation appeared in the mixture. It was filtered and dried. Yield: 70 mg (28 %) white powder. HPLC purity: 94.3 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{19}\text{H}_{27}\text{N}_4\text{O}_3\text{S}$ expected: 391.1803 measured: 391.1807. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 9.43 (s, 1H), 7.18 (d, $J = 8.4$ Hz, 2H), 7.14 (d, $J = 8.4$ Hz, 2H), 7.05 (s, 2H), 7.03–6.96 (m, 2H), 6.96–6.88 (m, 2H), 3.79 (d, $J = 1.0$ Hz, 3H), 3.35–3.24 (m, 2H), 3.08–3.01 (m, 2H). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 152.31, 139.98, 138.69, 131.06, 129.50, 123.87, 121.33, 118.98, 112.45, 56.96, 55.86, 51.64, 47.39, 45.81, 29.10.

5.35. [(4-{2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl}phenyl)sulfamoyl](methyl)amine (39b)

Amine **33** (200 mg, 0.64 mmol) was dissolved in DCM (5 mL) and TEA (0.278 mL) and methylsulfamoyl chloride (0.06 mL, 0.7 mmol) were added to the mixture. Then it was stirred at RT overnight. The solvent was evaporated and the crude product was purified with flash chromatography (DCM: MeOH) and with preparative TLC (CHCl_3 : MeOH -10:1 + 1 % TEA) Yield: 28.5 mg (11 %) white powder HPLC purity: 99.0 %. HRMS $[\text{M}+\text{H}]^+$ $\text{C}_{20}\text{H}_{29}\text{N}_4\text{O}_3\text{S}$ expected: 405.1960 measured: 405.1956. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 7.77 (s, 1H), 7.67–7.55 (m, 3H), 7.43 (d, $J = 8.2$ Hz, 2H), 7.37 (d, $J = 8.0$ Hz, 2H), 4.23–3.92 (m, 3H), 3.91–3.80 (m, 2H), 3.61 (d, $J = 64.3$ Hz, 2H), 3.46–3.36 (m, 2H), 3.22 (s, 6H), 3.13–3.05 (m, 2H), 2.99 (s, 3H). ^{13}C NMR (126 MHz $\text{DMSO}-d_6$, TFA- d_1) δ 150.44, 137.73, 130.56, 124.01, 122.28, 118.27, 116.58, 116.30, 113.90, 56.28, 50.81, 44.86, 42.52, 29.31.

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5.36. [(4-(2-[4-(2-Methoxyphenyl)piperazin-1-yl]ethyl)phenyl)sulfamoyl]dimethylamine (39c)

Amine **33** (200 mg, 0.64 mmol) was dissolved in DCM (5 mL) and TEA (0.278 mL) and dimethylsulfamoyl chloride (0.107 mL, 0.7 mmol) were added to the mixture. Then it was stirred at RT overnight. The solvent was removed under reduced pressure and the crude product was purified with flash chromatography (DCM:MeOH). Yield: 64 mg (24 %) white powder HPLC purity: 99.5 %. HRMS $C_{21}H_{30}N_4O_3S$ expected: 419.2101 measured: 419.2090. 1H NMR (500 MHz, DMSO- d_6) δ 9.86 (s, 1H), 8.15 (s, 1H), 7.21 (q, J = 8.3 Hz, 4H), 7.08–6.89 (m, 4H), 3.81 (s, 3H), 3.64 (d, J = 11.9 Hz), 3.54 (d, J = 12.6 Hz, 2H), 3.36 (t, J = 8.5 Hz, 2H), 3.25 (d, J = 12.8 Hz, 2H), 2.70 (s, 6H), 2.55 (s, 1H) ^{13}C NMR (126 MHz, DMSO- d_6) δ 163.42, 152.36, 139.85, 137.89, 132.20, 129.81, 123.99, 121.34, 120.17, 118.81, 112.47, 56.76, 55.87, 51.76, 47.49, 29.20.

5.37. tert-Butyl[(4-(2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl)phenyl)sulfamoyl]amine (39d)

Amine **33** (200 mg, 0.64 mmol) was dissolved in DCM (5 mL) and TEA (0.278 mL) and *N*-tert-butylsulfamoyl chloride (0.09 mL, 0.7 mmol) were added to the mixture. Then it was stirred at rt. overnight. The solvent was evaporated and the crude product was purified with flash chromatography (DCM:MeOH) and with preparative TLC (SiO₂, eluent: EtOAc+1 % TEA) Yield: 22.9 mg (8 %) yellowish-white powder. HPLC purity: 93.2 %. HRMS $[M+H]^+$ $C_{23}H_{35}N_4O_3S$ expected: 447.2424 measured: 447.2415. 1H NMR (500 MHz, DMSO- d_6 , TFA- d_1) δ 7.34–7.16 (m, 2H), 7.12 (d, J = 8.3 Hz, 1H), 7.08–7.01 (m, 1H), 7.01–6.95 (m, 1H), 6.92 (t, J = 7.5 Hz, 1H), 3.81 (s, 3H), 3.64 (d, J = 10.1 Hz, 2H), 3.55 (d, J = 12.4 Hz, 2H), 3.38 (dq, J = 17.0, 9.6 Hz, 2H), 3.25 (s, 2H), 3.08–2.90 (m, 4H), 1.17 (s, 9H). ^{13}C NMR (75 MHz, DMSO- d_6 , TFA- d_1) δ 163.32, 126.80, 118.79, 117.40, 116.03, 114.34, 112.37, 72.41, 55.76, 51.75, 47.51, 29.82.

5.38. N-(4-(2-(4-(3-Cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)phenyl)-aminosulfonamide (40a)

Amine **34** (200 mg, 0.53 mmol) was dissolved in DCM (6 mL) and TEA (0.112 mL) and NH_2SO_2Cl (92 mg, 0.80 mmol) were added to the mixture. Then it was stirred at RT for 24 h. During the reaction precipitation appeared in the mixture. It was filtered and the crude product was purified with flash chromatography (DCM: MeOH). Yield: 68 mg (28 %) white powder. HPLC purity: 98.9 %. HRMS $[M+H]^+$ $C_{20}H_{23}N_5O_2F_3S$ expected: 454.1524 measured: 454.1515. 1H NMR (500 MHz, DMSO- d_6) δ 8.14 (s, 1H), 8.02 (s, 1H), 7.68 (d, J = 8.7 Hz, 1H), 7.60–7.48 (m, 2H), 7.18–6.93 (m, 4H), 5.26 (s, 2H), 3.53–3.38 (m, 5H), 2.77 (s, 5H). ^{13}C NMR (126 MHz, DMSO- d_6 , TFA- d_1) δ 150.43, 138.70, 134.91, 130.58, 129.48, 127.80, 122.22, 120.37, 118.97, 116.33, 115.46, 113.91, 56.79, 54.35, 50.80, 44.86, 29.21.

5.39. N-(4-(2-(4-(3-cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)phenyl)-methanesulfonamide (40b)

Amine **34** (200 mg, 0.53 mmol) was dissolved in DCM (15 mL) and TEA (0.161 mL) and MsCl (0.089 mL, 1.15 mmol) were added to the mixture. The solution was stirred for 2 h at reflux temperature. Then further MsCl (0.045 mL, 0.31 mmol) and TEA (0.081 mL) were added to the mix. It was stirred overnight at RT. Water was added to the solution. The two phases were separated. The aqueous phase was washed with DCM and the combined organic phase was washed with 5 % HCl aqueous solution. Organic phases were dried over Na_2SO_4 and the solvent was evaporated. The crude product was purified with flash chromatography (DCM: MeOH). Yield: 200 mg (68 %) white powder HPLC purity: 97.6 %. HRMS $[M+H]^+$ $C_{21}H_{25}N_5O_2F_3S$ expected: 468.1681 measured: 468.1667. 1H NMR (500 MHz, DMSO- d_6 , TFA- d_1) δ 7.19 (d, J

= 8.3 Hz, 2H), 7.12 (d, J = 8.3 Hz, 2H), 7.04–6.97 (m, 1H), 6.94 (dd, J = 7.8, 1.5 Hz, 2H), 6.91–6.85 (m, 1H), 3.76 (s, 3H), 3.44 (d, J = 7.1 Hz, 3H), 3.35–3.07 (m, 4H), 3.11–2.91 (m, 2H), 2.44 (s, 3H). ^{13}C NMR (126 MHz, DMSO- d_6 , TFA- d_1) δ 152.30, 139.24, 137.92, 131.15, 129.72, 124.36, 121.24, 119.27, 118.80, 112.25, 57.09, 55.51, 51.87, 47.58, 29.16, 28.43.

5.40. 3-[4-(2-{4-[(Methylsulfamoyl)amino]phenyl}ethyl)piperazin-1-yl]-5-(trifluoromethyl)benzonitrile (40c)

Amine **34** (239.6 mg, 0.64 mmol) was dissolved in DCM (5 mL) and TEA (0.278 mL) and *N*-methylsulfamoyl chloride (0.06 mL, 0.7 mmol) were added to the mixture. Then it was stirred at rt. overnight. The solvent was evaporated, and the crude product was purified with flash chromatography (DCM: MeOH). Yield: 29.9 mg (10 %) white powder. HPLC purity: 90.5 %. HRMS $[M+H]^+$ $C_{22}H_{27}N_5O_2F_3S$ expected: 482.1837 measured: 482.1839. 1H NMR (500 MHz, DMSO- d_6 , TFA- d_1) δ 8.13 (s, 1H), 7.77 (s, 1H), 7.61 (d, J = 3.9 Hz, 2H), 7.20 (q, J = 8.4 Hz, 4H), 4.15 (d, J = 10.1 Hz, 2H), 3.69–3.63 (m, 2H), 3.38 (dd, J = 10.6, 6.5 Hz, 2H), 3.22–3.20 (m, 2H), 3.03–2.96 (m, 2H), 2.69 (s, 6H). ^{13}C NMR (126 MHz, DMSO- d_6 , TFA- d_1) δ 163.25, 150.46, 137.84, 132.03, 129.78, 124.74, 124.00, 122.28, 120.11, 118.29, 116.31, 113.90, 56.63, 50.78, 44.85, 38.08, 29.21.

5.41. 3-[4-(2-{4-[(tert-Butylsulfamoyl)amino]phenyl}ethyl)piperazin-1-yl]-5-(trifluoromethyl)benzonitrile (40d)

Amine **34** (239.6 mg, 0.64 mmol) was dissolved in DCM (5 mL) and TEA (0.278 mL) and *N*-tert-butylsulfamoyl chloride (0.09 mL, 0.7 mmol) were added to the mixture. Then it was stirred at RT overnight. The solvent was removed under reduced pressure and the crude product was purified with flash chromatography (DCM: MeOH). Yield: 29.4 mg (9 %) white powder HPLC purity: 93.2 %. HRMS $[M+H]^+$ $C_{24}H_{31}N_5O_2F_3S$ expected: 510.2150 measured: 510.2164. 1H NMR (500 MHz, DMSO- d_6 , TFA- d_1) δ 8.13 (s, 1H), 7.77 (s, 1H), 7.61 (d, J = 3.9 Hz, 2H), 7.20 (q, J = 8.4 Hz, 4H), 4.15 (d, J = 10.1 Hz, 2H), 3.69–3.63 (m, 2H), 3.38 (dd, J = 10.6, 6.5 Hz, 2H), 3.22–3.20 (m, 2H), 3.03–2.96 (m, 2H), 2.69 (s, 6H). ^{13}C NMR (126 MHz, DMSO- d_6 , TFA- d_1) δ 163.25, 150.46, 137.84, 132.03, 129.78, 124.74, 124.00, 122.28, 120.11, 118.29, 116.31, 113.90, 56.63, 50.78, 44.85, 38.08, 29.21.

5.42. N-(4-(2-(4-(2,3-Dichlorophenyl)piperazin-1-yl)ethyl)phenyl)methanesulfonamide (41)

Amine **32** (200 mg, 0.57 mmol) was dissolved in DCM (20 mL) and TEA (0.086 mL) and MsCl (0.048 mL, 0.62 mmol) were added to the mixture. The solution was stirred for 2 h at reflux temperature. Then further MsCl (0.048 mL, 0.32 mmol) and TEA (0.086 mL) were added to the mixture. It was stirred for another 2 h. Product was filtered and dried. Yield: 30.0 mg (12 %) white powder. HPLC purity: 95.1 %. HRMS $[M+H]^+$ $C_{19}H_{24}N_3O_2S_2Cl_2$ expected: 428.0966 measured: 428.0945. 1H NMR (500 MHz, Methanol- d_4) δ 7.34–7.29 (m, 4H), 7.25 (d, J = 8.6 Hz, 2H), 7.18 (dt, J = 7.1, 1.9 Hz, 1H), 3.64 (d, J = 95.3 Hz, 3H), 3.49–3.42 (m, 3H), 3.42–3.31 (m, 3H), 3.18 (d, J = 20.1 Hz, 2H), 3.15–3.08 (m, 2H), 2.95 (d, J = 1.2 Hz, 3H). ^{13}C NMR (126 MHz, Methanol- d_4) δ 149.23, 145.13, 137.34, 129.46, 127.94, 125.74, 120.78, 119.15, 57.48, 56.05, 52.21, 37.84, 29.22.

5.43. N-(4-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl)phenyl)methanesulfonamide (42)

Amine **33** (200 mg, 0.64 mmol) was dissolved in DCM (15 mL) and TEA (0.191 mL) and MsCl (0.106 mL, 1.37 mmol) were added to the mixture. The solution was stirred for 2 h at reflux temperature. Product was filtered and dried. Yield: 49.8 mg (20 %) yellowish-white powder. HPLC purity: 95.4 %. HRMS $C_{20}H_{28}N_3O_3S$ expected: 390.1851

measured: 390.1843. ¹H NMR (500 MHz, DMSO-*d*₆, TFA-*d*₁) δ 7.27 (d, *J* = 8.2 Hz, 2H), 7.22 (d, *J* = 8.2 Hz, 2H), 7.07–6.89 (m, 4H), 3.81 (s, 3H), 3.66 (d, *J* = 11.9 Hz, 2H), 3.55 (d, *J* = 12.8 Hz, 2H), 3.40 (dq, *J* = 9.2, 4.7 Hz, 2H), 3.25 (s, 1H), 3.04–2.98 (m, 2H), 2.97 (s, 4H). ¹³C NMR (126 MHz, DMSO-*d*₆, TFA-*d*₁) δ 152.38, 139.76, 137.55, 132.81, 130.05, 124.03, 121.31, 120.81, 118.83, 112.43, 56.70, 55.79, 51.79, 47.54, 39.55, 29.27.

5.44. 3-[4-(2-(4-[(Dimethylsulfamoyl)amino]phenyl)ethyl)piperazin-1-yl]-5-(trifluoromethyl)-benzonitrile (43)

Amin **34** (239.6 mg, 0.64 mmol) was dissolved in DCM (5 mL) and TEA (0.278 mL) and dimethylsulfamoyl chloride (0.107 mL, 0.7 mmol) were added to the mixture. Then it was stirred at r.t. overnight. The mixture was extracted DCM, water. The combined organic phases were dried over Na₂SO₄ and the solvent was evaporated. The crude product was purified with flash chromatography (DCM: MeOH). The crude product was purified with preparative TLC (SiO₂, eluent: DCM:MeOH-20:1 + 1 % TEA). Yield: 21.6 mg (7 %) white powder. HPLC purity: 96.5 %. HRMS [M+H]⁺ C₂₁H₂₄F₃N₄O₂S expected: 453.1566 measured: 453.1560. ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.65 (s, 1H), 7.49 (d, *J* = 15.1 Hz, 2H), 7.22 (d, *J* = 8.2 Hz, 2H), 7.13 (d, *J* = 8.1 Hz, 2H), 3.33 (d, *J* = 5.5 Hz, 4H), 2.94 (s, 3H), 2.74 (t, *J* = 7.7 Hz, 2H), 2.58 (t, *J* = 5.1 Hz, 4H), 2.55 (s, 3H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 151.81, 136.65, 131.76, 129.91, 121.48, 120.81, 118.54, 117.41, 115.46, 113.73, 59.88, 52.63, 47.41, 32.45, 29.01.

5.45. N-(4-(2-(4-(2,3-Dichlorophenyl)piperazin-1-yl)ethyl)phenyl)acetamide (44)

Amine **32** (200 mg, 0.57 mmol) was dissolved in AcOH (0.32 mL) and Ac₂O (0.32 mL, 3.38 mmol) was added to the solution. Then the mixture was stirred for 2 h at reflux temperature. The solution was poured onto ice and EtOAc was added to the mixture. The organic phase was dried over Mg₂SO₄ and evaporated. Yield: 52.7 mg. HPLC purity: 98.1 %. HRMS [M+H]⁺ C₂₀H₂₄N₃OCl₂ expected: 392.1296 measured: 392.1287. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.96 (s, 1H), 7.56 (d, *J* = 8.1 Hz, 2H), 7.37 (d, *J* = 7.7 Hz, 2H), 7.22 (t, *J* = 8.7 Hz, 3H), 3.67 (s, 2H), 3.19 (d, *J* = 77.7 Hz, 5H), 2.98 (t, *J* = 8.6 Hz, 2H), 2.04 (s, 3H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 149.82, 138.52, 133.31, 129.26, 128.78, 126.69, 125.71, 120.11, 119.73, 56.80, 51.74, 48.26, 29.34, 24.00.

5.46. N-(4-(2-(4-(2-Methoxyphenyl)piperazin-1-yl)ethyl)phenyl)acetamide (45)

Amine **33** (200 mg, 0.64 mmol) was dissolved in AcOH (0.32 mL) and Ac₂O (0.32 mL, 3.60 mmol) was added to the solution. Then the mixture was stirred for 2 h on the boiling point of the solvent and 1 night at RT. The solution was poured onto ice and EtOAc was added to the mixture. The organic phase was dried over Mg₂SO₄ and evaporated. Yield: 38.5 mg (17 %) yellowish-white powder. HPLC purity: 95.0 %. HRMS [M+H]⁺ C₂₁H₂₈N₃O₂ expected: 354.2176 measured: 354.2171. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.54 (d, *J* = 7.9 Hz, 1H), 7.37 (d, *J* = 7.6 Hz, 1H), 7.21 (dd, *J* = 16.0, 7.9 Hz, 2H), 6.95 (dt, *J* = 18.2, 10.1 Hz, 4H), 3.78 (s, 3H), 3.73–3.48 (m, 2H), 3.46–3.17 (m, 1H), 3.10 (s, 3H), 2.16 (d, *J* = 8.9 Hz, 5H), 2.01 (s, 1H), 1.88 (s, 1H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 163.42, 152.36, 139.85, 137.89, 132.20, 129.81, 123.99, 121.34, 120.17, 118.81, 112.47, 56.76, 55.87, 51.76, 47.49, 29.20.

5.47. N-(4-(2-(4-(3-cyano-5-(trifluoromethyl)phenyl)piperazin-1-yl)ethyl)phenyl)acetamide (46)

Amine **34** (200 mg, 0.53 mmol) was dissolved in AcOH (0.32 mL) and Ac₂O (0.32 mL, 3.60 mmol) was added to the solution. Then the mixture was stirred for 2 h on the boiling point of the solvent. Then the mixture was stirred for 2 h on the boiling point of the solvent and 1 night

at RT. The solution was poured onto ice and EtOAc was added to the mixture. The organic phase was dried over Mg₂SO₄ and evaporated. Yield: 31.6 mg (14 %) white powder. HPLC purity: 99.6 %. HRMS [M+H]⁺ C₂₂H₂₄N₄O₂F₃ expected: 417.1896 measured: 417.2013. ¹H NMR (500 MHz, CDCl₃) δ 7.43 (d, *J* = 8.1 Hz, 2H), 7.30–7.26 (m, 3H), 7.18 (d, *J* = 8.1 Hz, 2H), 3.34–3.29 (m, 4H), 2.81 (dd, *J* = 9.7, 6.1 Hz, 2H), 2.71–2.62 (m, 6H), 2.17 (s, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 168.23, 151.46, 136.09, 132.93, 129.15, 120.63, 120.13, 118.06, 115.42, 114.01, 60.06, 52.60, 47.81, 32.95, 24.51.

6. Modelling

All calculations were performed using the Schrödinger Small-Molecule Drug Discovery Suite 2024-4, Schrödinger, LLC, New York, NY, 2024.

6.1. Protein preparation

The three-dimensional structures of the inactive and active human dopamine D₂ and D₃ receptors were downloaded from the Protein Data Bank (PDB IDs: 6CM4, 3PBL, 7JVR and 9F33) [52–55]. The structures were prepared using the Schrödinger Protein Preparation Wizard with default settings, except for using simplified rules at neutral pH for H-bond assignment. The restrained minimization step was included with default settings (OPLS4 force field [103]), and all waters were removed subsequently. In the D₂ structure Ala3.40 was mutated back to the wild-type isoleucine, using the rotameric state of Ile3.40 found in the D₃ crystal structure. The active structures were refined with the GPCRdb protocol [92].

6.2. Docking

Ligands were protonated at the arylpiperazine basic amine moiety. Subsequently all ligands were prepared for docking using Schrödinger LigPrep with the OPLS4 force field [103] in the single known stereoisomer. Schrödinger Glide Single Precision (SP) [104] was used for docking the ligands. In the experimental structures the grid was placed on the centroid of the co-crystallized ligand. The inner box size was set to 12x12x12 Å³, the outer box size to the diameter of the ligands. A H-bond constraint was defined with the characteristic Asp3.32 residue of aminergic receptors. In all these cases, 15 poses were included in post-docking minimization and 5 poses were saved, which were ranked by the docking score separately for each ligand. Binding modes were analyzed in Schrödinger Maestro. The best scoring binding modes are shown in the figures.

7. Pharmacology

7.1. Chemicals

Tris-HCl, EDTA, NaHEPES were obtained from Sigma-Aldrich (Budapest, Hungary), D3R agonist, 7-OH-DPAT was obtained from Tocris Bioscience (Bristol, United Kingdom). CHO-hD3 cell line, the radiolabelled [³H]7-OH-DPAT (specific activity: 100 Ci/mmol) and the Ultima GoldTM MV harmless scintillation cocktail were acquired from PerkinElmer (Boston, USA).

8. D₂R/D₃R measurement

8.1. Preparation of cell lines for binding assays and competitive radioligand binding experiment

Membrane preparation of CHO cells stably expressing human D_{2short} (D₂R) and D₃ receptor (D₃R), as well as the radioligand displacement assay, were conducted as previously described [93].

Briefly, for radioligand binding assays, membrane preparations (25

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$\mu\text{g/well}$ for D₂R and 20 $\mu\text{g/well}$ for D₃R in 200 μl final volume) were co-incubated with [³H]-spiperone (0.2 nM final concentration) and varying concentrations of the test ligand for 120 min at room temperature. Each assay was conducted in triplicate, with 7 concentrations of test compound between 0.01 nM and 100 μM . Non-specific binding was determined using 10 μM haloperidol. Bound radioligand was separated from free radioligand via filtration using a 96-well cell harvester. Radioactivity was then measured by liquid scintillation counting.

Experimental data are presented as means $\text{pK}_i \pm \text{S.E.M.}$ The data analysis of at least three independent experiments was performed with GraphPad Prism 6 (San Diego, CA, USA) using non-linear regression (one-site competition). IC₅₀ values were transformed to the equilibrium binding affinity (K_i value) by using the Cheng-Prusoff equation.

9. 5-HT_R measurements

9.1. Cell culture and preparation of cell membranes for radioligand binding assays

HEK293 cells with stable expression of human 5-HT_{1A}, 5-HT₆ and 5-HT_{7B} (prepared with the use of Lipofectamine 2000) or CHO-K1 cells with plasmid containing the sequence coding for the human serotonin 5-HT_{2A} receptor (PerkinElmer) were maintained at 37 °C in a humidified atmosphere with 5 % CO₂ and grown in Dulbecco's Modified Eagle Medium containing 10 % dialyzed fetal bovine serum and 500 $\mu\text{g/ml}$ G418 sulfate. For membrane preparation, cells were subcultured in 150 cm² flasks, grown to 90 % confluence, washed twice with prewarmed to 37 °C phosphate buffered saline (PBS) and pelleted by centrifugation (200×g) in PBS containing 0.1 mM EDTA and 1 mM dithiothreitol. Prior to membrane preparation, pellets were stored at −80 °C.

9.2. Radioligand binding assays

Cell pellets were thawed and homogenized in 10 vol of assay buffer using an Ultra Turrax tissue homogenizer and centrifuged twice at 35000×g for 15 min at 4 °C, with incubation for 15 min at 37 °C in between. The composition of the assay buffers was as follows: for 5-HT_{1A}R: 50 mM Tris HCl, 0.1 mM EDTA, 4 mM MgCl₂, 10 μM pargiline and 0.1 % ascorbate; for 5-HT_{2A}R: 50 mM Tris HCl, 0.1 mM EDTA, 4 mM MgCl₂ and 0.1 % ascorbate; for 5-HT₆R: 50 mM Tris HCl, 0.5 mM EDTA and 4 mM MgCl₂; for 5-HT_{7B}R: 50 mM Tris HCl, 4 mM MgCl₂, 10 μM pargiline and 0.1 % ascorbate. All assays were incubated in a total volume of 200 μl in 96-well microtiter plates for 1 h at 37 °C, except 5-HT_{1A}R and 5-HT_{2A}R which were incubated at room temperature and 27 °C, respectively. The process of equilibration was terminated by rapid filtration through Unifilter plates with a 96-well cell harvester and radioactivity retained on the filters was quantified on a Microbeta plate reader (PerkinElmer, USA). For displacement studies the assay samples contained as radioligands (PerkinElmer, USA): 2.5 nM [³H]-8-OH-DPAT (135.2 Ci/mmol) for 5-HT_{1A}R; 1 nM [³H]-ketanserin (53.4 Ci/mmol) for 5-HT_{2A}R; 2 nM [³H]-LSD (83.6 Ci/mmol) for 5-HT₆R or 0.8 nM [³H]-5-CT (39.2 Ci/mmol) for 5-HT₇R. Non-specific binding was defined with 10 μM of 5-HT in 5-HT_{1A}R and 5-HT₇R binding experiments, whereas 20 μM of mianserin or 10 μM of methiothepine were used in 5-HT_{2A}R and 5-HT₆R, respectively. Each compound was tested in triplicate at 7 concentrations (10^{−10}–10^{−4} M). The inhibition constants (K_i) were calculated from the Cheng-Prusoff equation [94]. Results were expressed as means of two separate experiments.

10. GPCR binding panel

10.1. Competition binding in human 5-HT_{2A} receptor

Serotonin 5-HT_{2A} receptor competition binding experiments were carried out in a polypropylene 96-well plate. In each well was incubated 70 μg of membranes from CHO-5-HT_{2A} cell line prepared in our

laboratory (Lot: A006/10-03-2020, protein concentration = 5134 $\mu\text{g/ml}$), 1 nM [³H]-Ketanserin (42.5 Ci/mmol, 1 mCi/ml, PerkinElmer NET791250UC) and compounds studied and standard. Non-specific binding was determined in the presence of Methysergide 1 μM (Sigma M137). The reaction mixture (Vt: 250 $\mu\text{l/well}$) was incubated at 37 °C for 30 min, 200 μl was transferred to GF/B 96-well plate (Millipore, Madrid, Spain) pretreated with 0.5 % of PEI and treated with binding buffer (Tris-HCl 50 mM, pH = 7.4), after was filtered and washed six times with 250 μl wash buffer (Tris-HCl 50 mM, pH = 6.6), before measuring in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain).

10.2. Competition binding in human 5-HT_{2B} receptor

Serotonin 5-HT_{2B} receptor competition binding experiments were carried out in a polypropylene 96-well plate. In each well was incubated 20 μg of membranes from CHO-5-HT_{2B} cell line prepared in our laboratory (Lot: A004/27-03-2012, protein concentration = 2775 $\mu\text{g/ml}$), 1 nM [³H]-LSD (78.9 Ci/mmol, 1 mCi/ml, PerkinElmer NET638250UC) and compounds studied and standard. Non-specific binding was determined in the presence of 5-HT 50 μM (Sigma H9523). The reaction mixture (Vt: 250 $\mu\text{l/well}$) was incubated at 37 °C for 30 min, 200 μl was transferred to GF/C 96-well plate (Millipore, Madrid, Spain) pretreated with 0.5 % of PEI and treated with binding buffer (Tris-HCl 50 mM, Ascorbic acid 0.1 %, CaCl₂ 4 mM, pH = 7.4), after was filtered and washed four times with 250 μl wash buffer (Tris-HCl 50 mM, pH = 7.4), before measuring in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain).

10.3. Competition binding in human 5-HT_{2C} receptor

Serotonin 5-HT_{2C} receptor competition binding experiments were carried out in a polypropylene 96-well plate. In each well was incubated 3 μg of membranes from HeLa-5-HT_{2C} cell line prepared in our laboratory (Lot: A002/20-06-2011, protein concentration = 2041 $\mu\text{g/ml}$), 1.25 nM [³H]-Mesulergine (77.4 Ci/mmol, 1 mCi/ml, PerkinElmer NET1148250UC) and compounds studied and standard. Non-specific binding was determined in the presence of Mianserin 10 μM (Sigma M2525). The reaction mixture (Vt: 250 $\mu\text{l/well}$) was incubated at 27 °C for 60 min, 200 μl was transferred to GF/C 96-well plate (Millipore, Madrid, Spain) pretreated with 0.5 % of PEI and treated with binding buffer (50 mM Tris-HCl, pH = 7.5), after was filtered and washed four times with 250 μl wash buffer (50 mM Tris-HCl, pH = 6.6), before measuring in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain).

10.4. Competition binding in human 5-HT₇ receptor

Serotonin 5-HT₇ receptor competition binding experiments were carried out in a polypropylene 96-well plate. In each well was incubated 2 μg of membranes from HEK-5-HT₇#14 cell line prepared in our laboratory (Lot: A006/21-07-2016, protein concentration = 3316 $\mu\text{g/ml}$), 2 nM [³H]-SB269970 (32.7 Ci/mmol, 0.25 mCi/ml, PerkinElmer NET1198U250UC) and compounds studied and standard. Non-specific binding was determined in the presence of clozapine 25 μM (Sigma C6305). The reaction mixture (Vt: 250 $\mu\text{l/well}$) was incubated at 37 °C for 60 min, 200 μl was transferred to GF/C 96-well plate (Millipore, Madrid, Spain) pretreated with 0.5 % of PEI and treated with binding buffer (50 mM Tris-HCl, 4 mM CaCl₂, 1 mM ascorbic acid, 0.1 mM pargiline, pH = 7.4), after was filtered and washed four times with 250 μl wash buffer (50 mM Tris-HCl, 4 mM CaCl₂, 1 mM ascorbic acid, 0.1 mM pargiline, pH = 7.4) before measuring in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain).

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10.5. Competition binding in human α_{1A} adrenergic receptor

α_{1A} adrenergic receptor competition binding experiments were carried out in a polypropylene 96-well plate. In each well was incubated 20 μ g of membranes from HEK- α_{1A} #5 cell line prepared in our laboratory (Lot: A005/17-10-2018, protein concentration = 6824 μ g/ml), 0.25 nM [3 H]-Prazosin (77.4 Ci/mmol, 1 mCi/ml, PerkinElmer NET823250UC) and compounds studied and standard. Non-specific binding was determined in the presence of prazosin 1 μ M (Sigma P115). The reaction mixture (Vt: 250 μ l/well) was incubated at 25 °C for 90 min, 200 μ l was transferred to GF/C 96-well plate (Millipore, Madrid, Spain) pretreated with 0.5 % of PEI and treated with binding buffer (50 mM Hepes, 5 mM MgCl₂, 1 mM CaCl₂, 0.2 % BSA, pH = 7.4), after was filtered and washed four times with 250 μ l wash buffer (50 mM Hepes, 500 mM NaCl, 0.1 % BSA, pH = 7.4) before measuring in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain).

10.6. Competition binding in human M₁ receptor

Muscarinic M₁ receptor competition binding experiments were carried out in a polypropylene 96-well plate. In each well was incubated 2.5 μ g of membranes from CHO-M1 cell line prepared in our laboratory (Lot: A001/12 05–2020), 7 nM [3 H]-Pirenzepine (85 Ci/mmol, 1 mCi/ml, PerkinElmer NET780250UC) and compounds studied and standard. Non-specific binding was determined in the presence of Pirenzepine 200 μ M (Sigma P7412). The reaction mixture (Vt: 250 μ l/well) was incubated at 25 °C for 90 min, 200 μ l was transferred to GF/C 96-well plate (Millipore, Madrid, Spain) pretreated with 0.1 % Tween 20 and treated with binding buffer (Hepes 10 mM, MgCl₂ 10 mM, NaCl 100 mM, BSA 0.2 %, pH = 7.4), after was filtered and washed four times with 250 μ l wash buffer (Hepes 10 mM, BSA 0.1 %, pH = 7.4), before measuring in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain).

10.7. Competition binding in human H₁ receptor

Histamine H₁ receptor competition binding experiments were carried out in a polypropylene 96-well plate. In each well was incubated 25 μ g of membranes from CHO-hH1 #31 cell line prepared in our laboratory (Lot: A008/21-09-2020, protein concentration = 4300.7 μ g/ml), 2 nM [3 H]-Pyrilamine (20.1 Ci/mmol, 1 mCi/ml, PerkinElmer NET594250UC) and compounds studied and standard. Non-specific binding was determined in the presence of Triprolidine 10 μ M (Sigma T6764). The reaction mixture (Vt: 250 μ l/well) was incubated at 27 °C for 60 min, 200 μ l was transferred to GF/B 96-well plate (Millipore, Madrid, Spain) pretreated with 0.1 % of Tween-20 and treated with binding buffer (0.0086M KH₂PO₄, 0.0414M Na₂HPO₄, pH = 7.5), after was filtered and washed six times with 250 μ l wash buffer ((0.0086M KH₂PO₄, 0.0414M Na₂HPO₄, pH = 7.5), before measuring in a microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain).

