

**Investigation of Novel Combination Treatments
with HDAC Inhibitors in Leukemia and Bladder
Cancer Cell Lines**

Inaugural-Dissertation

for the attainment of the title of doctor
in the Faculty of Mathematics and Natural Sciences
at the Heinrich Heine University Düsseldorf

presented by

Lukas Michael Bollmann
from Bocholt

Düsseldorf, January 2026

from the Institute for Pharmaceutical and Medicinal Chemistry at the
Heinrich Heine University Düsseldorf

Published by permission of the
Faculty of Mathematics and Natural Sciences at
Heinrich Heine University Düsseldorf

Supervisor: Prof. Dr. Matthias U. Kassack

Co-supervisor: Prof. Dr. Holger Gohlke

Date of the oral examination: 10.06.2026

Contents

Abbreviations.....	5
List of Illustrations.....	9
Abstract.....	12
1. Introduction.....	14
1.1 Malignant Tumor Development.....	14
1.2 Tumor Classification.....	15
1.3 New Therapeutic Approaches for Acute Myeloid Leukemia and Bladder Cancer.....	16
1.3.1 Acute Myeloid Leukemia.....	16
1.3.2 Treatment Options of Acute Myeloid Leukemia.....	18
1.3.3 Bladder Carcinoma.....	20
1.3.4 Treatment Options of Bladder Cancer.....	20
1.3.5 Cisplatin.....	22
1.3.6 Cisplatin Resistance.....	26
1.4 Epigenetics and the Proteasome as Target of Cancer Therapy.....	28
1.4.1 Histone Deacetylases.....	29
1.4.2 Histone Deacetylases and Cancer.....	32
1.4.3 Bromodomain and Extra-Terminal Motif Proteins.....	33
1.4.4 Ubiquitin-Proteasome System.....	35
1.5 Epigenetic Modulators and the Proteasome.....	36
1.5.1 Histone Deacetylase Inhibitors.....	37
1.5.2 Multi-Target Histone Deacetylase Inhibitors.....	38
1.5.3 Bromodomain and Extra-Terminal Motif Inhibitors.....	40
1.5.4 Proteasome Inhibitors.....	42
2. Aim of the study.....	44
3. Publications.....	46
3.1 Publication I.....	46
3.2 Publication II.....	95
3.3 Publication III.....	127
4. Discussion and Conclusion.....	203
4.1 Combination of Class IIa HDACi with the PI BTZ.....	203
4.2 Combination of the Class I HDACi Entinostat, a BETi, and Cisplatin.....	213
4.3 Conclusion.....	224

5. References.....	225
List of Publications.....	239
Conference Poster.....	240
Acknowledgement	241
Eidesstattliche Erklärung	243

Abbreviations

2-HG	2-hydroxyglutarate
ADC	antibody-drug conjugate
AML	acute myeloid leukemia
AML-MRC	AML with myelodysplasia related changes
APL	acute promyelocytic leukemia
ATM	ataxia-telangiectasia mutated protein
ATR	ataxia-telangiectasia mutated protein and RAD3-related protein
ATRA	all-trans-retinoic acid
BCa	bladder carcinoma
BCL-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma extra-large protein
BET	bromodomain and extra terminal motif
BETi	bromodomain and extra-terminal motif inhibitor
BID	basic residue-enriched interaction domain
BD	bromodomain
BRD	bromodomain-containing protein
BTZ	bortezomib
bZIP	basic leucine zipper
CD33	sialic acid-binding immunoglobulin-like lectin
cDDP	cis-diamminedichloroplatinum(II) (cisplatin)
CEBPA	CCAAT/enhancer-binding protein α
CK2	casein kinase II
CR	complete response
CTR1	copper transporter 1
DNA	deoxyribonucleic acid
DNMTi	DNA methyltransferase inhibitor
EAU	European Association of Urology

Abbreviations

EMA	European Medicine Agency
ERBB2	erb-b2 receptor tyrosine kinase 2
ERCC1	excision repair cross-complementation group 1
EV	enfortumab vedotin
Fas	Fas Cell Surface Death Receptor
FDA	Food and Drug Administration
FLIP	fllice-like inhibitory protein
FLT3	FMS-like tyrosine kinase 3
FLT3wt	FMS-like tyrosine kinase 3 wild-type
GADD45	Growth Arrest and DNA Damage-inducible
GC	gemcitabine and cisplatin
GO	gemtuzumab ozogamicin
GSH	glutathione
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HDM	histone demethylase
HER2	human epidermal growth factor receptor 2
HMG	high mobility group
HMT	histone methyltransferase
ICC	International Consensus Classification
ICR	interstrand crosslink repair
IDH	isocitrate dehydrogenase
IDHi	isocitrate dehydrogenase inhibitor
I κ B α	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
MAPK	mitogen-activated protein kinase
Mdm2	mouse double minute 2

Abbreviations

MIBCa	muscle-invasive bladder carcinoma
MLH1	MutL protein homolog 1
MSH2	MutS homolog 2
MMR	mismatch repair
MRP2	multidrug resistance protein 2
MVAC	methotrexate, vinblastine, adriamycin, and cisplatin
NAD ⁺	nicotinamide adenine dinucleotide
NER	nucleotide excision repair
N-CoR	nuclear receptor corepressor
NES	nuclear export signal
NF-κB	nuclear factor κB
NLS	nuclear localization signal
NMIBCa	non-muscle invasive bladder carcinoma
NPM1	nucleophosmin 1
NPS	N-terminal phosphorylation site
NUT	nuclear protein in testis
ORR	overall response rate
OS	overall survival
PI	proteasome inhibitor
PIDD	p53-induced protein with a death domain
PD-L1	programmed death-ligand 1
PROTAC	proteolysis targeting chimeras
p-TEFb	positive transcription elongation factor b
PTEN	phosphatase and tensin homologue protein
PUMA	p53 upregulated modulator of apoptosis
ROS	reactive oxygen species
SIR2	sirtuin; silent information regulator 2
SMRT	silencing mediator for retinoid and thyroid receptors

Abbreviations

tAML	therapy-related acute myeloid leukemia
TETi	ten-eleven translocation enzyme inhibitor
TFIIB	transcription factor IIB
TFMO	5-(trifluoromethyl)-1,2,4-oxadiazole
TMT	trimodality therapy
TNF- α	tumor necrosis factor α
TUR	transurethral resection
UPS	ubiquitin-proteasome system
WHO	World Health Organization
ZBG	zinc-binding group

List of Illustrations

Figure 1. Hallmarks of cancer.....	14
Figure 2. Drivers and pathways in acute myeloid leukemia pathogenesis	17
Figure 3. Bladder cancer staging.....	20
Figure 4. Cisplatin	22
Figure 5. DNA adducts of cisplatin	23
Figure 6. Overview of mechanisms of action of cisplatin and anticancer activity.....	24
Figure 7. The fate of cisplatin in human cells.....	26
Figure 8. Targets of the epigenome.....	29
Figure 9. Interplay of HDACs and HATs in gene transcription.	30
Figure 10. Localization of zinc-dependent HDACs in the cell.....	31
Figure 11. Schematic structure of the human BET proteins BRD2, BRD3, BRD4 isoforms (BRD4-L, BRD4-B, BRD4-S) and BRDT	33
Figure 12. Influence of BET proteins on gene transcription in cancer cells	34
Figure 13. Ubiquitin-proteasome system.....	36
Figure 14. Structural examples for histone deacetylase inhibitors.....	37
Figure 15. Examples of multi-target inhibitors based on different design strategies.....	39
Figure 16. Structure of the pan-BETi	40
Figure 17. Proteasome inhibitors approved by FDA.....	42
Figure 18. Class IIa HDACi TMP269, YAK540 and FFK24.....	44
Figure 19. The most meaningful results of the combination of YAK540 and bortezomib in HL-60 cell line	47
Figure 20. The most meaningful results of the combination of FFK24 and bortezomib in THP-1 cells.....	96
Figure 21. The most meaningful results of the triple combination for J82 and J82 cisR	128
Figure 22. Caspase 3/7-activation in THP-1 cells	204
Figure 23. Synergy levels of combination of BTZ and YAK540 or TMP269.....	205
Figure 24. Western Blot analysis of HL-60, K562 and MONO-MAC-6.....	206
Figure 25. Comparison of cytotoxicity of the combination BTZ and class IIa HDACi YAK540 or TMP269 in HL-60 and HEK293.....	207
Figure 26. Synergy levels, caspase 3/7-activation and Western Blot of combinations of BTZ and FFK24 or YAK540 in THP-1 cell line	209
Figure 27. Chorio-allantoic membrane assay of FFK24	210
Figure 28. Structures of class IIa HDACi	211
Figure 29. Synergy levels of the triple combination studies experiments.....	215
Figure 30. Caspase 3/7-activation and double-strand breaks of triple combination in J82 and J82 cisR	217
Figure 31. Relative protein expression of FOXO1, Bim and p21	218
Figure 32. Concentration-effect-curves of cisplatin.....	219
Figure 33. Caspase 3/7 activity and relative protein expression of FOXO1, Bim and p21 in J82 and J82 cisR.....	220
Figure 34. Comparison of cytotoxicity of the triple combination in HEK293 cells and J82 cisR cells.....	221

Figure 35. Structure of compound 20	221
Figure 36. Caspase 3/7 activation and Western blot of compound 20	222
Figure 37. Overview of the results of triple combination of entinostat, a BETi and cisplatin in bladder cancer cell lines.....	223

Notes on the illustrations:

Figure 1 was reprinted from *Cancer Discovery*, 2022, 12(1): 31–46., Hanahan, Douglas, *Hallmarks of Cancer: New Dimensions*, with permission from AACR.

Figure 2 was reprinted from *Cells*, 2024, 13(16), Nong, T., S. Mehra, and J. Taylor, *Common Driver Mutations in AML: Biological Impact, Clinical Considerations, and Treatment Strategies*. The article and its associated figures have been published under a CC BY license.

Figure 3 was reprinted from *Nature Reviews Disease Primers*, 2023, 9(1): p. 58, Dyrskj t, L., et al., *Bladder cancer*. Reproduced with permission from Springer Nature.

Figure 5 and 6 was reprinted and minimally modified from *Bioorganic Chemistry*, 88, Ghosh, S., *Cisplatin: The first metal based anticancer drug*, p. 102925., 2019, with permission from Elsevier.

Figure 7 was reprinted from *Pharmacological Research*, Vol 106, Amable, L., *Cisplatin resistance and opportunities for precision medicine*, 27-36., Copyright (2016), with permission from Elsevier.

Figure 8 was reprinted from *Nature Reviews Clinical Oncology*, 2019, 17, p. 91-107, Morel, D., Jeffery, D., Aspeslagh, S. et al. *Combining epigenetic drugs with other therapies for solid tumours — past lessons and future promise*. Reproduced with permission from Springer Nature.

Figure 9 was reprinted from *The Journal of Antibiotics*, 2021, 74(10): p. 667–676., Maemoto, Y., et al., *Naturally occurring small molecule compounds that target histone deacetylases and their potential applications in cancer therapy*. Reproduced with permission from Springer Nature.

Figure 10 was reprinted from *Bioorganic & Medicinal Chemistry*, 2019, 27(22):115087, Asfaha, Y. et al., *Recent advances in class IIa histone deacetylases research*.

Figure 11 was reprinted from *Pharmacology & Therapeutics*, 243., Guo, J., Q. Zheng, and Y. Peng, *BET proteins: Biological functions and therapeutic interventions*, p. 108354, 2023, with permission from Elsevier.

Figure 12 was reprinted from *Oncotarget*, 2016, 7(33): p. 53997–54009, Sahai, V., et al., *Targeting BET bromodomain proteins in solid tumors*. The article has been published under a Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction.

List of Illustrations

Figure 13 was reprinted from *Cancers*, 2023, 15(8), Wang, J., et al., The Ubiquitin-Proteasome System in Tumor Metabolism. The article and its associated figures have been published under a CC BY license.

Figure 19 and Figure 22 to 25 were reprinted and partially modified from *International Journal of Molecular Sciences*, 2022, 23(21), Bollmann, L.M., et al., The Novel Class IIa Selective Histone Deacetylase Inhibitor YAK540 Is Synergistic with Bortezomib in Leukemia Cell Lines. The article and its associated figures have been published under a CC BY license.

Figure 20, Figure 26 and Figure 27 were reprinted and partially modified from *European Journal of Medicinal Chemistry*, 2024, 263: p. 115907, Asfaha, Y., et al., 5-(Trifluoromethyl)-1,2,4-oxadiazole (TFMO)-based highly selective class IIa HDAC inhibitors exhibit synergistic anticancer activity in combination with bortezomib.

Figure 21 and Figure 29 to 34 and Figure 36 to 37 were reprinted and partially modified from *Cancers*, 2024, 16(19): p. 3374, Bollmann, L.M., et al., Triple Combination of Entinostat, a Bromodomain Inhibitor, and Cisplatin Is a Promising Treatment Option for Bladder Cancer. The article and its associated figures have been published under a CC BY license.

Abstract

Despite major advances in oncology, the 5-year survival rate for some cancers like acute myeloid leukemia (AML) or advanced muscle-invasive bladder cancer is low. Among the main reasons for the low survival rates in AML and bladder cancer are the heterogeneity, the chemoresistance and the epigenetic dysregulation of the cancer cells.

To face these problems and increase survival rates, combination treatments can be utilized. Combination treatments have several advantages, including targeting multiple targets and synergistic effects between the compounds, leading to a more potent and possibly more selective cytotoxic effect in the cancer cells.

This thesis aimed to identify new combination partners targeting epigenetic dysregulation for improved treatment of AML and muscle-invasive bladder cancer. Several AML cell lines and two pairs of native and cisplatin-resistant muscle-invasive bladder cancer cell lines were used.

One important group of epigenetic regulators are histone deacetylases (HDACs), impacting a variety of intracellular processes. Dysregulation of HDACs in cells has emerged as a contributor to the development of cancer and chemoresistance. The HDAC family is classified into five distinct classes, comprising a total of 11 HDAC and 7 sirtuin enzymes. The use of selective histone deacetylase inhibitors (HDACi) should enable more targeted and efficient therapy than the use of broad-spectrum HDACi.

The first part of the thesis focuses on AML. Herein, the new class IIa specific HDACi YAK540 was intensively characterized. Subsequently, YAK540 in combination with the proteasome inhibitor (PI) bortezomib (BTZ) was analyzed in the AML cell lines THP-1, HL-60, and MONO-MAC-6 as well as in the chronic myeloid leukemia cell line K562. BTZ is used clinically for the treatment of multiple myeloma and is currently in clinical trials for the treatment of AML. The first study showed that the combination of the novel class IIa specific HDACi YAK540 with BTZ induced a synergistic cytotoxic effect with increased expression of p21 and enhanced caspase 3/7-mediated apoptosis. In a follow-up study, FFK24, a more potent class IIa HDACi was characterized and evaluated in combination with BTZ in the AML cell line THP-1, again demonstrating increased expression of p21 and a synergistic cytotoxic effect.

The second part of the thesis addresses the problem of cisplatin resistance in bladder cancer. The triple combination of the class I HDACi entinostat, a bromodomain and extra-terminal motif inhibitor (BETi; JQ1, OTX015) and cisplatin was investigated in two pairs of sensitive and cisplatin-resistant muscle-invasive bladder cancer cell lines (J82, J82 cisR and T24, T24 LTT). In addition, the dual inhibitor **compound 20**, (a combined HDAC- and BET-inhibitor) was investigated in combination with cisplatin.

Entinostat plus JQ1 or OTX015 as well as **compound 20** showed complete (J82 cisR) or partial (T24 LTT) reversal of cisplatin resistance. The combinations were highly synergistic and resulted in increased caspase-mediated apoptosis most likely triggered by enhanced expression of p21, Bim, and FOXO1.

In conclusion, HDACi are promising combination partners for different drug classes, such as PI, BETi and cisplatin. These combinations could contribute to the development of new therapeutic options for AML or bladder cancer, including overcoming chemoresistance.

1. Introduction

1.1 Malignant Tumor Development

Dysregulation of cell growth and cell death represents a critical mechanism underlying tumor development. It is primarily caused by mutations that alter gene expression [1]. These mutations can be induced by radiation, mutagenic chemicals, viruses or replication errors, which are normally repaired by the deoxyribonucleic acid (DNA) repair systems. However, mutations can accumulate in cells. At crucial positions, they lead to sustained proliferative signaling that supports the initiation of tumor development [2, 3].

Cancer cells exhibit distinctive biological capabilities (Figure 1).

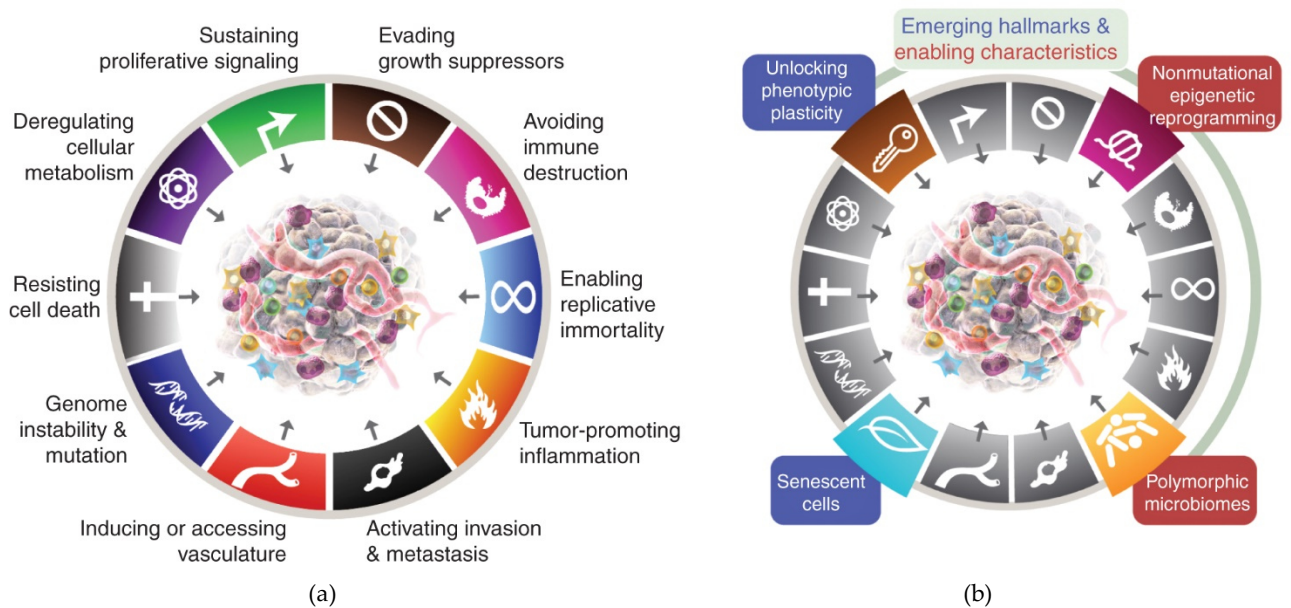


Figure 1. Hallmarks of cancer. (a) The eight hallmarks of cancer for tumor growth and progression. The enabling characteristics genome instability as well as mutation and tumor-promoting inflammation are involved in activating the eight hallmarks. (b) Additional hallmarks and enabling characteristics of cancer that are currently emerging in research. Figure taken from [4] with permission from AACR.