10.8. Competition binding in human D₁ receptor

Dopamine D₁ receptor competition binding experiments were carried out in a polypropylene 96-well plate. In each well was incubated 12 μ g of membranes from CHO-D1 cell line (PerkinElmer ES-172-M400UA, protein concentration = 4000 μ g/ml), 0.7 nM [3 H]-SCH23390 (83.2 Ci/mmol, 1 mCi/ml, PerkinElmer NET930250UC) and compounds studied and standard. Non-specific binding was determined in the presence of (+)-Butaclamol Hydrochloride 1 μ M (Sigma D033). The reaction mixture (Vt: 250 μ l/well) was incubated at 27 °C for 60 min, 200 μ l was transferred to GF/C 96-well plate (Millipore, Madrid, Spain) pretreated with 0.5 % of PEI and treated with binding buffer (50 mM Tris-HCl, 5 mM MgCl₂ pH = 7.4), after was filtered and washed four times with 250 μ l wash buffer (50 mM Tris-HCl pH = 7.4), before measuring in a

microplate beta scintillation counter (Microbeta Trilux, PerkinElmer, Madrid, Spain).

10.9. Bioluminescence Resonance Energy Transfer (BRET) Assays

The following BRET-based biosensor components have been previously described: human G protein subunits, p63-RlucII [95] Rap1GAP-RlucII [95]; PDZ-RlucII [95]; β -arrestin1-RlucII, β -arrestin2-RlucII, rGFP-CAAX [96,97]. The details of the transfection protocol have been previously described [71,98].

On the day of the BRET experiments, the cell culture medium was removed, and the cells were washed twice with PBS and treated with Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 12 mM NaHCO₃, 5.6 mM D-glucose, 0.5 mM MgCl₂, 0.37 mM NaH₂PO₄, 25 mM HEPES [pH 7.4]) and incubated at 37 °C for at least 15 min.

Compounds were tested in the agonist mode (in the absence of the endogenous agonist) and antagonist mode (in the presence of the endogenous agonist at its EC₈₀) using the EMTA biosensor suite. For the concentration response curves of the endogenous agonist and the ligands, cells were stimulated with different concentrations of the ligands and incubated for 5 min after which the substrate Prolume Purple (1.3 μ M) was added and incubated for an additional 6 min. BRET signal was measured on the Spark® multimode microplate reader (Tecan) (agonist mode) (acceptor, 515 $\pm$ 20 nm; and donor, 400 $\pm$ 70 nm filters). Cells were thereafter treated with a submaximal concentration of the endogenous agonist (EC₉₀ concentration at the respective pathways) and incubated for an additional 5 min after which a second BRET read (antagonist mode) was conducted.

10.10. In vivo testing of pharmacokinetics and antidepressant-like action of 20b in comparison with cariprazine

10.10.1. Animals

In vivo experiments were performed on male NMRI mice (12–16 weeks old, weighing 25–35 g). This outbred mouse strain is widely used for preclinical investigation in psychopharmacology [99]. Since there is no genotypic and phenotypic alteration of this strain, it is particularly suitable for testing of potential antidepressant compounds. This was already demonstrated shortly after introducing tail suspension test as a classical examination method of depression-like behavior [100]. The animals were bred and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs (temperature: 24–25 °C, humidity 50–60 %), provided with standard chow and water *ad libitum* and maintained under a 12-h light-dark cycle.

10.10.2. Ethics

All *in vivo* experimental procedures were performed according to the 40/2013 (II.14.) Government Regulation on Animal Protection and the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988), as well as the Directive 2010/63/EU of the European Parliament and the ARRIVE (Animal Research: Reporting *In vivo* Experiments) guidelines. Studies were approved by the Animal Welfare Committee of the University of Pécs and the National Scientific Ethical Committee on Animal Experimentation of Hungary and licensed by the Government Office of Baranya County (license number: BA02/2000-09/2023).

10.10.3. Drug treatments

Cariprazine and **20b** were suspended in 2.5 % Tween 80 and administered orally (p.o.) in the following doses: 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, 1 mg/kg. The control group received the same volume of the vehicles (2.5 % Tween 80). Oral treatments were carried out 60 min before starting the TST measurements.

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10.10.4. Pharmacokinetic analysis

As the most relevant pharmacokinetic parameters, blood and brain concentrations of compound **20b** were determined. 1 or 2 h after the oral treatment with the most effective, 0.1 mg/kg dose of **20b**, mice were deeply anaesthetized with i.p. injection of ketamine and xylazine (100 mg/kg and 5 mg/kg, respectively). Blood samples were taken using ethylenediamine tetra acetic acid (EDTA) as anticoagulant agent and centrifuged (1000g, 4 °C, 5 min and 10.000g, 4 °C, 5 min). Brain samples were weighed (ADAM SAB 125i), put in Direct-Q 3UV water and homogenized with Ultrasonic Homogenizer 4710. Homogenates were mixed with acetonitrile and centrifuged (10.000g, 4 °C, 10 min) (MPW-352R). In both cases supernatants were used for HPLC analysis.

10.10.5. Quantitation of EA-Ri-44

Mass spectrometric measurements were performed on a Sciex 6500QTrap (Sciex, Framingham, MA, USA) hybrid quadrupole-ion trap mass spectrometer equipped with Turbo-V ion source. HPLC separations were carried out on an Agilent 1100 system consisting of a binary pump, an autosampler and a column compartment. Analyst 1.6.3. (Sciex, Framingham, MA, USA) software was used for controlling the instrument and for data processing. Quantitation was performed by using the Sciex MultiQuant software.

HPLC separation of the samples was performed on a Kinetex EVO C18 (50 × 2.1 mm, 5 µm). The mobile phases were: water containing 0.1 % formic acid (eluent A) and acetonitrile containing 0.1 % formic acid (eluent B). The separation was performed in gradient elution mode. The initial eluent B composition was 5 % and kept for 0.5 min. A linear gradient was applied to reach the 95 % of eluent B by 4.5 min and kept there for 0.5 min. The initial solvent composition was set at 0.5 min and the equilibration time was 3 min. The overall run time was 9 min. The flow rate was 0.6 mL/min and the injection volume was 5 µL. The column temperature was ambient.

MS detection in positive ion MRM mode was applied for quantitation of the target molecule. Source parameters were: curtain gas (CUR), nebulizer gas (GS1) and drying gas (GS2) values were set at 40, 40 and 40 arbitrary unit, respectively. The spray voltage was 5500V. Source temperature was set at 450 °C. Declustering potential was set to 246V. The target molecule was detected in MRM mode with transitions of 449.3/242.8 (quantifier) and 449.3/199.8 (qualifier) (Q1/Q3) with dwell time of 150 ms. Collision energy was 41 and 51 eV, respectively.

10.10.6. Tail suspension test

TST is a widely used and validated test for determining depression-like behavior or to test the effect of antidepressant in rodents [77], where the animals can react to an acute inescapable stress exposure either with struggling or with immobility. Immobility time, defined as the complete lack of escape-oriented movements - during the final 4 min of the 6-min-long stress exposure [101], is directly proportional with the extent of depression-like behavior. This test has served as a suitable tool for preclinical testing of antidepressant drug candidates [102]. Mice are suspended by their tail 50 cm above the desk with the help of an adhesive tape. The behavior of the mice was detected and recorded by the EthoVision XT 11 Software (Noldus Information Technology, Netherlands) and the immobility time - defined as the complete lack of escape-oriented movements - during the final 4 min of the 6-min-long stress exposure [101] was determined in seconds.

10.11. Statistical analysis

In *in vivo* models, data are expressed as the mean ± SEM of n = 4–16 mice per group and analyzed with one-way ANOVA followed by Bonferroni's post hoc test. Statistical analysis was carried out with GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA), *p < 0.05 was considered as significant.

CRediT authorship contribution statement

Attila Egyed: Formal analysis, Methodology. **Dóra Judit Kiss:** Formal analysis, Methodology, Software, Writing – original draft. **Zsófia Pável:** Investigation. **Keve Rajna:** Investigation. **Supriya A. Gaitonde:** Data curation, Formal analysis, Investigation, Methodology, Visualization. **Mariam Dubiel:** Investigation, Methodology, Visualization. **Mária Isabel Loza:** Formal analysis, Investigation, Methodology, Visualization. **Edina Szűcs:** Formal analysis, Investigation, Visualization. **Éva Borbély:** Formal analysis, Methodology, Visualization. **Zsófia Hajna:** Formal analysis, Investigation, Visualization. **Grzegorz Satala:** Conceptualization, Investigation, Methodology, Visualization. **Márton Vass:** Formal analysis, Investigation, Software. **Pál Szabó:** Conceptualization, Formal analysis, Investigation, Validation. **Katalin Jósavay:** Investigation, Methodology. **Andrzej J. Bojarski:** Formal analysis, Supervision. **Holger Stark:** Formal analysis, Supervision. **Zsuzsanna Helyes:** Conceptualization, Resources, Supervision. **Erika Pintér:** Methodology, Resources, Supervision. **Michel Bouvier:** Methodology, Resources, Validation. **György M. Keserü:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

György M Keserü is a shareholder of Gedeon Richter plc and SAB member of Cytocast, ChemPass and Molecule Therapeutics. The remaining authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmcr.2025.100302>.

Data availability

Data will be made available on request.

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**The impact of secondary site interactions on the
binding specificity of bitopic dopamine D₂/D₃
receptor ligands**

Supporting Information

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Supplementary Table 1 Functional activity of compounds **20b**, **21b** and **22b** measured pathways in agonist and antagonist modes using BRET assays. The antagonist mode of the receptors shown in the main text are collected separately for easier comparison. logIC₅₀/logEC₅₀ ±SEM

GPCR pathway (antagonist- mode)	D ₃ R (G _q)		D ₂ (G ₀₈)		D ₂ R (β-arr2)		5HT _{1A} (G ₀₈)		5HT _{1A} R (G ₁₄)		5HT _{2A} R (G _q)		5HT _{2A} R (β-arr1)	
	logIC ₅₀	Inh.%	logIC ₅₀		logIC ₅₀	Inh.%	logIC ₅₀		logIC ₅₀	Inh.%	logIC ₅₀		logIC ₅₀	Inh.%
3	NA		NA		-8.73 ± 0.14	87.92 ± 6.80	NA		-6.37 ± 0.09	111.10 ± 5.10	NA		-5.80 ± 0.10	97.19 ± 5.15
20b	NA		NA		-7.54 ± 0.17	76.81 ± 6.83	NA		-5.52 ± 0.80	13.46 ± 7.62	NA		-5.75 ± 0.20	59.81 ± 6.21
21b	-7.08 ± 0.24	96.38 ± 12.33	NA		-6.88 ± 0.23	97.40 ± 11.32	NA		IC ₅₀ > 10 μM		NA		IC ₅₀ > 10 μM	
22b	-7.06 ± 0.21	107.20 ± 11.61	NA		-7.13 ± 0.26	59.40 ± 8.06	NA		IC ₅₀ > 10 μM		NA		IC ₅₀ > 10 μM	

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GPCR (pathway) (agonist)	5HT _{1A} (GoB)		5HT _{1B} (Gi2)		5HT _{1D} (GoB)		5HT _{1E} (Gi3)		5HT _{1F} (Gz)		5HT _{1G} (Gq)		5HT _{1H} (Gz)		D ₁ (GoB)		D ₂ (Gz)		D ₄ (GoB)		M ₂ (GoB)		(inverse agonism)	
	logEC ₅₀	E _{max}	logEC ₅₀	E _{max}	logEC ₅₀	E _{max}	logEC ₅₀	E _{max}	logEC ₅₀	E _{max}	logEC ₅₀	E _{max}	logEC ₅₀	E _{max}	logEC ₅₀	E _{max}	logEC ₅₀	E _{max}	logEC ₅₀	E _{max}	logEC ₅₀	E _{max}	logEC ₅₀	% response
3	8.44 0.22	61.52 6.27	8.05 0.25	65.28 7.64	7.61 0.33	68.39 11.46	6.11 0.26	67.75 9.90	5.21 0.07	52.11 1.97	7.05 0.05	80.42 2.16	8.97 0.79	19.87 7.26	8.44 0.22	61.52 6.27	7.47 0.31	61.74 9.91	7.25 0.17	108.9 9.86	5.76 0.89	108.9 23.10	% response	
22b	6.96 0.15	73.98 5.69	5.73 0.13	87.21 7.30	6.93 1.09	31.61 17.68	NA		NA		5.51 0.12	43.02 3.31	EC ₅₀ μM	10 (inverse agonism)	6.96 0.15	73.98 5.69	NA		6.95 0.26	80.84 10.70	6.02 0.16	80.84 10.70	% response	
21b	6.26 0.11	82.01 4.93	EC ₅₀ , 10 μM		NA		NA		NA		5.86 0.10	43.77 2.63	5.51 0.66	% response 34.4 ± 16.33	6.26 0.11	82.01 4.93	NA		7.01 0.10	109.80 5.67	6.34 0.46	109.80 5.67	% response	
20b	7.29 0.13	80.61 5.72	7.32 0.13	87.68 5.78	7.99 0.24	96.35 11.23	NA		NA		7.49 0.05	88.87 2.38	8.31 0.38	29.62 5.17	7.29 0.13	80.61 5.72	8.75 0.32	51.53 7.46	8.07 0.12	129.40 7.23	NA	NA	% response	

GPCR (pathway) (antagonist)	5HT _{1A} (G14)		5HT _{1A} (Barr1)		5HT _{1B} (G11)		3WAY-5HT _{1B} (G11)		D ₁ (G4)		D ₂ (Barr2)		D ₃ (G4)		D ₄ (G11)		M ₁ (G4)		M ₂ (G4)		M ₃ (G11)		H ₁ (G11)		H ₂ (G4)		H ₃ (G4)		H ₄ (G11)		H ₅ (G4)		H ₆ (G11)		H ₇ (G4)		H ₈ (G11)		H ₉ (G4)		H ₁₀ (G11)		H ₁₁ (G4)		H ₁₂ (G11)		H ₁₃ (G4)		H ₁₄ (G11)		H ₁₅ (G4)		H ₁₆ (G11)		H ₁₇ (G4)		H ₁₈ (G11)		H ₁₉ (G4)		H ₂₀ (G11)		H ₂₁ (G4)		H ₂₂ (G11)		H ₂₃ (G4)		H ₂₄ (G11)		H ₂₅ (G4)		H ₂₆ (G11)		H ₂₇ (G4)		H ₂₈ (G11)		H ₂₉ (G4)		H ₃₀ (G11)		H ₃₁ (G4)		H ₃₂ (G11)		H ₃₃ (G4)		H ₃₄ (G11)		H ₃₅ (G4)		H ₃₆ (G11)		H ₃₇ (G4)		H ₃₈ (G11)		H ₃₉ (G4)		H ₄₀ (G11)		H ₄₁ (G4)		H ₄₂ (G11)		H ₄₃ (G4)		H ₄₄ (G11)		H ₄₅ (G4)		H ₄₆ (G11)		H ₄₇ (G4)		H ₄₈ (G11)		H ₄₉ (G4)		H ₅₀ (G11)		H ₅₁ (G4)		H ₅₂ (G11)		H ₅₃ (G4)		H ₅₄ (G11)		H ₅₅ (G4)		H ₅₆ (G11)		H ₅₇ (G4)		H ₅₈ (G11)		H ₅₉ (G4)		H ₆₀ (G11)		H ₆₁ (G4)		H ₆₂ (G11)		H ₆₃ (G4)		H ₆₄ (G11)		H ₆₅ (G4)		H ₆₆ (G11)		H ₆₇ (G4)		H ₆₈ (G11)		H ₆₉ (G4)		H ₇₀ (G11)		H ₇₁ (G4)		H ₇₂ (G11)		H 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3 Publications

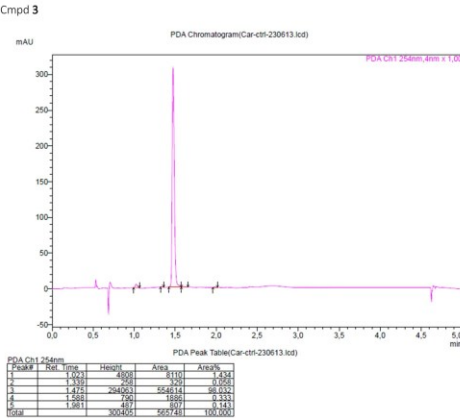
Supplementary Table 2 Reference compounds are used for 5-HT_{1A}, 5-HT_{2A}, 5-HT₆ and 5-HT₇ serotonin receptors radioligand binding assay

Cmpd	K _i ± SD [nM]			
	5-HT _{1A}	5-HT _{2A}	5-HT ₆	5-HT ₇
Buspirone	30 ± 1	NA	NA	NA
Clozapine	NA	39 ± 9	NA	NA
Olanzapine	NA	NA	12 ± 3	NA
Clozapine	NA	NA	NA	62 ± 4

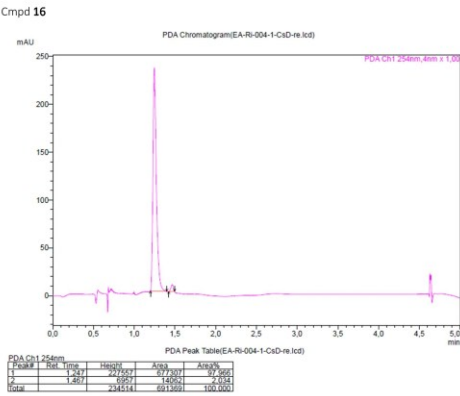
Supplementary Table 3 Pharmacokinetic parameters, blood and brain concentrations (ng/ml) of compound **20b**

EA-Ri-44	Blood	Brain	
Sample Name	Conc (ng/ml)	Conc (ng/ml)	Time (h)
Sample #1	1.44	0.79	1
Sample #2	0.69	0.36	1
Sample #3	5.73	3.71	1
Sample #4	1.88	1.24	1
Sample #5	3.03	1.69	1
Average	2.554	1.558	
Sample #6	0.58	0.55	2
Sample #7	3.09	2.77	2
Sample #8	3.49	2.53	2
Sample #9	5.64	4.85	2
Sample #10	4.22	2.8	2
Average	3.404	2.7	

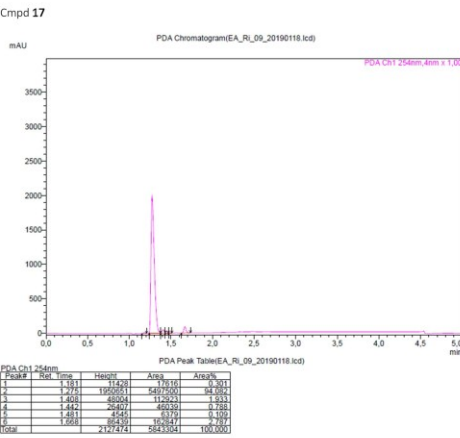
HPLC chromatogram and characterisation of new compounds
3, 18a, 18b, 20a, 21a, 21c, 22a, 22b, 24a, 24b, 32, and 33 are known compounds. The HPLC chromatograms required to verify their adequate purity are shown here. All of the new compounds were characterized by HR-MS, ¹H and ¹³C NMR spectra.



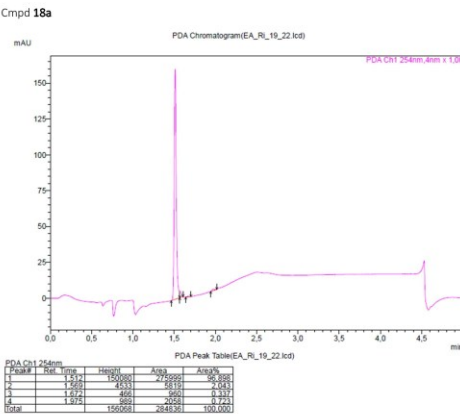
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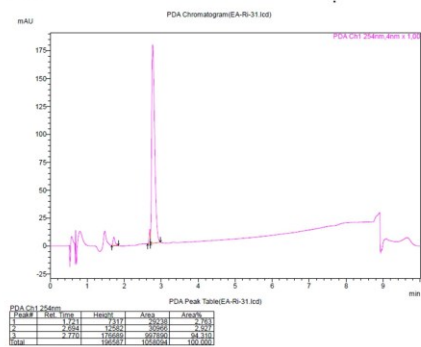
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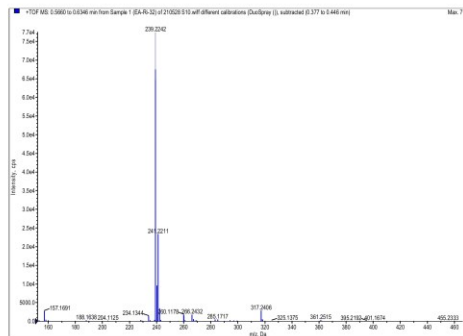
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3 Publications

Cmpd 18b

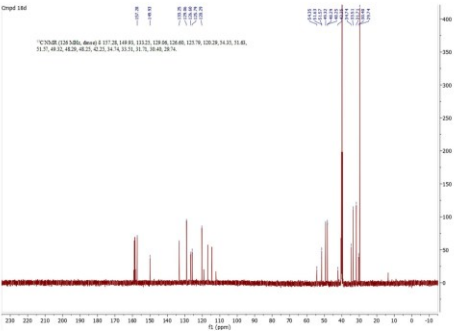
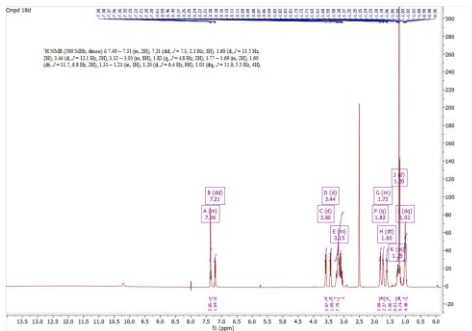


Cmpd 18d



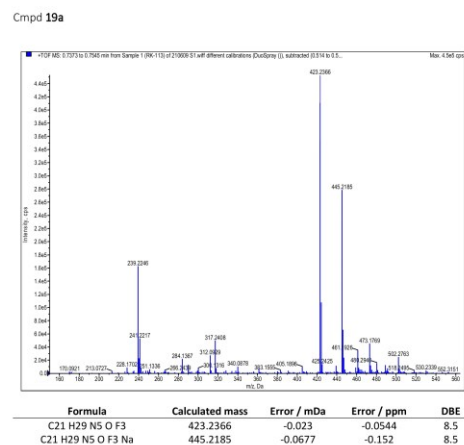
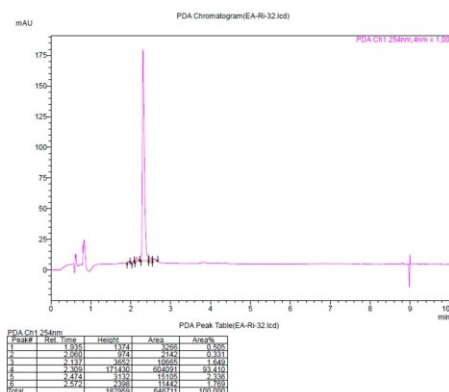
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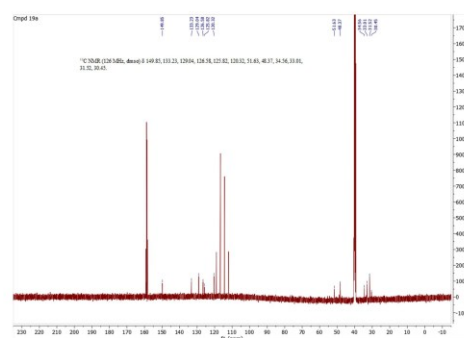
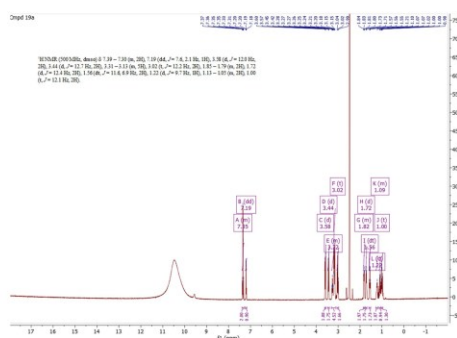
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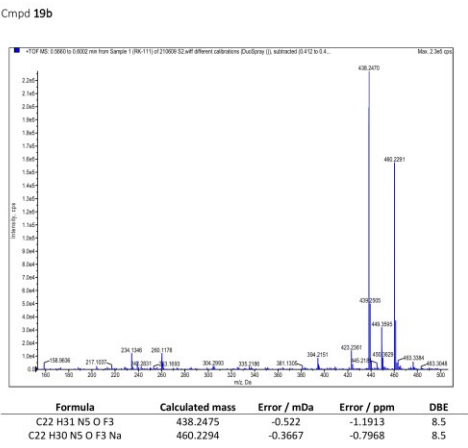
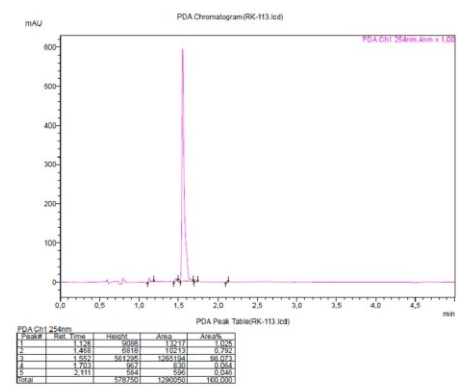
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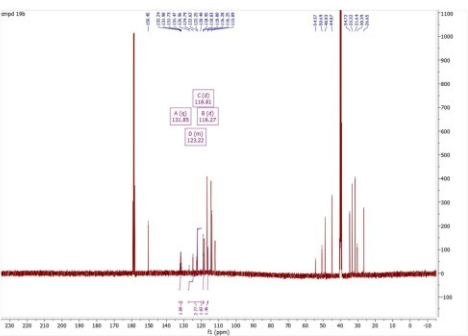
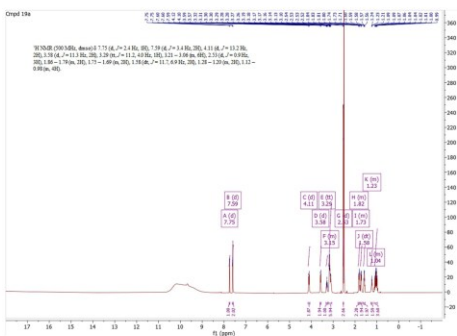
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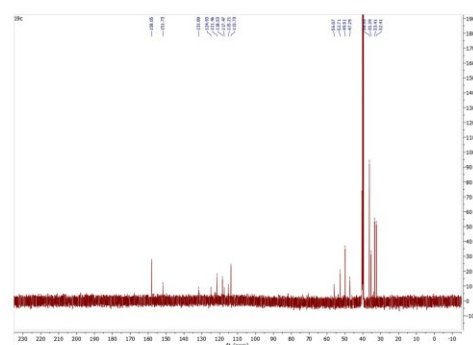
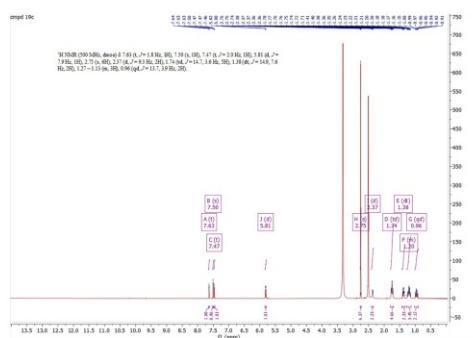
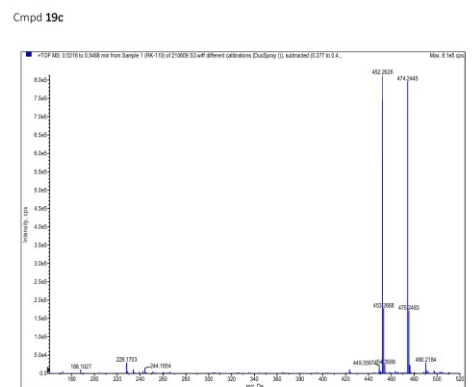
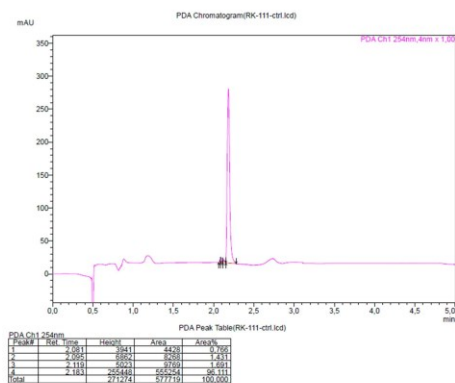
3 Publications



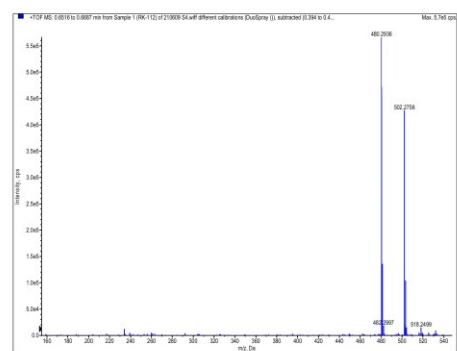
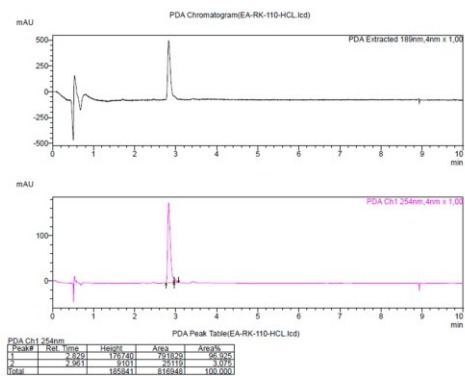
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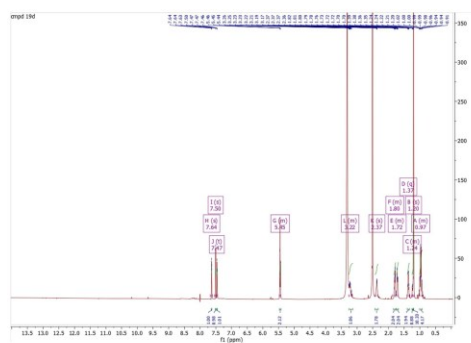
3 Publications



Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C25 H37 N5 O F3	480.2944	-0.8723	-1.8162	8.5
C25 H36 N5 O F3 Na	502.2764	-0.8169	-1.6265	8.5

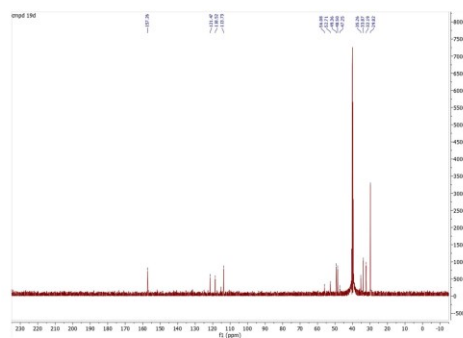
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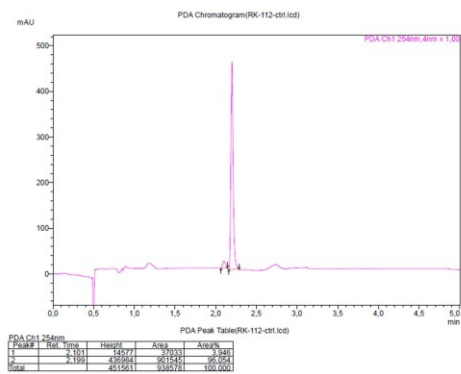
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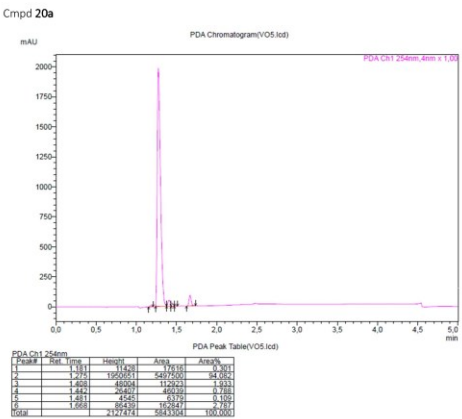
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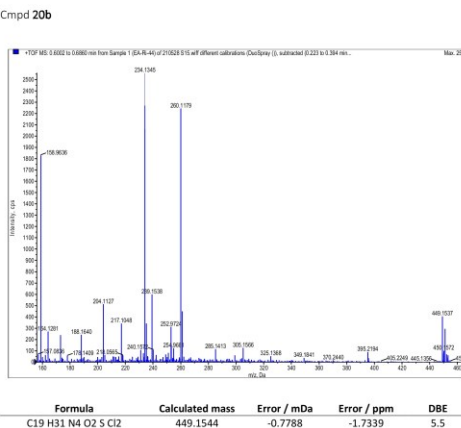




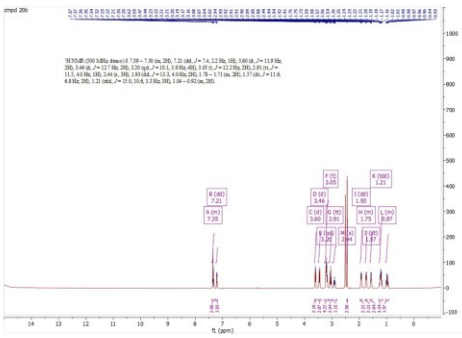
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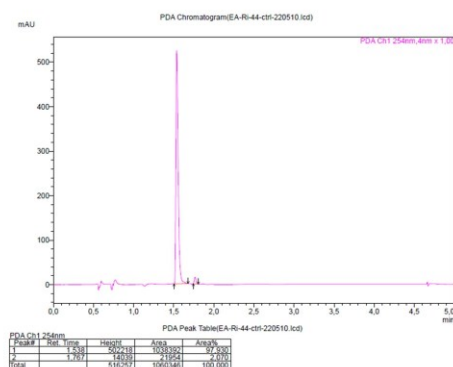
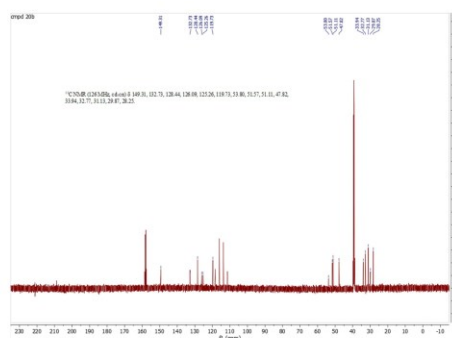