Such capabilities include the ability to sustain proliferative signaling by switching off tumor suppressor genes and the inhibition of cell death, which ultimately results in unregulated growth. Overcoming the process of cellular senescence allows for the proliferation of cells without limit. This in turn induces a sufficient supply of new blood vessels through the process of angiogenesis. Cancer cells are capable of invading deeper tissue layers and of causing metastasis by spreading to other organs. Additional characteristics include the reprogramming of energy metabolism and the evasion of destruction by the immune system. Two enabling characteristics have been identified: tumor promoting inflammation and genome instability (Figure 1a). Recently, four further potential parameters have been proposed: unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, senescent cells and,

polymorphic microbiomes (Figure 1b) [4]. It is yet to be determined whether these parameters could be incorporated into the model of hallmarks [4-6].

The development of cancer is a multistage process that occurs in a series of complex, sequential steps. It begins with random mutations that can induce uncontrolled cell division. Frequent cell division can yield an accumulation of mutations in the daughter cells. This process results in a genetic diversification of the aberrant cells. If cells acquire advantageous mutations that enhance their survival prospects, they can proliferate more rapidly. Consequently, the cancer cells that are best suited to survive in the given environment are clonally selected. The reiteration of these processes—uncontrolled cell division, the accumulation of mutations (genetic diversification), and clonal selection—leads to the generation of progressively aberrant cells, ultimately resulting in cancerous manifestations [2, 5, 7, 8].

Metastasis, the final stage of cancer development, requires further acquisitions of the aforementioned hallmarks of cancer (Figure 1a). Each metastatic cell must pass through the metastatic cascade, acquiring four essential characteristics: motility and invasion, the ability to alter the secondary microenvironment, plasticity, and the capacity to colonize secondary tissue [9, 10].

1.2 Tumor Classification

Tumors can be classified as either benign or malignant. Benign tumors typically exhibit a phenotype similar to that of normal cells albeit with mutations that influence their proliferation. They remain local and do not invade surrounding tissue or metastasize. Although most benign tumors are stable, there is a potential for malignant transformation. It can be facilitated by the accumulation of further mutations or by the influence of the tumor microenvironment. Malignant tumors spread invasively into the surrounding tissue. The tumor cells transform the surrounding tissue into a tumor microenvironment that promotes tumor growth. They tend to form metastases, which are spread through the blood and lymphatic system or migrate along nerves or the basal side of endothelial cells [9, 11, 12].

The nomenclature for tumors varies depending on the tissue of origin. The type of cancer this study is focussed on is referred to as leukemia and carcinoma. Leukemias specifically affect the blood or the hematopoietic system, with the presence of cancer cells in the blood system. Carcinomas are tumors derived from epithelial cells [13-15].

1.3 New Therapeutic Approaches for Acute Myeloid Leukemia and Bladder Cancer

Cancer treatment has improved over the past few decades. However, patients with advanced or recurrent cancer are still confronted with a poor prognosis of survival. To complicate matters, the development of chemoresistance to first-line therapies decreases the successful cancer treatment even further [16, 17]. As a consequence, new therapeutic strategies are urgently needed against cancer including acute myeloid leukemia (AML) and bladder cancer to improve the prognosis.

The five-year survival rate for AML is 30%, though the percentage varies according to the patients age. Younger patients have an overall survival (OS) rate of up to 50%. The survival quota of people older than 60 years, however, rests at 10% [18]. Especially with regards to the latter group, novel therapeutic strategies are required to enhance the 5-year survival rate.

Bladder cancer has a high incidence as sixth most common cancer in men and the tenth most common cancer worldwide [19]. Cisplatin, a well-established and highly effective cytostatic drug, is used in combination regimens to treat advanced stages of bladder cancer. During treatment, patients can develop resistance to cisplatin, limiting further treatment [20]. Therefore, new approaches for advanced bladder cancer are needed to provide patients with a long-term treatment option despite chemoresistance.

1.3.1 Acute Myeloid Leukemia

AML is a heterogeneous disease affecting the bone marrow. This results in the uncontrolled production of neoplastic clonal myeloid stem cells. The primary reason for AML is the presence of diverse genetic alterations (Figure 2) [21, 22].

1. Introduction

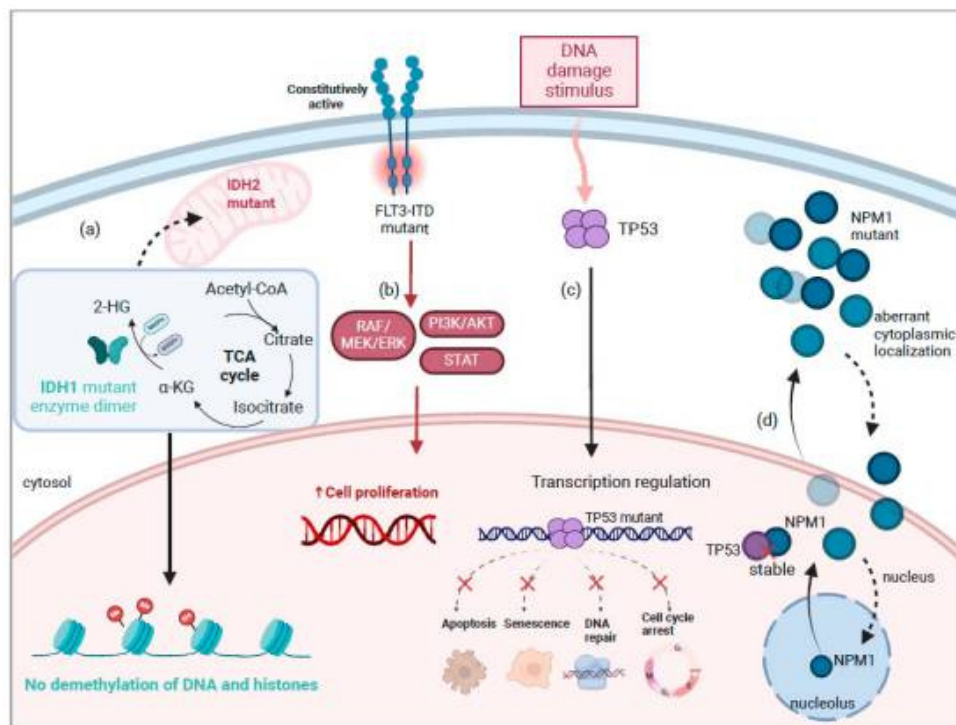


Figure 2. Drivers and pathways in acute myeloid leukemia pathogenesis. **(a)** Mutated isocitrate dehydrogenase (IDH) 1 and IDH2 enzymes lead to increased levels of 2-hydroxyglutarate (2-HG). 2-HG acts as an oncometabolite by inhibiting chromatin-modifying enzymes such as histone and deoxyribonucleic acid (DNA) demethylases, resulting in a blockade of cellular differentiation. **(b)** Constitutive activation of the FMS-like tyrosine kinase 3 (FLT3) receptor is induced by FLT3-internal tandem duplication mutations. Downstream signaling pathways such as RAF/MEK/ERK, PI3K/AKT and STAT are upregulated, promoting cell proliferation and survival as well as blocking differentiation. **(c)** p53 mutations affect the function of p53. Induction of apoptosis, senescence, DNA repair and cell cycle arrest is disrupted, leading to uncontrolled cell proliferation, genomic instability and increased leukemogenesis. **(d)** A mutated nucleophosmin 1 (NPM1) gene causes NPM1 to localize primarily in the cytoplasm. In the context of cellular stress, NPM1 is unable to bind mouse double minute 2 (Mdm2) in the nucleoplasm, thereby preventing the Mdm2-mediated degradation of p53. Consequently, the lower p53 activity results in a deficiency in cell cycle regulation and induction of apoptosis [23, 24]. Figure taken from [23].

The genetic heterogeneity of AML requires different therapeutic approaches to provide targeted and effective therapy. The choice of therapy must take into account the genetic alterations caused by mutations. In 2022, two classification systems – the updated classification system of the World Health Organization (WHO) and the International Consensus Classification (ICC) - were published for AML. Both of them were based on the concept of integrated molecular analysis. They likewise accord priority to genetic modifications, with AML sometimes being defined independently of the number of blasts [18, 25, 26]. Genetic abnormalities in AML are categorized into the three prognostic risk classification groups: favorable, intermediate, and adverse, according to the European LeukemiaNet [27, 28]. The application of classification systems in the diagnosis of AML serves to specify the diagnosis by determination of the AML subgroup and thereby contributes to the development of more effective and more specific treatment regimens.

1.3.2 Treatment Options of Acute Myeloid Leukemia

As discussed above, the treatment of AML is dependent on its specific subtype. The diagnosis and classification of AML is based on the criteria established by the WHO or the ICC. Prior to the initiation of therapy, a comprehensive evaluation is conducted to determine the urgency of treatment, the necessity of fertility preservation measures, and the patient's fitness for therapy. The genetic subtype is then considered during the selection of first-line therapy. Acute promyelocytic leukemia (APL) follows its own treatment algorithm [29].

The standard treatment regime for most AML subtypes includes the combination of seven days of cytarabine and three days of daunorubicin (7+3). Different approaches were investigated to improve the 7+3 regime. One of these improvements is CPX-351, which contains as nano-scale liposome a fixed 5:1 molar ratio of cytarabine and daunorubicin. In a phase III randomized trial, CPX-351 showed a significantly longer survival rate in comparison to the 7+3 regime with newly diagnosed secondary AML. CPX-351 is approved for therapy-related acute myeloid leukemia (tAML) and AML with myelodysplasia related changes (AML-MRC) [29, 30].

The antibody-drug conjugate (ADC) gemtuzumab ozogamicin (GO) is indicated as a first-line therapy in combination with the 7+3 regimen for patients diagnosed with CD33-positive AML, as well as for patients with basic leucine zipper (bZIP) in-frame mutated CCAAT/enhancer-binding protein α (CEBPA). CD33-positive AML subtypes include CD33-positive core-binding factor AML, CD33-positive nucleophosmin (NPM1) mutation with FMS-like tyrosine kinase 3 wild-type (FLT3wt), and CD33-positive AML [29]. The ADC GO uses an anti-CD33 antibody to specifically address the cytostatic ozogamicin. The ALFA0701 study showed that additional treatment with GO, resulted in increased event-free survival. However, the OS benefit was limited to AML patients with favorable and intermediate risk classification subtypes (European LeukemiaNet classification) [31, 32].

In some cases, the 7+3 regimen is contraindicated due to advanced age or the presence of comorbidities. Here, the combination of venetoclax with either azacitidine or low-dose cytarabine is an alternative treatment option. Venetoclax is a selective B-cell lymphoma 2 (BCL-2) inhibitor that demonstrated a survival benefit in a phase III study when combined with the aforementioned drugs [29, 33].

Approximately 30% of newly diagnosed cases of AML involve an activating mutation of the FMS-like tyrosine kinase 3 (FLT3). The addition of the type I FLT3 inhibitor midostaurin to the 7+3 regimen resulted in a significant improvement in the complete response (CR) rate, median survival, and estimated 5-year survival rate. In a randomized phase III trial, the CR rate increased from 54% to 59%, the median survival period from 25.6 months to 74.7 months, and the estimated 5-year survival rate from 42% to 50%. Another type I FLT3 inhibitor is gilteritinib, which was investigated in cases of relapsed or refractory FLT3-mutated AML. It was observed to increase the

1. Introduction

survival and CR rates of the therapy, and it remains effective even after first-line treatment with midostaurin [31, 32, 34].

The occurrence of mutations in isocitrate dehydrogenase 1 and 2 (IDH-1, IDH-2) is less prevalent in AML, accounting for 20% of cases [35]. The IDH inhibitors enasidenib and ivosidenib have been approved by the Food and Drug Administration (FDA) for the treatment of relapsed isocitrate dehydrogenase-mutated AML. Ivosidenib is specific for IDH-1, whereas enasidenib targets IDH-2. The two IDH inhibitors demonstrated comparable CR rates as single compounds, at 21.6% and 20.6% respectively. They also exhibited favorable median OS at 8.8 and 9.3 months. Both compounds are currently being investigated in combination with the 7+3 regimen in a large randomized phase III study (HOVON150AML; NCT03839771) [36-38].

Another approach is the selective hedgehog pathway inhibitor glasdegib, which was used in combination with low-dose cytarabine. This combination resulted in prolonged survival (median survival 8.8 months versus 4.9 months) and a higher 12-month survival rate (59.8% versus 38.2%). Glasdegib has been approved for newly diagnosed AML in patients aged 75 years or older, who are unsuitable for intensive induction chemotherapy [31].

In 2020, the FDA approved the combination therapy of decitabine (a 100% absorbable oral formulation) and cedazuridine, a cytosine deaminase inhibitor. The dose of decitabine is bioequivalent to that administered intravenously. This progress may pave the way for further investigation into effective oral therapies (such as oral decitabine-cedazuridine plus venetoclax) for older AML patients, with the potential to enhance tolerance and quality of life in patients [31].

The 7+3 regime is not employed in the treatment of the subtype APL, which represents 5-10% of AML. In contrast, APL is treated with a combination of all-trans-retinoic acid (ATRA) and either arsenic trioxide or ATRA and anthracyclines, specifically idarubicin. The combination treatment of ATRA and arsenic trioxide has been demonstrated to result in high cure rates of over 80% [31, 39-41].

For years, the proteasome inhibitor (PI) bortezomib (BTZ) has been investigated in AML as a single agent or in combination with other drugs. Clinical studies have shown both benefits [42-44] and non-benefits of the addition of BTZ in AML patients [43, 45]. Further studies are needed to determine the full therapeutic potential of BTZ and other PI in AML.

In contrast to the extensive research conducted on immunotherapies in solid tumors, investigations into similar approaches in AML are still in their infancy. Checkpoint inhibitors including antibodies targeting programmed death-ligand 1 (PD-L1) and chimeric antigen receptor-T-cell therapies are currently being explored. But it will take time for these treatment options to become established in AML [36].

1.3.3 Bladder Carcinoma

Bladder carcinomas (BCa) are malignant tumors that develop in the bladder wall. They are categorized into different stages based on the extent of invasion into the bladder wall (Figure 3).

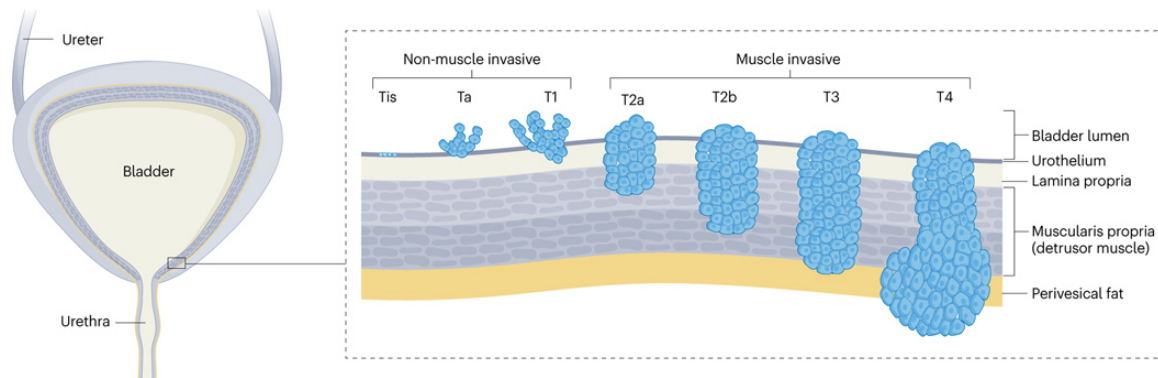


Figure 3. Bladder cancer staging. Figure taken from [46]. Reproduced with permission from Springer Nature.

The majority of cases of urothelial bladder cancer are limited to the mucosa (non-muscle invasive bladder carcinoma (NMIBCa)) encompassing all tumors until stage T1. Stage T2 or higher (muscle invasive bladder carcinoma, (MIBCa)), is reached by approximately 25-30% of patients. In these cases, tumor cells have already invaded deeper layers of the bladder tissue [47-49].

1.3.4 Treatment Options of Bladder Cancer

The specific therapeutic approach varies depending on the classification of bladder cancer (NMIBCa or MIBCa). The primary treatment for NMIBCa is transurethral resection (TUR), meaning the removal of the affected tissue from the bladder in a minimally invasive procedure [47]. The risk classification of recurrence or progression of NMIBCa determines whether additional forms of therapy, such as chemotherapy or immunotherapy are recommended in conjunction with TUR. Immunotherapy with bacillus Calmette-Guérin is indicated for patients at intermediate or high risk, in accordance with various guidelines [50]. Intravesical bacillus Calmette-Guérin treatment has been demonstrated to reduce the risk of progression of superficial bladder cancer and to be effective for prophylaxis of tumor recurrence [19, 47, 51, 52].

In accordance with the European Association of Urology (EAU) Guidelines on muscle-invasive and metastatic bladder cancer, the standard surgical treatment of MIBCa was the radical removal of the bladder for decades. Conversely, there has been a recent focus on enhancing patients' quality of life, which has led to the development of bladder-sparing approaches such as trimodality therapy (TMT) or radiation therapy. The TMT approach encompasses TUR, chemotherapy and radiotherapy, aiming to preserve the bladder while treating the cancer. The selection of the primary treatment is dependent on several factors, including the patient's life expectancy and the performance status [53]. Additionally, perioperative chemotherapy is employed to enhance five-year survival rates and mitigate the issue of micrometastases [47].

Neoadjuvant cisplatin-based combination chemotherapy has been proven to prolong survival and has been incorporated into standard care [53, 54]. This includes the combination of methotrexate, vinblastine, adriamycin, and cisplatin (MVAC) or gemcitabine and cisplatin (GC) [53, 55]. Both combination therapies demonstrated comparable efficacy. MVAC may have a superior survival outcome. GC on the other hand exhibited a higher tolerability [55, 56]. Since it is an element of high significance in the treatment of MIBCa, cisplatin is discussed in more detail in the following chapter.

Another therapeutic approach has been introduced through the use of Bacillus Calmette-Guerin. Recent studies have indicated that it might serve as a "proof of principle" for treating urothelial carcinoma with immunotherapy. Based on these findings, checkpoint inhibitors have been introduced as a novel therapeutic option for BCa. Two programmed cell death protein 1 inhibitors - pembrolizumab and nivolumab - and three PD-L1 inhibitors, - namely atezolizumab, durvalumab, and avelumab - , have been approved by the FDA and the European Medicine Agency (EMA) for advanced or refractory metastatic urothelial carcinoma [57, 58]. The checkpoint inhibitors showed favorable outcomes in clinical trials so far. In a phase III trial, pembrolizumab was found to have a significantly longer OS and a lower rate of treatment-associated adverse events than second-line chemotherapy in platinum-refractory advanced urothelial carcinoma [59]. In a phase 2 study, nivolumab demonstrated an objective response rate of 19.6%, which is a notable improvement over the historical objective response rate of 10% observed with secondary single-drug chemotherapy in a comparable setting [60]. Durvalumab exhibited a promising overall response rate (ORR) of 31% in advanced urothelial bladder cancer in a phase 1/2 trial [61]. According to the EAU Guidelines on muscle-invasive and metastatic bladder cancer, the first-line treatment of metastatic urothelial carcinoma was fundamentally changed by the introduction of the checkpoint inhibitors [53]. Checkpoint inhibitors are recommended as first-line therapy in combination with other drugs, such as cisplatin, carboplatin, or gemcitabine, or as a single treatment in cases of high PD-1 expression, depending on the patient's condition [53]. Further, the administration of adjuvant nivolumab is recommended in selected cases for patients with pathological T3/4 and/or pathological N+ (lymph node metastases) disease, who are not suitable candidates for or have declined adjuvant chemotherapy based on cisplatin [53]. More research is required to ascertain the full potential of checkpoint inhibitors in the treatment of bladder cancer, both as single agent and in combination with other drugs [57, 62].

Another therapeutic option is the administration of enfortumab vedotin (EV). It is an ADC that specifically binds to the transmembrane protein nectin-4, which is often overexpressed in urothelial bladder cancer. This results in internalization and release of monomethyl auristatin E into the cell. Monomethyl auristatin E is a microtubule inhibitor that leads to cell cycle arrest and subsequent apoptosis. EV has been approved for the treatment of locally advanced or metastatic urothelial carcinoma,

provided that prior platinum-based chemotherapy and PD-1/L1 inhibitor treatment has been undertaken. In a phase 3 study, a significantly longer OS was proven in comparison to standard chemotherapy (12.88 months vs. 8.97 months) [63-65]. The combination of pembrolizumab and EV is used in place of platinum-based combination therapies as first-line therapy for patients with metastatic urothelial cancer who are eligible for combination therapy, in accordance with the EAU Guidelines on muscle-invasive and metastatic bladder cancer [53].

1.3.5 Cisplatin

Cisplatin (cis-diamminedichloroplatinum(II), cDDP) is a well-established and approved cytostatic drug that is utilized in the treatment of various cancer types (Figure 4) [66, 67].

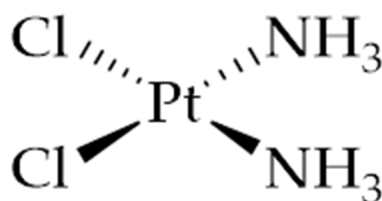


Figure 4. Cisplatin

Cisplatin currently is most often administered in combination with other pharmaceutical active ingredients. The main reasons are to circumvent cisplatin resistance, improve prognosis, and reduce serious side effects such as nephrotoxicity, ototoxicity, hepatotoxicity, and gastrointestinal toxicity. Cisplatin is dissolved in a sterile saline solution and administered intravenously to the patient. It is a prodrug that is activated intracellularly. Due to the high chloride concentration of approximately 100 mM in human blood, cisplatin remains unaltered and neutral in blood. The majority of cisplatin is inactivated through binding to albumin. Unbound cisplatin can enter cancer cells by passive diffusion or via the copper transporter 1 (CTR1). Once within the cell, one or two chloride ligands of cisplatin are replaced by a water ligand, resulting in the activated form of cisplatin. Cisplatin exhibits a higher degree of reactivity with a single water ligand than with two water ligands. This replacement of ligands is dependent on an intracellular chloride ion concentration that is lower than that of the extracellular environment. The activated form of cisplatin can react with numerous nucleophilic groups, including those found in proteins (sulfhydryl groups) and nucleic acids (nitrogen donor atoms). This is due to the fact that water ligands can leave the molecule more easily than the chloride ions. Approximately 1% of intracellular cisplatin binds to genomic DNA. Cisplatin can bind to different positions of the DNA, but it prefers the nitrogen at position 7 of the guanine. The result of this connection is the formation of monoadducts and intrastrand

1. Introduction

or interstrand crosslinks (Figure 5). If DNA is in its lightly packed euchromatin form, cisplatin can target DNA more effectively [66-69].

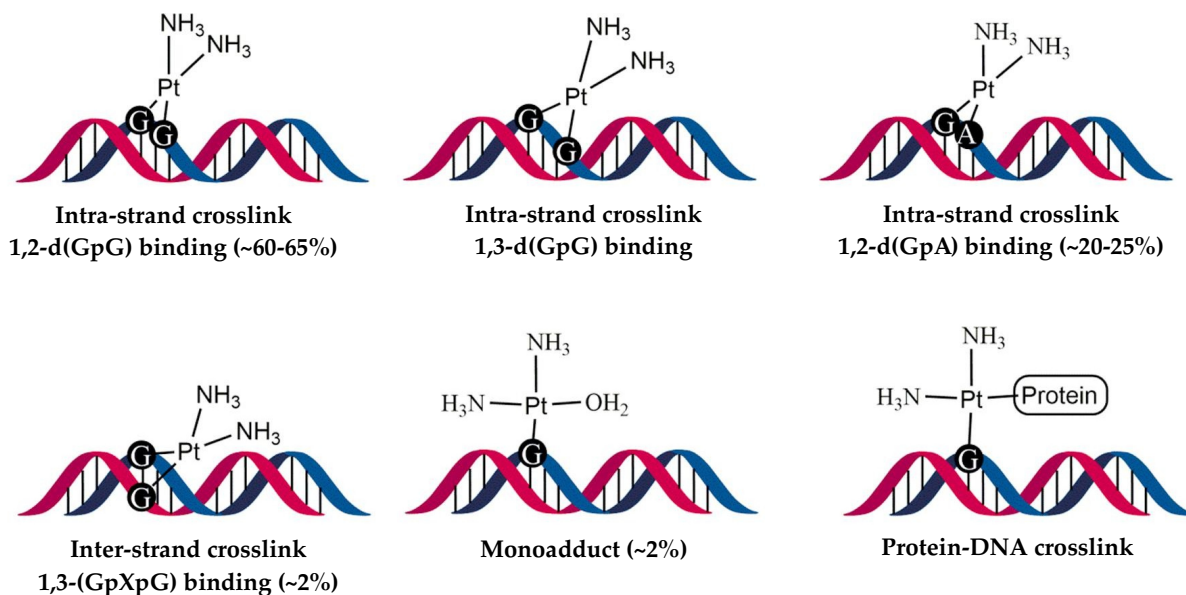


Figure 5. DNA adducts of cisplatin. Figure taken and minimally modified from [66] with permission from Elsevier.

The majority of cisplatin bonds lead to intrastrand crosslinks, accounting for 90-95% of cases. The most prevalent of these is 1,2-d(GpG), occurring in 60-65% of instances, followed by 1,2-d(ApG), which occurs in 20-25% of cases. It is assumed that the 1,2 intrastrand adducts have a stronger cytotoxic effect than the 1,3 intrastrand adducts. However, it has not yet been conclusively determined which cisplatin-DNA adducts have the greatest influence on cell death. In general, 1,3 intrastrand adducts are repaired at a faster rate than 1,2 intrastrand adducts. This may contribute to the stronger cytotoxic effect observed with 1,2 intrastrand adducts. Cisplatin-DNA adducts, such as 1,2 cisplatin-DNA intrastrand crosslinks, can be recognized and bound by high mobility group (HMG) proteins forming a complex (Figure 6).

1. Introduction

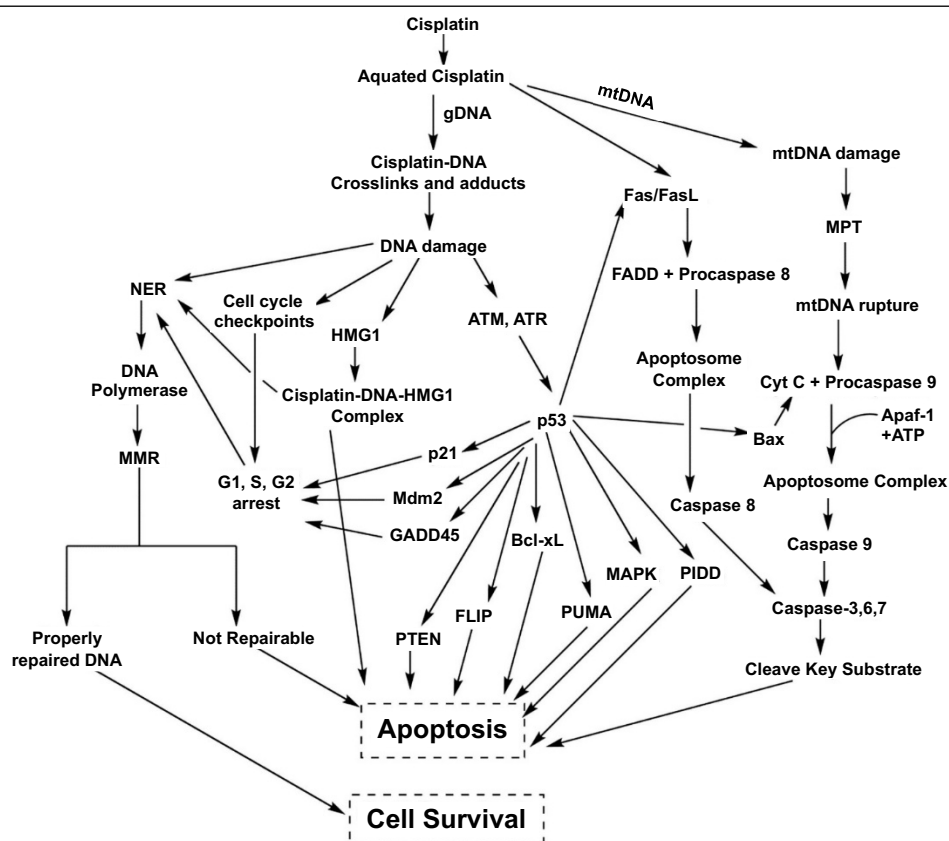


Figure 6. Overview of mechanisms of action of cisplatin and anticancer activity. Figure taken and minimally modified from [66] with permission from Elsevier.

Subsequently, the cisplatin-DNA-HMG1 complex can proceed along three pathways that have been described in the existing literature. Firstly, the nucleotide excision repair (NER) mechanism may be activated, which ultimately results in DNA repair and cell survival [66]. Secondly, the HMG protein has been shown to impede the repair of the cisplatin-DNA adduct by protecting against recognition by DNA repair enzymes [66, 70]. The third possibility is that HMG proteins, such as SSRP1, can alter the cell cycle and initiate cell death [66, 70]. According to the extant literature, HMG proteins exhibit both repair-promoting and repair-inhibiting functions [71]. It can be posited that the interaction of HMG proteins with the NER mechanism is intricate and can be influenced by the plethora of disparate experimental conditions. Consequently, these factors must be considered when interpreting the function of HMG proteins to gain a more precise understanding of their role in interaction with the NER mechanism [71]. Nevertheless, studies have demonstrated that the overexpression of HMG proteins can induce chemoresistance in cancer cells [72, 73].

Furthermore, cisplatin has been demonstrated to activate checkpoint kinases 1 and 2, leading to cell cycle arrest in G- or S-phase (Figure 6). During this period of arrested proliferation, the cell is afforded the opportunity to repair the damaged DNA or induce apoptosis [66, 67]. The opportunity for repair can be beneficial to cancer cells and contribute to their survival. However, if cell cycle arrest caused by checkpoint

kinases 1 and 2 is reversed, e.g., by checkpoint kinase inhibitors, cancer cells enter the cell cycle with damaged DNA, which can induce apoptosis via the NER signaling pathway [66, 74].

p53 is a pivotal enzyme in the process of cisplatin-induced apoptosis (Figure 6). Cisplatin activates the ATR kinase (ataxia-telangiectasia mutated protein (ATM) and RAD3-related protein), which results in the phosphorylation of p53 at serine-15. Subsequently, p53 activates the genes of p21, mouse double minute 2 (Mdm2), and growth arrest and DNA damage-inducible protein family (GADD45), which are responsible for cell cycle arrest. During the cell cycle arrest, the cell can repair the DNA damage. There are three common DNA repair pathways including direct repair, base excision repair and mismatch repair (MMR).

If the repair process during cell cycle arrest is unsuccessful, the initiation of apoptosis is facilitated via the MMR system pathway (Figure 6) [75-77]. The MMR is responsible for the repair of incorrect insertions, deletions, and false incorporation of bases that occur during the process of DNA replication. Two models have been proposed to account for the MMR-dependent apoptosis: the futile cycle model and the direct signaling model. The futile cycle model postulates the programmed cell death resulting from the creation of DNA strand breaks, which are the consequence of a futile attempt by the MMR pathway to repair alkylated DNA (e. g. O⁶-methylguanine) [78-80]. In contrast, the direct signaling model suggests that MMR complexes are capable of recognizing platinated or alkylated DNA and activating the DNA damage signaling network via c-Abl/p73 α /GADD45 α activation, which ultimately results in cell death [79-82].

Similarly, p53 causes apoptosis via the degradation of fllice-like inhibitory protein (FLIP), which functions as an antagonist to the antiapoptotic function of B-cell lymphoma extra-large protein (Bcl-xL). Moreover, p53 leads to apoptosis by over-expression of phosphatase and tensin homologue protein (PTEN) [66, 67]. p53 can also activate p53 upregulated modulator of apoptosis (PUMA), p53-induced protein with a death domain (PIDD) and mitogen-activated protein kinase (MAPK), which also play a role in cell apoptosis (Figure 6) [66].

Another mechanism of action of cisplatin is the induction of oxidative stress in the cell. Depending on the exposure time, reactive oxygen species (ROS) such as hydroxyl radicals and superoxides are generated. These ROS can lead to DNA damage and, ultimately to apoptosis by affecting various signaling transduction pathways including the intrinsic apoptotic pathway initiated by the release of cytochrome C from the mitochondria, and the extrinsic pathway, which is triggered by activation of Fas cell surface death receptor (Fas) [66, 67, 69].

1.3.6 Cisplatin Resistance

Cancer cells can be intrinsically resistant to cisplatin or develop resistance during treatment. Both types of resistance are a major problem in cisplatin-based chemotherapy.

The development of cisplatin resistance is attributed to a multitude of factors. The underlying molecular mechanisms have been meticulously elucidated in recent decades. These mechanisms can be broadly classified into three categories: pre-target resistance, on-target resistance, and post-target resistance [83].

Pre-target resistance is defined as the inhibition of the cytotoxic potential of cisplatin prior to its binding to cytoplasmic targets and DNA. This could happen due to a reduction in the intracellular accumulation of cisplatin, as well as an increase in the capture of cisplatin by glutathione (GSH), metallothioneins, or other cytoplasmic molecules with nucleophilic properties (Figure 7) [83-85].

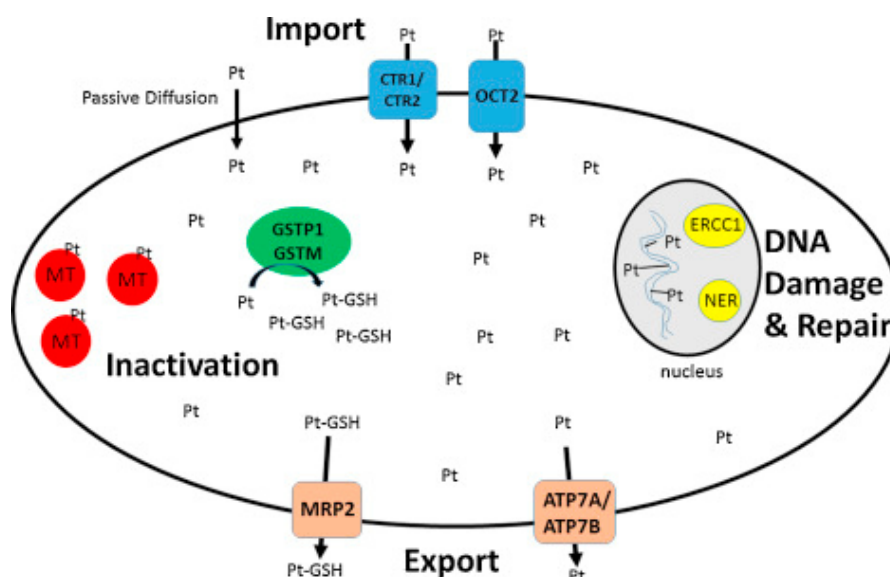


Figure 7. The fate of cisplatin in human cells. Cisplatin enters cells via the copper transporter 1/2 (CTR1/CTR2), the solute carrier family 22 member 2 (OCT2), or by passive diffusion. Inside the cell, cisplatin is activated and binds to DNA, forming crosslinks. The repair of these crosslinks is carried out by excision repair cross-complementation group 1 (ERCC1) and the nucleotide excision repair (NER) pathway. Further, cisplatin can be inactivated by binding to glutathione (GSH) through glutathione S-transferases or metallothionein proteins (MTs). Finally, cisplatin can be exported by copper-extruding P-type ATPases (ATP7A and ATP7B). Multidrug resistance-associated protein 2 (MRP2) exports conjugated cisplatin-GSH complexes. Figure taken from [86] with permission from Elsevier.

The accumulation of cisplatin can be decreased in two ways: by reducing its uptake or by increasing its efflux. The downregulation of CTR1 results in a reduction in cisplatin uptake (Figure 7). The increased efflux of cisplatin is driven by the action of the copper-extruding P-type ATPases ATP7A and ATP7B. In addition, conjugated cisplatin is exported out of the cell by the transporter multidrug resistance protein 2 (MRP2, Figure 7). The transporter MRP2 belongs to the adenosine triphosphate binding cassette family of plasma membrane transporters, which also includes P-glycoprotein [83-86].

Activated cisplatin has the potential to bind to cytoplasmic nucleophiles, including GSH, methionine, metallothioneins, and other cysteine-rich proteins. This results in the depletion of antioxidant reserves, thereby inducing oxidative stress within the cell. During this process, the proportion of active cisplatin is reduced massively. Consequently, the overexpression of glutathione synthase (GSH synthesis) or glutathione S-transferase (GSH and cisplatin coupling catalysis) may be associated with cisplatin resistance (Figure 7). GSH-cisplatin conjugates are more easily transported out of the cell by MRP1 or MRP2. MRP2 plays the most significant role in cisplatin efflux [69, 83-87].