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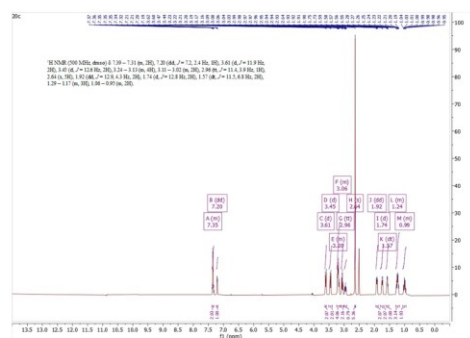
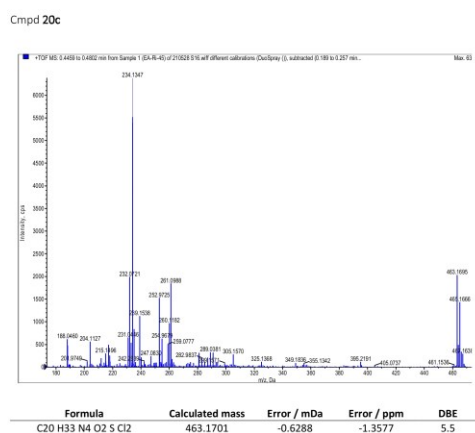
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3 Publications



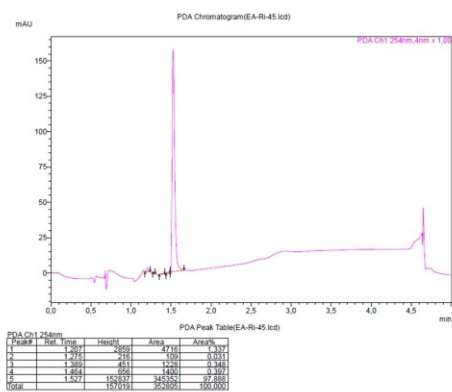
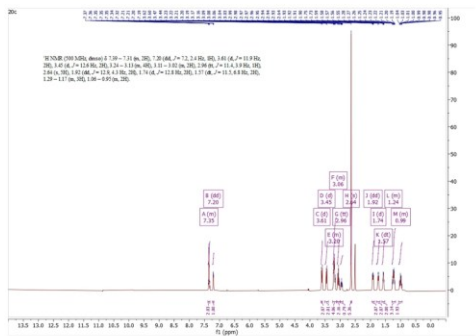
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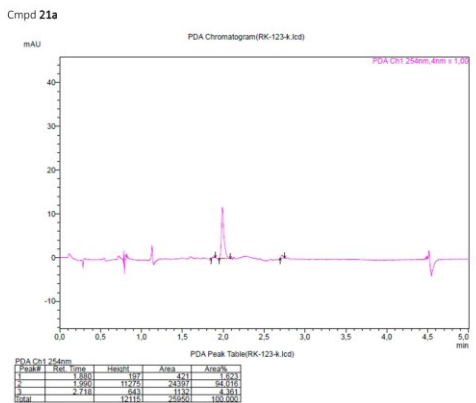
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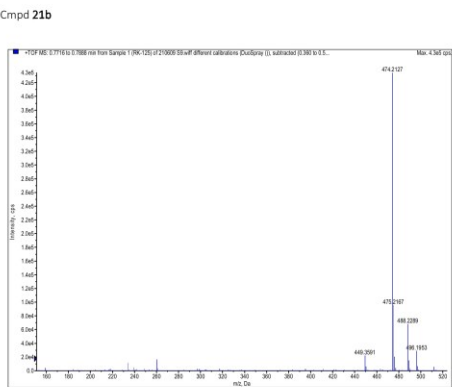
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2	1.26	1.26	10	10	6.90
3	1.36	1.36	10	10	6.90
4	1.46	1.46	10	10	6.90
5	1.56	1.56	10	10	6.90
6	1.66	1.66	10	10	6.90
7	1.76	1.76	10	10	6.90
8	1.86	1.86	10	10	6.90
9	1.96	1.96	10	10	6.90
10	2.06	2.06	10	10	6.90
11	2.16	2.16	10	10	6.90
12	2.26	2.26	10	10	6.90
13	2.36	2.36	10	10	6.90
14	2.46	2.46	10	10	6.90
15	2.56	2.56	10	10	6.90
16	2.66	2.66	10	10	6.90
17	2.76	2.76	10	10	6.90
18	2.86	2.86	10	10	6.90
19	2.96	2.96	10	10	6.90
20	3.06	3.06	10	10	6.90
21	3.16	3.16	10	10	6.90
22	3.26	3.26	10	10	6.90
23	3.36	3.36	10	10	6.90
24	3.46	3.46	10	10	6.90
25	3.56	3.56	10	10	6.90
26	3.66	3.66	10	10	6.90
27	3.76	3.76	10	10	6.90
28	3.86	3.86	10	10	6.90
29	3.96	3.96	10	10	6.90
30	4.06	4.06	10	10	6.90
31	4.16	4.16	10	10	6.90
32	4.26	4.26	10	10	6.90
33	4.36	4.36	10	10	6.90
34	4.46	4.46	10	10	6.90
35	4.56	4.56	10	10	6.90
36	4.66	4.66	10	10	6.90
37	4.76	4.76	10	10	6.90
38	4.86	4.86	10	10	6.90
39	4.96	4.96	10	10	6.90
40	5.06	5.06	10	10	6.90

39

40



Peak#	Ret	Time	Height	Area	Area%
1	1.16	1.16	145	145	100.00
2	1.26	1.26	10	10	6.90
3	1.36	1.36	10	10	6.90
4	1.46	1.46	10	10	6.90
5	1.56	1.56	10	10	6.90
6	1.66	1.66	10	10	6.90
7	1.76	1.76	10	10	6.90
8	1.86	1.86	10	10	6.90
9	1.96	1.96	10	10	6.90
10	2.06	2.06	10	10	6.90
11	2.16	2.16	10	10	6.90
12	2.26	2.26	10	10	6.90
13	2.36	2.36	10	10	6.90
14	2.46	2.46	10	10	6.90
15	2.56	2.56	10	10	6.90
16	2.66	2.66	10	10	6.90
17	2.76	2.76	10	10	6.90
18	2.86	2.86	10	10	6.90
19	2.96	2.96	10	10	6.90
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21	3.16	3.16	10	10	6.90
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23	3.36	3.36	10	10	6.90
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27	3.76	3.76	10	10	6.90
28	3.86	3.86	10	10	6.90
29	3.96	3.96	10	10	6.90
30	4.06	4.06	10	10	6.90
31	4.16	4.16	10	10	6.90
32	4.26	4.26	10	10	6.90
33	4.36	4.36	10	10	6.90
34	4.46	4.46	10	10	6.90
35	4.56	4.56	10	10	6.90
36	4.66	4.66	10	10	6.90
37	4.76	4.76	10	10	6.90
38	4.86	4.86	10	10	6.90
39	4.96	4.96	10	10	6.90
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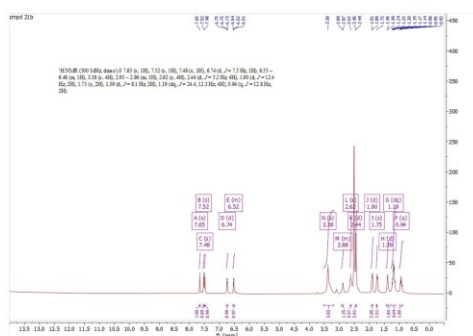


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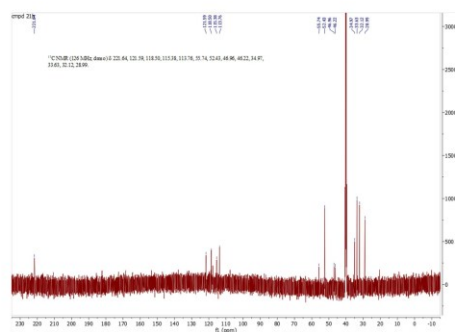
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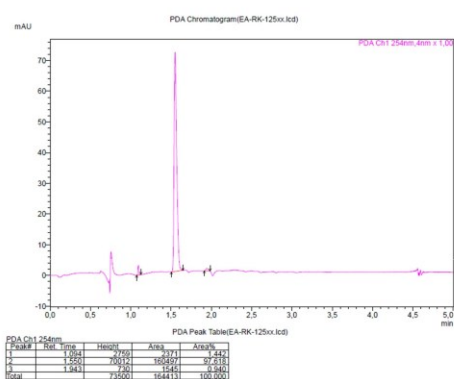
3 Publications



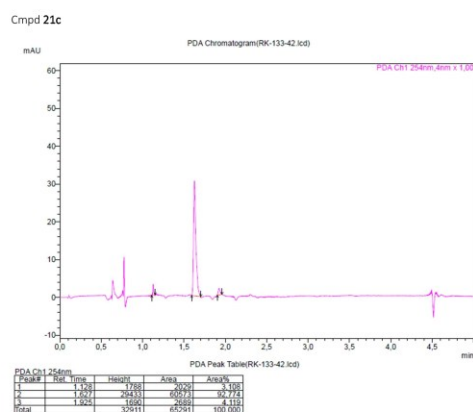
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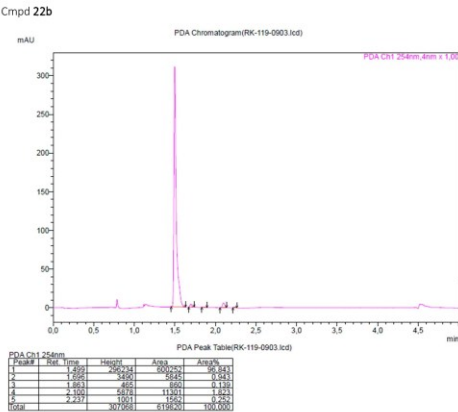
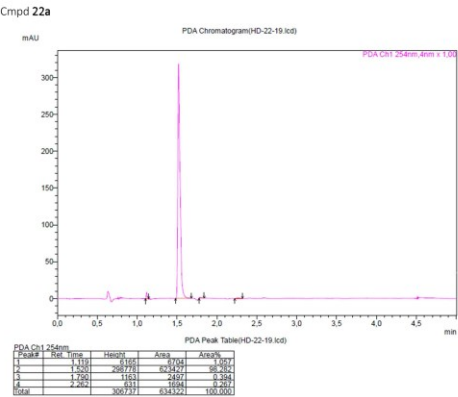
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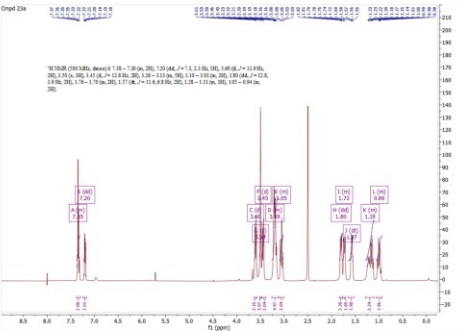
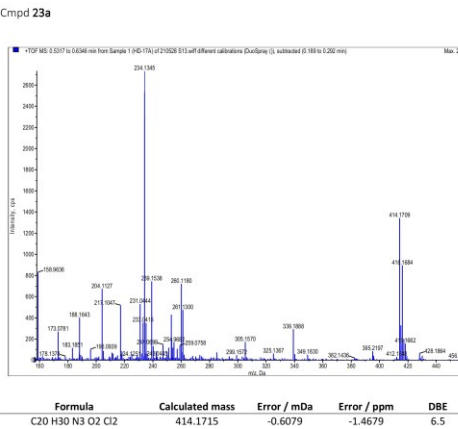


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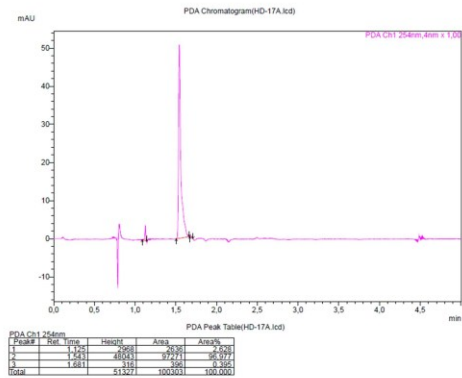
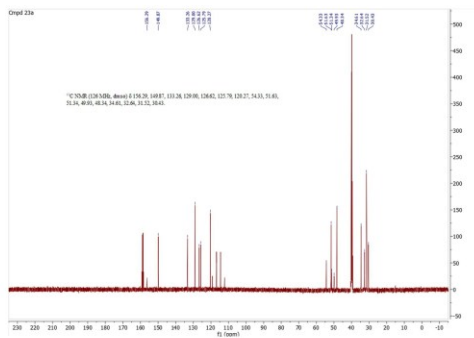
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49

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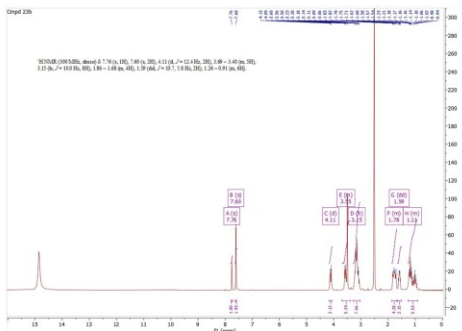
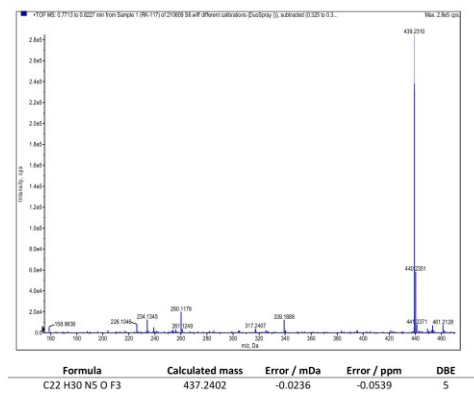
3 Publications



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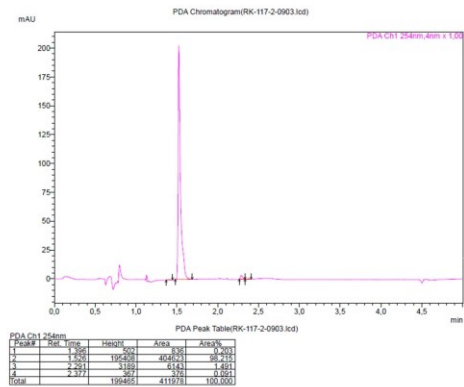
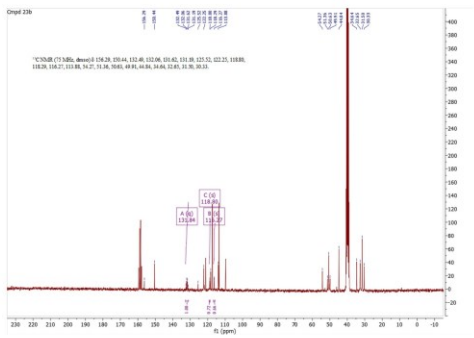
52

Cmpd 23b



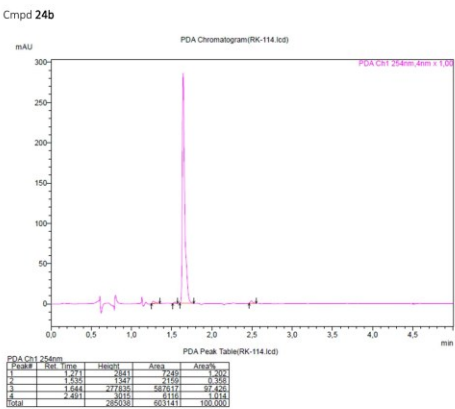
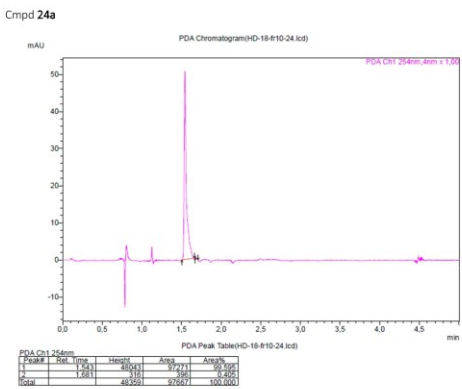
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54



55

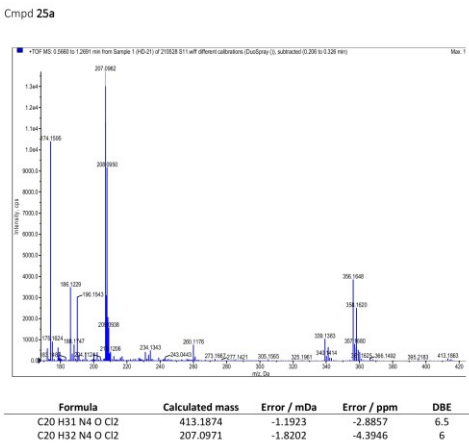
56



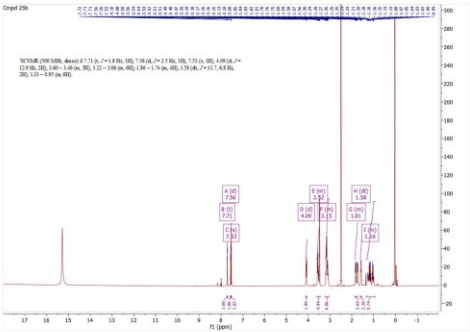
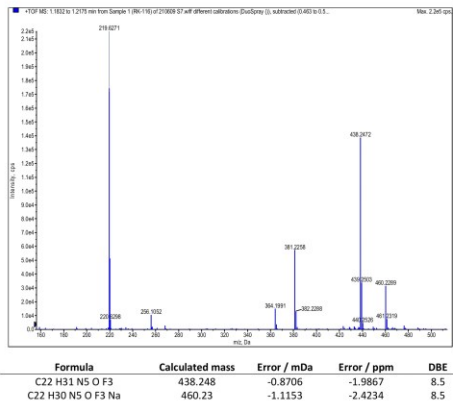
57

58

3 Publications

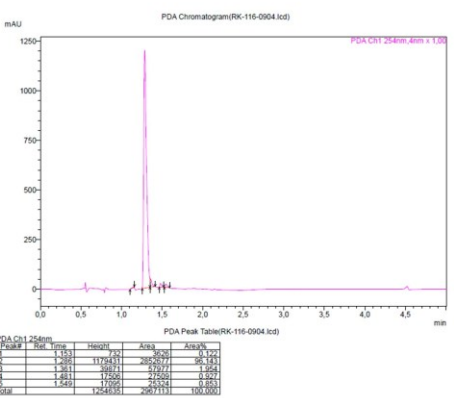
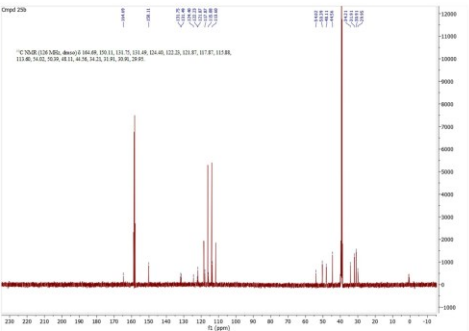


Cmpd 25b



63

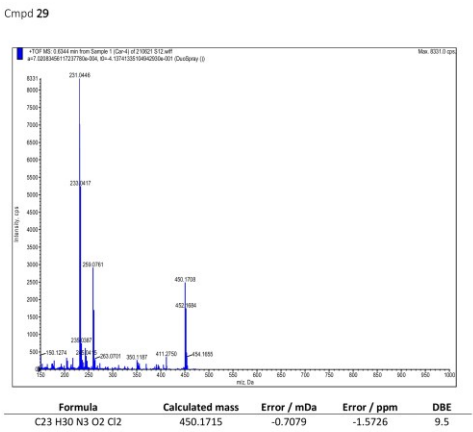
64



65

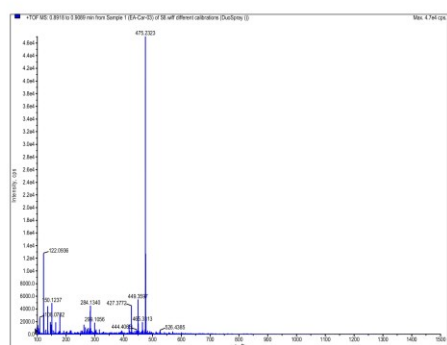
66

3 Publications



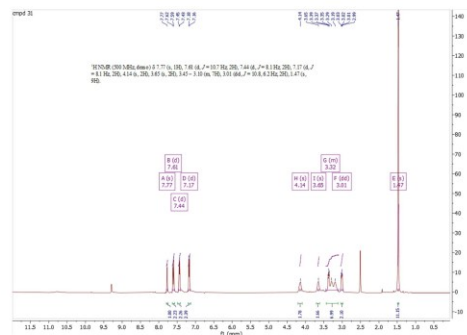
3 Publications

Cmpd 31

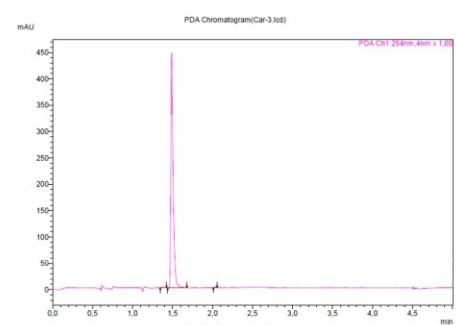
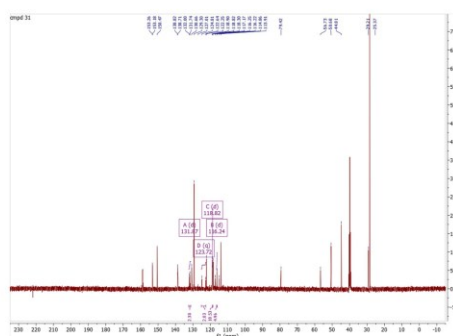


Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C25 H30 N4 O2 F3	475.2315	0.7623	1.604	11.5

75



76



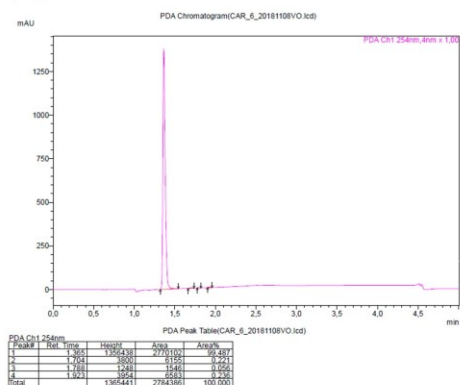
POA Ch1 254nm			POA Peak Ta	
Peak#	Ret. Time	Height	Area	Area%
1	1.367	981	2487	0.278
2	1.485	414060	892146	99.564
3	2.027	918	1419	0.158
Total		415955	895052	100.000

77

78

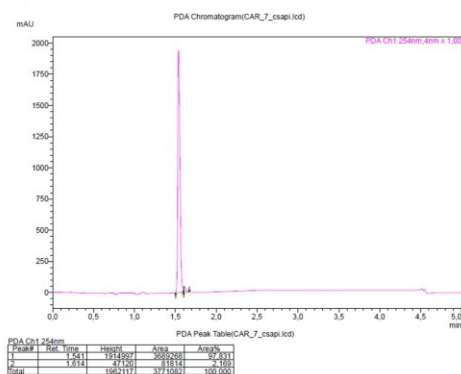
3 Publications

Cmpd 32



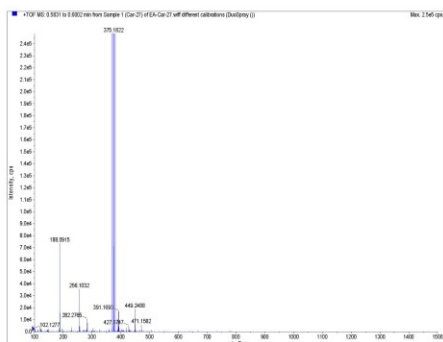
79

Cmpd 33



80

Cmpd 34



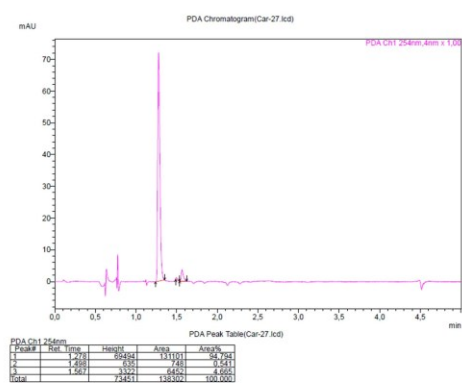
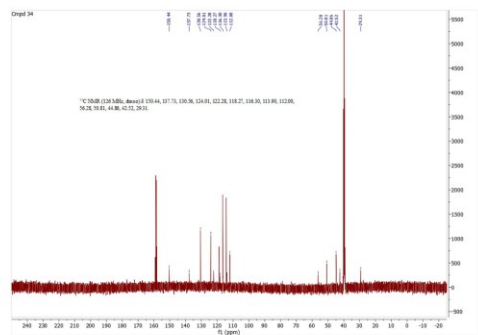
Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C20 H22 N4 F3	375.1791	3.0919	8.241	10.5

10
 ^1H NMR (200 MHz, CDCl_3): δ 12.5 (s, 1H), 7.5 (m, 1H), 7.2 (m, 1H), 6.8 (m, 1H), 5.5 (m, 1H), 4.8 (m, 1H), 4.2 (m, 1H), 3.8 (m, 1H), 3.2 (m, 1H), 2.8 (m, 1H), 2.2 (m, 1H), 1.8 (m, 1H), 1.2 (m, 1H), 0.8 (m, 1H).

81

82

3 Publications

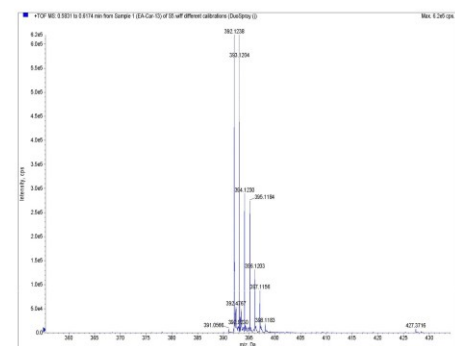


Peak#	Ret. Time	Height	Area	Area%
1	1.30	70.00	111.01	94.94
2	1.40	1.00	7.81	6.67
3	1.50	1.00	6.67	5.67
Total			115.49	100.00

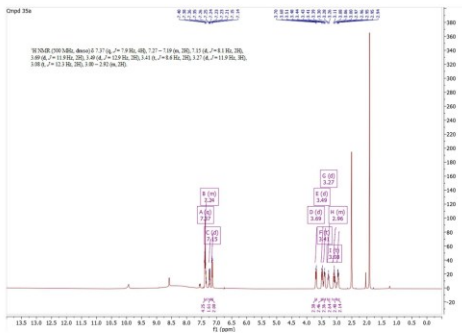
83

84

Compound 35a

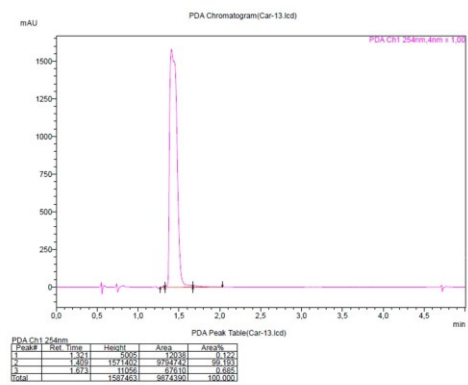
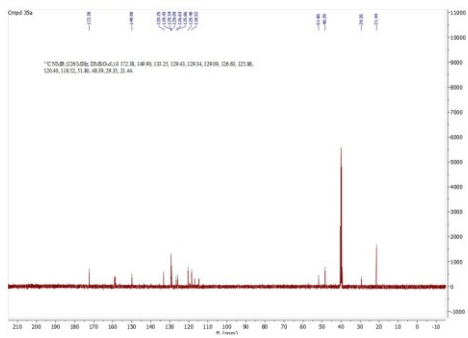


Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C19 H23 N4 O Cl2	393.1248	-4.492	-11.4266	9.5

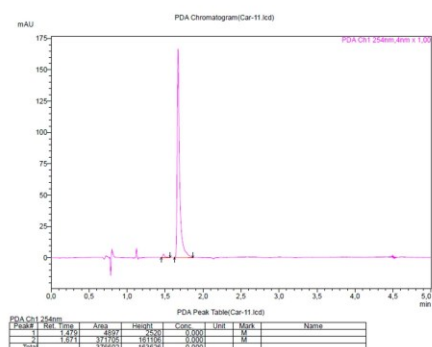
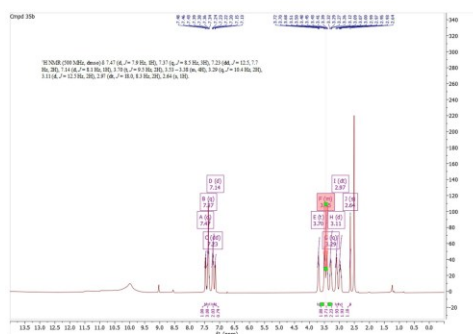


85

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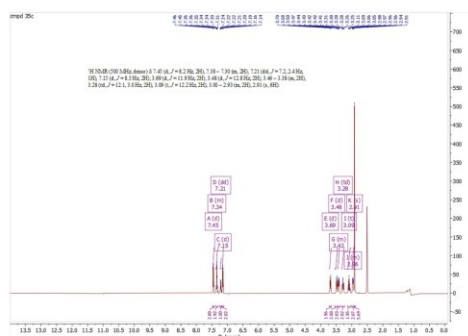
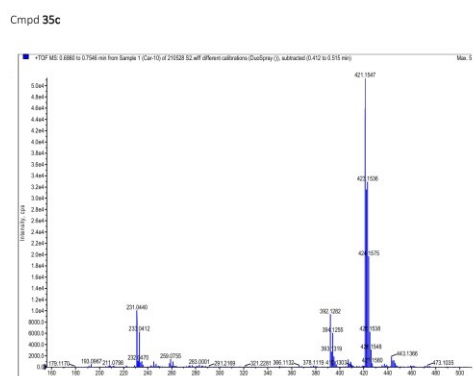


3 Publications



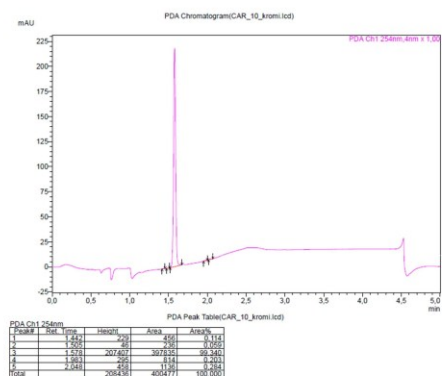
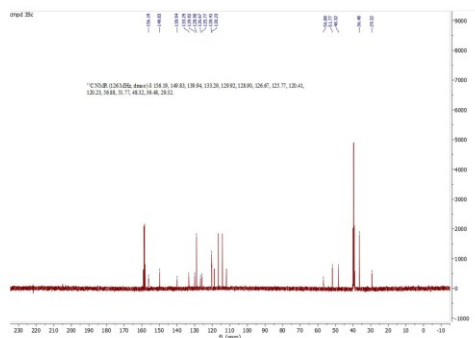
91

92



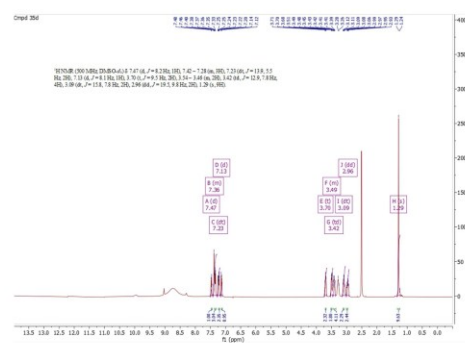
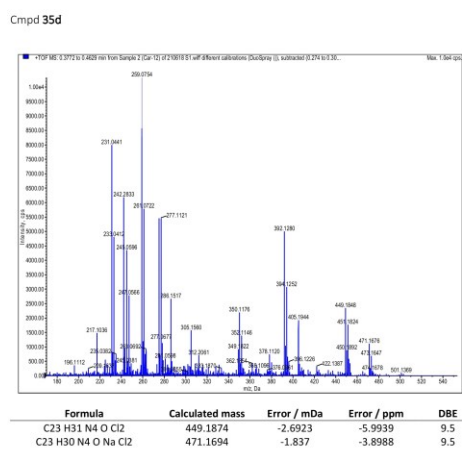
93

94



95

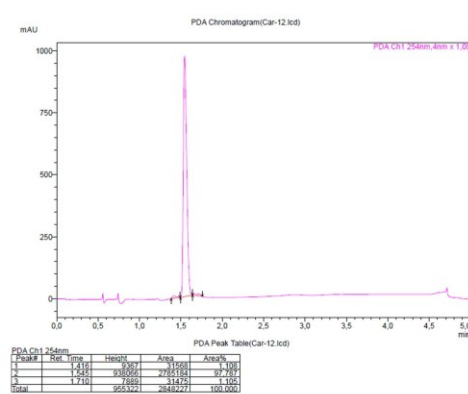
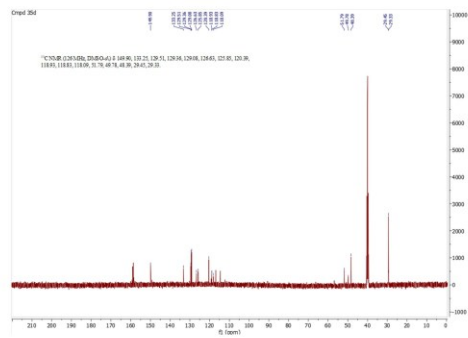
96



97

98

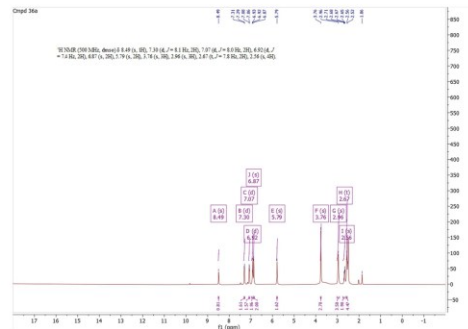
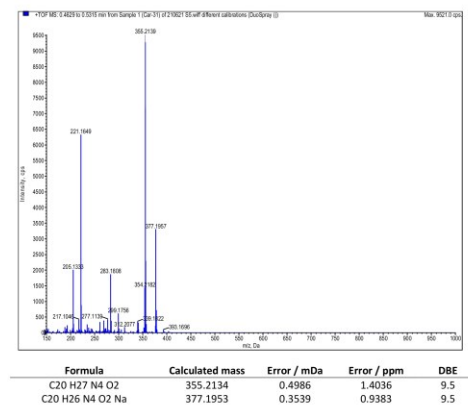
3 Publications



99

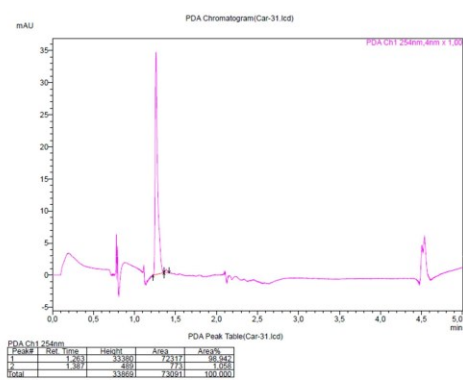
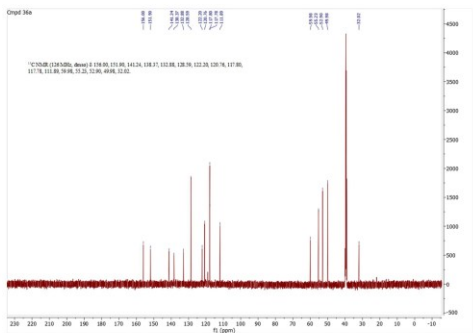
100

Compound 36a



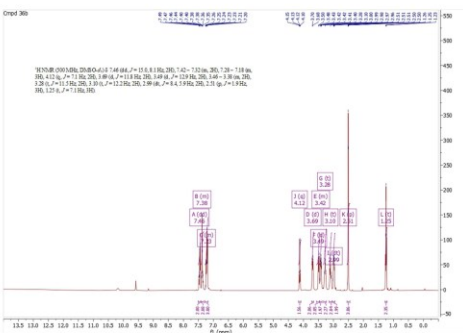
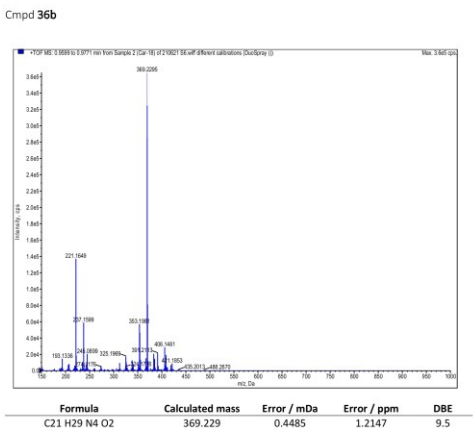
101

102



103

104



105

106

Sample 300

Scan 1000

2300
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1100
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700
600
500
400
300
200
100
0

m/z

210 200 180 160 140 120 100 80 60 40 20 0

12.0188, 12.0188m, 20.0420, 41.0 178.45, 131.04, 148.91, 158.43, 133.22, 133.89, 129.46, 129.56, 124.62, 121.15, 121.09, 114.46, 86.71, 73.76, 46.29, 29.15, 14.9



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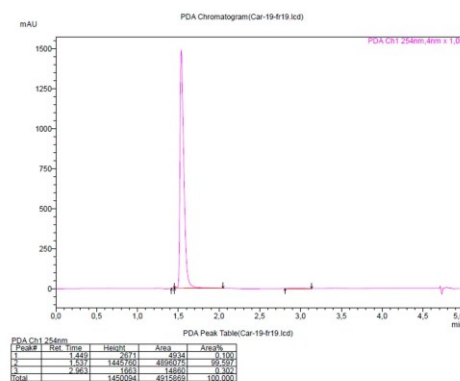
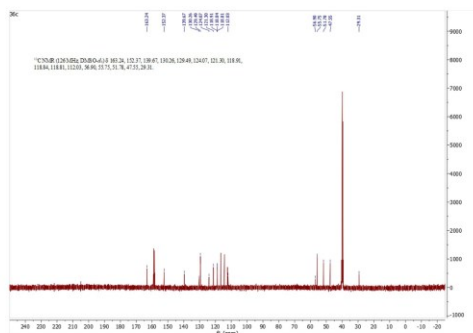
Figure 1: Mass spectrum of the sample. The x-axis represents the mass-to-charge ratio (m/z) from 100 to 500, and the y-axis represents the relative intensity from 0 to 100%. The base peak is at m/z 329.1991. Other labeled peaks include m/z 156.1057, 192.1025, 221.1638, 245.0985, 266.1022, 277.2011, 312.2087, 321.1039, 343.1040, 345.2054, 360.2175, 380.2175, 408.1959, 425.1982, 444.2020, and 462.2020.