One mechanism of on-target resistance to cisplatin is an increased ability either to repair DNA lesions or to tolerate them in a certain quantity. The cisplatin-DNA adducts are predominantly repaired by the NER system. DNA lesions are excised and replaced by new synthesis (Figure 7). The NER comprises at least 20 different proteins with diverse functions [83]. For instance, the single-stranded endonuclease excision repair cross-complementation group 1 (ERCC1) forms a complex with ERCC4 to create the ERCC1-XPF enzyme complex. In addition to its role in the NER system, studies have indicated that this complex may also play a role in other DNA repair pathways, including the repair of single-strand breaks, double-strand breaks, and interstrand cross-links [83, 88]. Therefore, ERCC1 plays a pivotal role in DNA repair (Figure 7). It has been observed that the absence of ERCC1 is consistently associated with cisplatin reactivity [83, 84]. Conversely, the overexpression of ERCC1 can but does not necessarily lead to reduced sensitivity to cisplatin [83, 89]. Nevertheless, ERCC1 is used as a biomarker for predicting cisplatin sensitivity in the clinical setting [83, 90].

Further, the development of on-target resistance to cisplatin can be attributed to the failure of cisplatin-DNA adducts to be recognized by the MMR system, which consequently results in the absence of an apoptotic signal due to a failed repair. GpG interstrand crosslinks can be recognized by proteins like MutS homolog 2 (MSH2) and MutL protein homolog 1 (MLH1) [83, 84]. The lack of MMR proteins has been related to resistance to alkylating agents and cisplatin, among various other anti-cancer drugs [79]. In cisplatin-resistant cells, the proteins MSH2 and MLH1 are often mutated or less expressed. This results in a lower detection of GpG interstrand crosslinks and thus an absence of an apoptotic signal [83, 84].

Another factor contributing to cisplatin resistance is the activity of specific DNA polymerases, including POLH, POLI, POLK, REV1, REV3, and REV7. These enzymes facilitate the DNA synthesis in the presence of lesions through a process known as translesion synthesis. The overexpression of REV3 has been demonstrated to confer increased resistance to cisplatin [69, 83-87].

Post-target resistance is caused by a multitude of alterations in diverse signaling pathways. These signal pathways typically induce apoptosis in response to DNA damage or regulate cell death in general. The presence of different genetic or epigenetic aberrations results in the development of varying degrees of cisplatin resistance. Approximately half of all human neoplasms are affected by p53 mutations. However, patients with wild-type p53 often demonstrate a superior response to cisplatin-based chemotherapy. A number of proteins that regulate or mediate apoptosis have the potential to influence the development of cisplatin resistance (Figure 6). These include death receptors, pro- and anti-apoptotic proteins of the BCL-2 family, caspase and mitochondrial intermembrane proteins, among numerous others. The increased expression of survivin, which inhibits caspases, has been demonstrated to correlate with a poorer response to cisplatin chemotherapy in various cancers [69, 83-85, 87].

Cisplatin resistance can also be mediated by alterations in signaling pathways that are not directly affected by cisplatin. This phenomenon is known as off-target resistance. For example, the pro-survival signaling through the overexpression of erb-b2 receptor tyrosine kinase 2 (ERBB2) or heat shock proteins has been proven in different cancer entities [83].

In response to stress conditions, autophagy can be upregulated in cancer cells, contributing to cisplatin resistance. Overall, the resistance against cisplatin is often modulated by multiple factors in cancer cells. Understanding how cancer cells become resistant to cisplatin may lead to new approaches to overcome said resistance [83].

1.4 Epigenetics and the Proteasome as Target of Cancer Therapy

In recent decades, the field of epigenetics has attracted a lot of attention due to its pivotal role in understanding the heritable alterations in gene expression that occur independently of changes to the DNA sequence [91]. These epigenetic alterations have been associated with a wide range of diseases. These include the development of early-stage neoplasia, through their influence on growth-related signaling pathways [92, 93]. A diverse array of epigenetic modifiers, comprising writers, readers, and erasers, has been identified. These modifiers add, read, or remove specific marks on DNA or histones, thereby remodeling chromatin and influencing gene expression (Figure 8) [93, 94].

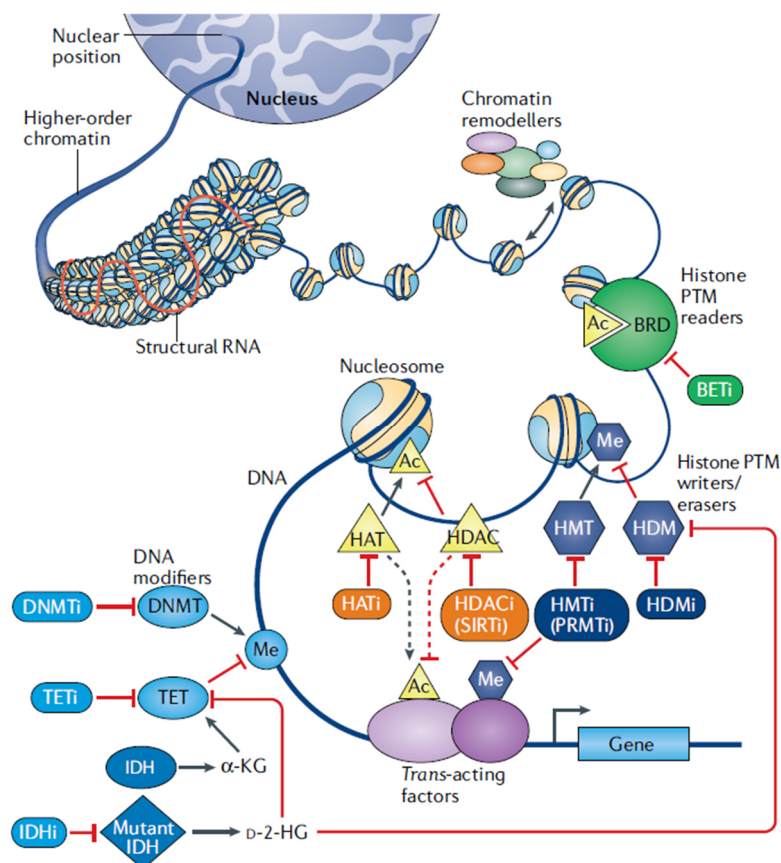


Figure 8. Targets of the epigenome. The epigenome is regulated by over 800 enzymes. These include epigenetic writers, readers, removers, and shapers. This process enables the remodeling of DNA or chromatin, which in turn affects gene expression. Epigenetic drugs, including DNA methyltransferase inhibitors (DNMTi) or ten eleven translocation enzyme inhibitors (TETi), as well as isocitrate dehydrogenase inhibitors (IDHi), have the capacity to modify the methylation status of DNA. In addition, other classes of epigenetic drugs have been shown to inhibit the post-translational modification of histone acetyltransferases (HAT), histone deacetylases (HDAC), histone methyltransferases (HMT), and histone demethylases (HDM). Moreover, inhibitors of the bromodomain and extra-terminal motif (BETi) has been demonstrated to inhibit the activity of readers of post-translational modifications. Figure reproduced with permission from Springer Nature [93].

The disruption of gene expression can cause various diseases, as well as chemoresistance in cancer treatment. Likewise, the influence of the proteasome on gene expression is a subject of considerable interest, as it can degrade tumor suppressor or growth-promoting proteins [95]. The following sections will provide a comprehensive overview of the epigenetic modulation of chromatin through histone deacetylases (HDACs) and the ubiquitin-proteasome system (UPS).

1.4.1 Histone Deacetylases

HDACs are important in the context of epigenetics. They catalyze the removal of acetyl groups from ϵ -amino lysines of proteins, with a particular impact on histones. The deacetylation of histones results in the formation of a condensed chromatin structure, which is referred to as heterochromatin. This process ultimately leads to the suppression of gene transcription. This gene suppression can influence a number of

different mechanisms and pathways, including those regulating the cell cycle, differentiation, and the induction of apoptosis. The counterparts of HDACs are the histone acetyltransferases (HATs). They cause an open chromatin structure (euchromatin) through acetylation. Overall, the interplay of HDACs and HATs plays an essential role in gene expression and associated processes such as DNA transcription, replication, repair, mitosis, and apoptosis (Figure 9).

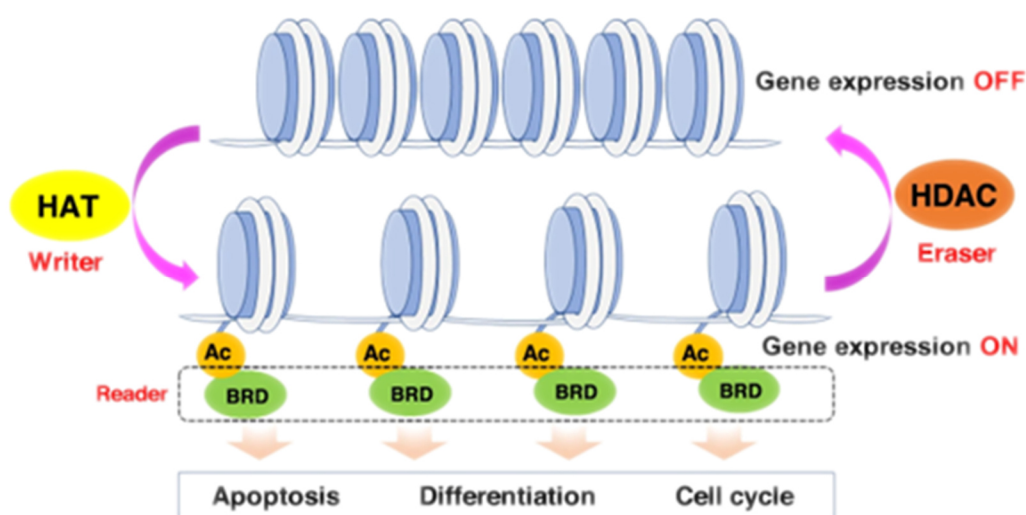


Figure 9. Interplay of HDACs and HATs in gene transcription. HATs acetylate (Ac) histone proteins, which activates gene expression by reading of bromodomain-containing proteins (BRD) of acetylated histones. HDACs remove the acetylation, thereby turning off gene expression. Figure taken from [96], reproduced with permission from Springer Nature.

Further, HDACs deacetylate non-histone proteins, such as the tumor suppressor protein p53. The activity of p53 can be decisively influenced. Specifically, the induction of apoptosis can be prevented by the deacetylation of certain lysine residues, such as K120 of p53. Consequently, p53 cannot induce the transcription of pro-apoptotic genes, such as PUMA. This is but one illustration of the profound significance of deacetylation and acetylation of non-histone proteins [94, 97-102].

HDACs are a family which can be classified into five distinct classes based on structural similarities: class I (HDAC1, 2, 3, 8); class IIa (HDAC4, 5, 7, 9); class IIb (HDAC 6, 10); class III (SIRT1-7) and class IV (HDAC11). Class I, IIa, IIb, and IV HDACs exhibit a high degree of structural similarity, whereas class III HDACs display structural homology to the SIR2 protein found in yeast. Class I, II, and IV HDACs are zinc-dependent enzymes, whereas class III HDACs utilize nicotinamide adenine dinucleotide (NAD⁺) as a cofactor [103]. The function of sirtuins in oncogenesis is complex, as some sirtuins exhibit a dual role as oncoproteins and tumor suppressors. In this thesis, only the zinc-dependent HDACs are object of study and thus further explained in their context to cancer [94, 104, 105].

1. Introduction

The different classes of HDACs are located in various parts of the cell, mainly the nucleus or the cytosol, or they shuttle between both (Figure 10) [104].

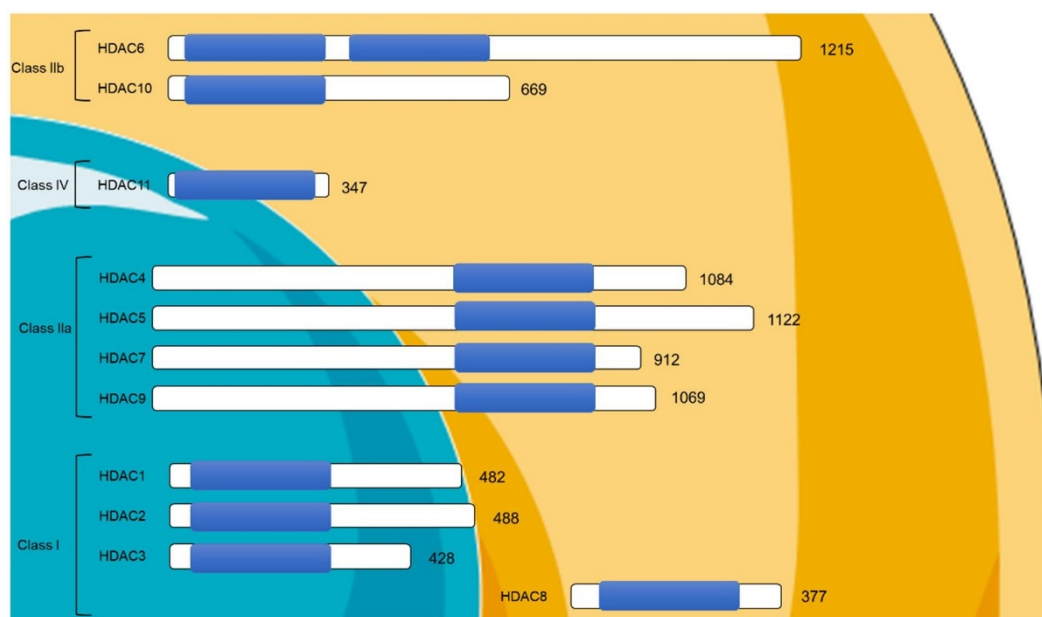


Figure 10. Localization of zinc-dependent HDACs in the cell. The blue background represents the nucleus and the yellow background represents the cytosol. The numbers on the white bars indicate the length of the amino acid sequence, including the catalytic domain (dark blue bars). Figure taken from [104] with permission from Elsevier.

The process of transport between the nucleus and the cytosol through nuclear pores is a regulated system with different possible pathways. One of such pathway involves the specific labeling of transported materials with nuclear export signals (NES) or nuclear localization signals (NLS) [104, 106, 107]. Class I HDACs primarily remain in the nucleus because they lack the aforementioned NES. Class IIa HDACs have the ability to shuttle between the nucleus and the cytosol due to the presence of both a NES and a NLS. The balance between the nuclear import and export of class IIa HDACs can be altered by the phosphorylation of conserved serine residues by several kinases, allowing the cell to adapt to cellular demands [104]. Class IIb HDACs are predominantly found in the cytosol due to the presence of a NES. In addition, HDAC11 is found in both the nucleus and the cytoplasm [104, 108].

HDACs are part of multiprotein complexes that influence the deacetylase activity and target substrates of the HDACs [94]. For class I HDACs, at least three stable protein complexes have been identified: the Sin3-, NuRD-, and CoREST complexes [94]. Another example is HDAC3, which forms a complex with the nuclear receptor co-repressor (N-CoR) and silencing mediator for retinoid and thyroid receptors (SMRT) that stimulates the HDAC3's enzymatic activity [94, 104].

Class IIa HDACs generally demonstrate lower deacetylase activity on acetyl-lysine than class I and class IIb HDACs due to a tyrosine-to-histidine substitution (Y976H) in their catalytic domain [104]. The importance of the role of the catalytic activity in their

biological function remains to be determined. It is assumed that they act as an acetyl-lysine recognition domain within large multiprotein complexes [104].

1.4.2 Histone Deacetylases and Cancer

A substantial body of research has demonstrated that HDAC activity is dysregulated in cancer cells. This dysregulation is reflected in the increased or decreased expression of HDACs. The altered expression pattern of HDACs results in a change of the acetylation status of histones or non-histone proteins. This change may enhance the transcription of oncogenes, growth-mediating factors and reduce the expression of tumor suppressor genes [94].

The overexpression of HDAC1 has been demonstrated in different types of carcinomas [94, 103, 109-112]. Other class I HDACs (HDAC1, HDAC2, HDAC3) have been found to be upregulated in renal cell carcinoma, Hodgkin's lymphoma, bladder cancer, and colorectal cancer [94, 113-116]. Elevated levels of single class IIa HDACs have been observed in diverse cancer types and are frequently linked to a worse prognosis. For example HDAC4 and HDAC9 were found to be overexpressed in the non-Hodgkin's lymphoma Waldenstrom's macroglobulinemia. Further examples include elevated levels of HDAC7 in patients with pancreatic cancer and increased expression of HDAC5 and HDAC9 in patients with high-risk medulloblastoma [117-119]. In addition, the overexpression of class IIa HDACs, particularly HDAC4 and HDAC9, was identified in bladder cancer cell lines [120].

A number of studies have indicated that class IIa HDACs can function as either proliferative factors or tumor suppressors, depending on the cellular context. According to the extant literature, there is a tendency for increased expression of class IIa HDACs to result in increased growth of cancer cells; however, further research is necessary to provide more definitive conclusions [104, 121-123].

One reason for the ambivalent function of HDAC4 is, that HDAC4 can modulate the expression of p21 in both directions. By interacting with the transcription factor specificity protein 1, HDAC4 can lead to the inhibition of p21, resulting in increased cell proliferation. However, HDAC4 can also increase the expression of p21 after DNA damage via recruitment by p53, causing cell cycle arrest and inhibition of cell proliferation [124, 125]. Studies have linked increased expression of HDAC4 with chemoresistance to platinum-based chemotherapeutic agents in ovarian cancer [104, 126].

Our working group has previously investigated the combination of histone deacetylase inhibitors (HDACi) and cisplatin in several cancer entities, including ovarian cancer and head and neck carcinoma in vitro [127, 128]. The combination of HDACi and cisplatin resulted in the resensitization of cisplatin-resistant cancer cells. These encouraging results, in combination with the previously mentioned reasons,

demonstrate that the dysregulation of HDACs emerges as a promising target for cancer therapy.

1.4.3 Bromodomain and Extra-Terminal Motif Proteins

Bromodomain-containing proteins (BRDs) play an important role in epigenetic regulation through their reader activity on acetylated lysine residues in chromatin. Thus, BRDs are directly involved in gene expression [129]. In the human proteome, there are 61 bromodomain (BD) modules distributed in 46 different proteins. A well-studied group of BRDs is the bromodomain and extra terminal motif (BET) protein family [130-132]. The BET protein family includes BRD2, BRD3, BRD4, and BRDT [133, 134]. BRDT is exclusively found in the testicles (Figure 11).

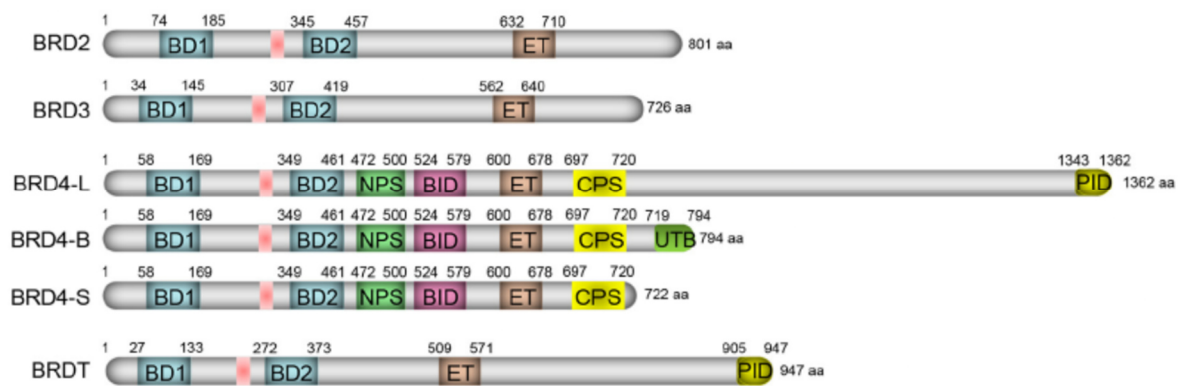


Figure 11. Schematic structure of the human BET proteins BRD2, BRD3, BRD4 isoforms (BRD4-L, BRD4-B, BRD4-S) and BRDT. The bromodomains (BD1 and BD2) detect acetylated lysine residues. The extraterminal (ET) domain facilitates the recruitment of transcription factors through protein-protein interactions. Ubiquitinylation of the substrate-binding consensus motifs (SRC, shown as pink rectangles) can trigger proteasomal degradation of BET proteins. The N-terminal phosphorylation site (NPS) is required for BRD4 binding to acetylated chromatin. The positively charged basic residue-enriched interaction domain (BID) can interact with the NPS to suppress interaction of BD2 with its acetylated substrates. The c-terminal phosphorylation site (CPS) is a functional motif of BRD4. The unique domain to isoform B (UTB) may prevent DNA repair of damaged DNA through interaction with condensin II complex, SMC2 and CAPD3. P-TEFb interaction domain (PID) occurs in BRD4-L and BRDT, leading to gene expression via activation of the elongation factor P-TEFb [133-135]. Figure taken from [133] with permission from Elsevier.

All BET protein family members possess two bromodomains (BD1 and BD2) in their amino-terminal region. Both BDs are composed of four alpha helices, with the helices separated by variable loop regions. This creates a hydrophobic pocket into which the acetylated histone tails of histones or non-histone proteins can enter [129, 130, 133]. BET proteins also possess an additional carboxy-terminal protein-protein interaction domain.

It has been demonstrated that BRD4 can contribute to the development of cancerous cells. Both, BRD4 gene amplification in 20 common cancer entities and higher BRD4 levels in various tumor entities were observed. It has been shown that the translocation

1. Introduction

of the BRD3 and BRD4 genes t(15;19) results in the formation of a fusion protein with the nuclear protein in testis (NUT). This fusion protein plays a key role in the development of NUT midline carcinoma [136]. Three isoforms of BRD4 - BRD4-long, BRD4-B, and BRD4-short - have been identified as results of an alternative splicing of pre-mRNAs. BRD4-long and BRDT possess an additional positive transcription elongation factor B (p-TEFb) interaction domain. It enables both proteins to interact with p-TEFb and thus activate gene expression. The N-terminal phosphorylation site (NPS) is situated near BD2 in BRD4 (Figure 11). The NPS is indispensable for the attachment of BRD4 to acetylated chromatin and the recruitment of transcription factors to target sequences. Consequently, the binding of BD2 in BRD4 to acetylated chromatin is mediated by the regulation of phosphorylation. In close proximity to the NPS is a domain that is rich in basic residues, called the basic residue-enriched interaction domain (BID). This positively charged domain can interact with the negatively charged NPS, thereby preventing the interaction of the second bromodomain with acetylated substrates. BRD4-B contains a unique domain specific to isoform B, which associates with the condensin II complex, SMC2, and CAPD3. This complex is capable of impeding DNA repair mechanisms in cases of DNA damage caused by radiation [129, 131, 133].

BET proteins influence gene expression through bromodomains. The bromodomains interact with acetylated lysine residues of histones, transcription factors and chromatin remodeling factors via protein-protein interaction (Figure 12).

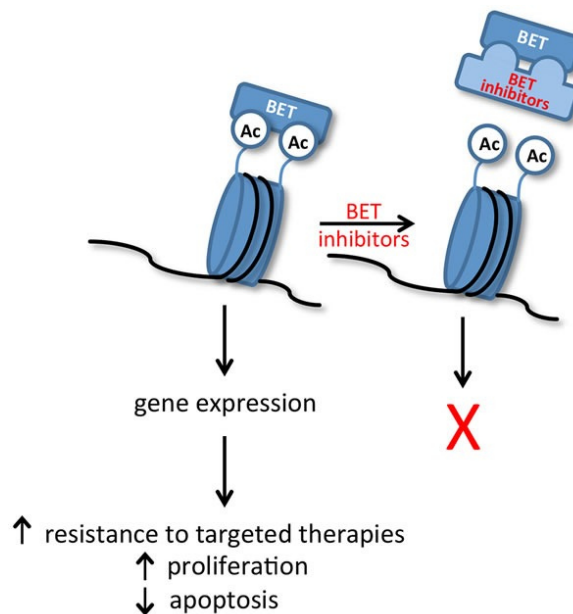


Figure 12. Influence of BET proteins on gene transcription in cancer cells [137].

This induces the recruitment of transcription factors, repressors, or the interaction with transcription factors via the extra-terminal domain. The transcription factors include c-Myc, E2F1, OCT4, EWS/ETS, and Twist, among others. Altered gene expression can

lead to resistance to chemotherapy, enhanced proliferation and reduced apoptosis of cancer cells.

Further, BRD4 interacts with transcription factor IIB (TFIIB), creating an open gene promoter region and thereby enabling c-Myc to enter and activate gene transcription. The interaction of BET proteins and transcription factors can also alter the acetylation status of chromatin through the recruitment of acetyltransferases. By recruiting HDACs or histone 4-specific acetyltransferases, BET proteins such as BRD2 or related proteins can themselves alter the acetylation status. Another important role of BET proteins is the control and regulation of the transcription of DNA repair factors. Finally, BET proteins initiate cell cycle checkpoints [129-133].

1.4.4 Ubiquitin-Proteasome System

Proteins are involved in many complex cellular processes and signaling pathways, including the regulation of gene expression. Thus, the regulation of protein synthesis and protein degradation is of paramount importance for the maintenance of intracellular homeostasis and regulation. Protein degradation involves lysosomes or the UPS [138]. Lysosomes are responsible for the elimination of long-lived proteins, insoluble protein aggregates, and organelles via a variety of pathways, such as phagocytosis and autophagy. In contrast, proteasomes degrade short-lived proteins and soluble misfolded proteins that are unfolded, useless, and damaged [139]. The proteasome exerts a profound impact on protein and amino acid homeostasis by mediating the degradation of regulatory proteins. This process can influence a multitude of cellular functions, such as the cell cycle, signal transduction, transcription, DNA replication, and stress responses. Consequently, any disruption to the UPS could disturb cellular metabolism, potentially leading to cell death [138, 140]. The proteasome controls gene transcription by regulating epigenetic modification by writers, readers, and erasers through their concentration. Therefore, the UPS plays a key role in epigenetic regulation [141].

The proteasome targets proteins for degradation by ubiquitination. In addition to the proteasome, ubiquitin ligases and deubiquitinating enzymes are also part of the UPS (Figure 13) [139].

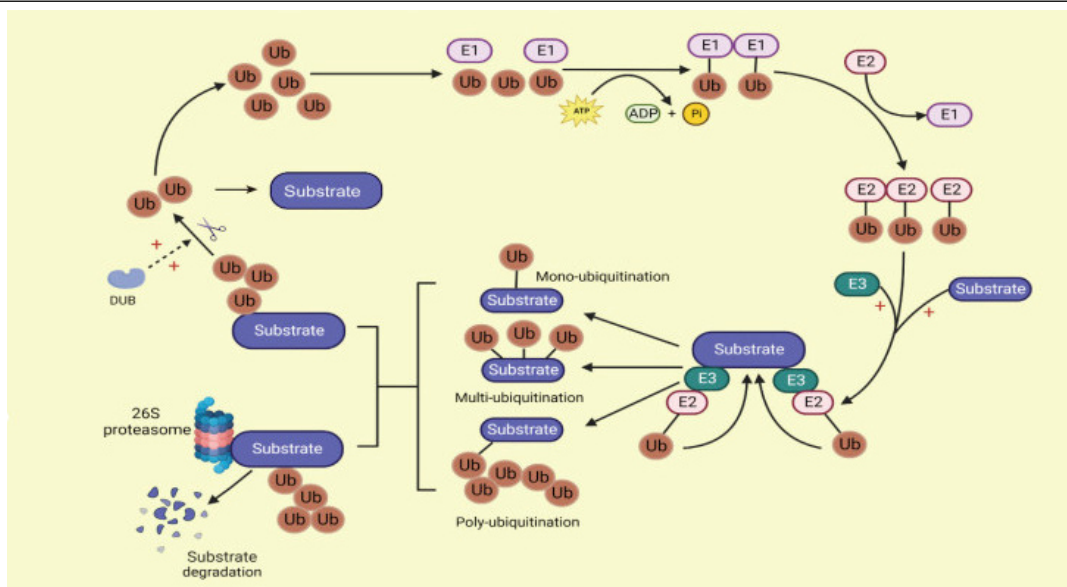


Figure 13. Ubiquitin-proteasome system. Ubiquitin (Ub) is transferred to the substrate by E1, E2 and E3 enzymes with the consumption of adenosine triphosphate (ATP). Ubiquitinated substrate is degraded by proteasome or deubiquitinated by deubiquitinating enzymes (DUB). Figure taken and modified from [142].

The ubiquitination process is catalyzed by the enzymes E1 (activating enzyme requiring adenosine triphosphate), E2 (conjugating enzyme), and E3 (ubiquitin ligase) [143]. The proteasome is only capable of degrading proteins that have been modified by the attachment of at least four ubiquitin molecules. The mono-ubiquitination or multi-ubiquitination of proteins serves as a catalyst for a multitude of additional functions, including endocytosis and histone regulation. Consequently, the ubiquitination process can be reversed by deubiquitinating enzymes [138, 142].

The 26S proteasome is composed of two distinct subunits: the 19S regulatory subunit and the 20S catalytic subunit. In the 19S regulatory subunit, the polyubiquitinated substrate is first recognized, then unfolded, deubiquitinated, and finally transferred to the 20S catalytic subunit. Here the protein is ultimately cleaved [138, 140].

1.5 Epigenetic Modulators and the Proteasome

Epigenetics plays a pivotal role in carcinogenesis. New therapeutic targets can be identified by continuously discovering and understanding the regulatory processes within cells. A substantial body of research has demonstrated that epigenetic modulators such as HDACs and BET proteins and the proteasome influence gene expression in cells [94, 100, 101, 129, 144]. Consequently, these modulators have emerged as potential targets for cancer treatment. The employment of suitable inhibitors of the epigenetic modulators or the proteasome aims to modify gene expression in cancer cells, thereby inducing apoptosis and eradicating them. Epigenetic inhibitors are often combined with established drug classes in preclinical or clinical studies. Through their impact on gene expression, they can, for example,

facilitate a reversal of chemoresistance, thereby enabling further treatment of chemoresistant cancer. The following section will introduce the HDACis, bromodomain and extra-terminal motif inhibitors (BETis) and PIs.

1.5.1 Histone Deacetylase Inhibitors

Based on their chemical structure, HDACis are classified into categories such as hydroxamates, o-aminoanilides, short-chain fatty acids and cyclic peptides (Figure 14) [145, 146]. In addition, a variety of further zinc-binding groups (ZBGs) were introduced in recent years, including hydrazides, imidazolethiones, thiols, epoxy ketones, and alkyl ketones, which vary in HDAC selectivity [147, 148].

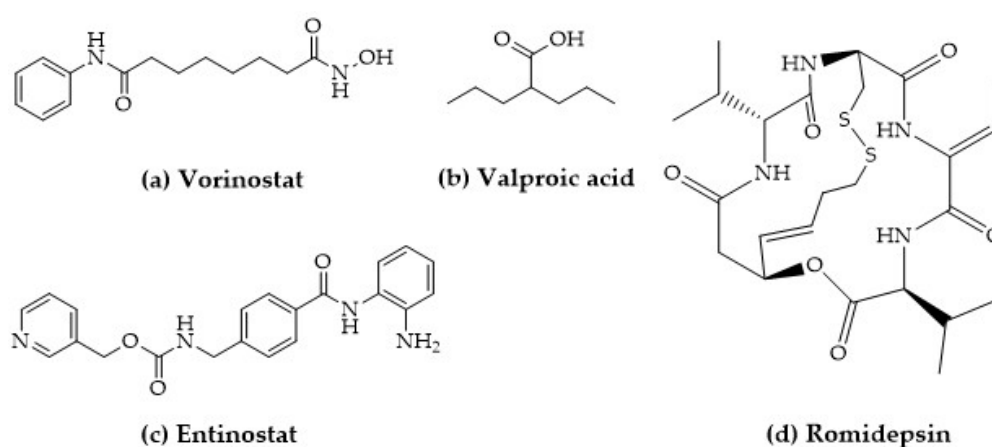


Figure 14. Structural examples for histone deacetylase inhibitors. (a) Vorinostat (hydroxamate), (b) valproic acid (short-chain fatty acid), (c) entinostat (benzamide), and (d) romidepsin (cyclic peptide).

Hydroxamates, o-aminoanilide, short-chain fatty acids and cyclic peptides may all be considered "classic" HDACi, as they inhibit class I, II, and IV HDACs through the binding of the zinc ion situated within the active center of the enzyme. Distinctions are made between non-selective HDACi (panobinostat, belinostat, vorinostat) and selective HDACi (romidepsin, entinostat) and multiparmacological HDACi (CUDC-101, CUDC-907) [149].

Four HDACi have been approved by the FDA for the treatment of hematological cancer [150]. These include vorinostat, panobinostat, belinostat, and romidepsin. Moreover, tucidinostat is approved in china for relapsed or refractory peripheral T-cell lymphoma and advanced breast cancer as well as in japan for relapsed or refractory adult T-cell leukemia-lymphoma [151]. Most recently, the HDACi givinostat was approved by the FDA and EMA for the treatment of Duchenne muscular dystrophy [152, 153]. A multitude of additional HDACi are presently undergoing clinical investigation. Since HDAC expression is frequently dysregulated in cancer cells, HDACi can modulate gene expression. By normalizing the acetylation status, cell cycle

arrest, apoptosis, differentiation or inhibition of angiogenesis and metastasis can be induced in cancer cells [149].

Recent years have seen a notable increase in research about selective HDACi. A more targeted inhibition of HDACs should result in a reduction of off-target effects and a more potent effect in cancer cells overexpressing certain HDAC enzymes. Romidepsin is the inaugural selective class I HDACi approved by the FDA for the treatment of cutaneous T-cell lymphomas and peripheral T-cell lymphoma [149, 154, 155]. Entinostat is an orally available class I HDACi with a benzamide structure and is currently being investigated in clinical trials for various cancer entities, primarily in combination therapies [156, 157]. These include phase II trials with the combination of entinostat and pembrolizumab in relapsed and refractory lymphomas and the combination of entinostat with nivolumab and ipilimumab in previously treated renal cell carcinoma [158, 159]. A randomized phase III trial evaluated the combination of entinostat plus exemestane versus exemestane plus placebo. The addition of entinostat to exemestane did not increase survival in patients with aromatase inhibitor-resistant, advanced hormone receptor-positive, human epidermal growth factor receptor 2 (HER2)-negative breast cancer [160].

The therapeutic potential of class IIa HDAC inhibition is as of yet scarcely researched. Therefore, more work has to be done on the development of specific class IIa HDACi. In 2013, TMP269 was introduced as the inaugural specific class IIa inhibitor comprising a trifluoromethyloxadiazole (TFMO) group as a pharmacophore [161]. Further optimization resulted in the synthesis of compound 12 and NT160, which exhibited enhanced solubility and a better pharmacokinetic profile [162, 163]. The combination of the class IIa HDACi TMP269 and the PI carfilzomib was found to enhance the induction of apoptosis through endoplasmic reticulum stress signaling in multiple myeloma [164].

1.5.2 Multi-Target Histone Deacetylase Inhibitors

Another prominent area of research into HDACi is the strategy of developing compounds with multiple targets [165]. A multitude of studies have demonstrated the synergistic effects of multi-target drugs, including those targeting HDACs and other targets such as kinases or topoisomerases [166]. Multi-target inhibitors can modulate multiple signal pathways, thereby enhancing therapeutic effects in cancer cells. The inhibition of several signaling pathways has the potential of reducing drug resistance. Another benefit of multi-target inhibitors is the simplification of medication regimes, enhancing patient compliance and reducing the risk of drug interactions [166-168].

In the following section, three distinct design strategies for multi-targeted HDACi are outlined. In the first one, both the HDACi and its partner are connected by a linker,

1. Introduction

which is cleavable under physiological conditions. In the second one, a stable linker is employed, enabling both molecules to interact with their respective targets. A third approach involves the fusion of two pharmacophores to create a single molecule comprising fragments capable of targeting their respective sites. The pharmacophores do not interfere with the binding mechanism of the other (Figure 15) [167, 168].