¹³C NMR (101MHz, *acetone*-d₆) δ 13.0 (CH₃), 31.9 (CH₂), 41.8 (CH₂), 62.1 (CH₂), 67.7 (CH₂), 69.2 (CH₂), 77.0 (CDCl₃), 119.1 (CH), 120.1 (CH), 121.6 (CH), 124.8 (CH), 125.1 (CH), 125.2 (CH), 125.3 (CH), 125.4 (CH), 125.5 (CH), 125.6 (CH), 125.7 (CH), 125.8 (CH), 125.9 (CH), 126.0 (CH), 126.1 (CH), 126.2 (CH), 126.3 (CH), 126.4 (CH), 126.5 (CH), 126.6 (CH), 126.7 (CH), 126.8 (CH), 126.9 (CH), 127.0 (CH), 127.1 (CH), 127.2 (CH), 127.3 (CH), 127.4 (CH), 127.5 (CH), 127.6 (CH), 127.7 (CH), 127.8 (CH), 127.9 (CH), 128.0 (CH), 128.1 (CH), 128.2 (CH), 128.3 (CH), 128.4 (CH), 128.5 (CH), 128.6 (CH), 128.7 (CH), 128.8 (CH), 128.9 (CH), 129.0 (CH), 129.1 (CH), 129.2 (CH), 129.3 (CH), 129.4 (CH), 129.5 (CH), 129.6 (CH), 129.7 (CH), 129.8 (CH), 129.9 (CH), 130.0 (CH).

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110

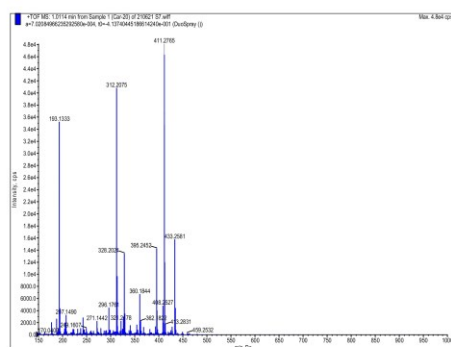
3 Publications



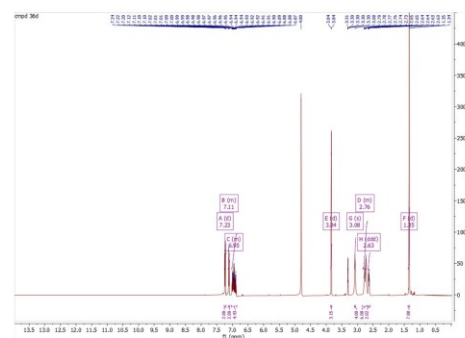
111

112

Cmpd 36d



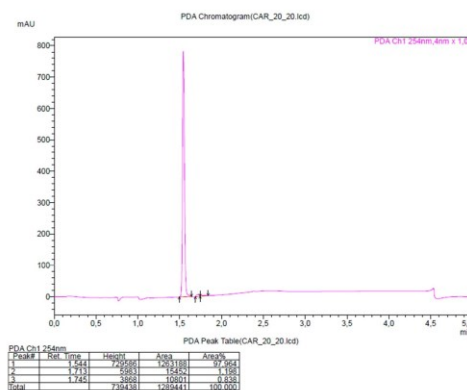
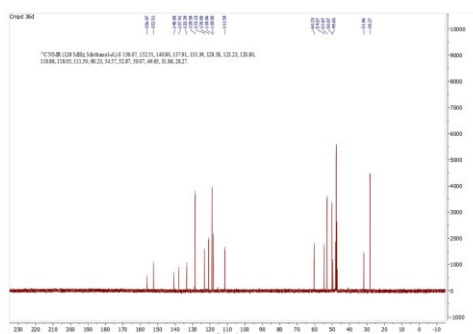
Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C24 H35 N4 O2	411.276	0.4982	1.2115	9.5
C24 H34 N4 O2 Na	433.2579	0.1536	0.3545	9.5



113

114

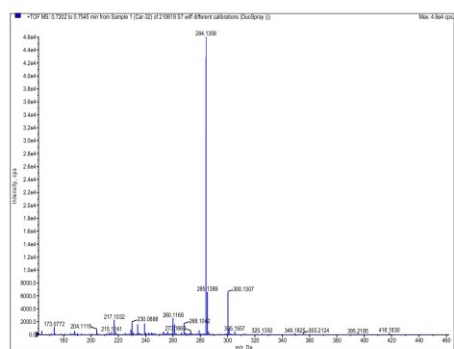
3 Publications



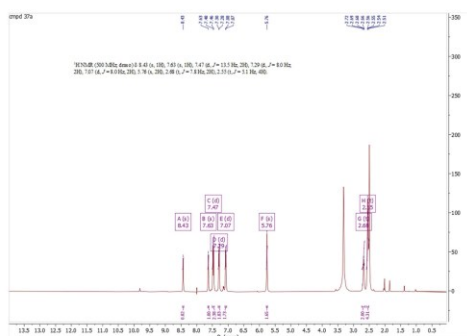
115

116

Cmpd **37a**

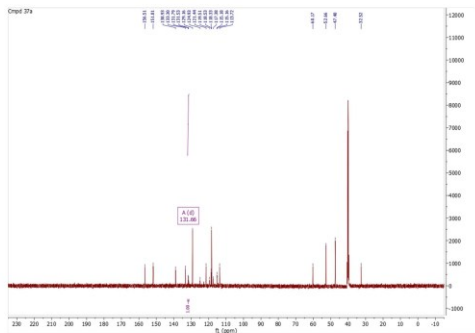


Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C21 H23 N5 O F3	418.1854	-2.4703	-5.9073	11.5

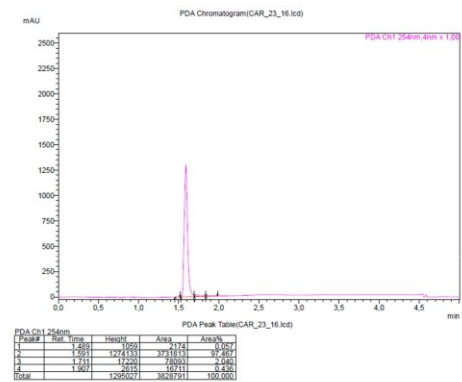
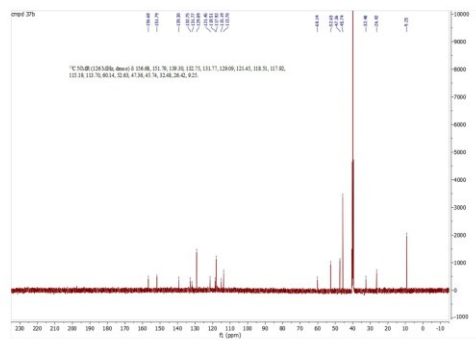


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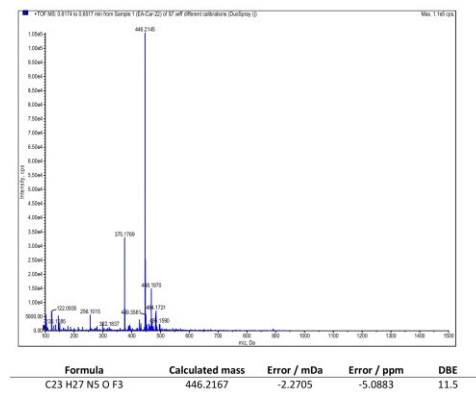
3 Publications

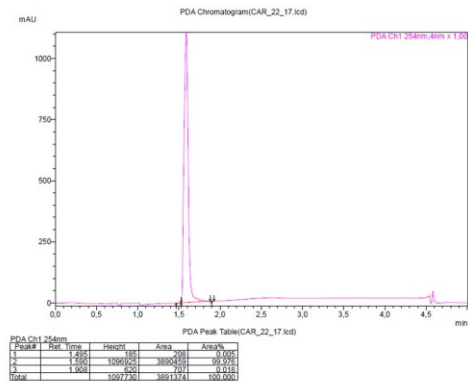
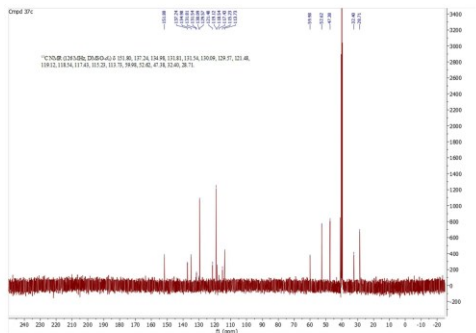


123

124

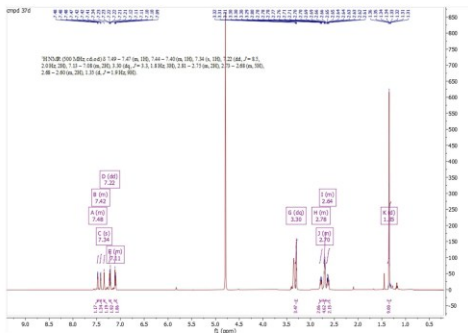
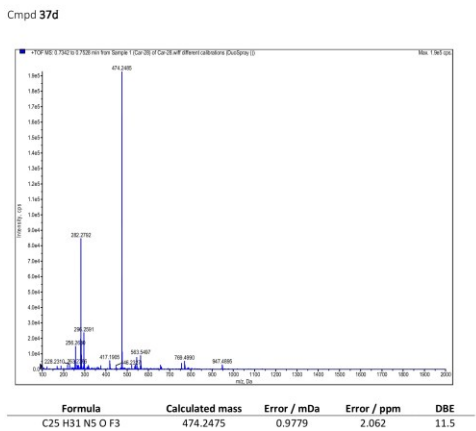
Cmpd 37c





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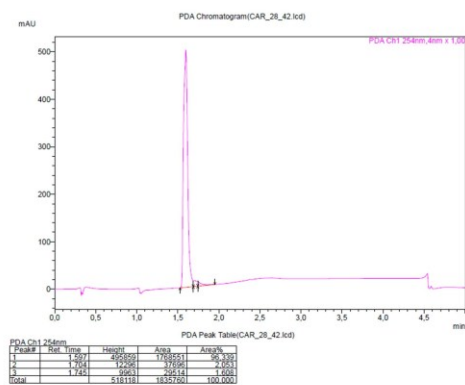
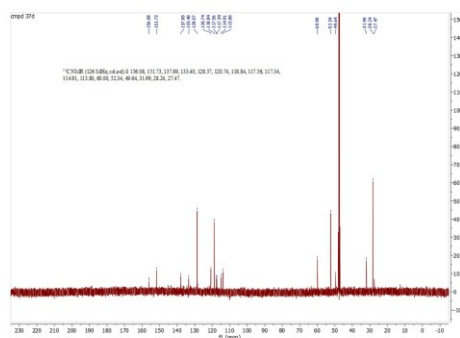
128



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130

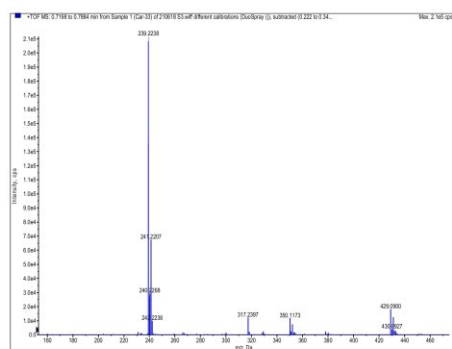
3 Publications



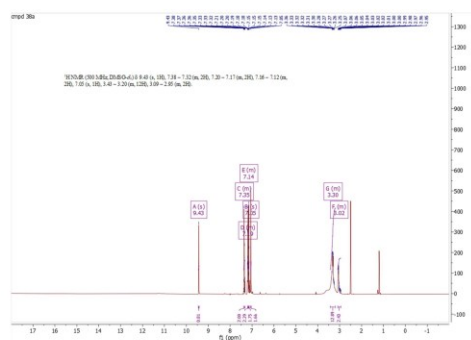
131

132

Cmpd 38a

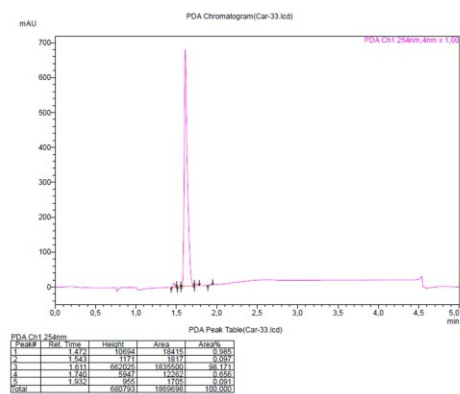
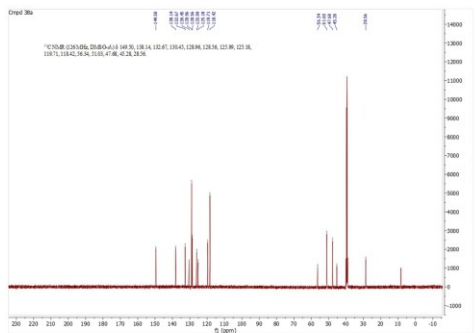


Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C18 H23 N4 O2 S Cl2	429.0918	-1.8784	-4.3778	8.5



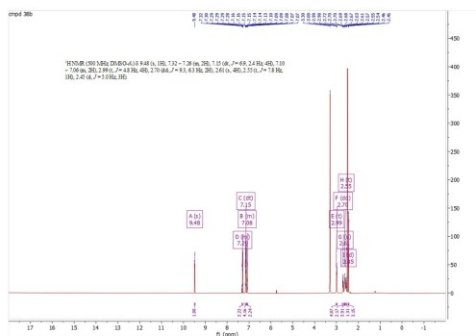
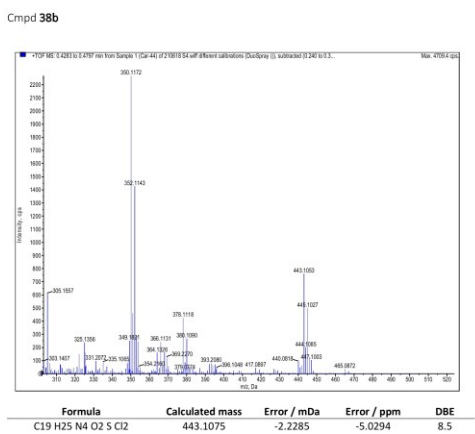
133

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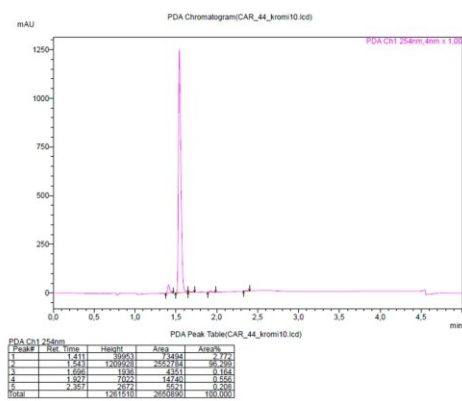
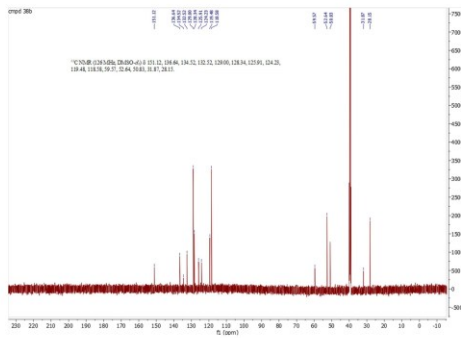
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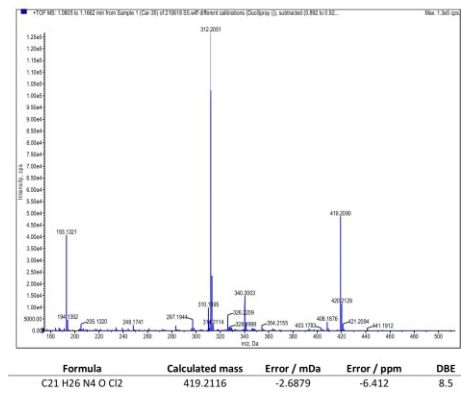
3 Publications



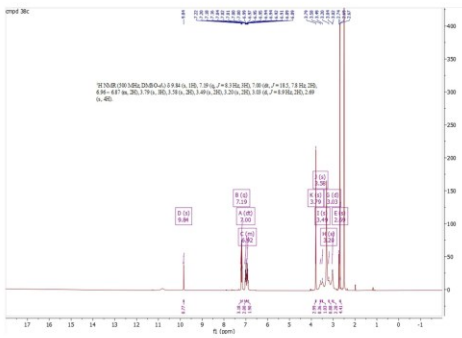
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140

Cmpd 38c

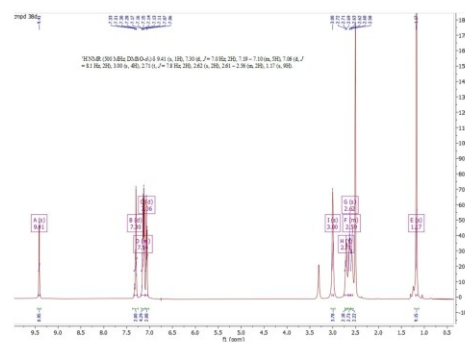
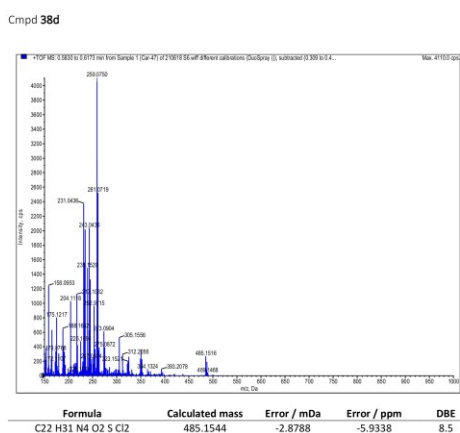
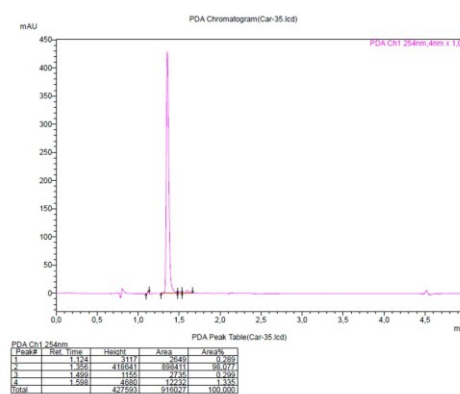
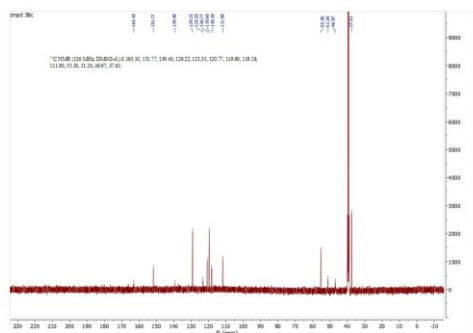


Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C21 H26 N4 O Cl2	419.2116	-2.6879	-6.412	8.5

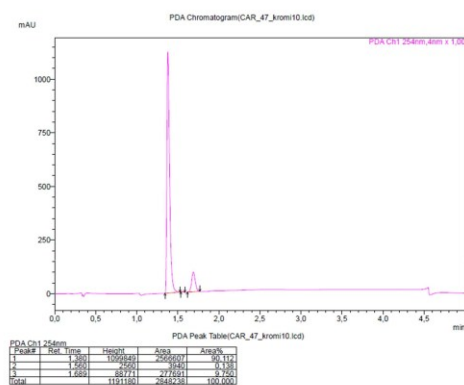
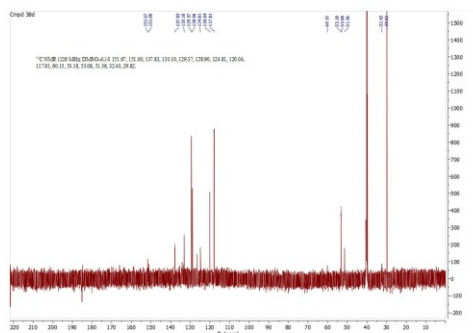


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142

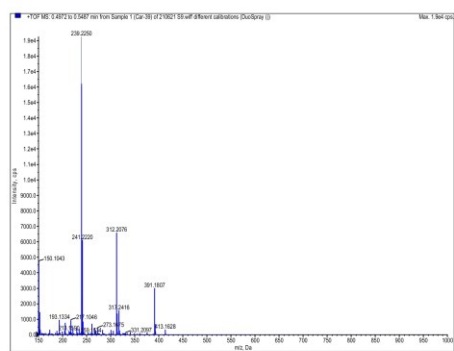


3 Publications

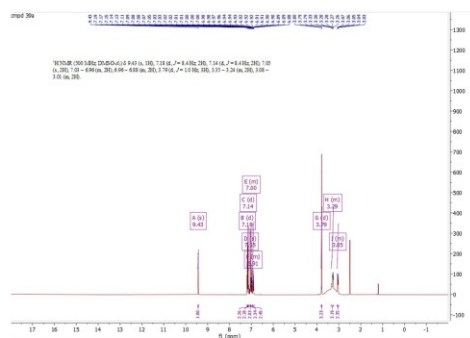


147

148

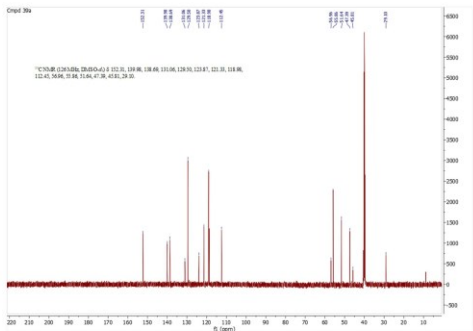


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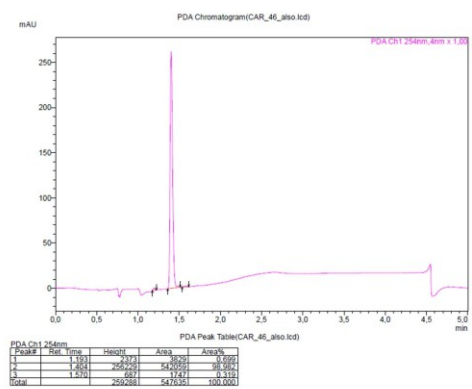
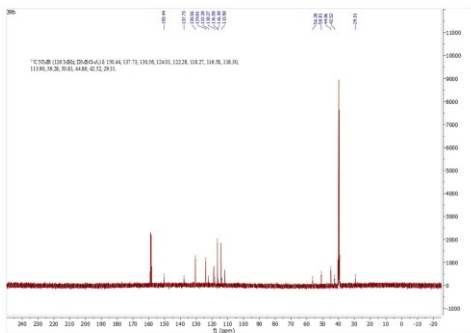


149

150



3 Publications

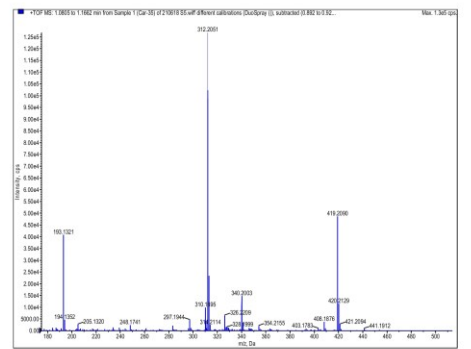


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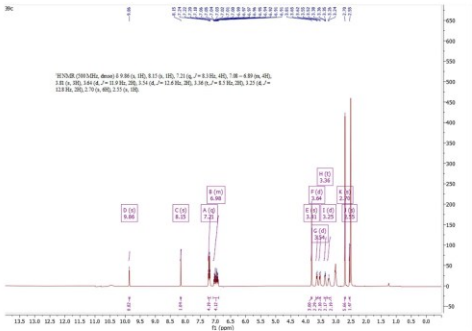
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156

Cmpd 39c

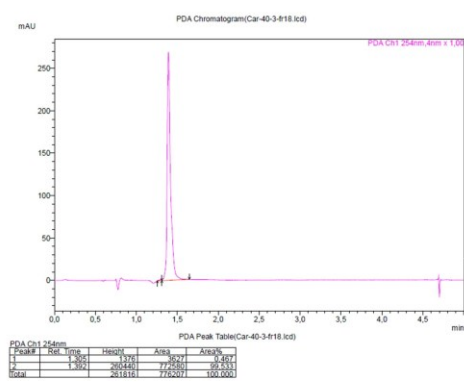
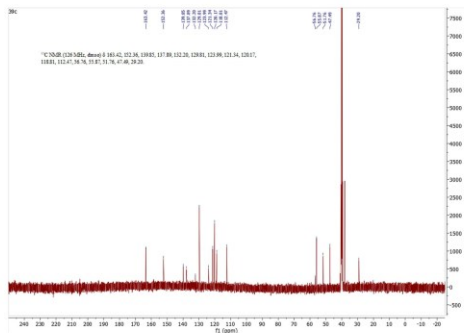


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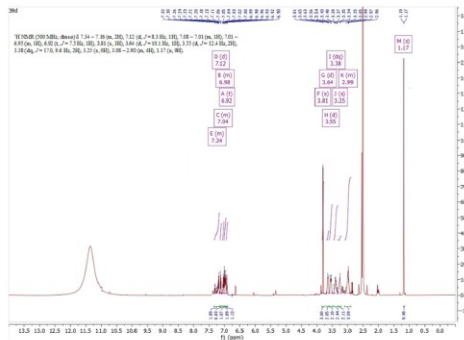
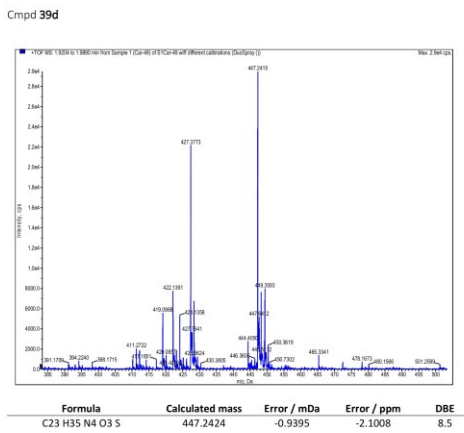
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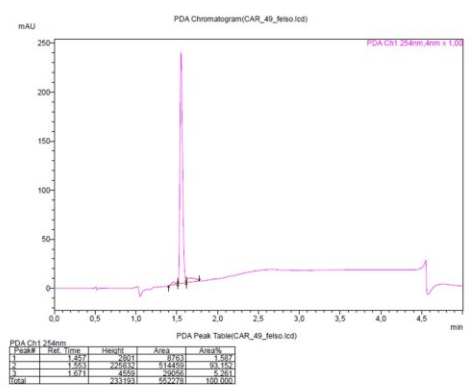
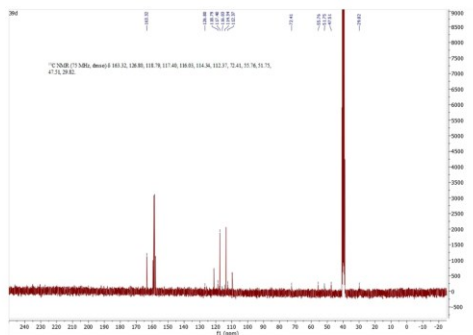
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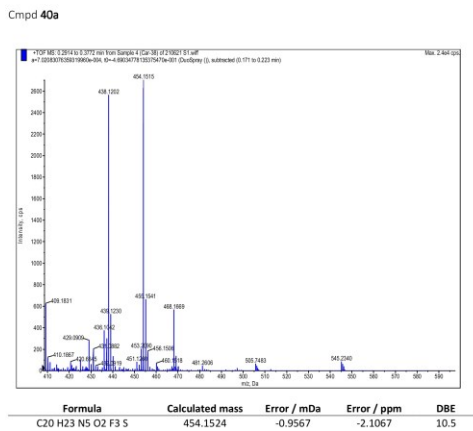
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3 Publications

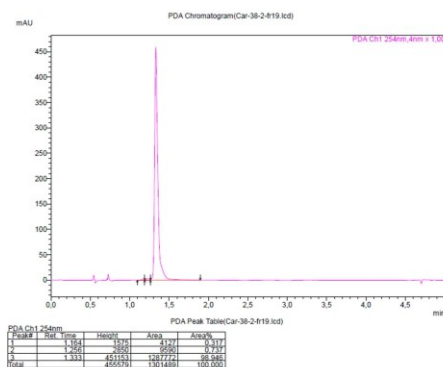
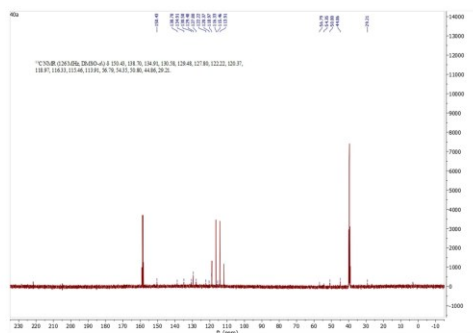


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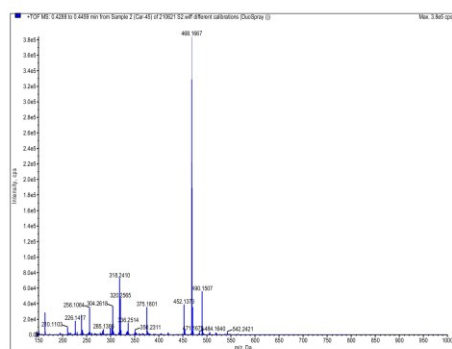