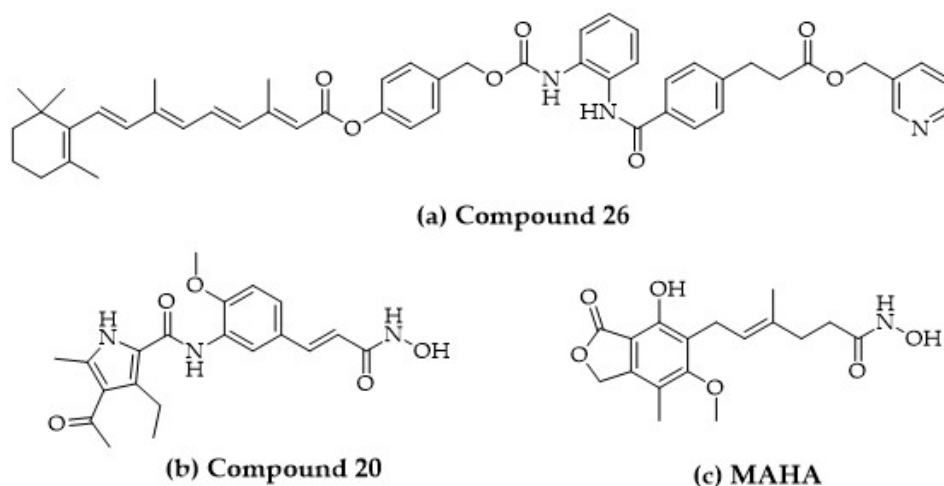


Figure 15. Examples of multi-target inhibitors based on different design strategies. **(a)** Compound 26, a physiologically cleavable prodrug designed from all-trans retinoic acid (ATRA) and HDACi entinostat. ATRA binds to the PML/RAR α fusion protein at the RAR α site, thereby inducing its degradation [167, 169, 170]. **(b)** Compound 20, a multi-target inhibitor with a stable linker composed of pan-bromodomain and extra-terminal motif inhibitor (BETi) XD14 and HDACi panobinostat [171]. **(c)** MAHA, merged pharmacophores of mycophenolic acid and HDACi (hydroxamic acid). Pharmacophore of mycophenolic acid targets the NAD site of inosine monophosphate dehydrogenase [167].

Multi-target inhibitors include a variety of inhibitors such as inhibitors of HDACs and BET proteins, estrogen receptors, retinoid X receptors, topoisomerase I/II, receptor tyrosine kinases, tubulin, DNA methyltransferases, 1 α , 25-vitamin D or nitric oxide donors, among others [171-180]. One of the most promising among these is CUDC-101, which has been demonstrated to exert an inhibitory effect on epidermal growth factor receptor, HER2, and HDACs [149]. In a phase I study, the maximum tolerated dose was determined to be 275 mg/m² [181]. Another phase I study investigated the combination of CUDC-101, high-dose cisplatin, and radiation in the head and neck region. However, the study was terminated due to one case of renal toxicity. Instead of further investigation, the sponsor opted to prioritize an oral formulation of CUDC-101 [182]. Further, CUDC-101 was tested in different cancer entities such as bladder or multiple myelom as single treatment or in combination with BTZ *in vitro* [183, 184].

1.5.3 Bromodomain and Extra-Terminal Motif Inhibitors

The role of BET proteins in cancer has been the subject of recent investigation. In a considerable number of solid tumors, BET proteins have demonstrated the ability to promote the transcription of c-Myc. In turn, the protein c-Myc has been shown to regulate the transcription of a multitude of genes, including those responsible for cell proliferation and cell cycle control. This has led to the development of BETi for the purpose of cancer therapy. The initial generation of BETi demonstrated low response rates in solid tumors and a number of serious adverse events, including thrombocytopenia [133].

One frequently utilized pan-BET inhibitor is JQ1, which exhibits equal potency in inhibiting BD1 and BD2 of the BET proteins (Figure 16).

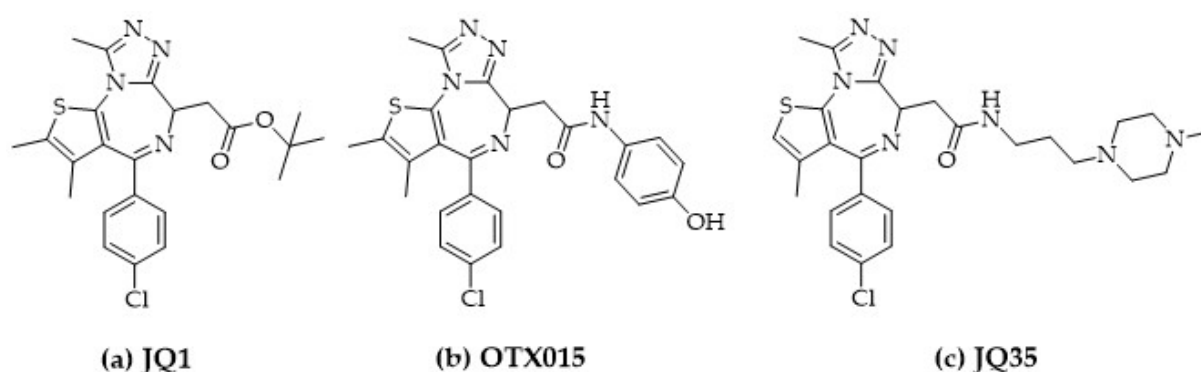


Figure 16. Structure of the pan-BETi. (a) JQ1, (b) OTX015 and (c) JQ35.

However, due to its relatively short half-life in vivo, alternative BETis with enhanced pharmacokinetic properties have been developed and clinically investigated for their efficacy in treating leukemias and solid tumors [130, 185]. These include ABBV-075, GSK525762, FT-1101, i-BET762, OTX015, and ZEN-3694, which demonstrated only low response rates of less than 30% [130, 133, 186]. The low response rates may be partially attributable to the intermittent administration strategy employed to mitigate the incidence of adverse effects, such as thrombocytopenia.

To improve efficiency and tolerability, selective BETi were developed. The initial selective BETi was RVX-208 [133]. The selective inhibition of BD2 resulted in more specific altered gene expression than that observed with pan-BETi, but the anticancer efficacy of RVX-208 was less pronounced. Subsequently, selective BD2 BETi included ABBV-744, which demonstrated robust antiproliferative effects in AML and prostate cancer. The BD2-selective BETi SJ432 showed greater potency than JQ1 in vivo using neuroblastoma models by inhibiting the expression of c-Myc. It is possible that BD2-selective inhibitors could reduce the unwanted side effects of pan-BETi [130, 133].

An alternative strategy for targeted inhibition with diminished adverse effects could be the utilization of BRD4-selective inhibitors. The BET proteins exhibit varying degrees of selectivity in recognizing acetylated histones. The targeted inhibition of the

1. Introduction

BDs however is challenging due to the highly conserved structures involved. Accordingly, the allosteric inhibition of casein kinase II (CK2)-mediated phosphorylation of specific sites between the BDs and the extra terminal domain could be employed to selectively inhibit BRD4. The phosphorylation enables the BD2 domain to interact with its target substrate. This is because the NPS keeps the BD2 domain inactive by masking, whereas phosphorylation enables interaction with the BID and facilitates access to the BD2 domain. Hyperphosphorylation of BRD4 has been implicated in cancer and in the development of resistance to BETi in lung adenocarcinomas, making blockade of CK2-mediated BRD4 phosphorylation a promising target for a cancer therapy.

A distinct approach to improve efficiency and tolerability is the degradation of BET proteins through the utilization of Proteolysis-Targeting Chimeras (PROTACs) [133, 187, 188]. The pharmacophore that binds to the desired target is linked to a recruiting molecule for protein degradation, such as an E3 ubiquitin ligase ligand. Subsequently, the target is bound by the pharmacophore and undergoes degradation. This represents a sustainable strategy for maintaining the effect of BETi. In 2015, three BET degrader compounds, namely dBET1, MZ1, and ARV-825, were introduced based on the pharmacophore of JQ1. The pharmacokinetic properties of ARV-825 were improved, resulting in the compound ARV-771. Subsequently, ARV-771 was shown to be effective in castration-resistant prostate cancer, AML, and mantle cell lymphoma. Currently, BET protein degraders are not in clinical trials [130, 133, 189-192].

Further recent approaches to develop new effective drugs include bivalent BETi, which bind both BDs of BET proteins simultaneously. AZD5153 is currently the most advanced bivalent BETi in phase I development [130, 193-195]. Covalent BETi are also being developed, though they are still in the early stages of development [130, 196].

1.5.4 Proteasome Inhibitors

The key role of the proteasome in the epigenetic regulation and degradation of regulatory proteins and its dysregulation in diseases such as cancer make it an interesting therapeutic target [144]. To date, three PIs, bortezomib (BTZ), carfilzomib and ixazomib, have been approved by the FDA for the treatment of multiple myeloma. BTZ was the first PI to be introduced into clinical practice (Figure 17).

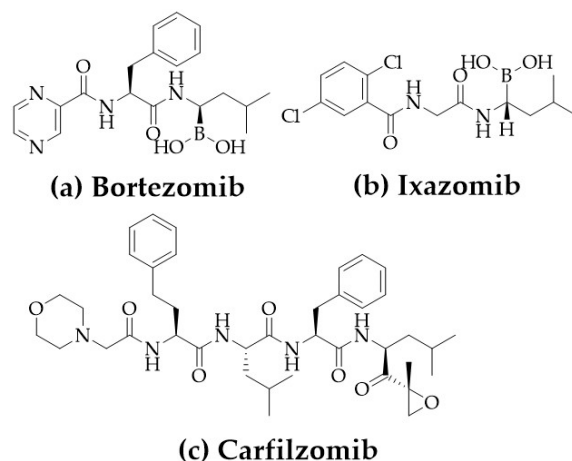


Figure 17. Proteasome inhibitors approved by FDA (a) bortezomib; (b) ixazomib and (c) carfilzomib.

BTZ binds reversibly to the 20S catalytic core of the 26S proteasome, thereby influencing a range of cellular processes [197]. For one, the accumulation of misfolded proteins results in the activation of the c-Jun N-terminal kinase, which may induce apoptosis. In contrast, tumor suppressor proteins such as p53 can accumulate leading to cell cycle arrest. Another aspect is the stabilization of the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha ($I\kappa B\alpha$), which has been demonstrated to reduce the increased nuclear factor- κB (NF- κB) activity. NF- κB activity, in turn, is crucial for the survival and proliferation of cells. This is of particular relevance in the context of AML cells, where NF- κB is known to be constitutively active. The constitutive activity of NF- κB is supported by tumor necrosis factor α (TNF- α), which phosphorylates the regulator $I\kappa B\alpha$. The phosphorylation of $I\kappa B\alpha$ results in ubiquitination and degradation by the proteasome. As a result, a greater quantity of NF- κB is released, which in turn increases the expression of target genes, including TNF- α . PIs disrupt this positive feedback loop between NF- κB and TNF- α , thereby inducing apoptosis via the accumulation of $I\kappa B\alpha$ in AML cells [95, 198-200].

As evidenced by a phase III study, BTZ exhibits superior outcomes in terms of time to progression, ORR, and OS compared to high-dose dexamethasone [201, 202]. The adverse effects of BTZ include neuropathy and hemotoxicity, which restrict its clinical application [203, 204]. Ixazomib is an orally bioavailable drug that demonstrated an overall rate of neuropathy of 20% and a response rate of less than 20%. These findings prompted further research into ixazomib [202]. Ixazomib was administered to patients diagnosed with refractory myeloma who had undergone one to three prior lines of therapy. The study showed that ixazomib in combination with lenalidomide and dexamethasone improved progression-free survival compared to lenalidomide plus

dexamethasone [205]. Carfilzomib is a second-generation PI that irreversibly binds to the 20S catalytic subunit of the 26S proteasome through its epoxyketone group. In contrast, BTZ and ixazomib bind this 20S catalytic subunit reversibly through their boronic acid group. In a single-arm study, carfilzomib provided an ORR of 23.7% in 266 patients, including one complete response and 13 very good partial responses [206]. Further PI are currently under clinical investigation as single or combination treatments with initial findings suggesting potential for further investigation [202].

2. Aim of the study

The emergence of chemoresistance in patients represents a significant concern in the field of oncology. Considerable therapeutic advancements in the treatment of cancer notwithstanding, the survival prognosis for advanced or recurrent cancer is still insufficient. This is evident in both acute myeloid leukemia (AML) and bladder carcinoma. To surmount chemoresistance to established therapies and provide patients with a superior therapy, novel treatment options or additional combination partners for approved drugs, such as cisplatin or bortezomib (BTZ), are necessary.

There has already been much research in the field of epigenetics. Histone modifications are integral to numerous intricate cellular processes and play a pivotal role in the pathogenesis of cancer and the development of chemoresistance. This encompasses the deacetylation of histones and non-histone proteins by histone deacetylases, which have been identified as a promising target for cancer therapy. The administration of histone deacetylase inhibitors (HDACi) is expected to positively influence gene transcription in cancer cells. HDACi can be used in combination with established drugs to target chemoresistant cancer cells or to treat native cancer cells more effectively. In recent years, the focus has shifted towards the investigation and development of subtype-selective HDACi, with the aim of enabling a more targeted therapy with fewer off-target effects.

The overall aim of this study was to investigate epigenetic modulators (novel HDACi, BETi) in combination with classical cytostatics (BTZ, cisplatin) for synergistic effects using the example of two severe cancers, AML and resistant bladder cancer.

The first objective of this work was the characterization of the novel class IIa-specific HDACi YAK540 and FFK24 (Figure 18).

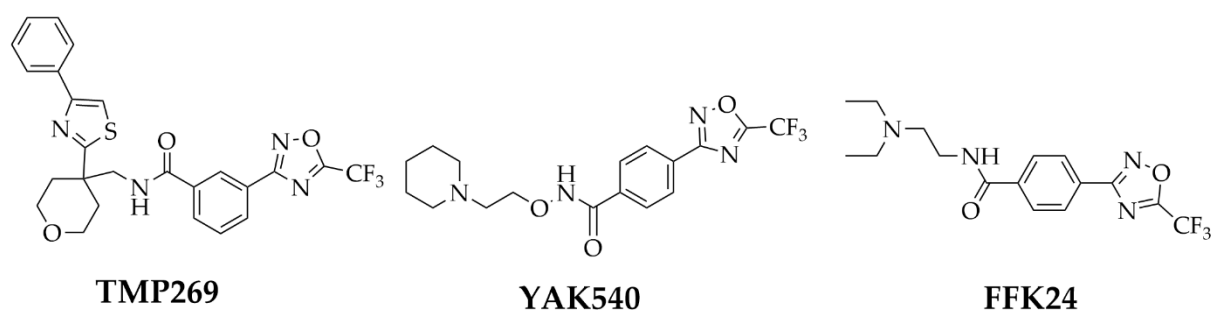


Figure 18. Class IIa HDACi TMP269, YAK540 and FFK24.

YAK540 and FFK24 should be evaluated in combination with the proteasome inhibitor BTZ in leukemia, focusing on AML (HL-60, MONO-MAC-6 and THP-1) and chronic myeloid leukemia (K562) cell lines in vitro. Despite the availability of a range of therapeutic options for AML, the 5-year survival rate remains disappointingly low, at 30%, particularly in older patients. It is therefore evident that new treatment options

2. Aim of the study

for AML are required. BTZ has been approved for the treatment of multiple myeloma and is currently under clinical investigation for use in AML. However, BTZ has a high toxicity profile, which results in limiting adverse effects such as hemotoxicity and neuropathy. One goal was to test the combination of BTZ with class IIa HDACi for a synergistic effect, with the potential clinical aim of reducing the dose of BTZ and thus reducing the toxicity of BTZ while maintaining efficacy.

In the second project, the combination of the class I HDACi entinostat (Figure 14) with the bromodomain and extra-terminal motif inhibitors (BETi) JQ1 and OTX015 (Figure 16) and cisplatin should be investigated in the bladder carcinoma cell lines J82, J82 cisR, T24 and T24 LTT. Cisplatin resistance is a prevalent challenge in the management of bladder cancer. Prior research in our research group has demonstrated the potential of HDACi to resensitize cisplatin-resistant cancers [127, 128, 207]. Other groups have investigated the combination of cisplatin and BETi or HDACi and BETi in various cancer entities, with promising results [208, 209]. Accordingly, the triple combination of HDACi, BETi, and cisplatin was selected for investigation of synergistic effects to reestablish cisplatin sensitivity, i.e. proving in vitro cytotoxic efficacy at clinically relevant concentrations of all utilized drugs. The objective of the triple treatment is to enhance the efficacy of cisplatin with low concentrations of the epigenetic inhibitors, particularly in cisplatin-resistant bladder cancer cells. Another novel approach investigated the dual HDACi and BETi **compound 20** (Figure 15) in combination with cisplatin.

In both parts, results show encouraging synergy in cytotoxicity and caspase-mediated apoptosis and gave insights into underlying mechanisms.

3. Publications

3.1 Publication I

The Novel Class IIa Selective Histone Deacetylase Inhibitor YAK540 Is Synergistic with Bortezomib in Leukemia Cell Lines

Lukas M. Bollmann¹, Alexander J. Skerhut¹, Yodita Asfaha¹, Nadine Horstick¹, Helmut Hanenberg², Alexandra Hamacher¹, Thomas Kurz¹, Matthias U. Kassack¹

¹ Institute of Pharmaceutical and Medicinal Chemistry, Heinrich-Heine-University Duesseldorf, 40225 Duesseldorf, Germany.

² Department of Otorhinolaryngology and Head/Neck Surgery, Heinrich Heine University, 40225 Duesseldorf, Germany.

published in: International Journal of Molecular Sciences, 02. November 2022

DOI: 10.3390/ijms232113398

The following content is derived from the aforementioned publication, entitled "The Novel Class IIa Selective Histone Deacetylase Inhibitor YAK540 Is Synergistic with Bortezomib in Leukemia Cell Lines." It is important to note that word-by-word citations are not explicitly highlighted in this section.

Authors Contribution

First authorship was shared by L.M.B, A.J.S., and Y.A. L.M.B. was responsible for conducting the majority of the biological experiments. A.J.S. and N.H. performed the enzyme inhibition experiments. Y.A. synthesized YAK540 for the experiments. L.M.B. wrote the original draft and visualized the data. A.H. and M.U.K. revised and edited the manuscript. The supervision was carried out by M.U.K., A.H., H.H., and T.K.

Background

The treatment of leukemias, especially acute myeloid leukemia (AML), is still a challenge as can be seen by poor 5-year survival of AML. Therefore, new therapeutic approaches are needed to increase the treatment success. Epigenetic aberrations play a role in pathogenesis and resistance of leukemia. Histone deacetylase (HDAC) inhibitors (HDACi) can normalize epigenetic dysbalance by affecting gene expression. In order to decrease side effects of so far mainly used pan-HDACi, this paper introduces the novel highly selective class IIa HDACi YAK540. Further, YAK540 was combined with the highly cytotoxic proteasome inhibitor bortezomib (BTZ), which is already approved by the FDA for multiple myeloma. The combination of YAK540 and BTZ has the potential to serve as a novel therapeutic approach for the treatment of AML. In addition, a synergistic effect of the two drugs could allow a dose reduction of BTZ to reduce therapy-limiting side effects.

Results

YAK540 showed excellent selectivity for HDAC4 against HDAC2, HDAC6 and HDAC8 (at least 82-fold). It had only a low cytotoxic effect in several leukemia cell lines (HL-60, THP-1, MONO-MAC-6, K562). The lowest IC₅₀ value of YAK540 was observed in MONO-MAC-6 (91.4 μ M). Synergism studies according to the Chou-Talalay method for the combination of YAK540 and BTZ revealed a synergistic cytotoxic effect in all four cell lines. Synergism levels were calculated applying the Bliss model (Figure 19a).

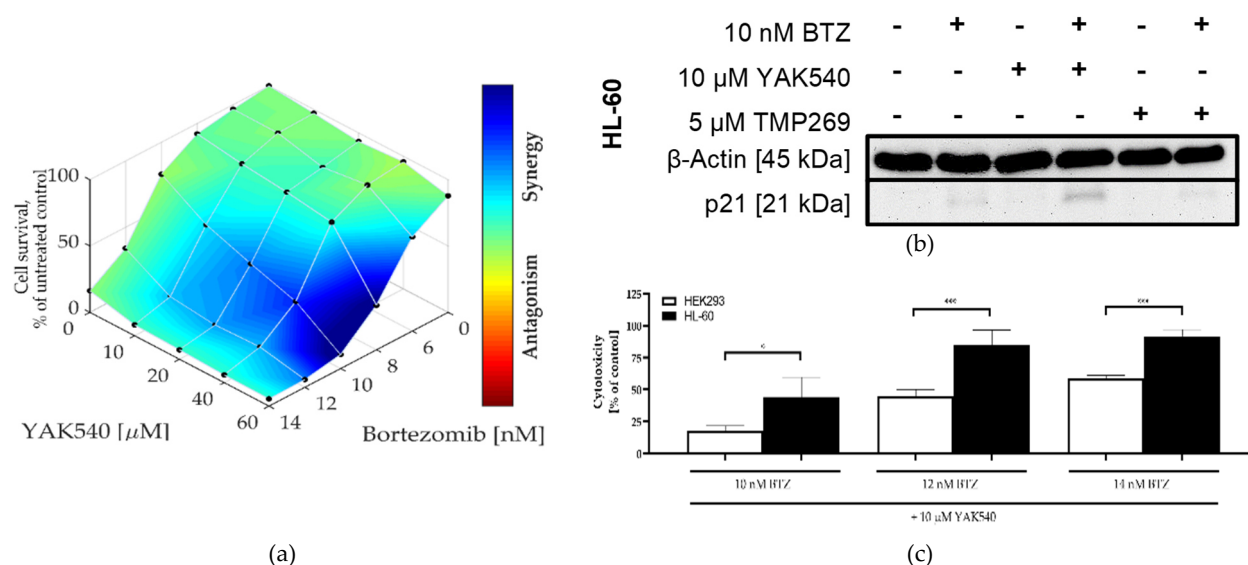


Figure 19. The most meaningful results of the combination of YAK540 and bortezomib (BTZ) in HL-60 cell line. **(a)** Synergy levels calculated applying the Bliss model. **(b)** p21 expression analyzed by Western Blot. **(c)** Comparison of the cytotoxicity of YAK540 and BTZ in HL-60 and the non-cancerous cell line HEK293. Figures taken and modified from [210].

The combination of YAK540 and BTZ showed generally increased proapoptotic gene expression in qPCR, increased *p21* expression on gene and protein level (Figure 19b), and synergistic, caspase 3/7-mediated apoptosis. Combinations of YAK540 and BTZ are clearly less toxic in non-cancer HEK293 compared to HL-60 leukemia cells (Figure 19c).

Conclusion

This study introduced and characterized the novel selective class IIa HDACi YAK540. Further, YAK540 was combined with the PI BTZ. In short, this study demonstrated:

- YAK540 as selective class IIa HDACi (at least 82-fold more selective for HDAC4 compared to HDAC2, HDAC6 and HDAC8)
- Synergistic cytotoxic effect of combination of YAK540 and BTZ through caspase-mediated apoptosis and increased p21 expression
- Lower cytotoxic effect of the combination in non-cancerous cell line HEK293 in comparison to HL-60

Thus, the synergistic combination of class IIa selective HDACi such as YAK540 and BTZ represents a promising approach against leukemias to increase the anticancer effect and to reduce the general toxicity of HDACi.



Article

The Novel Class IIa Selective Histone Deacetylase Inhibitor YAK540 Is Synergistic with Bortezomib in Leukemia Cell Lines

Lukas M. Bollmann ^{1,†}, Alexander J. Skerhut ^{1,†}, Yodita Asfaha ^{1,†}, Nadine Horstick ¹, Helmut Hanenberg ², Alexandra Hamacher ¹, Thomas Kurz ^{1,*,‡} and Matthias U. Kassack ^{1,*,‡}

¹ Institute of Pharmaceutical and Medicinal Chemistry, Heinrich-Heine-University Duesseldorf, 40225 Duesseldorf, Germany

² Department of Otorhinolaryngology and Head/Neck Surgery, Heinrich Heine University, 40225 Duesseldorf, Germany

* Correspondence: thomas.kurz@hhu.de (T.K.); matthias.kassack@uni-duesseldorf.de (M.U.K.)

† These authors contributed equally to this work.

‡ Shared senior authorship.

Abstract: The treatment of leukemias, especially acute myeloid leukemia (AML), is still a challenge as can be seen by poor 5-year survival of AML. Therefore, new therapeutic approaches are needed to increase the treatment success. Epigenetic aberrations play a role in pathogenesis and resistance of leukemia. Histone deacetylase (HDAC) inhibitors (HDACIs) can normalize epigenetic disbalance by affecting gene expression. In order to decrease side effects of so far mainly used pan-HDACIs, this paper introduces the novel highly selective class IIa HDACI YAK540. A synergistic cytotoxic effect was observed between YAK540 and the proteasome inhibitor bortezomib (BTZ) as analyzed by the Chou-Talalay method. The combination of YAK540 and BTZ showed generally increased proapoptotic gene expression, increased *p21* expression, and synergistic, caspase 3/7-mediated apoptosis. Notably, the cytotoxicity of YAK540 is much lower than that of pan-HDACIs. Further, combinations of YAK540 and BTZ are clearly less toxic in non-cancer HEK293 compared to HL-60 leukemia cells. Thus, the synergistic combination of class IIa selective HDACIs such as YAK540 and proteasome inhibitors represents a promising approach against leukemias to increase the anticancer effect and to reduce the general toxicity of HDACIs.

Keywords: leukemia; acute myeloid leukemia; histone deacetylase (HDAC) inhibition; proteasome inhibitor; bortezomib; YAK540; TMP269; class IIa HDAC inhibition; epigenetics



Citation: Bollmann, L.M.; Skerhut, A.J.; Asfaha, Y.; Horstick, N.; Hanenberg, H.; Hamacher, A.; Kurz, T.; Kassack, M.U. The Novel Class IIa Selective Histone Deacetylase Inhibitor YAK540 Is Synergistic with Bortezomib in Leukemia Cell Lines. *Int. J. Mol. Sci.* **2022**, *23*, 13398. <https://doi.org/10.3390/ijms232113398>

Academic Editors: Wolfgang Sippl and Hany S. Ibrahim

Received: 29 September 2022

Accepted: 30 October 2022

Published: 2 November 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Leukemia is the summary of various forms of blood cancer. The four main forms of leukemia are acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML). The pooled 5-year survival rate from all leukemias is 65.7% over 2012–2018 in the United States [1]. This demonstrates that leukemias can be treated reasonably well, although a more nuanced view shows that, for example, the 5-year survival rate differs greatly between acute myeloid leukemia and chronic myeloid leukemia. For example, the 5-year survival rate for acute myeloid leukemia is only 30.5% between 2012 and 2018 in the United States, whereas the 5-year survival rate for chronic myeloid leukemia is 70.4% over the same period [2,3]. The unmet medical need to improve AML treatment is also reflected in the recorded deaths from AML (51689) compared to 5653 in chronic myeloid leukemia between 2014 and 2018 in the United States, and furthermore, the incidence of AML along with chronic lymphatic leukemia, is among the leaders in the field [4]. For this reason, it is important to develop new and even better therapeutic concepts to enable more successful treatment of leukemias, especially AML.

In the past, proteasome inhibitors, such as bortezomib (BTZ), have been shown to be a promising approach for the treatment of hematological disorders. Proteasome inhibitors are approved for the treatment of multiple myeloma [5]. Bortezomib-induced inhibition of the proteasome leads to the accumulation of ubiquitinated, damaged proteins affecting various cellular processes such as cell cycle, DNA repair, transcription, cell signaling pathways, cell survival and apoptosis [6]. Due to the high proliferation rate and increased protein synthesis rate, malignant cells are more sensitive to inhibition of the proteasome [7]. The use of BTZ is however limited by severe side effects such as neuropathy and hemotoxicity [8,9]. Therefore, a potential dose reduction of BTZ to minimize its side effects or, alternatively, less toxic proteasome inhibitors are highly desirable. A recent study investigated the proteasome inhibitor ixazomib with an improved safety profile, such as reduction of peripheral neuropathy, and BTZ on ALL and AML cell lines [10]. BTZ is more potent than ixazomib, but this may be compensated by an improved pharmacokinetic and toxicological profile of ixazomib [10–13]. Several studies investigated BTZ in combination with other cytotoxic agents against AML from the groups of anthracyclines (e.g., doxorubicin), DNA methyltransferase inhibitors (e.g., azacitidine), and histone deacetylase inhibitors (HDACI) such as vorinostat [6]. In addition, carfilzomib, another proteasome inhibitor, showed superadditive cytotoxic effect on multiple myeloma cells in vitro in combination with the class IIa HDACI TMP269 [14].

Through epigenetic modulation, histone deacetylases (HDACs) have an impact on various cellular processes [15]. HDACs can be divided into four classes. Class I includes HDAC 1, 2, 3, and 8, which are present in the nucleus. Class II can be divided into IIa (HDAC 4, 5, 7 and 9) and IIb (HDAC 6 and 10). These HDACs are present in both the nucleus and cytoplasm [16]. Class III do not contain zinc dependent HDACs. Class IV contains HDAC 11, which has properties of class I and II [15]. Dysregulation of HDACs play a role in the development of certain cancers by altering transcription. The use of HDACIs is intended to restore the dysregulation of HDACs in cancer cells to normal. By regulating pro- and anti-apoptotic genes, HDACIs can induce apoptosis [15]. Another point is that the combination treatment of HDACIs with, for example, bortezomib could increase the sensitivity of cancer cells, so that a dose reduction of bortezomib would be possible.

To minimize the potential side effects of BTZ by dose reduction on the one hand and to reduce the potential side effects of unselective (pan) HDACIs on the other hand, the use of selective class IIa inhibitors in combination with the proteasome inhibitor was tested. One of the first potent class IIa HDACIs is TMP269 which has however the disadvantage of poor solubility [17]. We intended to improve the physicochemical properties and potency of TMP269 and thus developed YAK540 as a new selective class IIa HDACI. In the meantime, further improved class IIa selective HDACIs were developed such as NT160 [18] and compound 12 [19]. Yet, in this study we compare the first well-established trifluoromethylloxadiazolyl (TFMO)-type class IIa selective inhibitor TMP269 with our novel inhibitor YAK540. Both, YAK540 and TMP269 were evaluated for a synergistic effect in combination with BTZ in various leukemia cell lines with a focus on AML.

The pharmacophore model for class IIa selective HDACI contains the following four elements: a zinc binding group (ZBG), a linker that interacts with the substrate binding tunnel, a lower pocket group (LP-group) that occupies the selectivity pocket and a cap, also known as surface recognition domain (Figure 1) [20]. In 2013, Lobera et al. and Novartis discovered simultaneously class IIa selective HDACIs, revealing a trifluoromethylloxadiazolyl moiety (TFMO) as a novel ZBG. Lobera et al. demonstrated that inhibitors exhibiting this novel ZBG showed up to 150–10,000 fold higher selectivity towards class IIa HDACs compared to their corresponding hydroxamates [21]. A crystallographic analysis confirmed that the TFMO group acts as a non-chelating ZBG via the coordination of the fluorine atoms and its oxygen to the zinc ion in the catalytic center. It is proposed that class IIa selectivity arises from the bulkiness but modest zinc binding ability of the TFMO moiety as well as the U-shaped conformation of these inhibitors. Lobera et al. reported that the TFMO-based HDACI display an improved pharmacokinetic profile compared

to hydroxamates [21]. Furthermore, it is presumed that the TFMO series has fewer pan inhibitor associated off-targets effects.

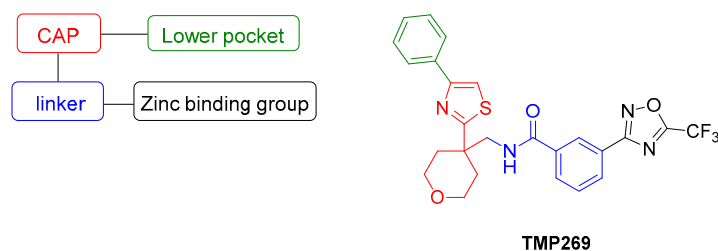
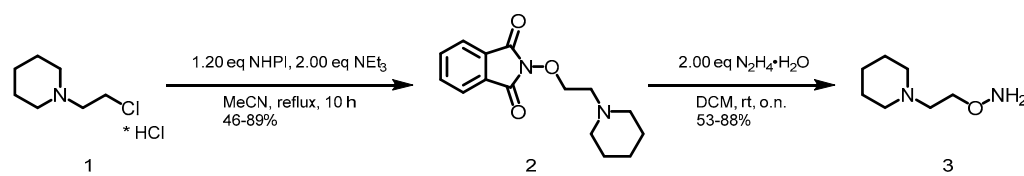


Figure 1. Pharmacophore model of class IIa histone deacetylase inhibitors (HDACIs) and the structure of TMP269 [21].

2. Results

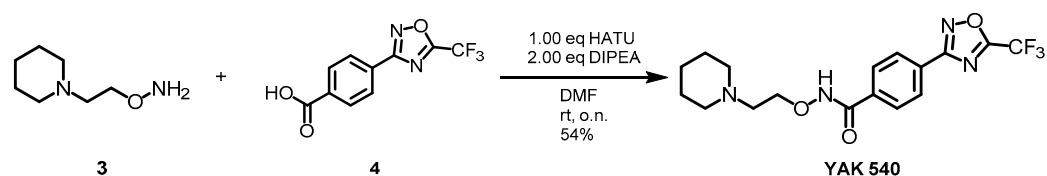
2.1. Synthesis of YAK540

The first building block was the respective hydroxylamine **3**. 1-(2-Chloroethyl)piperidine hydrochloride (**1**) was converted with N-hydroxyphthalimide (NHPI) to generate **2**. Subsequently, the deprotection was performed with 2.00 eq of hydrazine monohydrate yielding the desired hydroxylamine **3** (Scheme 1).



Scheme 1. Synthesis of hydroxylamine **3**.

YAK540 was generated, via a HATU-mediated coupling of the respective acid **4** and the hydroxylamine **3** in moderate yield (Scheme 2, Table S1).



Scheme 2. Synthesis of YAK540.

2.2. Investigation of Cytotoxicity and HDAC Inhibitory Effect

First, the cellular HDAC inhibition activity and cell permeability of YAK540 and TMP269 were determined. For this purpose, cellular HDAC assays were performed using a class IIa HDAC and HDAC 8 specific substrate Boc-Lys-(TFa)-AMC in addition to a class I and IIb HDAC substrate Boc-Lys (Ac)-AMC [22,23]. This allowed differentiating the cellular activity of class IIa HDACs from the remaining HDACs. The results for YAK540 and TMP269 are shown in Figure 2.

The IC₅₀ value of YAK540 using the class IIa selective substrate Boc-Lys-(TFa)-AMC was 590 nM whereas the IC₅₀ value using the class I/IIb substrate Boc-Lys-(Ac)-AMC was 64.2 μM. This result showed a high (factor 108) cellular selectivity of YAK540 for class IIa HDACs. In contrast to YAK540, TMP269 appeared with a much lower cellular class IIa HDAC inhibition (IC₅₀ value around 100 μM) and no class I/IIb HDAC inhibition up to 100 μM.

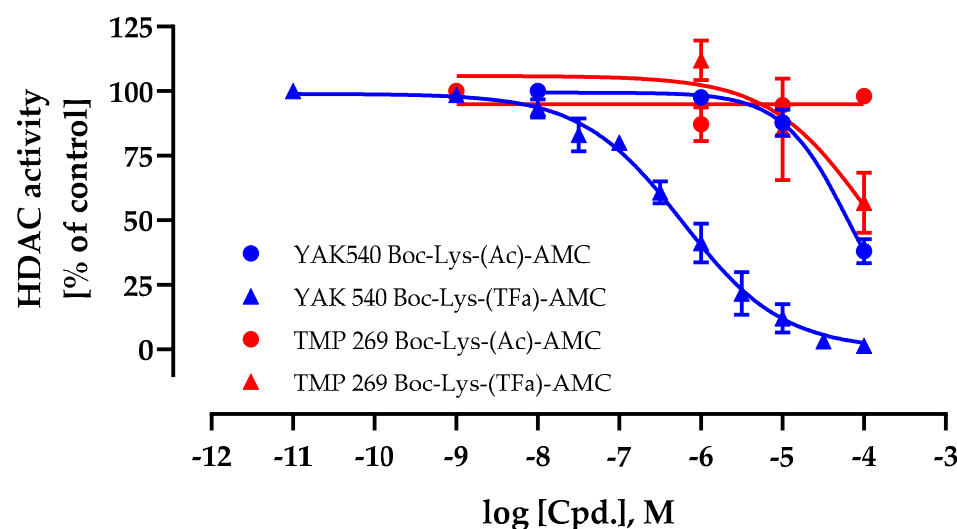


Figure 2. Whole-cell histone deacetylase (HDAC) activity assay of YAK540 and TMP269. Cells were treated with increasing concentrations of YAK540 or TMP269 for 24 h. Data shown are normalized to vehicle control and the mean of at least two independent experiments each carried out in triplicates.

Next, YAK540 and TMP269 were investigated in HDAC enzyme assays using HDAC 2, HDAC 4, HDAC 6, and HDAC 8 as representatives for their respective HDAC classes (Table 1).

Table 1. HDAC enzyme inhibitory activity of YAK540, and TMP269.

HDAC Enzyme	YAK540		TMP269 *	
	IC ₅₀ (pIC ₅₀ ± SEM) [μM]	Selectivity Index to HDAC 4	IC ₅₀ [μM]	Selectivity Index to HDAC 4
HDAC 2	30.2 (4.52 ± 0.03)	265	>100	>637
HDAC 4	0.114 (6.95 ± 0.03)	1	0.157	1
HDAC 6	11.4 (4.94 ± 0.12)	100	8.2	52
HDAC 8	9.39 (5.03 ± 0.03)	82	4.2	27

Data shown are mean of pooled data from at least two experiments with duplicates. * The values of TMP269 are taken from the literature [22].