3 Publications

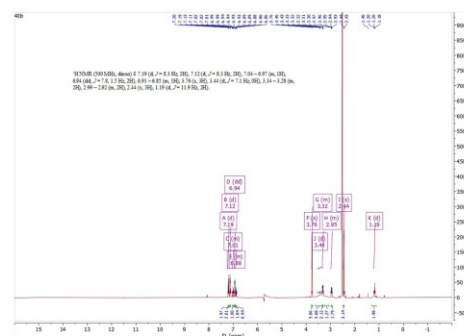


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168



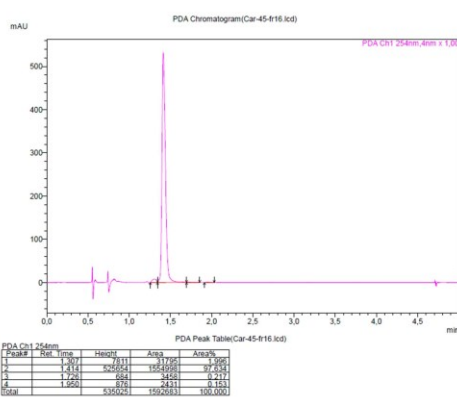
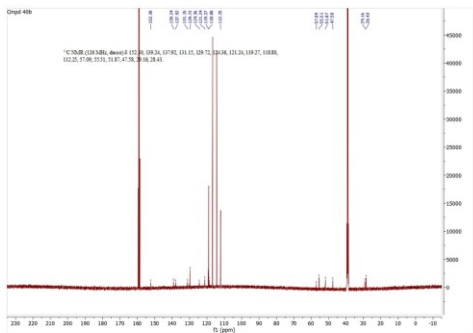
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169

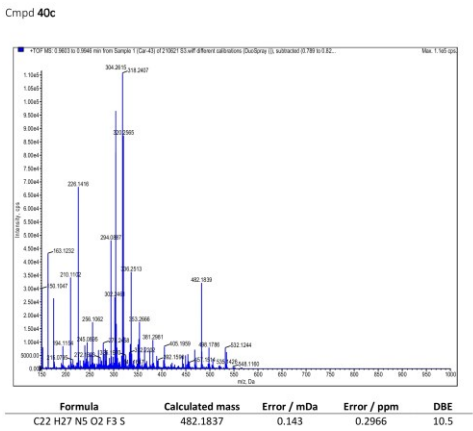
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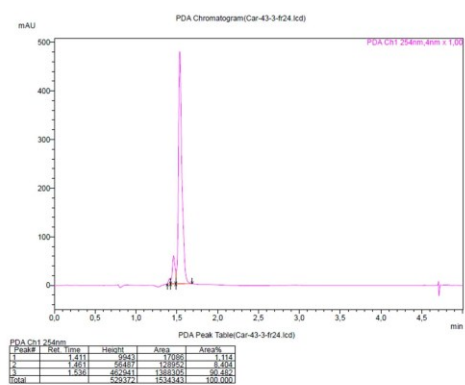
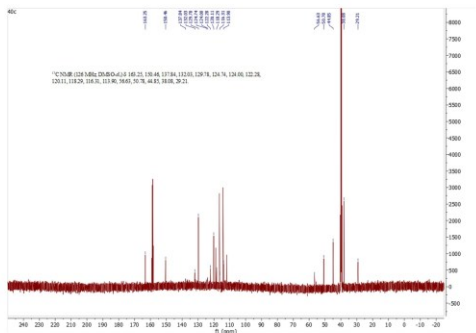
3 Publications



171

172

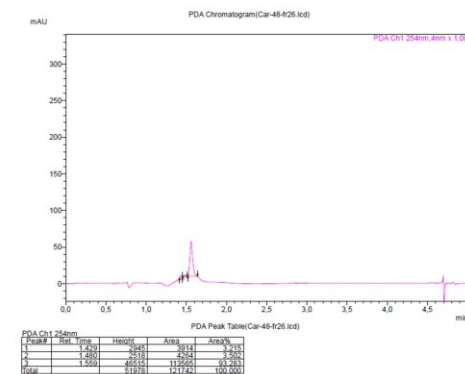
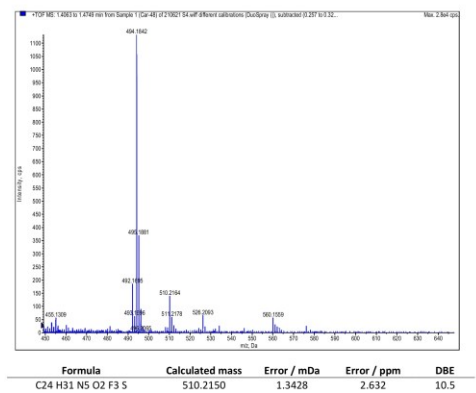




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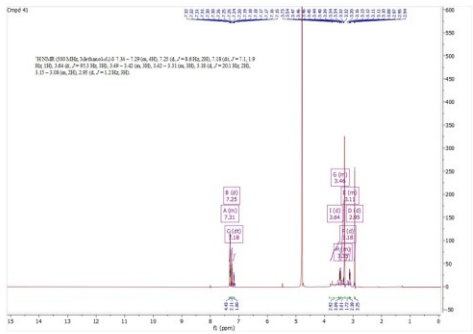
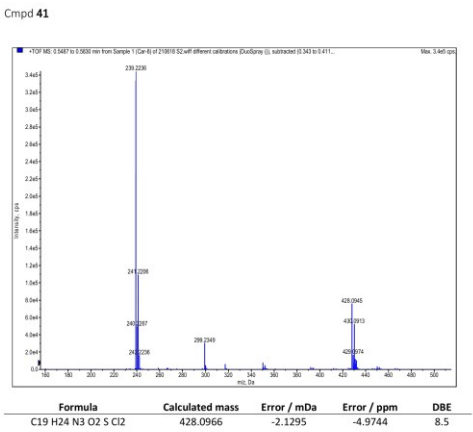
Cmpd 40d



177

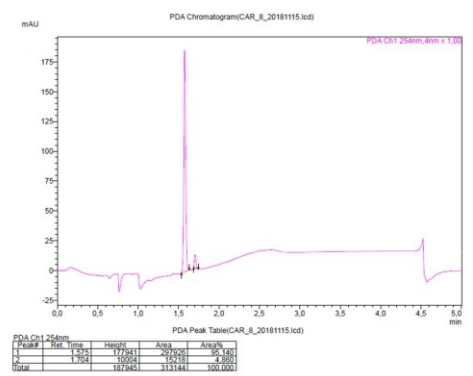
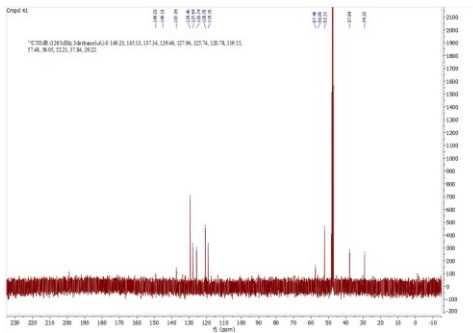
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3 Publications



179

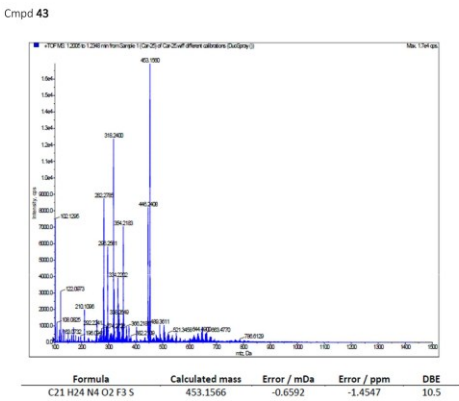
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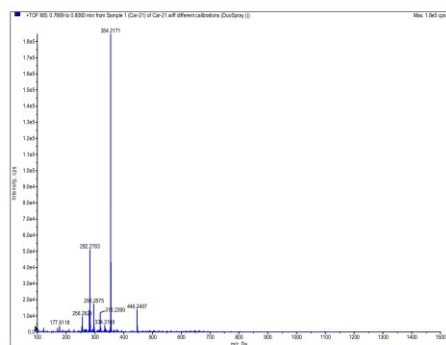
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3 Publications

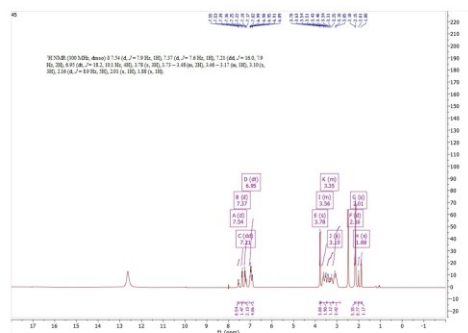


3 Publications

Cmpd 45

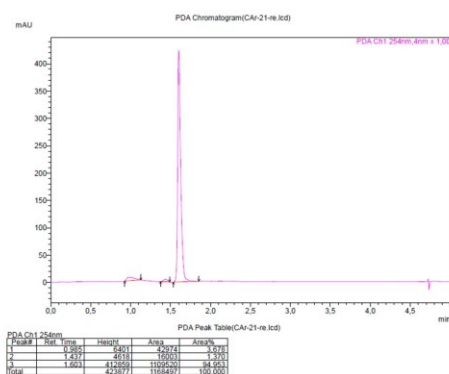
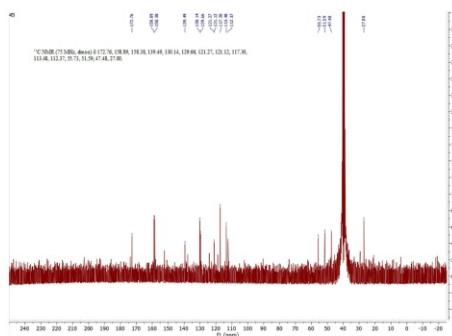


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195

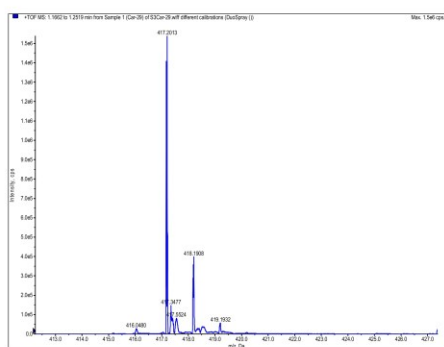
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197

198

Cmpd 46

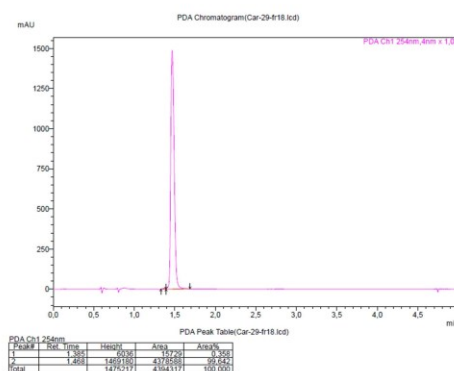


Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C22 H24 N4 O F3	417.1896	-3.6728	-8.8037	11.5

Opel 46

元	号	重量	直径	长度
1	1	1.1	1.1	1.1

107506 (120.7846, 4484.61, 148.31, 120.46, 134.96, 132.41, 129.11, 120.49, 120.43, 108.98, 107.42, 104.00, 89.08, 76.86, 67.93, 58.43, 34.31)



Peak#	Ret. Time	Height	Area	Area%
1	1.385	6036	15728	0.358
2	1.468	1469180	4378588	99.642
Total		1475217	4394317	100.000

3 Publications

3.5 PUBLICATION 5

Design and Synthesis of Arylpiperazine Serotonergic/Dopaminergic Ligands with Neuroprotective Properties

Margherita Mastromarino¹, Mauro Niso¹, Carmen Abate¹, Ewgenij Proschak², Mariam Dubiel³, Holger Stark³, Marián Castro⁴, Enza Lacivita^{1,*} and Marcello Leopoldo¹

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Contribution: Co-authorship. M.D. prepared, planned, and conducted dopamine D1R, D2R, D3R, and D5R binding studies and selectivity screenings. She performed the corresponding data analysis, contributed to writing parts of the radioligand binding studies section, and participated in the proofreading process.

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Article

Design and Synthesis of Arylpiperazine Serotonergic/Dopaminergic Ligands with Neuroprotective Properties

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Abstract: Long-chain arylpiperazine scaffold is a versatile template to design central nervous system (CNS) drugs that target serotonin and dopamine receptors. Here we describe the synthesis and biological evaluation of ten new arylpiperazine derivatives designed to obtain an affinity profile at serotonin 5-HT_{1A}, 5-HT_{2A}, 5-HT₇ receptor, and dopamine D₂ receptor of prospective drugs to treat the core symptoms of autism spectrum disorder (ASD) or psychosis. Besides the structural features required for affinity at the target receptors, the new compounds incorporated structural fragments with antioxidant properties to counteract oxidative stress connected with ASD and psychosis. All the new compounds showed CNS MultiParameter Optimization score predictive of desirable ADMET properties and cross the blood–brain barrier. We identified compound **12a** that combines an affinity profile compatible with antipsychotic activity (5-HT_{1A} K_i = 41.5 nM, 5-HT_{2A} K_i = 315 nM, 5-HT₇ K_i = 42.5 nM, D₂ K_i = 300 nM), and compound **9b** that has an affinity profile consistent with studies in the context of ASD (5-HT_{1A} K_i = 23.9 nM, 5-HT_{2A} K_i = 39.4 nM, 5-HT₇ K_i = 45.0 nM). Both compounds also had antioxidant properties. All compounds showed low in vitro metabolic stability, the only exception being compound **9b**, which might be suitable for studies in vivo.

Keywords: serotonin; dopamine; 5-HT_{1A}; 5-HT_{2A}; 5-HT₇; D₂; oxidative stress; arylpiperazine

1. Introduction

The long-chain arylpiperazine structure is a versatile model that has allowed the identification of several drugs over the years, such as antipsychotics (aripiprazole, ziprasidone, and lurasidone) [1], anxiolytics (buspirone, tandospirone) [2], and antiparkinsonian drugs (piribedil) [3] (Figure 1). This variety of actions is due to the possibility of modulating the pharmacological profile of the arylpiperazine derivatives through structural variations. Even if more than 30 years have passed since the introduction of buspirone in the market, the long-chain arylpiperazine model is still valid, so that, in the last five years, new drugs with this structure have been introduced on the market [4].

The above-mentioned drugs owe their action to the modulation at the central nervous system (CNS) level of one or more serotonin and/or dopamine receptors that are relevant to the specific pathology. For example, aripiprazole owes its antipsychotic action to the blocking of dopaminergic D₂ and serotonergic 5-HT_{2A} receptors. The anxiolytic action of buspirone is due to a partial agonist activity on the 5-HT_{1A} receptors, while the antiparkinsonian effect of piribedil is due to dopaminergic D₂ receptors activation.

Recently, we reported on a set of arylpiperazine derivatives with an activity profile on a subgroup of serotonin receptors (5-HT₇ and 5-HT_{1A} agonist and 5-HT_{2A} antagonist) specifically designed to obtain prospective drugs for the treatment of the core symptoms of autism spectrum disorder [5]. Over the last few years, several studies have highlighted that besides an adequate modulation of the neuroreceptors relevant in the pathology, a neuroprotective action due to a reduction of oxidative stress might have a beneficial effect on these pathologies. In fact, schizophrenia, depression, and autism spectrum disorder (ASD) have a significant neuroinflammatory component linked to oxidative stress [6]. Therefore, the advantage of intervening in targeting a set of receptors and oxidative stress is evident. Of note, recent studies have shown that the activation of the 5-HT_{1A} [7] and 5-HT₇ receptors [8] produces neuroprotective effects. Thus, for the present study, we have designed a set of new arylpiperazine derivatives with the structural characteristics to target a group of neuroreceptors relevant to treating schizophrenia or ASD and producing neuroprotective effects.

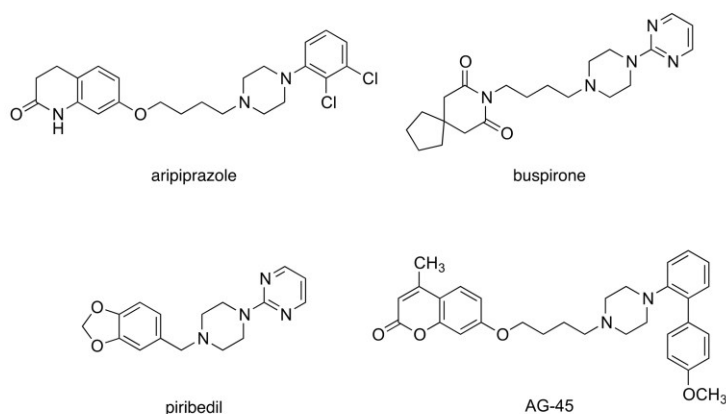


Figure 1. Representative arylpiperazine derivatives.

2. Results

2.1. Study Design

The starting point for our study was AG-45 (compound 20b in Ref. [5], Figure 1), which has an affinity for 5-HT_{1A}, 5-HT_{2A}, and 5-HT₇ receptors. We specifically searched this affinity profile to obtain prospective drugs for the treatment of the core symptoms of ASD. Thus, we introduced structural fragments known for their antioxidant properties, such as the pyridyl [9], 1,2,4-triazine [10], 1,4-benzoxazine [11], and benzopyrone [12], and left unchanged the features responsible for the affinity profile (i.e., the biphenyl-like structure linked to the piperazine ring, and a trimethylene or tetramethylene spacer between the piperazine ring and the terminal fragment). The design of the target compounds is graphically illustrated in Figure 2. Interestingly, AG-45 showed a measurable affinity for the D₂ receptor, and this prompted us to evaluate structural modifications to increase D₂ receptor affinity in the prospect of obtaining antipsychotic compounds with antioxidant properties. Of note, while the affinity for D₂ and 5-HT_{2A} receptors is a crucial feature of atypical antipsychotics, targeting 5-HT_{1A} and 5-HT₇ receptors have been variously correlated to beneficial effects in the context of psychosis [13,14].

The discovery of CNS drugs also faces the challenge of controlling physicochemical properties. CNS Multi-Parameter Optimisation (MPO) scoring tool is an approach that can facilitate the design of CNS drugs [15]. This tool gives a desirability score (0.05–1) for six physicochemical properties: molecular weight (MW); calculated partition coefficient (cLogP); calculated distribution coefficient at pH = 7.4 (cLogD_{7.4}); acid dissociation constant

(pK_a); topological polar surface area (TPSA); number of hydrogen bond donors (HBD). The sum of these scores yields the CNS MPO score on a 0.3–6 scale. A CNS MPO desirability score higher than 4 is predictive of desirable ADMET properties and cross the blood–brain barrier [15]. As shown in Table 1, all the newly designed compounds display favorable characteristics for a prospective CNS drug.

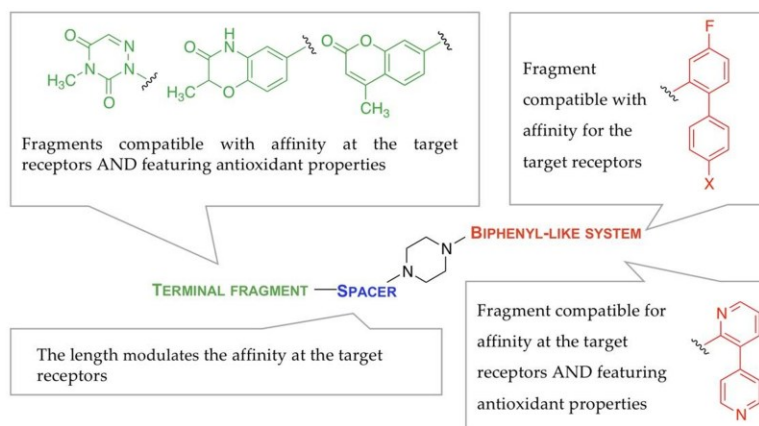


Figure 2. Design Strategy for the Target Compounds.

Table 1. Lipophilicity (cLogP), CNS MPO, and Metabolic Stability (MS) of the Target Compounds.

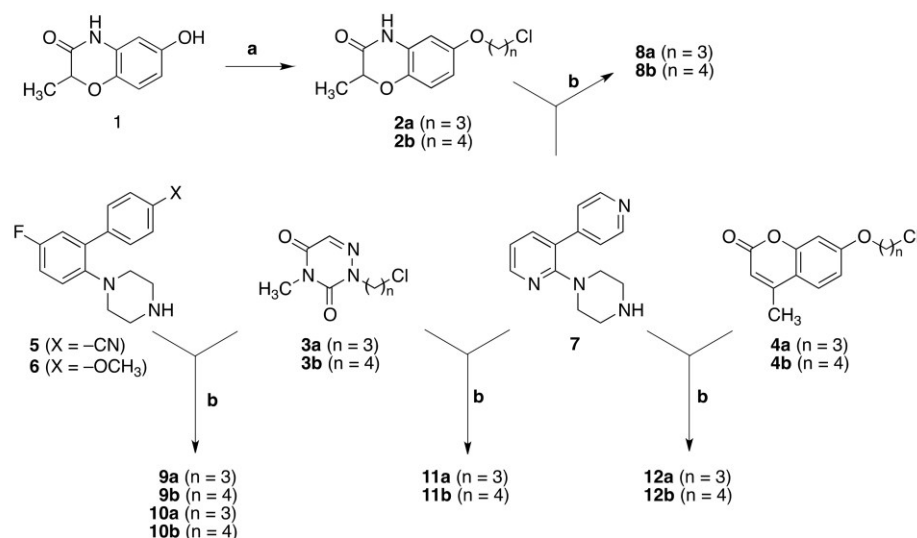
Compd		Structure	cLogP ¹	MPO ²	MS ³
8a	n = 3		2.79	4.98	4%
8b	n = 4		3.31	4.54	14%
9a	n = 3		3.06	5.05	<3%
9b	n = 4		3.58	4.47	45%
10a	n = 3		3.08	5.07	5%
10b	n = 4		3.60	4.49	26%
11a	n = 3		1.24	5.66	4%
11b	n = 4		1.76	5.44	3%
12a	n = 3		3.55	4.52	19%
12b	n = 4		4.07	3.97	14%

¹ cLogP values were calculated with ChemAxon software; ² Multi-Parameter Optimisation; ³ Metabolic Stability (percentage of recovery of the parent compound after incubation for 30 min with rat microsomes).

2.2. Chemistry

The synthesis of the target compounds **8a,b–12a,b** was accomplished through the reaction of the appropriate chloroalkyl derivatives **2a,b–4a,b** with arylpiperazines **5–7**

(Scheme 1). Both chloroalkyl and arylpiperazine intermediates were synthesized with literature methods (as detailed in the Materials and Methods) except alkyl chloride **2b** which was prepared by alkylating 6-hydroxy-2-methyl-2*H*-benzo[b][1,4]oxazin-3(4*H*)-one (**1**) [16] with 1-bromo-4-chlorobutane via the Williamson's synthesis under basic conditions.



Scheme 1. Reagents and conditions: (a) 1-bromo-3-chloropropane or 1-bromo-4-chlorobutane, NaH, anhydrous DMF, r.t., 12h; (b) Na₂CO₃, acetonitrile, reflux, overnight.

2.3. Radioligand Binding Experiments

The final compounds were tested for their affinity at serotonin 5-HT_{1A}, 5-HT_{2A}, and 5-HT₇ receptors and at dopamine D₁, D₂, D₃, and D₅ receptors. As a first step, competition radioligand binding assays were carried out for all the compounds at the single concentration of 10 μ M, on membrane preparations from stable cell lines heterologously expressing the human cloned 5-HT_{1A}, 5-HT_{2A}, or 5-HT₇ receptors. Affinity was determined for those compounds showing % of displacement of the specific binding of the radioligand over 65% at 10 μ M concentration, by means of competition binding curves of six concentration datapoints (typically from 0.1 nM/1 nM to 10 μ M/100 μ M). The affinity of the compounds, expressed as equilibrium dissociation constant (*K_i*) value, is shown in Table 2. For the affinity determination at dopamine receptor subtypes, membrane fractions of human dopamine D_{2s}, D₃, D₁, and D₅ receptor-expressing cells were used. They were co-incubated with [³H]spiperone (for D_{2s} and D₃ receptor) or [³H]SCH23390 (for D₁ and D₅ receptor) and appropriate concentrations of test compound for 120 min. Bound radioligand was separated from the free ligand using filtration. Radioactivity was measured by liquid scintillation counting. The resulting *K_i* values with 95% confidence intervals are listed in Table 2.

Table 2. Affinity and Cytotoxicity Data of the Target Compounds.

Compd	Affinity						Cytotoxicity EC ₅₀ [μM] ± S.E.M.	
	K _i [nM] ± S.E.M.			K _i [nM] (CI 95%)				
	5-HT _{1A} ¹	5-HT _{2A} ¹	5-HT ₇ ¹	D ₂	D ₃	D ₁ ¹	D ₅	
8a	13.1 ± 2.3	782 ± 119	10.7 ± 0.7	388 (249; 606)	1223 (684; 2185)	6290 (1608; 24,595)	7412 (2038; 26,956)	32.9 ± 3.5
8b	17.6 ± 0.9	611 ± 87	9.38 ± 0.42	9.08 (5.07; 16.2)	691 (516; 926)	1711 (981; 2983)	8205 (2475; 27,203)	25.5 ± 2.2
9a	653 ± 95	49.1 ± 12	60.8 ± 7.1	>10,000	>10,000	193 (137; 271)	413 (199; 856)	nd
9b	23.9 ± 4.9	39.4 ± 7.4	45.0 ± 3.4	>10,000	>10,000	88.3 (35.7; 218)	222 (76.0; 646)	51.9 ± 4.3
10a	1091 ± 154	96.8 ± 11	45.7 ± 1.3	>10,000	>10,000	104 (33.0; 331)	180 (96.2; 338)	28.2 ± 3.2
10b	43.4 ± 6.2	80.7 ± 3.1	36.5 ± 5.2	>10,000	>10,000	140 (73.4; 268)	101 (33.8; 299)	34.3 ± 2.5
11a	269 ± 18	>10,000	44.5 ± 2.2	>10,000	>10,000	>10,000	>10,000	>100
11b	5.19 ± 0.12	>10,000	79.4 ± 9.0	>10,000	>10,000	>10,000	>10,000	>100
12a	41.5 ± 3.0	315 ± 44	42.5 ± 6.2	300 (210; 430)	1075 (651; 1776)	2198 (780; 6191)	>10,000	35.2 ± 4.1
12b	11.3 ± 0.4	~1419 ²	52.2 ± 10.6	17.0 (4.56; 63.5)	424 (201; 960)	1527 (354; 6586)	2733 (683; 10,929)	47.9 ± 3.5

¹ Average data from n = 3 independent experiments performed in duplicate. ² Full displacement of specific binding not achieved at the maximum concentration assayed (100 μM), so K_i value might not be accurately estimated; maximum displacement achieved was 84%.

2.4. Docking Studies

Docking studies were performed to rationalize the different binding profiles of the compounds. Representative compounds were docked at the serotonin 5-HT_{1A} and 5-HT_{2A}, and dopamine D₂ and D₁ using the available crystal structures of the receptors (Figures 3 and 4).

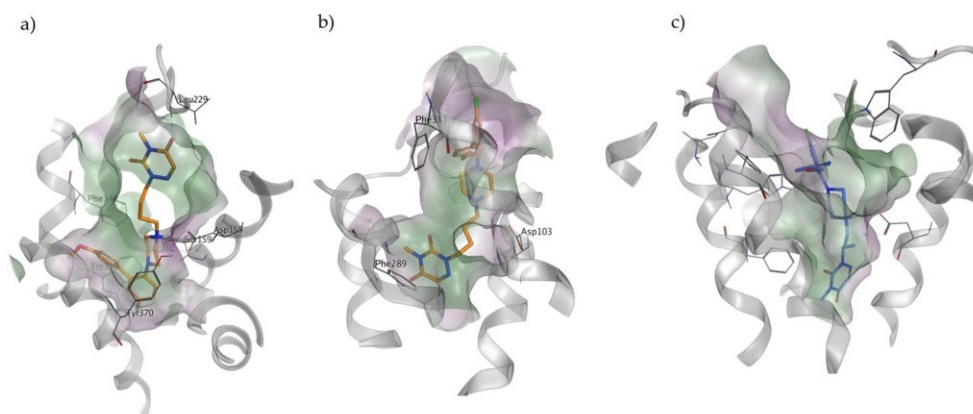


Figure 3. Docking poses of compound **9b** at (a) serotonin 5-HT_{2A} receptor; (b) dopamine D₁ receptor; (c) dopamine D₂ receptor.

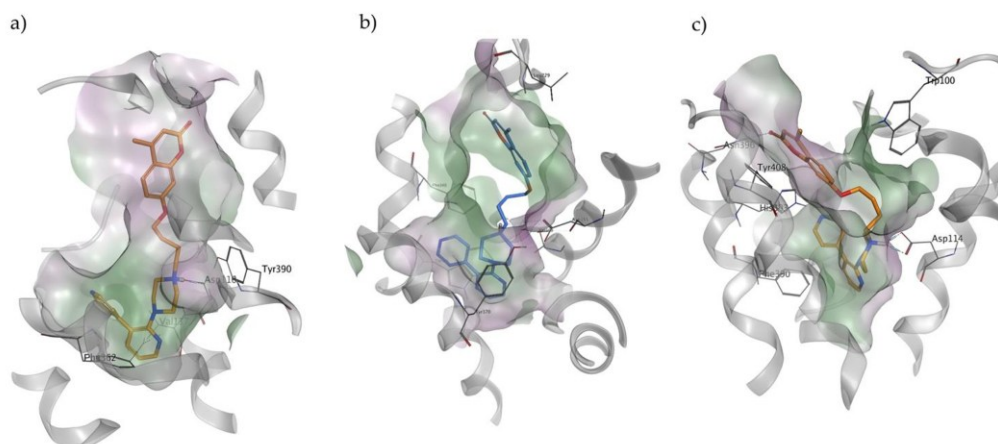


Figure 4. Docking poses of compound **12b** at (a) serotonin 5-HT_{1A} receptor; (b) serotonin 5-HT_{2A} receptor; (c) dopamine D₂ receptor.

2.5. In Vitro Metabolic Stability

To evaluate the susceptibility to first-pass oxidative metabolism, the leading cause of metabolic degradation in vivo [17] the target compounds were incubated with rat liver microsomes. The turnover of the parent compound was assessed as the percentage of the parent compound recovered after 30 min of incubation with microsomes in the presence of an NADPH-regenerating system [18]. The percentages of the recovered unchanged parent compound are listed in Table 1.

2.6. Neuroprotection against H₂O₂ in SHSY-5Y Cell Line

Initially, the cytotoxicity of the target compounds was assessed by treating SH-SY5Y cells for 48 h with various concentrations of compounds (0.1 to 100 μ M). Compounds **11a** and **11b** were not cytotoxic at the tested doses, while the remaining were cytotoxic, exhibiting EC₅₀ values between 20–50 μ M (Table 2). Cells treatment with 1 μ M or 5 μ M of each compound for 24 h did not result in an effect on cell viability (<4% and <8% decrease at a concentration of 1 μ M and 5 μ M, respectively).

Treatment of SH-SY5Y cells with 400 μ M H₂O₂ for 24 h led to a reduction of cell viability of 72–76% (Figure 3). The protective effect of each compound was determined by pretreating the cells with the test compound for 3 h at the non-toxic doses of 1 μ M and 5 μ M, and subsequent treatment with 400 μ M H₂O₂ for an additional 24 h. Pre-treatment of SH-SY5Y cells with compounds **8a,b**, **9b**, **10a**, **11a**, and **12a** resulted in a 14–20% recovery of cell viability with respect to treatment with the H₂O₂ alone (Figure 5).

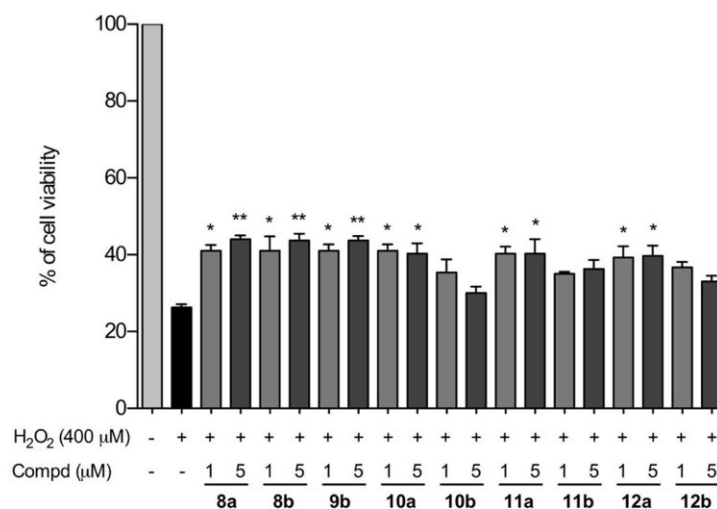


Figure 5. Cell viability of SH-SY5Y cells pretreated with 1 μ M or 5 μ M of the test compound for 3 h, and subsequently exposed to 400 μ M H₂O₂ for 24 h. The data are shown as the mean $\pm$ S.E.M. of three independent experiments (** $p < 0.001$ * $p < 0.05$ vs. 400 μ M H₂O₂-treated group).

3. Discussion

The binding affinities of the target compounds are listed in Table 2. The analysis of the binding data at the screened receptors provides information regarding the role of the three structural elements that compose the structure, i.e., biphenyl-like system, spacer, and terminal fragment.

Considering the affinities for the 5-HT_{1A} receptor, we can note that both biphenyl-like systems and terminal fragments selected for the study are compatible with high affinity at the receptor. In fact, four compounds out of 10 show K_i values lower than 17.6 nM (compounds **8a,b**, **11b**, **12b**). The differences in affinity are related to the length of the spacer between the piperazine and the terminal fragment. Examining the compounds characterized by the oxotriazinyl terminal fragment (compounds **9a,b–11a,b**), the derivatives with a tetramethylene spacer have higher affinity than the trimethylene counterparts. The influence of the spacer length on affinity is related to the nature of the terminal fragment. In fact, considering the compounds featuring the bipyridyl system linked to the piperazine, the K_i ratios of the trimethylene and tetramethylene homologs are variable (**11a/11b** = 52; **12a/12b** = 3.7; **8a/8b** = 0.7).

As for the 5-HT_{2A} receptor, the compounds featuring the bipyridyl system linked to the piperazine display moderate to low affinity. Within this group of compounds, the terminal fragment plays a role on affinity. In fact, the compounds with the oxotriazinyl fragment have a low affinity, whereas a bicyclic terminal fragment imparts comparatively higher affinity. The replacement of the bipyridyl with a biphenyl system has a favorable effect on the affinity. In fact, for the compounds with a tetramethylene spacer, **9b** has a slightly higher affinity than **10b** which shows much higher affinity than **11b**. A similar trend can be observed for the trimethylene spacer derivatives **9a**, **10a**, and **11a**.

The affinity values of the target compounds at 5-HT₇ receptor are distributed in a narrower range as compared to the other receptors under study. In fact, the highest affinity values were shown by **8b** (K_i = 9.38 nM) and the lowest by **11b** (K_i = 79.4 nM). Consequently, the differences in affinity between homologs (trimethylene or tetramethylene spacer derivatives) or analogs (i.e., pair of compounds featuring the same biphenyl-like system and a different terminal fragment) are always lower than 2-fold.

As for the D₂-like receptors (D₂ and D₃), it is immediately evident that the oxotriazinyl terminal fragment leads to compounds devoid of affinity (compounds **9a,b–11a,b**). By contrast, the bicyclic terminal fragments lead from moderate to high affinity (compounds **8a,b, 12a,b**) to both D₂ and D₃ receptors, being the affinity for the former receptor slightly higher. For these compounds, the tetramethylene derivatives have slightly higher affinity than the trimethylene analogs.

Regarding the D₁-like receptors (D₁ and D₅), the biphenyl-like system influences the affinity to a greater extent than the terminal fragment. In fact, the bipyridyl fragment leads to low affinity for D₁ and D₅ receptors, whereas the biphenyl fragment to moderate affinity. Considering compounds **9a,b** and **10a,b**, the trimethylene or tetramethylene homologs display a difference in affinity lower than 2-fold.

Collectively, the binding data of the target compounds revealed that the three structural elements that compose the structure have different effects on the affinity for the receptors under study. For 5-HT_{1A} receptor, variations of length of the spacer result in the highest variations in affinity. For 5-HT_{2A} receptors, the nature of the biphenyl-like system has high influence on the affinity. For 5-HT₇ receptor, the biphenyl-like systems linked to the piperazine ring confirmed their favorable role on affinity, with no substantial effects of both the spacer length and the terminal fragments. For D₂-like receptor, the terminal fragment has a pronounced impact on affinity, whereas for D₁-like receptor biphenyl-like system has a greater effect.

To identify molecular contacts responsible for the observed affinity profile, compounds **9b** and **12b** were docked at serotonin 5-HT_{1A} and 5-HT_{2A} and dopamine D₂ and D₁ receptors. We found that compounds orientations were analogous for all the studied receptors, except for the dopamine D₁ receptor, with the piperazine moiety forming a strong charged-assisted hydrogen bond with the highly conserved aspartate residue on the third transmembrane helix and the aryl substituent linked to the piperazine ring deeply buried in the binding site. By comparing the docking poses at serotonin 5-HT_{2A} receptor of compounds **9b** (5-HT_{2A} K_i = 39.4 nM) and **12b** (5-HT_{2A} K_i = 1492 nM), it is evident that the dimension of the terminal fragment impacts the interaction of the molecule with the receptor. In fact, the oxotriazinyl terminal fragment of compound **9b** formed an additional contact with Leu229 of ECL2. In contrast, the bicyclic terminal fragment of compound **12b** is too big to allow a proper accommodation in the binding pocket and leads to a steric clash. Similarly, compounds **9b** and **12b** showed very different binding poses at the dopamine D₂ receptor, which agree with the very different K_i values (compound **9b** K_i = >10,000 nM, compound **12b** K_i = 17.0 nM). In fact, while the arylpiperazine moiety of compound **12b** is located deeper into the binding site and the terminal fragment is orientated towards the extracellular milieu forming an additional contact with Asn396, compound **9b** showed an opposite orientation with the arylpiperazine pointing towards the extracellular milieu that is not in agreement with the observed structure–activity relationships.

Binding affinity data and docking poses indicated that, when the structural requirements for high affinity for a given set of receptors overlap, compounds with different profiles can be identified. In fact, compounds **9b** and **10b** have a balanced profile concerning the affinity for 5-HT_{1A}, 5-HT_{2A}, and 5-HT₇ receptors, which is the desired affinity profile to obtain prospective drugs for the treatment of the core symptoms of ASD. In addition, the selectivity over D₂ receptors of both **9b** and **10b** is indeed favorable in terms of potential extrapyramidal side effects. On the other hand, compounds **8b** and **12a** have an affinity profile compatible with antipsychotic activity, as they have an affinity for the D₂ receptor, being **12a** preferable for the balanced affinity for D₂ and 5-HT_{2A} receptors, accompanied by a comparable affinity for 5-HT_{1A} and 5-HT₇ receptors.

Next, we studied the potential of the target compounds to protect against apoptosis induced by H₂O₂ using SH-SY5Y neuroblastoma cells as an experimental model.

We first evaluated the cytotoxicity of the target compounds alone and found that compounds **11a** and **11b** were not cytotoxic in the dose range of 0.1 to 100 µM, while the remaining compounds showed moderate cytotoxicity, having EC₅₀ values between

20–50 μM (Table 2). Of note, SH-SY5Y cells treatment with each compound at 1 μM or 5 μM concentration for 24 h had a marginal effect on cell viability, with a decrease lower than 4% or 8% at 1 μM or 5 μM , respectively.

Next, the protective effect of each compound was determined by pretreating the cells with the test compound for 3 h at the non-toxic doses of 1 μM and 5 μM , and subsequent treatment with 400 μM H_2O_2 for an additional 24 h. Treatment of SH-SY5Y cells with 400 μM H_2O_2 for 24 h caused a reduction of cell viability of 72–76% (Figure 3). Pretreatment of SH-SY5Y cells with compounds **8a,b**, **9b**, and **10a–12a** resulted in a statistically significant recovery of cell viability with respect to treatment with the 400 μM H_2O_2 alone at both concentrations studied (Figure 3). Thus, compounds **8b**, **9b**, and **12a** stand out of the set because they combine a favorable affinity profile with antioxidant properties.

The target compounds were screened for their in vitro metabolic stability to evaluate their liability to metabolic degradation by first-pass oxidative metabolism—the main cause of metabolic degradation in vivo [17]. The metabolic stability was assessed as the percentage of the parent compound recovered after 30 min of incubation with rat microsomes in the presence of an NADPH-regenerating system (Table 1). Compound **9b** was the most stable compound as it showed a percentage of recovery (45%) that is predictive of low clearance in vivo, based on our previous data on a broad set of analog arylpiperazine derivatives [5,18]. Among the remaining compounds, **10b** and **12a** showed borderline stability value, while the others were massively metabolized. By comparing the cLogP values and metabolic stability of each compound, it emerges that the compound's oxidative liability is not related to lipophilicity, as shown by pairs of compounds **9a,b** and **10a,b** (Table 1).

Finally, the solubility of compounds **9b** and **12a**, which showed the best combination of biological activity and metabolic stability, were calculated using the Swiss ADME algorithm [19]. Both compounds were predicted as moderately soluble showing solubility values in the same range of buspirone and aripiprazole (solubility **9b**: 5.08×10^{-3} mg/mL; **12a**: 5.06×10^{-3} mg/mL; buspirone: 8.64×10^{-2} mg/mL; aripiprazole: 1.88×10^{-3} mg/mL).

4. Materials and Methods

4.1. Chemicals

Chemicals were purchased from Sigma-Aldrich, Alfa Aesar, TCI Chemicals. Unless otherwise stated, all chemicals were used without further purification. Thin layer chromatography (TLC) was performed using plates from Merck (silica gel 60 F254). Column chromatography was performed with 1:30 Merck silica gel 60 Å (63–200 μm) as the stationary phase. Flash chromatographic separations were performed on a Biotage SP1 purification system using flash cartridges pre-packed with KP-Sil 32–63 μm , 60 Å silica. ^1H NMR spectra were recorded on a Varian Mercury-VX spectrometer (300 MHz) or on a 500-vnmrs500 Agilent spectrometer (500 MHz). All chemical shift values are reported in ppm (δ). Recording of mass spectra was done on an HP6890-5973 MSD gas chromatograph/mass spectrometer; only significant m/z peaks, with their percentage of relative intensity in parentheses, are reported. HRMS-ESI analyses were performed on a Bruker Daltonics MicrOTOF-Q II mass spectrometer, mass range 50–800 m/z , electrospray ion source in positive or negative ion mode. All spectra were in accordance with the assigned structures. Elemental analyses (C,H,N) of the target compounds were performed on a Eurovector Euro EA 3000 analyzer. Analyses indicated by the symbols of the elements were within $\pm 0.4\%$ of the theoretical values. The purity of the target compounds listed in Table 1 was assessed by RP-HPLC and combustion analysis. All compounds showed $\geq 95\%$ purity. RP-HPLC analysis was performed on an Agilent 1260 Infinity Binary LC System equipped with a diode array detector using a Phenomenex Gemini C-18 column (250 mm $\times$ 4.6 mm, 5 μm particle size). All target compounds (Table 1) were eluted with CH_3CN /ammonium formate 50 mM pH 5, 8:2 (v/v) at a flow rate of 1 mL/min.

The following compounds were prepared as described in the literature: 6-hydroxy-2-methyl-2H-benzo[b][1,4]oxazin-3(4H)-one (**1**) [20], 6-(3-chloropropoxy)-2-methyl-2H-benzo[b][1,4]oxazin-3(4H)-one (**2a**) [5], 2-(3-chloropropyl)-4-methyl-1,2,4-triazine-3,5(2H,4H)-dione (**3a**) [21], 2-(4-chlorobutyl)-4-methyl-1,2,4-triazine-3,5(2H,4H)-dione (**3b**) [21], 7-(3-chloropropoxy)-4-methyl-2H-chromen-2-one (**4a**) [5], 7-(4-chlorobutoxy)-4-methyl-2H-chromen-2-one (**4b**) [5], 5'-fluoro-2'-(piperazin-1-yl)-[1,1'-biphenyl]-4-carbonitrile (**5**) [18], 1-(5-fluoro-4'-methoxy-[1,1'-biphenyl]-2-yl)piperazine (**6**) [18], 2-(piperazin-1-yl)-3,4'-bipyridine (**7**) [22].

4.2. Synthesis

6-(4-Chlorobutoxy)-2-methyl-2H-benzo[b][1,4]oxazin-3(4H)-one (2b). To a suspension of NaH (0.11 g, 4.47 mmol) in anhydrous DMF (3 mL), 6-hydroxy-2-methyl-2H-benzo[b][1,4]oxazin-3(4H)-one (**1**) (0.61 g, 3.43 mmol) was added portionwise. The mixture was stirred at room temperature for 10 min, then a solution of the appropriate 1-bromo-4-chlorobutane (0.70 g, 4.47 mmol) in the same solvent (2 mL) was added. The reaction mixture was stirred at room temperature for 12 h, poured on ice water and extracted with AcOEt (3 × 20 mL). The organic layers were collected, washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. The crude was purified on a silica gel column by gradient elution from *n*-hexane/AcOEt 7:3 to *n*-hexane/AcOEt, 1:1, to obtain the pure compound as a yellow solid (0.31 g, 36% yield). ¹H NMR (CDCl₃): δ 1.56 (d, 3H, *J* = 6.8 Hz), 1.95 (qn, 2H, *J* = 6.0 Hz), 2.21 (qn, 2H, *J* = 6.0 Hz), 3.43 (t, 2H, *J* = 6.4 Hz), 3.73 (t, 2H, *J* = 6.4 Hz), 4.59 (q, 1H, *J* = 6.8 Hz), 6.37 (d, 1H, *J* = 2.5 Hz), 6.52 (dd, 1H, *J* = 2.9 and 8.8 Hz), 6.89 (d, 1H, *J* = 8.8 Hz), 8.14 (s, 1H, D₂O exchanged). GC/MS *m/z* 271 (M⁺+2, 20), 269 (M⁺, 60), 179 (100), 1336 (65), 55 (50).

General Procedure for the Preparation of the Final Compounds **8a,b–12a,b**

A stirred mixture of the alkylating agent **2a,b–4a,b** (0.9 mmol), the piperazine **6–7** (1.08 mmol) and K₂CO₃ (0.15 g, 1.08 mmol) in acetonitrile (20 mL) was refluxed overnight. After cooling, the mixture was evaporated to dryness and H₂O (20 mL) was added to the residue. The aqueous phase was extracted with AcOEt (3 × 20 mL). The collected organic layers were dried over Na₂SO₄ and evaporated under reduced pressure. The crude residue was purified by chromatographic column using CHCl₃/MeOH, 19:1 to give the pure target compound.

6-(3-(4-([3,4'-Bipyridin]-2-yl)piperazin-1-yl)propoxy)-2-methyl-2H-benzo[b][1,4]oxazin-3(4H)-one (8a). Yellow solid, 41% yield. ¹H NMR (CDCl₃): δ 1.55 (d, 3H, *J* = 6.4 Hz), 1.89–1.93 (m, 2H), 2.42 (br s, 4H), 2.48–2.53 (m, 2H), 3.13 (br s, 4H), 3.92 (t, 2H, *J* = 6.4 Hz), 4.53–4.60 (m, 1H), 6.33 (d, 1H, *J* = 2.3 Hz), 6.47 (dd, 1H, *J* = 2.3 Hz and 8.8 Hz), 6.86 (d, 1H, *J* = 8.8 Hz), 6.95 (dd, 1H, *J* = 4.7 Hz and 7.6 Hz), 7.47 (dd, 1H, *J* = 1.8 Hz and 7.6 Hz), 7.55 (d, 2H, *J* = 5.9 Hz), 8.17 (br s, 1H), 8.27 (dd, 1H, *J* = 1.8 Hz and 4.7 Hz), 8.65 (d, 2H, *J* = 4.68 Hz). HRMS (ESI⁺) calcd for [(C₂₆H₂₉N₅O₃)+Na]⁺: 482.2163, found: 482.2169. ESI⁺/MS *m/z* 482 (M⁺). ESI⁺/MS/MS *m/z* 482 (100); Mp 179–180 °C (from CHCl₃/*n*-hexane).

6-(4-(4-([3,4'-Bipyridin]-2-yl)piperazin-1-yl)butoxy)-2-methyl-2H-benzo[b][1,4]oxazin-3(4H)-one (8b). Yellow solid, 33% yield. ¹H NMR (CDCl₃): δ 1.55 (d, 3H, *J* = 7.0 Hz), 1.60–1.67 (m, 2H), 1.71–1.80 (m, 2H), 2.36–2.41 (m, 6H), 3.11–3.14 (m, 4H), 3.89 (t, 2H, *J* = 6.4 Hz), 4.57 (q, 1H, *J* = 6.4 Hz), 6.32 (d, 1H, *J* = 2.3 Hz), 6.47 (dd, 1H, *J* = 2.9 Hz and 8.8 Hz), 6.87 (d, 1H, *J* = 8.8 Hz), 6.95 (dd, 1H, *J* = 7.0 Hz and 7.61 Hz), 7.47 (dd, 1H, *J* = 1.8 Hz and 7.6 Hz), 7.54–7.57 (m, 2H), 8.11 (br s, 1H), 8.27 (dd, 1H, *J* = 1.8 Hz and 4.7 Hz), 8.65 (d, 2H, *J* = 5.9 Hz). HRMS (ESI⁺) calcd for [(C₂₇H₃₁N₅O₃)+Na]⁺: 496.2319, found: 496.2319. ESI⁺/MS *m/z* 496 (M+Na)⁺. ESI⁺/MS/MS *m/z* 496 (100), 295 (66), 226 (56). Mp 174–175 °C (from CHCl₃/*n*-hexane).

2-(3-(4-(5-Fluoro-4'-methoxy-[1,1'-biphenyl]-2-yl)piperazin-1-yl)propyl)-4-methyl-1,2,4-triazine-3,5(2H,4H)-dione (9a). Yellow oil, 17% yield. ¹H NMR (CDCl₃): δ 1.68–1.80 (m, 2H), 2.31–2.35 (m, 6H), 2.77–2.80 (m, 4H), 3.32 (s, 3H), 3.82 (s, 3H), 3.97 (t, 2H, *J* = 7.0 Hz), 6.90–6.98 (m, 5H), 7.37 (s, 1H), 7.54–7.56 (m, 2H). HRMS (ESI⁺) calcd for [(C₂₄H₂₈FN₅O₃)+Na]⁺: 476.2068, found: 476.2068. ESI⁺/MS *m/z* 476 (M+Na)⁺. ESI⁺/MS/MS *m/z* 476 (100).

2-(4-(4-(5-Fluoro-4'-methoxy-[1,1'-biphenyl]-2-yl)piperazin-1-yl)butyl)-4-methyl-1,2,4-triazine-3,5(2H,4H)-dione (9b). Dark brown oil, 24% yield. ¹H NMR (CDCl₃): δ 1.49–1.54 (m, 2H),

1.67–1.80 (m, 2H), 2.31–2.36 (m, 6H), 2.77–2.80 (m, 4H), 3.32 (s, 3H), 3.85 (s, 3H), 3.97 (t, 2H, $J = 7.0$ Hz), 6.90–6.98 (m, 5H), 7.37 (s, 1H), 7.54–7.56 (m, 2H). HRMS (ESI⁺) calcd for [(C₂₅H₃₀FN₅O₃)+Na]⁺: 490.2225, found: 490.2210. ESI⁺/MS m/z 490 (M+Na)⁺. ESI⁺/MS/MS m/z 341 (14).

5'-Fluoro-2'-(4-(3-(4-methyl-3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl)propyl)piperazin-1-yl)-[1,1'-biphenyl]-4-carbonitrile (**10a**). Yellow oil, 51% yield. ¹H NMR (CDCl₃): δ 1.83–1.93 (m, 2H), 2.29 (br s, 4H), 2.36–2.40 (m, 2H), 2.71 (app t, 4H), 3.31 (s, 3H), 4.02 (t, 2H, $J = 7.0$ Hz), 6.92–6.95 (m, 1H), 7.00–7.06 (m, 2H), 7.36 (s, 1H), 7.66–7.76 (m, 4H). HRMS (ESI⁺) calcd for [(C₂₄H₂₅FN₆O₂)+Na]⁺: 471.1915, found: 471.1888. ESI⁺/MS m/z 471 (M+Na)⁺. ESI⁺/MS/MS m/z 471 (100), 316 (53).

5'-Fluoro-2'-(4-(4-(4-methyl-3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl)butyl)piperazin-1-yl)-[1,1'-biphenyl]-4-carbonitrile (**10b**). Brown oil, 48% yield. ¹H NMR (CDCl₃): δ 1.44–1.53 (m, 2H), 1.71–1.81 (m, 2H), 2.30–2.35 (m, 6H), 2.75–2.78 (m, 4H), 3.31 (s, 3H), 3.97 (t, 2H, $J = 7.0$ Hz), 6.92–6.98 (m, 1H), 7.00–7.06 (m, 2H), 7.37 (s, 1H), 7.66–7.74 (m, 4H). HRMS (ESI⁺) calcd for [(C₂₅H₂₇FN₆O₂)+Na]⁺: 485.2072, found: 485.2058. ESI⁺/MS m/z 485 (M+Na)⁺. ESI⁺/MS/MS m/z 336 (100).

2-(3-(4-(3,4'-Bipyridin)-2-yl)piperazin-1-yl)propyl)-4-methyl-1,2,4-triazine-3,5(2H,4H)-dione (**11a**). Yellow oil, 29% yield. ¹H NMR (CDCl₃): δ 1.84–1.92 (m, 2H), 2.34–2.38 (m, 4H), 2.40 (t, 2H, $J = 7.0$ Hz), 3.06 (app t, 4H), 3.31 (s, 3H), 4.03 (t, 2H, $J = 6.4$ Hz), 6.93 (dd, 1H, $J = 7.0$ Hz and 7.6 Hz), 7.35 (s, 1H), 7.46 (dd, 1H, $J = 1.8$ and 7.6), 7.51 (dd, 2H, $J = 1.8$ Hz and 4.7 Hz), 8.25 (dd, 1H, $J = 1.8$ Hz and 4.7 Hz), 8.63 (d, 2H, $J = 5.9$ Hz). HRMS (ESI⁺) calcd for [(C₂₁H₂₅N₇O₃)+Na]⁺: 430.1962, found: 430.1960. ESI⁺/MS m/z 430 (M+Na)⁺. ESI⁺/MS/MS m/z 430 (100), 64 (26).

2-(4-(4-(3,4'-Bipyridin)-2-yl)piperazin-1-yl)butyl)-4-methyl-1,2,4-triazine-3,5(2H,4H)-dione (**11b**). Brown solid, 10% yield. ¹H NMR (CDCl₃): δ 1.50–1.52 (m, 2H), 1.70–1.78 (m, 2H), 2.33–2.38 (m, 6H), 3.30–3.32 (m, 4H), 3.31 (s, 3H), 3.97 (t, 2H, $J = 7.0$ Hz), 6.93 (dd, 1H, $J = 7.0$ Hz and 7.6 Hz), 7.37 (s, 1H), 7.45–7.48 (m, 1H), 7.51–7.56 (m, 2H), 8.26–8.29 (m, 1H), 8.63–8.68 (m, 2H). HRMS (ESI⁺) calcd for [(C₂₂H₂₇N₇O₂)+Na]⁺: 444.2118, found: 444.2111. ESI⁺/MS m/z 444 (M+Na)⁺. ESI⁺/MS/MS m/z 444 (100), 226 (46). Mp 165–167 °C (from CHCl₃/n-hexane).

7-(3-(4-(3,4'-Bipyridin)-2-yl)piperazin-1-yl)propoxy)-4-methyl-2H-chromen-2-one (**12a**). Yellow oil, 41% yield. ¹H NMR (CDCl₃): δ 1.92–2.01 (m, 2H), 2.38 (s, 3H), 2.39 (app t, 4H), 2.51 (t, 2H, $J = 7.61$ Hz), 3.13 (app t, 4H), 4.06 (t, 2H, $J = 5.9$ Hz), 6.12 (s, 1H), 6.80–6.84 (m, 2H), 6.96 (dd, 1H, $J = 5.3$ Hz and 7.6 Hz), 7.45–7.48 (m, 2H), 7.53–7.6 (m, 2H), 8.26 (dd, 1H, $J = 1.8$ Hz and 4.7 Hz), 8.64–8.66 (m, 2H). HRMS (ESI⁺) calcd for [(C₂₇H₂₈N₄O₃)+Na]⁺: 479.2054, found: 479.2052. ESI⁺/MS m/z 479 (M+Na)⁺. ESI⁺/MS/MS m/z 479 (100), 281 (55), 84 (23).

7-(4-(4-(3,4'-Bipyridin)-2-yl)piperazin-1-yl)butoxy)-4-methyl-2H-chromen-2-one (**12b**). Yellow oil, 18% yield. ¹H NMR (CDCl₃): δ 1.62–1.69 (m, 2H), 1.80–1.86 (m, 2H), 2.39 (s, 3H), 2.40–2.43 (m, 4H), 3.13 (t, 2H, $J = 5.4$ Hz), 3.14–3.19 (m, 4H), 4.02 (t, 2H, $J = 6.9$ Hz), 6.12 (s, 1H), 6.78 (d, 1H, $J = 2.4$ Hz), 6.83 (dd, 1H, $J = 2.4$ Hz and 8.8 Hz), 6.93 (dd, 1H, $J = 4.9$ Hz and 7.3 Hz), 7.46–7.48 (m, 2H), 7.53–7.55 (m, 2H), 8.28 (dd, 1H, $J = 4.9$ Hz and 9.3 Hz), 8.65 (d, 1H, $J = 5.9$ Hz), 8.67 (d, 1H, $J = 5.9$ Hz). HRMS (ESI⁺) calcd for [(C₂₈H₃₀N₄O₃)+Na]⁺: 493.2210, found: 493.2214. ESI⁺/MS m/z 493 (M+Na)⁺. ESI⁺/MS/MS m/z 295 (100), 226 (90).

4.3. Radioligand Binding Assays

4.3.1. 5-HT_{1A} Receptor

The affinity of the compounds for serotonin 5-HT_{1A} receptor was evaluated in membrane preparations from HEK293 cells stably expressing the human cloned receptor, following previously described procedures [23] with minor modifications. Competition binding experiments were performed using 1 nM [³H]-8-Hydroxy-DPAT (1 mCi/mL, PerkinElmer NET929250UC) as radioligand. Nonspecific binding was assessed in the presence of 10 μ M serotonin. 5-Carboxamidotryptamine (5-CT) was included in the assays as reference compound. Assays were carried out in duplicate in 96-well assay plates, in assay buffer (50 mM Tris-HCl, 5 mM MgSO₄, pH = 7.4). Assay mixtures were incubated at 37 °C for 120 min,

followed by filtration through GF/C glass filter plates, and washed with ice-cold wash buffer (50 mM Tris-HCl, pH = 7.4). Competition binding curves, typically constructed with six different concentrations of the compounds, were fitted to a one-site competition model using Prism 6 software (GraphPad, San Diego, CA, USA), and equilibrium dissociation constant (K_i) of the compounds was calculated according to the Cheng–Prusoff equation. K_i value of the reference ligand 5-CT was 0.32 ± 0.02 nM.

4.3.2. 5-HT_{2A} Receptor

The affinity of the compounds for serotonin 5-HT_{2A} receptor was evaluated in membrane preparations from CHO-K1 cells stably expressing the human cloned receptor, following previously described procedures [23] with minor modifications. Competition binding experiments were performed using 1 nM [³H]-ketanserin (50.3 Ci/mmol, 1 mCi/mL, PerkinElmer NET791250UC) as radioligand. Nonspecific binding was assessed in the presence of 1 μ M methysergide. Risperidone was included in the assays as reference compound. Assays were carried out in duplicate in 96-well assay plates, in assay buffer (50 mM Tris-HCl, pH = 7.4). Assay mixtures were incubated at 37 °C for 30 min, followed by filtration through GF/B glass filter plates, and wash with ice-cold wash buffer (50 mM Tris-HCl, pH = 6.6). Affinity (equilibrium dissociation constant, K_i) of the compounds was calculated from competition binding curves as indicated above. K_i value of the reference ligand risperidone was 0.20 ± 0.04 nM.

4.3.3. 5-HT₇ Receptor

The affinity of the compounds for serotonin 5-HT₇ receptor was evaluated in membrane preparations from HEK293 cells stably expressing the human cloned 5-HT_{7A} receptor, following previously described procedures [24] with minor modifications. Competition binding experiments were performed using 2 nM [³H]-SB269970 (34.5 Ci/mmol, 0.25 mCi/mL, Perkin Elmer NET1198U250UC) as radioligand. Nonspecific binding was assessed in the presence of 25 μ M clozapine. Methiothepine was included in the assays as reference compound. Assays were carried out in duplicate in 96-well assay plates, in assay buffer (50 mM Tris-HCl, 4 mM MgCl₂, 1 mM ascorbic acid, 0.1 mM pargyline, pH = 7.4). Assay mixtures were incubated at 37 °C for 60 min, followed by filtration through GF/C glass filter plates, and washing with ice-cold assay buffer. Affinity (equilibrium dissociation constant, K_i) of the compounds was calculated from competition binding curves as indicated above. K_i value of the reference ligand methiothepin was 1.33 ± 0.32 nM.

4.3.4. Dopamine Receptors

Competition binding experiments at dopamine D_{2s} and D₃ receptor were performed to determine the affinity of the compounds. Cell culture conditions are described in Frank et al. [25]. Membrane preparations of transfected CHO-K1 cells stably expressing the human D_{2s} or D₃ receptor were performed according to Bautista-Aguilera et al. [26]. The competition binding experiments were conducted as previously reported [26,27]. Briefly, membrane fractions (D_{2s}R: 25 μ g/200 μ L; D₃R: 20 μ g/200 μ L) were incubated with [³H]spiperone (0.2 nM) and test compound for 120 min at room temperature. Seven appropriate concentrations of compound between 100 μ M and 0.01 nM were used. Nonspecific binding was determined in the presence of 10 μ M haloperidol. Haloperidol was also used as reference compound. Separation of the bound ligand from the free ligand was conducted by filtration through GF/B filters using deionized water.

The affinity for dopamine D₁ and D₅ receptors was evaluated in membrane fractions of HEK-293 cells stably expressing the human dopamine D₁ or D₅ receptor. HEK-293-D₁ cells were cultured in DMEM/F12 (15 mM HEPES, 1.2 g/L NaHCO₃) with 10% (v/v) FBS and 1% (v/v) L-glutamine. HEK-293-D₅ cells were cultured in DMEM/F12 (with L-glutamine, 1.2 g/L NaHCO₃) with 20% (v/v) FBS. Preparation of membrane fractions and radioligand competition assays were performed as previously described [26]. Briefly, membrane fractions were co-incubated with [³H]-SCH23390 (0.3 nM) and different concentrations

of test compound for 120 min at room temperature. Fluphenazine (100 μ M) was used to determine non-specific binding and as reference compound.

Data of at least three independent experiments in triplicate were analyzed using GraphPad Prism 8.1 (San Diego, CA, USA). Binding curves were fitted to a non-linear regression model (one-site competition). K_i values were calculated from IC_{50} values using the Cheng–Prusoff equation. The statistical calculations were performed on $-\log(K_i)$. Results are presented in mean K_i (nM) with 95% confidence intervals (nM).

4.4. Docking Studies

The structures of compounds **8b**, **9b**, **12a**, and **12b** were loaded into MOE 2019.0102 (Chemical Computing Group, Montreal, Canada). Subsequently, the protonation state was adjusted to dominant at pH = 7.0 and the compounds were subjected to energy minimization. X-ray structures of 5-HT_{1A} (PDB code: 7E2Z [28]), 5-HT_{2A} (PDB code: 6WHA [29]), D₁ (PDB code: 7JOZ [30]), and D₂ (PDB code: 6CM4 [31]) were loaded into MOE 2019.0102 and subjected to “QuickPrep” routine, which includes correction of missing loops, adjustment of protonation state and energy minimization. For docking, the initial placement was performed by setting the central interaction towards the acidic aspartate as essential. Initial scoring of 30 poses was performed using London dG scoring function, while refinement was performed with a rigid receptor, and the five best poses were selected based on GBVI/WSA dG scoring function. The best pose was subjected to energy minimization and inspected manually.

4.5. Stability Assays in Rat Liver Microsomes

Test compounds were pre-incubated at 37 °C with rat liver microsomes (Tebu-Bio, Milan, Italy) (1.0 mg/mL microsomal protein) at 10 μ M final concentration in 100 mM potassium phosphate buffer (pH 7.4) for 10 min. Metabolic reactions were initiated by the addition of the NADPH regenerating system (containing 10 mM NADP, 50 mM glucose-6-phosphate, and 10 unit/mL glucose-6-phosphate dehydrogenase, final glucose-6-phosphate dehydrogenase concentration, 1 unit/mL). After 30 min incubation, the reaction mixture was quenched by adding an equal volume of cold acetonitrile containing the internal standard. A test compound incubated with microsomes without NADPH regenerating system was included. Quenched samples were centrifuged at 4500 rpm for 15 min and the supernatants were injected for quantification analysis. Samples (100 μ L) were analyzed by using an Agilent 1260 Infinity Binary LC System equipped with a diode array detector (Open Lab software was used to analyze the chromatographic data) and a Phenomenex Gemini C-18 column (250 mm $\times$ 4.6 mm, 5 μ m particle size). The samples were eluted using CH₃CN/20 mM ammonium formate pH 5.5 (7:3, v/v) as eluent (volumetric flow rate = 1 mL/min). Concentrations were quantified by measuring the area under the peak.

The percentage of the parent compound remaining after a 30-min incubation was calculated according to the equation:

$$\% \text{ of parent compound remaining after 30 min} = C_{\text{parent}}/C_{\text{control}} \times 100 \quad (1)$$

where C_{parent} is ligand concentration after incubation with microsome fraction and NADPH regenerating system and C_{control} is ligand concentration after incubation with microsome fraction only.

4.6. Evaluation of Cell Viability

4.6.1. Cell Culture

SH-SY5Y neuroblastoma cells (cat. CRL-2266, ATCC) were cultured in a 1:1 mixture of Eagle’s Minimum Essential Medium (cat. 15-010-CVR, Corning) and Ham’s F12 medium (cat. 10-080-CVR, Corning). This medium was supplemented with 10% (v/v) Fetal Bovine Serum (cat. 35-079-CV, Corning), 1% (v/v) Glutamine (cat. ECB3000D, Euro Clone) and 1% (v/v) Penicillin–Streptomycin (cat.30-002-CI, Corning). Cells were cultivated at 37 °C with 5% CO₂ at saturated humidity.

4.6.2. Cell Viability

Determination of cell growth was performed using the MTT assay at 48 h. [32] On day 1, 25,000 cells/well were seeded into 96-well plates in a volume of 100 μ L. On day 2, the various drug concentrations (1 μ M–100 μ M) were added. In all the experiments, the various drug-solvents (EtOH, DMSO) were added in each control to evaluate possible solvent cytotoxicity. After the established incubation time with drugs (48 h), MTT (0.5 mg/mL) was added to each well, and after 3–4 h incubation at 37 $^{\circ}$ C, the supernatant was removed. The formazan crystals were solubilized using 100 μ L of DMSO/EtOH (1:1, *v/v*) and the absorbance values at 570 and 630 nm were determined on the microplate reader Victor 3 (PerkinElmer Life Sciences, Waltham, MA, USA).

4.6.3. Evaluation of Cell Viability

Determination of neuroprotection against H₂O₂ was performed using the MTT assay at 24 h. [33] On day 1, 25,000 cells/well were seeded into 96-well plates in a volume of 100 μ L. On day 2, the cells were pretreated (3 h) with two drug concentrations (1 μ M and 5 μ M) before being incubated with 400 μ M H₂O₂ for 24 h. In all the experiments, the various drug-solvents (EtOH, DMSO) were added in each control to evaluate a possible solvent cytotoxicity. After 24 h incubation, MTT (0.5 mg/mL) was added to each well, and after 3–4 h incubation at 37 $^{\circ}$ C, the supernatant was removed. The formazan crystals were solubilized using 100 μ L of DMSO/EtOH (1:1, *v/v*) and the absorbance values at 570 and 630 nm were determined on the microplate reader Victor 3 from PerkinElmer Life Sciences (Waltham, MA, USA).

4.6.4. Statistical Analysis

Data were analyzed by applying the one-way repeated measures analysis of variance, and Bonferroni's multiple comparison test followed as a post hoc test. Results are reported as mean SD of at least two to three independent experiments, performed in triplicate. Statistical significance was accepted at $p < 0.05$.

5. Conclusions

In the present study, we have described the design, synthesis, and biological evaluation of a set of long-chain arylpiperazine derivatives. The structural modification led to the identification of new compounds displaying an array of affinity for serotonin 5-HT_{1A}, 5-HT_{2A}, 5-HT₇ receptors, and dopamine D₂ receptor and, in some cases, antioxidant properties. Binding affinity data evidenced that: (i) the nature of the terminal fragment had an impact mostly on the affinity at dopamine D₁- and D₂-like receptors; (ii) the length of the linker influenced the affinity at serotonin 5-HT_{1A} receptor; (iii) the nature of the biphenyl-like system linked to the piperazine ring influenced the affinity at serotonin 5-HT_{2A} and 5-HT₇ receptors. The most interesting compounds were: **12a** that combines an affinity profile compatible with antipsychotic activity (affinity for dopamine D₂ (K_i = 300 nM) and 5-HT_{2A} (K_i = 315 nM) receptors, accompanied by an affinity for 5-HT_{1A} (K_i = 41.5 nM) and 5-HT₇ (K_i = 42.5 nM) receptors) and antioxidant properties; **9b** that has an affinity profile compatible for studies in the context of ASD (affinity for serotonin 5-HT_{1A} (K_i = 23.9 nM), 5-HT_{2A}, (K_i = 39.4 nM), 5-HT₇ (K_i = 45.0 nM) receptors and selectivity over dopamine D₂ receptors) and antioxidant properties. Even though the new compounds showed CNS MultiParameter Optimization score predictive of desirable ADMET properties and cross the blood–brain barrier, they fail to achieve in vitro metabolic stability suitable for studies in vivo. The only exception is compound **9b** that therefore deserves further characterization.

Author Contributions: E.L. and M.L. designed the research. M.M. synthesized the compounds. E.L. and C.A. supervised the synthesis, purification, and characterization of the target of compounds. E.P. performed docking studies. M.C., M.D. and H.S. performed the binding assays. M.N. cytotoxicity assays. E.L. conducted the microsomal stability experiments. M.L., E.L., M.C. and H.S. contributed

to the writing, review, and revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Samples of all target compounds are available from E.L. and M.L.

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3 Publications

3.6 PUBLICATION 6

BOPPY-based novel fluorescent dopamine D₂ and D₃ receptor ligands

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journal homepage: www.elsevier.com/locate/bmclBOPPY-based novel fluorescent dopamine D₂ and D₃ receptor ligandsMilica Elek^a, Mariam Dubiel^a, Laura Mayer^b, Aleksandra Zivkovic^a, Thomas J.J. Müller^b, Holger Stark^{a,*}^a Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Universitätsstrasse 1, 40225 Duesseldorf, Germany^b Institute of Organic Chemistry, Heinrich Heine University Düsseldorf, Universitätsstrasse 1, 40225 Duesseldorf, Germany

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ABSTRACT

Dopamine is one of the crucial neurotransmitters in the human brain. Its out-of-range concentration can lead to various neurological diseases with special interest for dopamine D₂ and D₃ receptor subtypes. Although BODIPY is a highly versatile structural moiety for fluorescence labeling, we have looked out for structurally related pyridine-based moieties. We used BOPPY labelling of well-described D₂R/D₃R pharmacophores to obtain ligands with moderate to low nanomolar binding affinities as well as low to excellent quantum yields for bright fluorescence ligands. To best of our knowledge, this is the first report on the application of BOPPY fluorophores to GPCR ligands. This approach offers a general applicable way for fluorescence labelling via primary aliphatic amine elements.

Dopamine represents one of the crucially important neurotransmitters in the human brain for maintaining physiological functions. Fluctuating dopamine concentration could cause severe neurological (e.g., Parkinson's Disease, schizophrenia, restless leg syndrome, addictive behavior, etc.) and ocular disorders (ocular hypertension or updating sensory representations after eye movements). All dopamine receptors are G-protein coupled receptors divided into two subfamilies: D₁-like (D₁ and D₅ subtype) and D₂-like (D₂, D₃, and D₄ receptors). [1,2]. D₁-like receptors signal through G_s and activate adenylyl cyclase. D₂-like receptors, on the other hand, signal through G_i and inhibit this enzyme. [3] D₂R can be divided in two isoforms, short D₂R and long D₂R generated by alternative intron splicing, located mainly pre- and post-synaptically respectively. However, due to high homology between receptor subtypes [4] (up to 78% identity in the transmembrane domains), [5] syntheses of selective ligand that will target only one subtype remained mainly unsolved.

The human D₃ receptor (D₃R) [4] can be an especially interesting target for antipsychotics and neuroleptics and a biomarker for Parkinson's disease. [6] Its distribution in the brain is mainly limited to limbic region [7,8] (islands of Calleja and shell division of nucleus accumbens). [9] Dopamine itself shows 100fold higher affinity at D₃R when compared to D₂ receptor (D₂R). [10] D₃R is highly occupied after acute treatment with commercially available antipsychotics, [11,12] and therefore various efforts have been made to develop novel, potent, and selective D₃R ligands. General pharmacophore for this receptor subtype

has been described in the early 2000s [13] containing aromatic region, amide moiety, linker, and a basic center with aryl substitution.

Fluorescence labeling is one of the most common techniques in bioimaging, [14,15] used to localize receptors and estimate their distribution, expressing advantages over the radio-ligand binding assays. (e.g., waste disposal). In the recent years various efforts were made to enlighten cell signaling via GPCRs. [14] Fluorescent ligands can be used for a broad range of experiments, from single molecule spectroscopy to the cutting edge technologies (e.g., BRET or FRET), that could enlighten these processes. [15] Even though dopaminergic involvement in severe neurological disorder is a well-known fact, up to date, only a few fluorophores which selectively target dopamine transporter, [16] receptors of interest and determine differences between receptor subtypes and isoforms. [17,18] were developed. These ligands contain commercially available or easily prepared fluorophore such as BODIPY, [19,20] coumarin, rhodamine, fluorescein, [19,21–23] and recently reported dansyl-, xanthene- or cyanine-based derivatives. [24]

BODIPY [25] gained overall recognition during the '90s, being the most chemically stable and versatile alternative compared to up to than described fluorophores. [26,25] It still represents a commonly used tool in bioimaging. [27] Nevertheless, BODIPY in a solid-state expressed weak fluorescence. [28] Efforts have been centered around the design of novel fluorophores containing heterocyclic systems and express better stability and higher emission wavelengths compared to BODIPY. This resulted in reporting ligands with highly beneficial fluorescent

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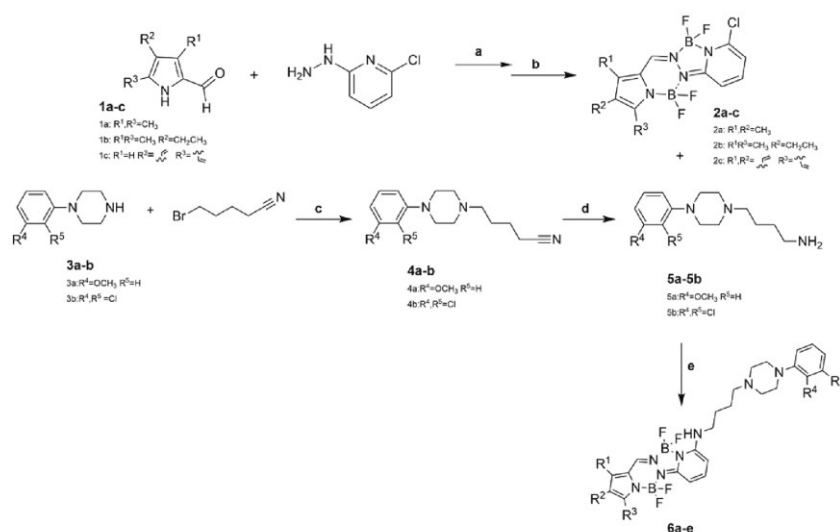


Fig. 1. Synthesis of compounds **6a-e**: a: PTSA, DCE, 16 h; b: BF₃ · Et₂O, DIPEA, c: K₂CO₃, KI, acetone, reflux 10 h; d: Raney-Ni, MeOH/NH₃ 10 h, H₂ 5 bar; e: appropriate compound from **2a-c**, DCE, Et₃N, DCE 10 h.

Table 1
Dopamine D_{2s} and D₃ receptor binding affinities of compounds **6a-e**.

No.	Structure	K _i (D _{2s} R) [nM] [95%CI]	K _i (D ₃ R) [nM] [95%CI]	SI (D ₂ R/D ₃ R)
Haloperidol		2.61 [2.02; 3.39]	13.5 [10.4; 17.4]	0.2
6a		328 [185; 585]	22.5 [12.7; 39.9]	14.6
6b		81.1 [45.5; 145]	27.2 [12.6; 56.6]	3.0
6c		309 [182; 524]	34.3 [19.7; 59.7]	9.0
6d		497.3 [121; 2044]	52.1 [32.3; 84.2]	9.5
6e		182 [129; 528]	16.7 [5.57; 49.8]	10.9

properties containing symmetrical pyrrole – BF₂ chromophore (BOPHY) [29] and unsymmetrical bis(BF₂) pyridine-based chromophore (BOPPY). BOPPY derivatives are physically and chemically more stable in solid-state as well in solution in comparison to these properties of almost all so far described fluorophores, which could expedite practical use of fluorescent dyes. [30] Moreover, they show good two photon absorption in the near infrared region. [30]

Our goal was to design, develop and evaluate selective, novel dopaminergic ligands with beneficial fluorescent properties by coupling BOPPY moiety with dopaminergic pharmacophores. This could further enlighten *in vivo* localization of receptors of interest.

Compounds **2a-c** have been synthesized in one pot-synthesis from commercially available pyrrole-2-aldehyde derivatives (**1a-c**) and 5-

chloro-2-hydrazinyl-pyridine in the presence of *p*-toluene sulfonic acid (PTSA) in an aprotic solvent. After the addition of BF₃ · Et₂O we obtained the versatile fluorescent tools for coupling (**2a-c**).

Compounds **3a** and **3b** have been alkylated under basic conditions with 4-bromobutyronitrile to obtain corresponding nitriles (**4a-b**). Nitriles were then reduced using Raney-Ni [31] as a catalyst to obtain primary aliphatic amines (**5a-b**). Finally, fluorophores (**2a-c**) underlaid nucleophilic substitution (**5a-b**) under basic conditions (Et₃N) to obtain final compounds **6a-e** (Figure 1)

Synthesized compounds have been further *in vitro* examined to estimate their affinities at D₂R and D₃R. Radioligand displacement assay was performed as described previously, with modifications. [32,33] Haloperidol was used as a standard. The binding affinities of synthesized

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Table 2
Fluorescence properties of compounds **6a–e**.

Structure	$\lambda_{\text{max,abs}}^{[a]}$ [nm] (ϵ [M ⁻¹ cm ² [1]])	$\lambda_{\text{max,em}}^{[b]}$ [nm] (Φ_F [a.u.])
6a	256 (16300), 297 (6800), 423 (32800), 444 (41700)	461, 490 (0.49)
6b	252 (12500), 291 (5700), 423 (25600), 444 (32500)	461, 489 (0.19)
6c	255 (21500), 293 (8500), 425 (45300), 448 (60100)	464, 490 (0.63)
6d	258 (21000), 305 (8000), 425 (44100), 448 (58700)	465, 490 (0.69)
6e	343 (9000), 445 (44300), 459 (44500)	523 (0.03)

compounds with corresponding confidence intervals (CI) and their receptor preferences presented as selectivity index (SI) are shown in Table 1.

We have also investigated the photophysical properties of compounds **6a–e** by UV/V is and static fluorescence spectroscopy (Table 2). In addition, the fluorescence quantum yields Φ_F were determined in dichloromethane with coumarin 343 as a standard ($\Phi_F = 0.63$ in ethanol) due to its comparable optical properties with so far described BOPPY derivatives. [34,35] Measurements were performed in dichloromethane (concentration range from 0.1 – 0.7 to 3 mM).

All novel compounds showed moderate to low nanomolar affinities at D₂R and D₃R, with a slight preference at D₃R. All compounds **6a–e** contain a four-carbon methylene linker between (2-methoxyphenyl) piperazino or (2,3-dichlorophenyl)piperazino moiety to the fluorophore, which is assumed to possess the optimal spacer length for the orthosteric binding pockets in the receptor and is in accordance with so far described structurally comparable compounds for dopamine D₃R. [13,36,37] Compound **6b** showed the highest affinity at D₂R ($pK_i = 7.09$) and compound **6e** to D₃R ($pK_i = 7.78$) in this small subset of compounds. Compounds that contain a (2,3-dichlorophenyl)piperazino moiety (**6a**, **6d**) show higher affinities at D₃R (pK_i values of 7.65 and 7.28, respectively) when compared to those of compounds containing (2-methoxyphenyl)piperazino moiety (**6b**, **6c**) and same fluorophore (pK_i values of 7.57 and 7.47). Replacement of pyrrol-2-yl by indol-2-yl (**6e**) derivatives increased selectivity between D₂R and D₃R, but strongly decreased quantum yield.

Compounds **6a–d** express four split different absorption maxima. Two of them in the range of 252–258 nm and 293–297 nm correspond to substituted phenyl piperazine moiety. Two others in the range of 423–425 nm and 444–448 nm belong to BOPPY chromophore. Extinction coefficients are in a range of $1.5\text{--}3.01 \cdot 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ [1]. The moderate quantum yield of compound **6b** (0.19) compared to those of other compounds of this set should be further explored, and conformational studies need to be performed to estimate the exact orientation of compounds. Compound **6e** containing an indole ring coupled to BOPPY moiety displays three absorption maxima at 343, 445, and 449 nm with an extinction coefficient of $1.7 \cdot 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ [1] and one emission maximum at 523 nm. Low quantum yield (0.03) can be explained with the photoinduced electron transfer process (PET), as similarly reported for various fluorophores. [38] Fluorescence quenching is observed in different fluorophores (oxazine, rhodamine, carbocyanine, coumarine, isoindole derivatives) and aromatic moieties (e.g. amino acids). Redox potential between these compounds enables PET and leads to low quantum yield and red shifted absorption maxima. [39]

Compounds **6c** and **6d** that combine high D₃R affinity and bright fluorescence properties have been chosen. Compounds **6c** and **6d** give high quantum yields of 0.63 and 0.69 and Stokes shifts of 770 cm⁻¹ [1] and 820 cm⁻¹ [1], respectively, and may be further utilized as fluorescent biomarkers for dopamine receptor localization in tissues.

To summarize, dopamine D₂ R and D₃ R pharmacophores coupled with different novel chemically and thermally stable BOPPY fluorophores were successfully established. These compounds could be

further examined for single fluorescence imaging experiments to examine their affinity with a fluorescence method and based on obtained results as potential tools for *in vivo* examination, localization, and distribution of D₂R and D₃R. To the best of our knowledge, this is the first report on the introduction of BOPPY in GPCR ligands. If further studies prove the application in tissues, this can be a general novel approach for the fluorescence labeling of new GPCR ligands at the aliphatic primary amino function.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bmcl.2022.128573>.

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Supporting Material

BOPPY-based novel fluorescent dopamine D₂ and D₃ receptor ligands

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Chemistry

Starting materials have been provided from Sigma Aldrich and been used without further purification. Analytical thin-layer chromatography was carried out on pre-coated TLC sheets ALUGRAM® Xtra SIL G/UV254 (Macherey-Nagel, Dueren, Germany) with visualization under UV light (254 and 365 nm). Melting points were determined at Büchi Schmelzpunkt M-565 (Büchi, Flawil, Switzerland) and they are uncorrected. Solvents have been evaporated at Rotavapor R II (Büchi, Flawil, Switzerland) with PC 3001 VARIO Chemie-Vacuum pump (Vacubrand, Wertheim, Germany) and CVC 3000 Vacuum controlling system. The compounds were dried at the high-vacuum pump (Vacubrand Chemie-Hybrid-Pumpe RC 6 (Vacubrand, Wertheim, Germany). Mass spectra have been determined using Advion Mass Express. Atmospheric-pressure chemical ionization (APCI) was used as a method of ionization, operating in a positive and negative mode. Data have been shown as [M+H]⁺. ¹H and ¹³C NMR spectra were measured at Bruker Avance III - 300 (Year 2010) and Bruker Avance III - 600 (Year 2011) Bruker, Germany. As solvents for NMR DMSO-*d*₆ and as standard tetramethylsilane (TMS) have been used. NMR spectra were interpreted with MestReNova 14.0 (Mestrelab Research, Campostela, Spain). Chemical shifts are given as parts per million (ppm) and been reported as s (singlet), d (doublet), dd (doublet of doublets), dt (doublet of triplet), ddd (doublet of doublets of doublets), t (triplet), q (quartet), quin (quintet) or m (multiplet), Coupling constant (*J*) are given in Hertz (Hz). Compounds were purified with flash column chromatography: Biotage Isolera™ Spektra Systems with ACI™ and Assist (Biotage, Uppsala, Sweden) and manually, using SNAP KP-Sil and SNAP KP-Sil ULTRA, SNAP-KP-SIL SFAER (Biotage, Uppsala, Sweden) as a stationary phase and mixtures of Hexane, DCM and MeOH in different ratios and as a mobile phase. Compounds purity was determined by liquid chromatography-mass spectrometry (LC-MS) Elute SP (HPG 700) Bruker Daltonics and amaZon speed ion Trap LC/MSn System (ESI-MS), Method: Alternating ion-Polarity : on; Scan Range: m/z: 80-1200; Nebulizer: Nitrogen, 15 Psi; Dry Gas: Nitrogen, 8 l/min, 200°C; Mass range Mode: Ultra Scan; Column: Intensity Solo 2 C18 (100 mm * 2.1 mm); Temperature: 50°C; Mobile phase: A. water hypergrade for LC-MS with 0.1 % formic acid (v/v) (Merck); B. Acetonitrile hypergrade for LC-MS (for LC-MS); Method of Analysis: 0-4 min 98 % A, 4-5 min gradient 95% A, 5-9 min 95 % A, 9-16 min gradient 5% A, 16-17 min. gradient to 0% A, reconditioning: 17-18 min. gradient to 98 % A, 18-21 min 98 % A.

General procedures

General procedures for the synthesis of BOPPY fluorophores (2a-c):

General procedure for the synthesis of BOPPY derivatives was conducted as described in the literature,¹ with modifications. To the mixture of hydrazine pyridine chloride (1.05 equiv) and corresponding pyrrole-2-aldehyde derivative (1 equiv.) in 1,2 dichloroethane (DCE) was added 0.05 mmol of *p*-toluene sulfonic acid. The reaction mixture was refluxed overnight. On the following day, 3 mL of diisopropylethylamine (DIPEA) was added to the reaction mixture and stirred another 15-20 min. Consequently, boron trifluoride diethyl etherate (BF₃ · Et₂O) was added and the reaction mixture was refluxed for another 4 h. TLC was used for control. The reaction mixture was left to cool down, 50 mL of dichloromethane (DCM) was poured into the reaction mixture, and all of it was poured into 100-150 mL of water. The organic phase was extracted with DCM, washed 3x with water, brine, and dried with anhydrous MgSO₄. The reaction mixture was concentrated to dryness. The crude product was purified using flash column chromatography (Sorbent: SiO₂ Eluent: Hex/DCM 1/1)

10-Chloro-5,5,12,12-tetrafluoro-1,3-dimethyl-5*H*,12*H*-5λ⁴,6λ⁴,12λ⁴,13λ⁴pyrido[1',2':4,5][1,2,4,3]triazaborolo[2,1-*a*]pyrrolo[1,2-*d*][1,2,4,3]triazaborinine (2a): Yellow powder. Yield 30%. ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.64 (s, 1H, 13*H*), 8.12 (dd, *J* = 8.9, 7.5 Hz, 1H, 7*H*), 7.36 (d, *J* = 8.9 Hz, 1H, 8*H*), 7.31 (d, *J* = 7.5 Hz, 1H, 9*H*), 6.31 (s, 1H, 2*H*), 2.40 (s, 3H, 1-CH₃), 2.34 (s, 3H, 3-CH₃). MS (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₁₂H₁₂B₂ClF₄N₄]⁺: 344.1; 345.1; 346.1 found: 345.4; 344.5; 346.5; 347.5.

10-Chloro-2-ethyl-5,5,12,12-tetrafluoro-1,3-dimethyl-5*H*,12*H*-5λ⁴,6λ⁴,12λ⁴,13λ⁴pyrido[1',2':4,5][1,2,4,3]triazaborolo[2,1-*a*]pyrrolo[1,2-*d*][1,2,4,3]triazaborinine (2b): Yellow powder. Yield 53%. ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.56 (s, 1H, 13*H*), 8.09 (dd, *J* = 8.9, 7.5 Hz, 1H, 7*H*), 7.34 (d, *J* = 8.9 Hz, 1H, 8*H*), 7.27 (dd, *J* = 7.5, 0.7 Hz, 1H, 9*H*), 2.43 (q, *J* = 7.5 Hz, 2H, -CH₃-CH₂), 2.37 (s, 3H, 1-CH₃), 2.28 (s, 3H-3-CH₃), 1.03 (t, *J* = 7.6 Hz, 3H, CH₂-CH₃). MS (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₁₄H₁₆B₂ClF₄N₄]⁺: 372.1; 371.1; 374.1 found: 373.6; 374.6.

11-Chloro-6,6,13,13-tetrafluoro-6*H*,13*H*-6λ⁴,7λ⁴,13λ⁴,14λ⁴pyrido[1'',2'':4',5'] [1,2,4,3]triazaborolo[2',1':1,2][1,2,4,3]triazaborinino[4,5-*a*]indole (2c): Orange powder. Yield 42%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.23 (s, 1H, 14*H*), 8.27 (dd, *J* = 8.9, 7.7 Hz, 1H, 4*H*), 7.84 (dt, *J* = 8.3, 1.1 Hz, 1H, 2*H*), 7.67 (d, *J* = 8.4 Hz, 1H, 14*H*), 7.60 (s, 1H, 10*H*), 7.55 – 7.42 (m, 3H, 8*H*, 9*H*, 16*H*), 7.21 (ddd, *J* = 8.0, 6.9, 0.9 Hz, 1H, 3*H*). MS (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₁₄H₁₀B₂ClF₄N₄]⁺: 366.1; 365.1; 368.1 found: 367.0; 367.9.

General procedure for the synthesis of ω-[(4-(2-methoxyphenyl)piperazinyl)butannitrile and ω-[(4-(2,3-dichlorophenyl)piperazin-1-yl)butannitrile (4a-b):

To a stirring solution of 1-(2-methoxyphenyl)piperazine (1 equiv) (**3a**) or 1-(2,3-dichlorophenyl)piperazine (**3b**) (1,1 equiv) in acetone, bromobutyl-nitrile (1 equiv.) and anhydrous K₂CO₃ (6-12 equiv) were added. The reaction mixture was refluxed for 10 h. TLC was used for control. The reaction mixture was left to cool down, filtered off, and the filtrate was concentrated to dryness. The crude product was partitioned between EtOAc (3x) and water. The organic phase was washed with a

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saturated solution of NaHCO₃, brine, and dried with anhydrous MgSO₄. Crude products have been purified using flash chromatography (sorbet: SiO₂, eluent: DCM/ MeOH/NH₃).

4-[4-(2-Methoxyphenyl)piperazin-1-yl]butannitrile 4a:² Yellow solid. **Yield** 69%. **¹H NMR** (300 MHz, DMSO-*d*₆) δ 7.00 – 6.85 (m, 4H), 3.78 (s, 3H), 2.97-3.02 (brs, 4H), 2.55 – 2.52 (m, 2H), 2.47 – 2.50 (m, 6H), 2.41 (t, *J* = 6.9 Hz, 2H), 1.76 (quin, *J* = 7.0 Hz, 2H). **MS** (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₁₅H₂₂N₃O]⁺: 260.2; 261.2 found: 260.4; 261.4

4-[4-(2,3-Dichlorophenyl)piperazin-1-yl]butannitrile (4b):² Yellow solid. **Yield** 95%. **¹H NMR** (300 MHz, DMSO-*d*₆) δ 7.34 – 7.27 (m, 2H), 7.14 (dd, *J* = 6.2, 3.5 Hz, 1H), 3.04 – 2.91 (brs, 4H), 2.57 – 2.52 (m, 4H), 2.50-2.51 (m, 2H), 2.42 (t, *J* = 6.8 Hz, 2H), 1.75 (quin, *J* = 6.9 Hz, 2H). **MS** (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₁₄H₁₈Cl₂N₃]⁺: 298.1; 300.1 found: 299.0; 300.0.

General procedure for synthesis amines of ω -[4-(2-methoxyphenyl)-piperazinyl]alkylamine and ω -[4-(2,3-dichlorophenyl)-piperazinyl]alkylamine (5a-b):

Obtained nitriles **4a** (1 equiv) and **4b** (1.1 equiv), were dissolved in 50 mL ammonia solution in methanol and consequently subjected to catalytic hydrogenation using freshly prepared Raney-Nickel³ with H₂ at 5 bar pressure, overnight. The reaction mixture was filtered off through celite and the filtrate was evaporated to dryness. The obtained amines were used without further purification into the next reaction step.

4-[4-(2-Methoxyphenyl)piperazin-1-yl]butan-1-amine (5a):⁴ Yellow oil. **Yield** 85%. **¹H NMR** (300 MHz, DMSO-*d*₆) δ 6.97 – 6.83 (m, 4H), 3.77 (s, 3H), 3.01 – 2.87 (brs, 4H), 2.61 – 2.53 (m, 2H), 2.44-2.52 (m, 6H), 2.30 (t, *J* = 7.2 Hz, 2H), 1.53 – 1.30 (m, 4H). **MS** (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₁₅H₂₆N₃O]⁺: 264.2 found: 265.2.

4-[4-(2,3-Dichlorophenyl)piperazin-1-yl]butan-1-amine (5b):⁵ Light yellow solid. **Yield** 59%. **¹H NMR** (300 MHz, DMSO-*d*₆) δ 7.34 – 7.24 (m, 2H), 7.13 (dd, *J* = 6.3, 3.3 Hz, 1H), 3.02 – 2.92 (brs, 4H), 2.62 – 2.51 (m, 4H), 2.45-2.47 (m, 4H), 2.31 (t, *J* = 7.1 Hz, 2H), 1.55 – 1.28 (m, 4H). **MS** (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₁₄H₂₂Cl₂N₃]⁺: 302.1; 304.1 found: 302.4; 304.4.

General procedures for synthesis of fluorescent dopamine receptor ligands 6a-e

To a stirring solution of **2a-c** (1 equiv) and **4a-b** (5-7 equiv) in DCE was added 0.1 mL of triethylamine (Et₃N). The reaction mixture was stirred overnight at 40 °C. TLC was used for the control. Consequently, 50 mL of water was poured into the reaction mixture and it was extracted 3 times with DCM. Organic layers were collected, washed with water, saturated solution of NaHCO₃, brine, and dried with anhydrous MgSO₄. Crude product was purified using flash column chromatography (sorbet: SiO₂ eluent: DCM/MeOH gradient: 100-95/0-5 %)

***N*-{4-[4-(2,3-Dichlorophenyl)piperazin-1-yl]butyl}-5,5,12,12-tetrafluoro-1,3-dimethyl-5*H*,12*H*-5 λ^4 ,6 λ^4 ,12 λ^4 ,13 λ^4 -pyrido[1',2':4,5][1,2,4,3]triazaborolo[2,1-*a*]pyrrolo[1,2-*d*][1,2,4,3]triazaborinin-10-amine (6a):** Yellow solid. **Yield** 85% **¹H NMR** (600 MHz, DMSO-*d*₆) δ 8.38 (s, 1H, 13*H*BP), 7.79 (t, *J* = 8.3 Hz, 1H, NH), 7.39 – 7.26 (m, 2H, 7*H*, BP, 8*H* BP), 7.14 (dd, *J* = 5.9, 3.9 Hz, 1H, 9*H*BP), 6.91 (d, *J* = 6.5 Hz, 1H, 4*H*diClppz), 6.37 (m, 2H, 5*H*, 6*H*diClppz), 6.21 (s, 1H, 2*H*BP), 3.45–3.35 (m, 4H, ppzN-CH₂NH-CH₂), 2.98 (s, 4H, ppz), 2.50–2.55 (bs, 4H, ppz) 2.36 (s, 3H, 1-CH₃), 2.31 (s, 3H, -CH₃), 1.68 – 1.43 (m, 4H, PipNC-CH₂-CH₂). **¹³C NMR** (151 MHz, CDCl₃) δ 151.50 (6C BP), 146.14 (3C BP), 144.64 (1C diClppz), 141.20 (13C BP), 135.10 (3C diClppz), 134.05 (8C BP), 127.47 (5C diClppz), 127.10 (2C diClppz), 124.59 (4C diClppz), 122.57 (1C BP), 118.61 (2C BP), 117.50 (7C BP), 96.50 (14C BP), 94.51 (9C BP), 57.81 (ppzN-C), 53.35 (2C, 6C ppz), 53.18 (3C, 5C ppz), 51.34 (NH-C), 26.72 (ppzNC-C), 23.92, (NHC-C) 13.94 (3-CH₃), 10.90 (1-CH₃). **MS** (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₂₆H₃₂B₂Cl₂F₄N₇]⁺: 610.1; 612.2; 609.2 found: 610.3; 611.3; 612.4; 613.3 **Melting point**: 160.9 °C. **LC-MS** (ESI(+)) = 95.48%

5,5,12,12-Tetrafluoro-*N*-{4-[4-(2-methoxyphenyl)piperazin-1-yl]butyl}-1,3-dimethyl-5*H*,12*H*-5 λ^4 ,6 λ^4 ,12 λ^4 ,13 λ^4 -pyrido[1',2':4,5][1,2,4,3]triazaborolo[2,1-*a*]pyrrolo[1,2-*d*][1,2,4,3]triazaborinin-10-amine (6b): Chartreuse solid **Yield** 88% **¹H NMR** (300 MHz, DMSO-*d*₆) δ 8.37 (s, 1H, 13*H*BP), 7.78 (t, *J* = 8.3 Hz, 1H, NH), 7.04 – 6.81 (m, 5H, 4*H*MeOph7*H* BP), 6.36 (dd, *J* = 21.8, 8.3 Hz, 2H, 8*H*, 9*H*BP), 6.21 (s, 1H, 2*H*BP), 3.76 (s, 3H, OCH₃), 3.35 – 3.30 (m, 2H, NH-CH₂), 2.99 – 2.86 (m, 4H, ppz), 2.48 (s, 4H, ppz), 2.36 (s, 3H, 1-CH₃), 2.30 (s, 3H, 3-CH₃), 1.74 – 1.38 (m, 4H, ppzN-CH₂-CH₂), 1.34 – 1.15 (m, 2H, 2H NHC-CH₂). **¹³C NMR** (75 MHz, DMSO-*d*₆) δ 152.01 (2C MeOph), 151.39 (13C BP), 149.24 (1C MeOph), 145.07 (14C BP), 144.11 (6C BP), 141.35 (8C BP), 134.58 (1C BP), 128.56 (2C BP), 122.25 (4C MeOph), 120.86 (3C MeOph), 117.88 (5C MeOph), 116.78 (6C MeOph), 112.09 (7C BP), 96.08 (10C BP), 93.27 (9C BP), 57.21 (ppzN-C), 55.33 (OCH₃), 52.95 (2C 6C ppz), 50.07 (3C 5C ppz), 41.83 (NH-C), 26.54 (ppz NC-C) 25.85 (ppzNCC-C), 13.47 (3-CH₃), 10.50 (1-CH₃). **MS** (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₂₈H₃₆B₂F₄N₇O]⁺: 571.3; 572.3; 573.3; found: 570.4; 571.4 **Melting point**: 198.2 °C. **LC-MS** (ESI(+)) = 95.59%

2-Ethyl-5,5,12,12-tetrafluoro-*N*-{4-[4-(2-methoxyphenyl)piperazin-1-yl]butyl}-1,3-dimethyl-5*H*,12*H*-5 λ^4 ,6 λ^4 ,12 λ^4 ,13 λ^4 -pyrido[1',2':4,5][1,2,4,3]triazaborolo[2,1-*a*]pyrrolo[1,2-*d*][1,2,4,3]triazaborinin-10-amine (6c): Yellow solid **Yield** 52% **¹H NMR** (300 MHz, DMSO-*d*₆) δ 8.31 (s, 1H, 13*H*BP), 7.77 (t, *J* = 8.3 Hz, 1H, NH), 7.01 – 6.77 (m, 5H, 4*H*MeOph, 7*H*BP), 6.39 (d, *J* = 8.1 Hz, 1H, 8*H*BP), 6.31 (d, *J* = 8.5 Hz, 1H, 9*H*BP), 3.76 (s, 3H, -OCH₃), 3.41 – 3.34 (m, 2H, NH₂-CH₂), 2.94 (br.s, 4H, ppz), 2.45–2.50 (m, 6H, 4*H*ppz pipN-CH₂), (2.44 – 2.36 (m, 2H, CH₃-CH₂), 2.33 (s, 3H, 1-CH₃), 2.25 (s, 3H 3-CH₃), 1.66 – 1.44 (m, 4H NHC-CH₂-CH₂), 1.02 (t, *J* = 7.5 Hz, 3H CH₃-CH₂). **¹³C NMR** (75 MHz, DMSO-*d*₆) δ 152.52 (2C MeOph), 151.86 (13C BP), 145.44 (14C BP), 141.85 (1C MeOph), 132.25 (3C BP), 129.78 (1C BP), 128.53 (8C BP), 122.74 (4C MeOph) 123.5 (3C MeOph), 121.37 (6C MeOph), 118.39 (5C MeOph), 112.60 (10C BP), 96.32 (7C BP), 93.80 (9C BP), 57.71 (OCH₃-c), 55.84 (ppz 2C 6C), 53.46 (3C, 5C ppz) 50.57 (ppzN-C) 42.32 (HN-C), 26.36 (ppzNC-C), 23.64 (CH₃-C), 17.12 (NHC-C), 15.21 (CH₃-C) 11.99, (3-CH₃), 9.11 (1-CH₃). **MS** (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₂₉H₄₀B₂F₄N₇O]⁺: 600.3; 599.3; 601.3 found: 599.9; 601.0; 601.9. **Melting point**: 201.7 °C. **LC-MS** (ESI(+)) = 100%

***N*-{4-[4-(2,3-Dichlorophenyl)piperazin-1-yl]butyl}-2-ethyl-5,5,12,12-tetrafluoro-1,3-dimethyl-5*H*,12*H*-5 λ^4 ,6 λ^4 ,12 λ^4 ,13 λ^4 -pyrido[1',2':4,5][1,2,4,3]triazaborolo[2,1-*a*]pyrrolo[1,2-*d*][1,2,4,3]triazaborinin-10-amine(6d):** Yellow solid **Yield**: 39%. **¹H NMR** (300 MHz, DMSO-*d*₆) δ 8.34 (s, 1H, 13*H*BP), 7.80 (t, *J* = 8.3 Hz, 1H, NH), 7.31 (d, *J* = 3.7 Hz, 2H, 5*H* 6*H* diClppz), 7.22 – 7.06 (m, 1H, 7*H*BP), 6.89 (m, 1H, 8*H*BP), 6.37 (dd, *J* = 23.4, 8.3 Hz, 2H, 9*H*BP, 4*H*, diClppz), 3.00 (s, 3H, 1-CH₃), 2.50–2.60 (m, 4H, ppzN-CH₂NH-CH₂), 2.45–2.50 (m, 4H ppz) 2.44 – 2.35 (m, 4H, ppz),

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2.36 (s, 3H, 3-CH₃), 2.27 (m, 2H, ppzNC-CH₂), 1.72 – 1.38 (m, 4H CH₃-CH₂, CH₂-CNH), 1.05 (t, *J* = 7.5 Hz, 3H, CH₃-CH₂). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 151.31 (13C BP), 151.19 (1C diClppz), 145.00 (3C BP), 132.58 (3C diClppz), 131.74 (8C BP), 129.28 (1C BP), 128.44 (2C BP), 125.97 (5C diClppz), 124.31 (4C diClppz), 121.34 (6C diClppz), 119.53 (6C diClppz), 95.88 (10C BP), 93.20 (9C BP), 57.05 (ppzN-C), 52.72 (2C 6C ppz), 50.96 (3C 5C ppz), 41.74 (NH-C), 25.78 (ppzNCC-C) 23.11 (ppzNC-C), 16.65 (CH₃-C) 14.81 (CH₃-C), 11.54 (3-CH₃), 8.74 (1-CH₃). **MS** (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₂₈H₃₆B₂Cl₄N₇]⁺: 637.3; 638.3; 640.24 found: 638.6; 639.5, 640.4; 641.5. **Melting point**: 176.0 °C. **LC-MS (ESI(+))**= 95.2%

6,6,12,12-Tetrafluoro-N-[4-[4-(2-methoxyphenyl)piperazin-1-yl]butyl]-6H,12H-6λ⁴,12λ⁴,13λ⁴-

benzo[4',5'] [1,2,3]diazaborolo[2',1':1,2][1,2,4,3]triazaborinino[4,5-*a*]indol-11-amine (6e): Orange solid. **Yield**: 40% **¹H NMR** (300 MHz, DMSO-*d*₆) δ 8.94 (s, 1H 16HBP), 7.90 (t, *J* = 8.3 Hz, 1H, NH), 7.80 (dd, *J* = 8.2, 1.2 Hz, 1H, 4HBP), 7.67 (d, *J* = 8.4 Hz, 1H, 14HBP), 7.46 – 7.31 (m, 3H, 1H, 2H, 8HBP), 7.18 (ddd, *J* = 8.1, 6.9, 1.0 Hz, 1H, 3HBP), 6.98 – 6.82 (m, 4H, MeOph), 6.54 (dd, *J* = 8.4, 5.7 Hz, 2H 10H,9HBP), 3.76 (s, 3H, OCH₃), 3.40 (d, *J* = 6.5 Hz, 2H, ppzN-CH₂), 2.90-3.00 (bs, 4H, ppz), 2.45 (m, 4H ppr) 2.37 (bs, 2H NH-CH₂), 1.68 – 1.45 (m, 4H ppzNCH₂-CH₂). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 152.02 (6C BP), 151.75, (3C BP) 145.92 (2C MeOph), 132.67 (1C MeOph), 129.16 (13C BP), 126.47 (8C BP), 122.67 (6C MeOph), 122.27 (5C MeOph) 121.30 (4C MeOph) 120.87 (1C BP), 117.90 (2C BP), 114.48 (3C MeOph), 113.30 (7C BP), 112.10 (14C BP), 98.39 (9C BP), 57.18 (ppzN-C), 55.35, (OCH₃), 52.96 (2C, 6C ppz), 50.06 (3C, 5C ppz), 41.95 (NH-C) 25.83, (ppzNC-C), 23.09 (ppzNCC-C). **MS** (APCI(+)) *m/z* [M+H]⁺: calcd. for [C₂₉H₃₄B₂F₄N₇O]⁺: 594.3; 593.3 found: 593.8; 594.5 **Melting point**: 197.8 °C. **LC-MS (ESI(+))**= 97.5%

General considerations for UV/Vis and fluorescent spectra

UV/Vis spectra were recorded in CH₂Cl₂ high performance liquid chromatography (HPLC) at 293 K on a Perkin–Elmer UV/Vis/NIR Lambda 19 spectrometer. Emission spectra were recorded in CH₂Cl₂ HPLC grade at 293 K on a Hitachi F-7000 spectrometer using the emission correction curve provided by the instrument manufacturer. Emission spectra were not corrected for the wavelength-dependent spectral responsivity of the fluorometer. All solution spectra were recorded with dyes dissolved in spectroscopic grade solvents at 293 K using 1 cm-quartz cuvette from Hellma GmbH. The molar absorption coefficients ϵ of dye solutions of known dye concentration were measured in a multipoint setup.

Radioligand displacement assay at hD₂R and hD₃R

Radioligand competition assay at hD₂R and hD₃R

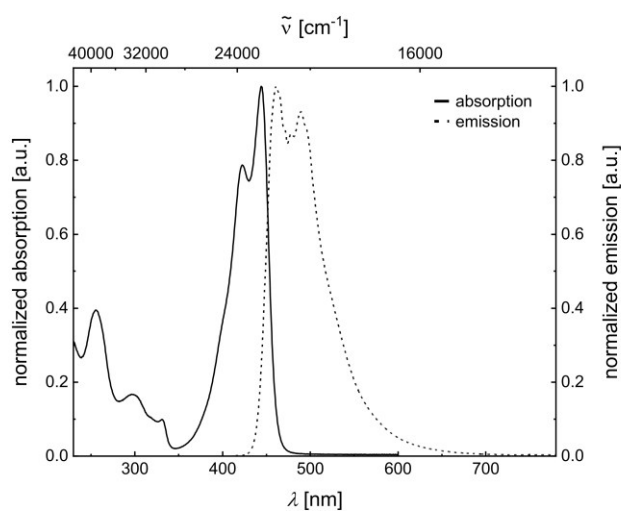
The affinity at human isoform dopamine D₂ and D₃ receptors was determined by radioligand competition assays. Cell cultivation and membrane preparations of transfected CHO-K1 cells were performed according to Frank *et al.*⁶.

Competition binding assays were conducted as previously reported with modifications.⁷ Various assay conditions have been tested during initial binding experiments to validate the binding assay.⁸ Parameters within the assay like buffer concentration, tissue preparation, incubation temperature or pH value has been chosen carefully. A suitable incubation time has been adapted and investigated according to literature protocols to ensure equilibrium conditions.^{9, 10, 11} Compounds were dissolved in DMSO to obtain 10 mM stock solutions. They were diluted within the assay with binding buffer. Membrane fractions (D₂R: 25 µg/200 µL; D₃R: 20 µg/200 µL) were co-incubated with [³H]spiperone (0.2 nM final concentration) and

test compound (seven concentrations between 100 μM and 0.01 nM) for 120 min at room temperature.. To determine non-specific binding, samples were incubated with 10 μM haloperidol. Bound ligand was separated from free ligand by filtration through GF/B glass fiber filters using a 96-well cell harvester and washed with 4 °C deionized water. Data of at least three independent experiments in triplicate was analysed using GraphPad Prism 6.1 (San Diego, CA, USA) using non-linear regression (one-site competition). The IC_{50} values were transformed to K_i values by using Cheng-Prusoff equation.¹²

Absorption and emission spectra

Fig. S1. Absorption and emission spectra of compound **6a** (recorded in dichloromethane, $T = 293\text{ K}$, $c_{\text{abs}}(\mathbf{4}) = 10^{-5}\text{ M}$, $c_{\text{em}}(\mathbf{4}) = 10^{-7}\text{ M}$, $\lambda_{\text{ex}} = 400\text{ nm}$)



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Fig. S2. Absorption and emission spectra of compound **6b** (recorded in dichloromethane, $T = 293\text{ K}$, $c_{\text{abs}}(\mathbf{4}) = 10^{-5}\text{ M}$, $c_{\text{em}}(\mathbf{4}) = 10^{-7}\text{ M}$, $\lambda_{\text{ex}} = 400\text{ nm}$)

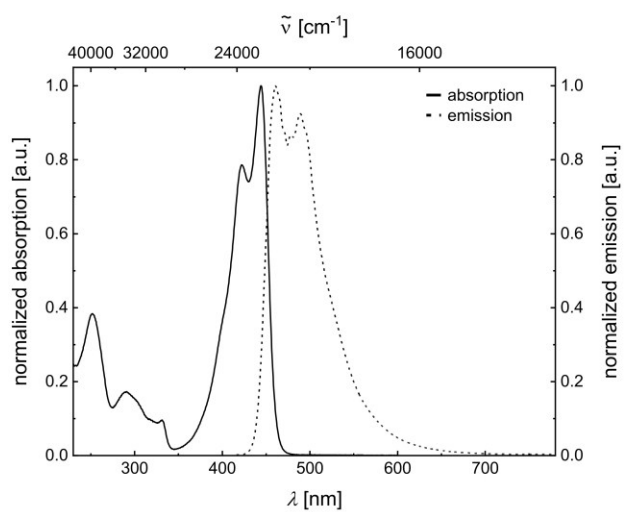


Fig. S3. Absorption (left) and emission (right) spectra of compound **6c**

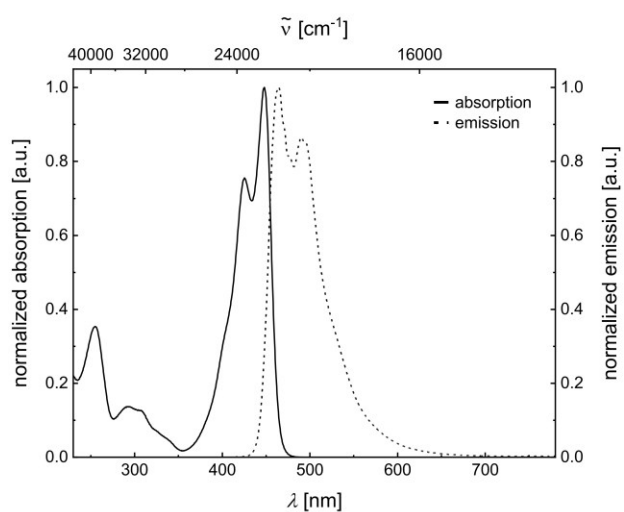


Fig. S4. Absorption and emission spectra of compound **6d** (recorded in dichloromethane, $T=293\text{ K}$, $c_{\text{abs}}(\mathbf{4})=10^{-5}\text{ M}$, $c_{\text{em}}(\mathbf{4})=10^{-7}\text{ M}$, $\lambda_{\text{ex}}=400\text{ nm}$)

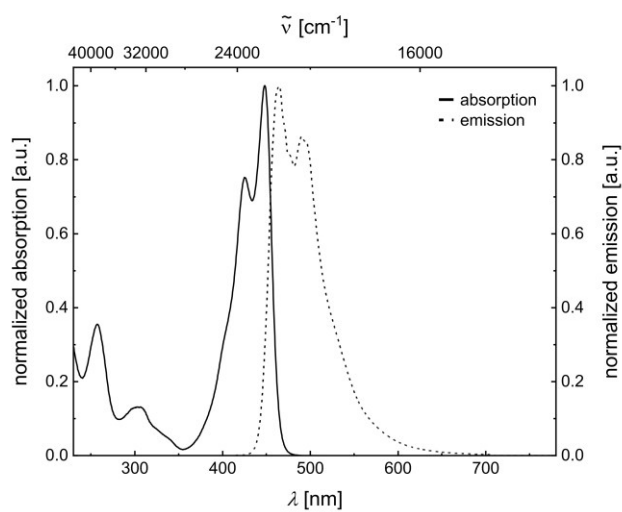
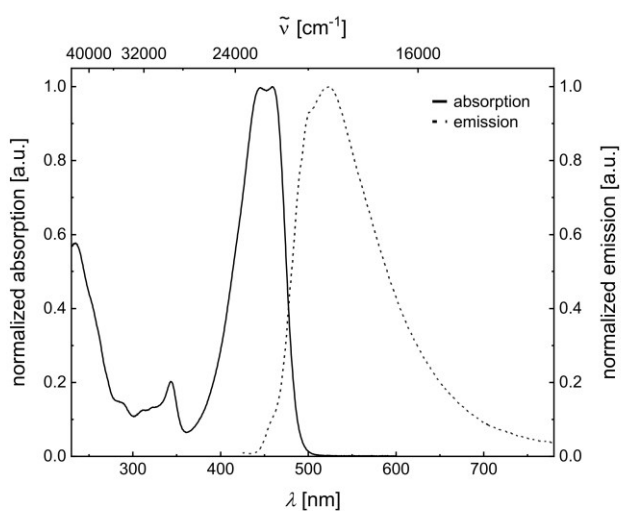
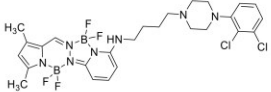
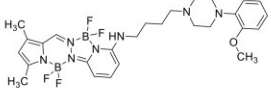
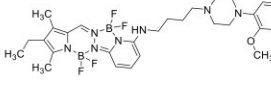
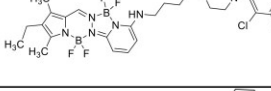
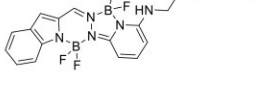


Fig. S5. Absorption and emission spectra of compound **6e** (recorded in dichloromethane, $T=293\text{ K}$, $c_{\text{abs}}(\mathbf{4})=10^{-5}\text{ M}$, $c_{\text{em}}(\mathbf{4})=10^{-7}\text{ M}$, $\lambda_{\text{ex}}=400\text{ nm}$)



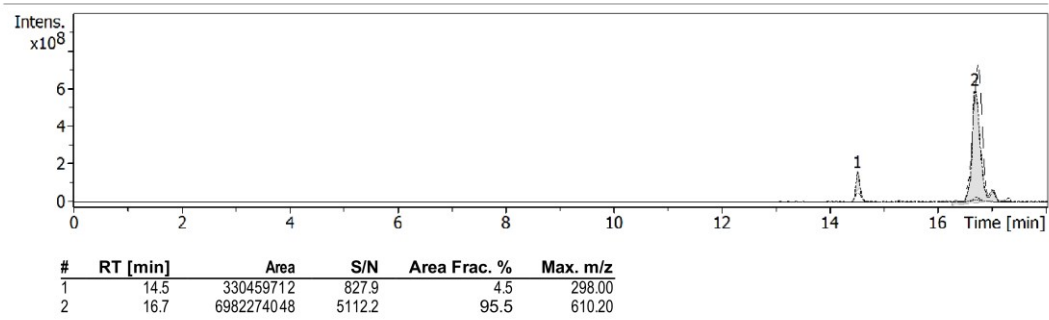
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Table S1: Synthesized compounds and their SMILES files

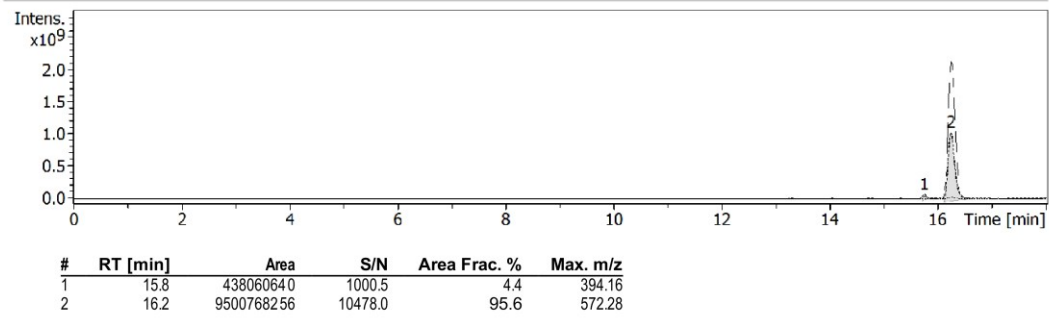
Name	Structure	SMILES
6a		<chem>FB1(F)N2C(C=CC=C2NCCCCN3CCN(C4=C(Cl)C(Cl)=CC=C4)CC3)=[N]5B(N6C(C)=CC(C)=C6C=[N]51)(F)F</chem>
6b		<chem>FB1(F)N2C(C=CC=C2NCCCCN3CCN(C4=C(OC)C=CC=C4)CC3)=[N]5B(N6C(C)=CC(C)=C6C=[N]51)(F)F</chem>
6c		<chem>CC1=C(CC)C(C)=C2N1B(F)(F)[N]3=C(C=CC=C4NCCCCN5CCN(C6=C(OC)C=CC=C6)CC5)N4B(F)(F)[N]3=C2</chem>
6d		<chem>FB1(F)N2C(C=CC=C2NCCCCN3CCN(C4=C(Cl)C(Cl)=CC=C4)CC3)=[N]5B(N6C(C)=C(C(C)C(C)=C6C=[N]51)(F)F</chem>
6e		<chem>FB1(F)N2C(C=CC=C2NCCCCN3CCN(C4=C(OC)C=CC=C4)CC3)=[N]5B(N6C7=C(C=CC=C7)C=C6C=[N]51)(F)F</chem>

LC-MS

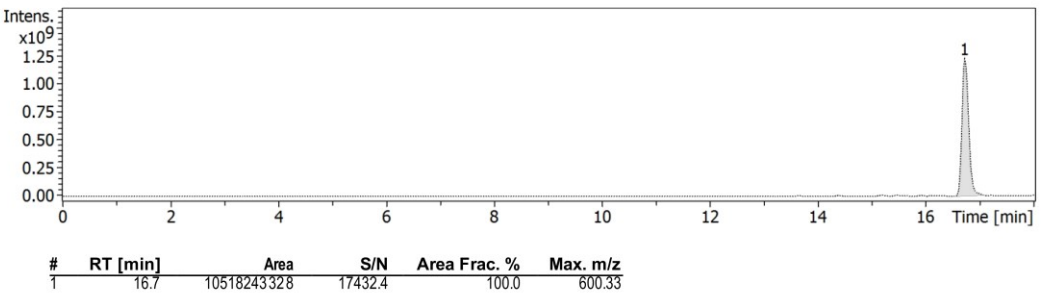
6a



6b

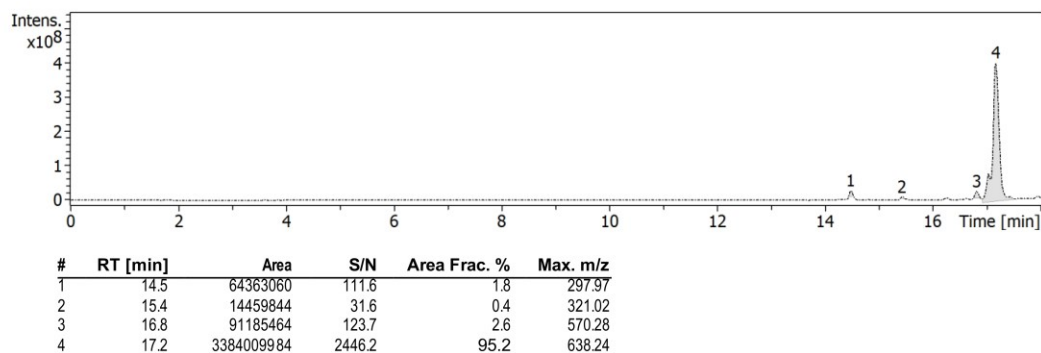


6c

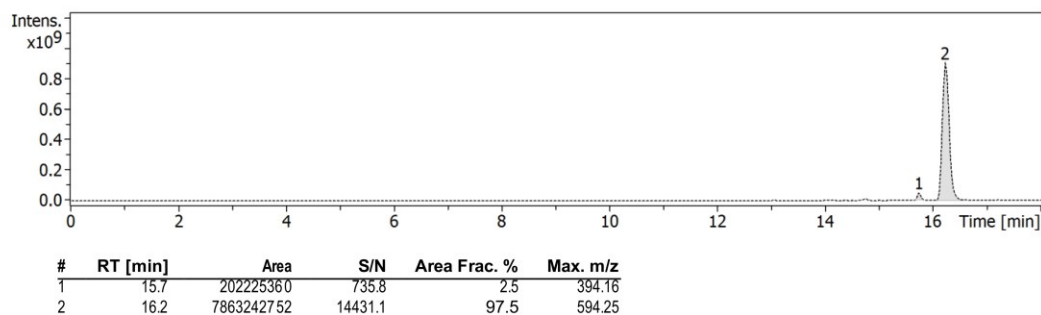


3 Publications

6d



6e



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3 Publications

4 CONCLUDING DISCUSSION AND PERSPECTIVES

Neurogenic diseases are complex, multifactorial conditions that place a significant burden on both patients and their families, highlighting the urgent need for effective treatments. The presented scientific work aims to contribute to the progress of drug development in the field of neurodegenerative, neuropsychiatric, and neurodevelopmental disorders. The MTDL approach is regarded as one of the most promising strategies in tackling multifactorial diseases.

Within the histaminergic research field, the first publication from Venkatachalam, Zhong, Dubiel et al. (2022) presents the novel compound ST-2300, a pimavanserin derivative and MTDL, targeting H3R and serotonin 5-HT_{2A} receptor simultaneously. The compound was designed to combine the procognitive effects of an H3R antagonist with the antidepressant effect of 5-HT_{2A} antagonist/inverse agonist properties. The design utilises the strategy of “merging” two pharmacophores within one molecule, which is the most promising option for ligands that aim to achieve CNS-penetrance and drug-like properties. The compound has been tested in various animal models mimicking depression and anxiety-like behaviours. The *in vivo* results suggest that ST-2300 induces antidepressant and anxiolytic effects, comparable or superior to the reference compound pimavanserin, which in parts might be due to its effect on H3R receptor. ST-2300 showed excellent affinity for H3R; however, 5-HT_{2A} affinity was found to be only moderate. Nevertheless, ST-2300 presents a promising compound for further lead optimisation in the field of depression and anxiety-related disorders.

In Publication 2 from Hafez, Dubiel, et al. (2023), several benzothiazole-based MTDLs are presented that aim to exhibit antagonist properties for H3R, and inhibitory properties towards AChE, BuChE, and MAO-B, combining the procognitive effects for the treatment of AD. The benzothiazole central core represents a privileged scaffold for a variety of substituents while maintaining receptor affinity. Among all tested compounds, six could be regarded as MTDL; however, affinity values are not equally balanced. For example, compound 3b, a pyrrolidine-substituted derivative with a H3R K_i of 0.036 μ M and IC₅₀ of 1.60 μ M for MAO-B, only achieved an IC₅₀ of 6.7 μ M for AChE. Additionally, some compounds exhibit a notable H1R inhibition. Further drug development should focus on improving desired affinities for a balanced multitargeting profile while reducing unfavourable affinities and maintaining druglike properties.

Publication 3 of Eissa et al. (2021) builds the bridge between the histaminergic and dopaminergic research fields by presenting an H3R and D2R/D3R MTDL. The compound is being explored in the context of autism-like repetitive behaviour. Autism

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spectrum disorder (ASD), a neurodevelopmental disorder with high heterogeneity, is accompanied by a pattern of restricted, repetitive behaviour, which is not sufficiently treated by current pharmacotherapies. Dopaminergic dysfunction is proposed to play a role in the pathophysiology of ASD, while genetic alterations in HNMT and HA receptors have been found. The presented study introduces the compound ST-2223, an MTDL merging the pharmacophores of H3R antagonists/inverse agonists with D2R/D3R antagonist properties. The compound has been assessed for the reduction of RRB in a mouse model of autism spectrum disorder. ST-2223 significantly reduced repetitive behaviour (marble burying and self-grooming), similar to antipsychotic aripiprazole and NMDAR antagonist memantine. Additionally, antioxidant properties and anxiolytic-like effects have been observed. ST-2223 might be a promising compound for the treatment of RRB in autism and associated comorbidities; however, further research and mechanism of action studies are necessary to confirm the insight gained from this *in vivo* study.

Within the dopaminergic research field, the publication by Egyed et al. (Publication 4) delves deeper into the role of D2R/D3R as drug targets for neuropsychiatric disorders such as SCH and depression. The presented compounds are bitopic ligands designed to interact with both the orthosteric binding pocket (OBP) and the secondary binding pocket (SBP) of D₂/D₃ receptors. Cariprazine served as the lead compound for the design and synthesis of this compound library, incorporating a cyclohexyl linker and variations in the SBP and OBP binding moieties. This study aimed to elucidate the impact of linker rigidity, investigate the influence of the SBP moiety on receptor affinity, selectivity, and functionality, and identify derivatives with the most promising polypharmacological properties relevant to antidepressant effects. A comprehensive SAR analysis of over 40 compounds confirmed that both the SBP moiety and the linker affect not only receptor affinity and selectivity but also signalling pathways and functional outcomes, as demonstrated by BRET studies. From this compound set, 20b, a D₃-preferring partial agonist with additional partial agonist activity at the 5-HT_{2A} receptor, was further evaluated in an *in vivo* model, where it demonstrated antidepressant-like effects. This SAR study provides valuable insights into the structural determinants governing receptor interaction, offering a foundation for the rational design of novel bitopic ligands with optimised therapeutic profiles for neuropsychiatric disorders.

Following the path of bitopic ligands, the publication from Mastromarino et al. (2022) explores long-chain arylpiperazine derivatives as multitarget compounds for neuropsychiatric disorders. The compounds were designed to modulate serotonin (5-HT_{1A}, 5-HT_{2A}, 5-HT₇) receptors and D2R while also demonstrating neuroprotective effects. Compound 12a was showing a pharmacological profile consistent with

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antipsychotic activity and antioxidant properties, while compound 9b demonstrated selectivity for serotonin receptors, making it a promising drug candidate for ASD. Despite favourable BBB penetration properties, most compounds lacked sufficient metabolic stability, except for 9b, which needs further investigation and lead structure optimisation.

With the publication of Elek et al. (2022), research on arylpiperazines continues, but with a different approach. This work explores the development of fluorescent dopaminergic ligands for imaging of D2R and D3R. The goal was to synthesise selective ligands with beneficial fluorescent properties by coupling dopamine pharmacophores with BOPPY, a chemically stable fluorophore. The synthesised compounds showed moderate to high affinities for D2R and D3R, with a slight preference for D3R. Among them, compound 6e demonstrated the highest selectivity for D3R. Structural variation influenced receptor selectivity and fluorescent properties. For example, compounds 6c and 6d combined strong D3R affinity with high quantum yield, making them promising candidates for fluorescent probes. This study represents the first application of BOPPY in GPCR ligand design. Upon further research, these compounds could prove their potential use for in vivo receptor localisation and tracking, as well as for fluorescent biophysical assays.

The presented scientific work represents one small part of extensive preclinical research on drug discovery and development for CNS diseases. Despite the vast amount of research, overall drug approvals have experienced a declining trend in recent years, with a particular drop in neurology compared to the five-year average [220]. This trend underscores the urgent need for improvement in the transition “from bench to bedside”. There are several barriers in academic, preclinical, and clinical research. Addressing translational barriers is crucial for ensuring that promising drug candidates advance successfully into clinical applications [221,222].

The development of H3R ligands faces early difficulties. Despite many promising preclinical effects of H3R antagonists, the outcome of marketed drugs is low. One issue is the high resemblance of the H3R and hERG K⁺ channels' pharmacophore, which can cause cardiotoxic effects [223]. Another concern is phospholipidosis, especially for ligands with two basic centres, or prolonged receptor blockage, which may cause insomnia [224]. Consequently, receptor occupation should be below 80% to reduce wake-inducing side effects, and the residence time of the ligand should be rather short [225]. Addressing these challenges early in development can help to improve a successful transition from the preclinical to the clinical phase.

Other generally applicable challenges are the lack of reproducibility of in vitro assays [226,227] and the limited predictive value of current preclinical models, as rodent

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models often fail to fully capture human disease complexity [228]. Efforts towards improving the reproducibility of in vitro assays, as well as refining and expanding human-relevant preclinical models, such as induced pluripotent stem cells (iPSCs)-derived neurons and brain organoids, will improve translational success [229]. The multitarget-directed approach should be further explored to address the complexity of CNS disorders. One of the most promising developments in the field of next-generation CNS therapeutics is the emergence of biased GPCR ligands. For instance, selective activation of the D1R β -arrestin pathway could improve normal movement and cognitive function while reducing the risk of side effects [230]. Other studies have demonstrated that D2R agonists with signalling bias for β -arrestin recruitment mitigate SCH-like behaviour in mice without inducing motor site effect [231,232]. Similarly, biased D3R ligands are being explored to enhance therapeutic benefits for neurodegenerative and neuropsychiatric disorders [168,233]. Advances in GPCR structural biology have also played a crucial role in modern drug discovery [177]. The identification of novel binding sites and secondary binding pockets has opened new possibilities for the design of highly selective and efficacious drug candidates. Another future trend is the utilisation of computational modelling and artificial intelligence-assisted drug discovery [234,235]. Together with advanced imaging technologies, these innovations will not only enhance drug screening but also provide valuable tools for diagnostic insights. The future of neuropsychiatric and neurodegenerative therapeutics lies in this interdisciplinary, multitargeting effort, aiming to transform promising molecular discoveries into effective, accessible treatments for patients in need.

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APPENDIX

ABBREVIATIONS

5-HT	serotonin
5-HT _{1A/2A/2C/7}	serotonin receptor subtype 5-HT _{1A} /5-HT _{2A} /5-HT _{2C} /5-HT ₇
AA	arachidonic acid
AADC	aromatic L-amino acid decarboxylase
AC	adenylyl cyclase
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
ADHD	attention deficit hyperactivity disorder
AKT	protein kinase B
ALDH	aldehyde dehydrogenase
ASD	autism spectrum disorder
Asp	aspartic acid
ATP	adenosine triphosphate
A β	amyloid beta
BACE1	β -site APP cleaving enzyme 1
BBB	blood-brain barrier
BuChE	butyrylcholinesterase
cAMP	cyclic adenosine monophosphate
CBT	cognitive-behavioural therapy
CNS	central nervous system
COMT	catechol-O-methyltransferase
CREB	cAMP response element-binding protein
Cryo-EM	cryo-electron microscopy
D1R/D2R/D3R/D4R/D5R	dopamine D ₁ /D ₂ /D ₃ /D ₄ /D ₅ receptors
D2 _L R	long isoform of dopamine D2R
D2 _S R	short isoform of dopamine D2R
DA	dopamine
DAO	diamine oxidase
DAR	dopamine receptors
DBH	dopamine β -hydroxylase
ECL2	extracellular loop 2
EPM	elevated plus maze
EPS	extrapyramidal symptoms
FGA	first-generation antipsychotic
FST	forced swim test
GABA	γ -aminobutyric acid
GAD	generalised anxiety disorder
GDP	guanosine diphosphate
GIRK	G protein-gated inwardly rectifying potassium channels
Glu	glutamic acid
GLU	glutamate
GRK	G protein-coupled receptor kinases
GSK3 β	glycogen synthase kinase-3 beta
GTP	guanosine triphosphate
H1R/H2R/H3R/H4R	histamine H ₁ /H ₂ /H ₃ /H ₄ receptor
HA	histamine

Appendix

HDC	histidine decarboxylase
hH3R	human isoform of H3R
HNMT	histamine N-methyltransferase
Hth	hypothalamus
HVA	homovanillic acid
IP3	inositol 1,4,5-trisphosphate
L-DOPA	levodopa
MAO	monoamine oxidase
MAO-B	monoamine oxidase B
MAPK	mitogen-activated protein kinase
MBT	marble burying test
MDD	major depressive disorder
MTD	Multitargeting drug
MTDL	Multitarget-directed ligand
NA	noradrenaline
NAc	nucleus accumbens
NHE	Na ⁺ /H ⁺ exchange
NMDA	<i>N</i> -methyl-D-aspartate
NMDAR	NMDA receptor
NSF	novelty suppressed feeding
OBP	orthosteric binding pocket
OBS	orthosteric binding site
OFT	open field test
PD	Parkinson's disease
PI3K	phosphatidylinositol 3-kinases
PIM	pimavanserin
PKA	protein kinase A
PLA ₂	phospholipase A ₂
PLC	phospholipase C
PNMT	phenyl ethanolamine N-methyltransferase
RAMH	(R)- α -methylhistamine
rH3R	rat isoform of H3R
ROS	reactive oxygen species
SAR	structure activity relationship
SBP	secondary binding pocket
SBS	secondary binding site
SCH	schizophrenia
SGA	second-generation antipsychotic
SNc	substantia nigra pars compacta
SNRIs	serotonin-norepinephrine reuptake inhibitors
SSRIs	selective serotonin reuptake inhibitors
TGA	third-generation antipsychotic
TH	tyrosine hydroxylase
TM	transmembrane
TMN	tuberomammillary nucleus
TST	tail suspension test
VGCC	voltage-gated calcium channels
VTA	ventral tegmental area
β 2AR	β 2-adrenergic receptor

LIST OF PUBLICATIONS AND PRESENTATIONS

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Appendix

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EIDESSTATTLICHE ERKLÄRUNG

Ich, Frau Mariam Dubiel, versichere an Eides Statt, dass die Dissertation „Aminergic Ligands for Multitargeting in Central Nervous System Disorders“ 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 25. November 2025

Mariam Dubiel

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 und dass die Disputation in englischer Sprache abgelegt wird. Hiermit beantrage ich den Ausschluss der Öffentlichkeit von der Befragung im Rahmen der Disputation. Auf den Ausschluss der Öffentlichkeit vom Vortrag wird verzichtet.

Düsseldorf, den 25. November 2025

Mariam Dubiel