YAK540 showed excellent potency at HDAC 4 as the major representative of class IIa HDACs with an IC₅₀ of 0.114 μM. Further, YAK540 indicated excellent selectivity for HDAC 4 against HDAC 2 (265-fold), against HDAC 6 (100-fold), and against HDAC 8 (82-fold). Thus, YAK540 presented at least 82-fold selectivity for HDAC 4 compared to the other enzymes. Compared to TMP269, YAK540 had improved selectivity. TMP269 had more than 600-fold selectivity for HDAC 4 compared to HDAC 2, but only 27-fold selectivity for HDAC 4 compared to HDAC 8, based on literature data for enzyme inhibition [22]. Another problem related to TMP269 is its poor solubility. We observed precipitations at higher concentrations of TMP269 in aqueous assay buffer, which was not the case for YAK540, requiring to add further solubilizers such as DMSO to TMP269 dilution series. YAK540 thus has improved aqueous solubility which clearly is advantageous over TMP269.

We next determined the cytotoxic effects of YAK540, TMP269, and BTZ on four different leukemia cell lines, namely the AML cell lines HL-60, THP-1, and MONO-MAC-6, and the CML cell line K562 (Figure 3). BTZ was expectedly cytotoxic in the double-digit nanomolar range. Interestingly, YAK540 had a significantly lower cytotoxic activity compared to TMP269. The IC₅₀ value of TMP269 was around 20 μM in all leukemia cell lines examined, whereas the IC₅₀ of YAK540 was predominantly above 100 μM. The IC₅₀ values determined are listed in Table 2.

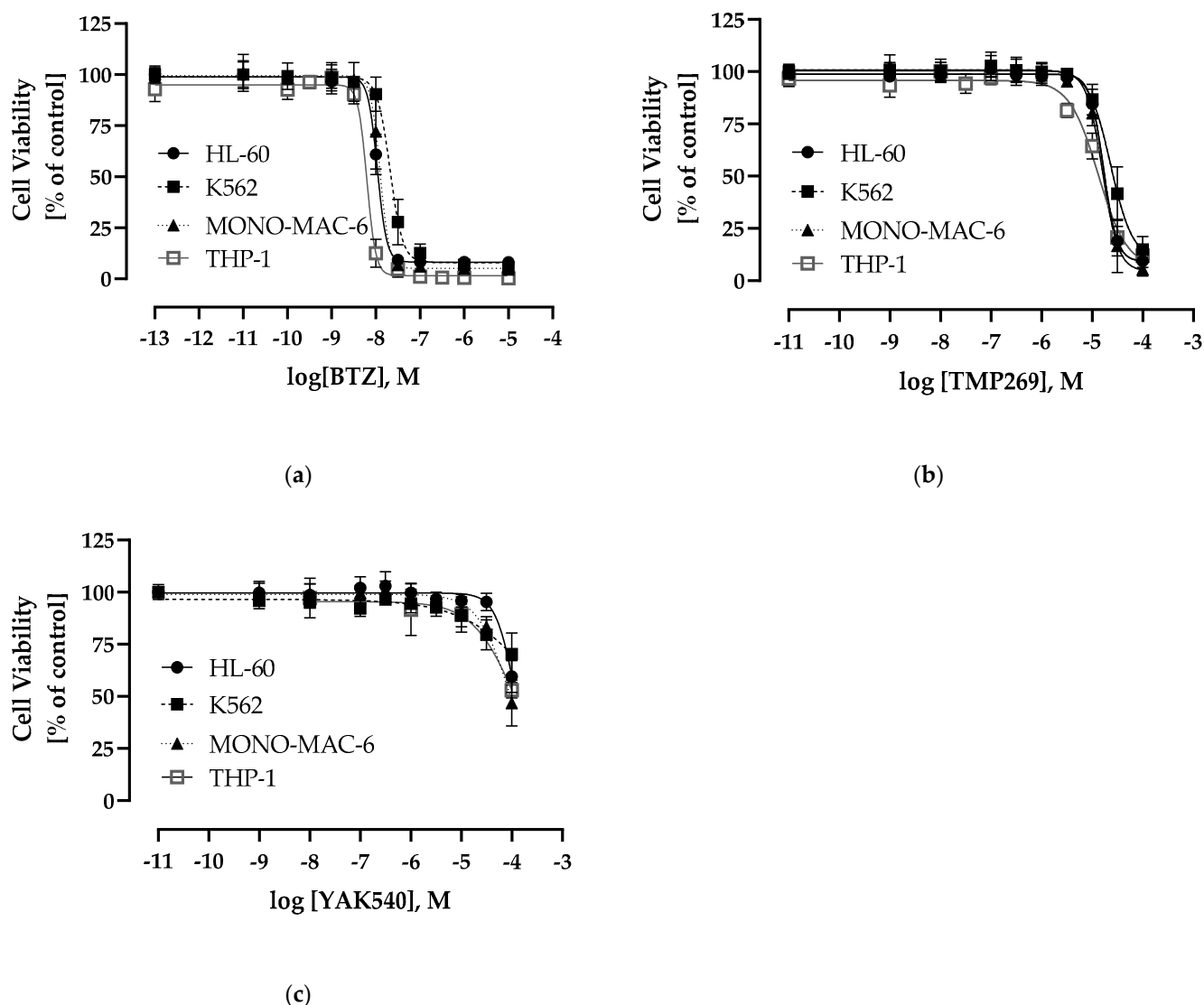


Figure 3. Concentration-response-curves determined by 72 h MTT-Assay (a) bortezomib (BTZ) (b) TMP269 (c) YAK540; Data shown are the mean of pooled data $n \geq 4$.

Table 2. Cytotoxic activity (MTT, 72 h) of BTZ, YAK540, TMP269. Data shown are the mean of pooled data from $n \geq 4$.

Cell Line	Bortezomib		50	YAK540IC		50	TMP269IC	
	IC ₅₀	pIC ₅₀ ± SEM [nM]		pIC ₅₀ ± SEM [μM]	pIC ₅₀ ± SEM [μM]			
HL-60	10.7	7.97 ± 0.006		> 100		16.7	4.78 ± 0.016	
K562	20.9	7.68 ± 0.014		> 100		23.0	4.64 ± 0.024	
MONO-MAC-6	12.4	7.91 ± 0.012	91.4	4.04 ± 0.022		15.9	4.80 ± 0.023	
THP-1	6.28	8.20 ± 0.020		> 100		13.0	4.89 ± 0.035	

2.3. Apoptosis Induction and Caspase Activation of Class IIa Inhibitors in Combination with Proteasom inhibitor Bortezomib

Next, we investigated combinations of BTZ and YAK540 or TMP269 and analyzed if the cytotoxic effect is based on caspase-mediated apoptosis. SubG1 analysis of BTZ, YAK540 and TMP269 alone and in combinations in THP-1 cells is illustrated in Figure 4. Whereas 5 μM TMP269 or 5.7 μM YAK540 showed only very little subG1 induction, combinations of

TMP269 or YAK540 with BTZ demonstrated over additive effects for both concentrations of BTZ used in this experiment (Figure 4a). Figure 4b displays the difference between an additive effect only (black bars) where the effects of single treatments with BTZ and YAK540 or TMP269 were simply added compared to the effect of the combination of BTZ with YAK540 or TMP269 (white bars). Incubation of the combination leads to a superadditive (=synergistic) effect.

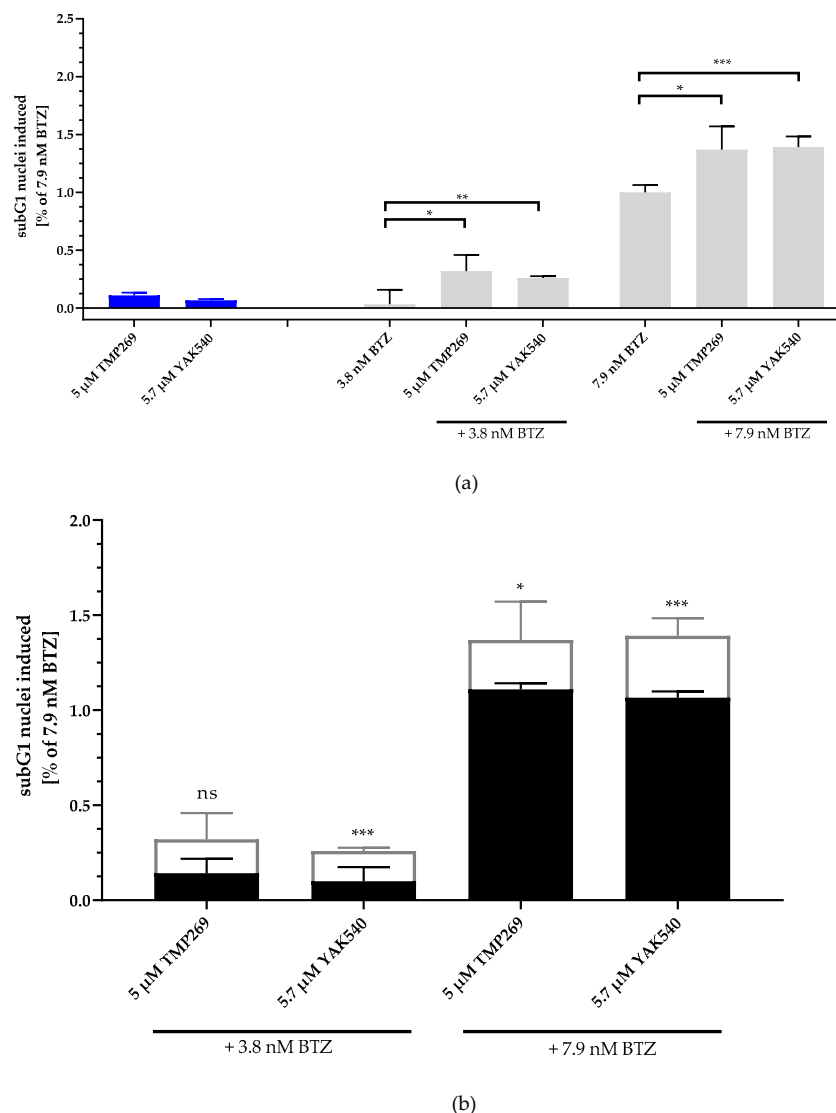
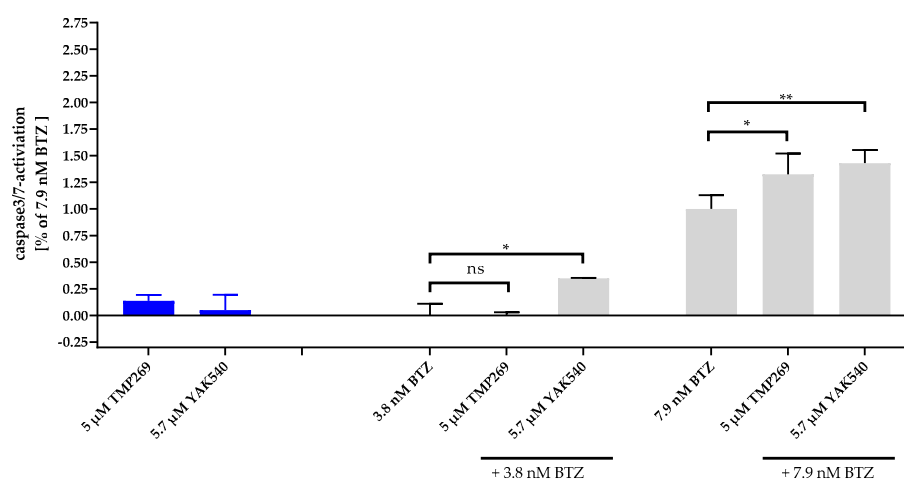


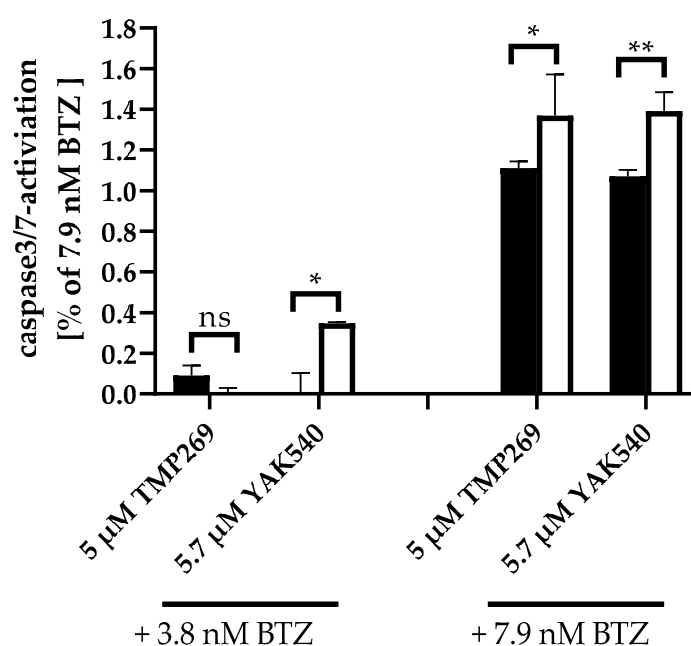
Figure 4. Incubation with class IIa inhibitors increases the induction of apoptosis by BTZ. The cell line THP-1 was incubated with the combination of class IIa HDACI and BTZ for 48 h. (a) Apoptosis was determined by subG1 fraction by flow cytometry. Control was subtracted and data normalized to 7.9 nM bortezomib. (b) Summary presentation of over additive subG1 induction. The black bars illustrate the additive effects of the individual treatments, the white overlapping bars demonstrate the over additive (=synergistic) effect of the combination treatment compared to the individual treatments. Data shown is the mean $\pm$ SD, $n \geq 2$. Statistical analysis was performed by *t*-test with significance level: ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$).

In the next step, we investigated whether apoptosis is induced in a caspase-dependent manner. For this purpose, caspase 3/7 activation was measured by fluorescence imaging of YAK540, TMP269, and BTZ treated THP-1 cells (Figures 5 and S23). Whereas YAK540, TMP269, and 3.8 nM BTZ alone showed (almost) no caspase activation, 7.9 nM BTZ indicated clear caspase activation and combination of 3.8 nM BTZ with YAK540 but not

TMP269 increased caspase activation. To analyze over-additive effects, single effects of the compounds were added (white bars) and compared to combination effects (YAK540 plus BTZ; TMP269 plus BTZ). Whereas the combinations of 5.7 μ M YAK540 plus BTZ illustrated over-additive (=synergistic) effects for both BTZ concentrations, only the combination of 5 μ M TMP269 with 7.9 nM BTZ showed a synergistic effect. Fluorescence images showing nuclear staining (Hoechst 33342) and cleaved fluorescent caspase substrate are available in the supplemental information (Figure S23).



(a)



(b)

Figure 5. Synergistic effect of class IIa HDACIs and BTZ in caspase 3/7 activation in THP-1 cells. Caspase activation is presented as a ratio of fluorescence data with the effect (fluorescence) of 7.9 nM bortezomib. (a) Caspase 3/7 activation of the single and combination treatments, 48 h incubation. Untreated Control was subtracted, and data normalized to 7.9 nM bortezomib. (b) Summary presentation of over additive caspase activation. The black bars illustrate the additive effects of the individual treatments, the white bars are the real measured data of the combination treatment. Data shown is the mean $\pm$ SD, $n \geq 2$. Statistical analysis was performed by t-test with significance level: ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$).

2.4. Chou-Talalay Synergism Studies in MTT Assays

To determine whether a synergistic effect exists, Chou-Talalay combination studies were performed using MTT assay [24]. The combination index (CI) was calculated for different concentrations of BTZ with the class IIa HDACs YAK540 and TMP269 (Table 3). Cells were co-incubated with BTZ and the HDACI. A synergistic effect is indicated by a CI value of less than 0.9. All cell lines exhibited CI values smaller than 0.9, and in some cases smaller than 0.5, indicating strong synergism. In addition, the synergy levels of the combination-concentration-response surface were graphically mapped by Combenefit. For calculating the synergy levels, the Bliss model was used. (Figure 5) [25,26].

Table 3. Synergism studies (CI-values) between BTZ and Class IIa HDACIs.

BTZ [nM]		YAK540 [μM]				TMP269 [μM]				
		10	20	40	60	2.5	5	7.5	10	
HL-60	8	○	○	3.43	0.512	○	○	2.82	1.84	
	10	1.11	0.791	0.576	0.188	1.96	1.47	1.22	0.744	
	12	0.301	0.237	0.260	0.136	0.631	0.451	0.386	0.279	
	14	0.206	0.185	0.158	0.150	0.286	0.244	0.275	0.260	
BTZ [nM]		YAK540 [μM]				TMP269 [μM]				
		10	15	20	25	30	2.5	5	7.5	10
K562	15	1.58	1.43	1.21	1.27	1.22	1.53	1.64	1.14	0.902
	20	0.920	0.900	0.883	0.727	0.677	0.957	1.05	0.836	0.675
	25	0.706	0.668	0.636	0.557	0.507	0.690	0.801	0.708	0.615
	30	0.646	0.630	0.566	0.536	0.512	n.p.	n.p.	n.p.	n.p.
BTZ [nM]		YAK540 [μM]				TMP269 [μM]				
		10	20	30	40	60	2.5	5	7.5	10
MONO-MAC-6	6	○	○	1.98	1.42	1.47	○	1.41	1.09	0.859
	8	1.72	1.69	0.694	0.476	0.416	1.45	1.09	0.560	0.480
	10	0.670	0.579	0.265	0.174	0.124	0.764	0.622	0.298	0.259
	12	0.277	0.177	0.070	0.075	0.075	0.385	0.290	0.203	0.205
	14	0.094	0.095	0.060	0.069	0.073	0.198	0.179	0.177	0.202
BTZ [nM]		YAK540 [μM]				TMP269 [μM]				
		10	15	20	25	30	35	7.5	10	12.5
THP-1	5	1.62	1.42	1.74	1.35	1.47	1.34	1.07	0.93	0.95
	10	0.92	0.62	0.81	0.67	0.73	0.73	1.00	1.46	0.86
	15	1.24	0.79	1.11	0.61	0.73	0.59	0.75	0.79	0.67

Synergism studies (CI-values) between BTZ and Class IIa HDACIs with 72 h incubation time. Combination indices (CI) were calculated using CompuSyn 1.0, which is based on the Chou-Talalay method [24]. CI > 1.1 indicates antagonism, CI = 1 indicates an additive effect, CI < 0.9 indicates synergism. Values marked in red ≥ 0.9, values marked in green < 0.9 (○ is fa ≤ 0.2, n.p. is not performed). Data for calculation are from at least two independent experiments each carried out at least in duplicates.

As can be seen in the combenefit graphs based on the Bliss model in Figure 6, HL-60 and MONO-MAC-6 show significantly stronger synergy with YAK540 plus BTZ than TMP269 plus BTZ.

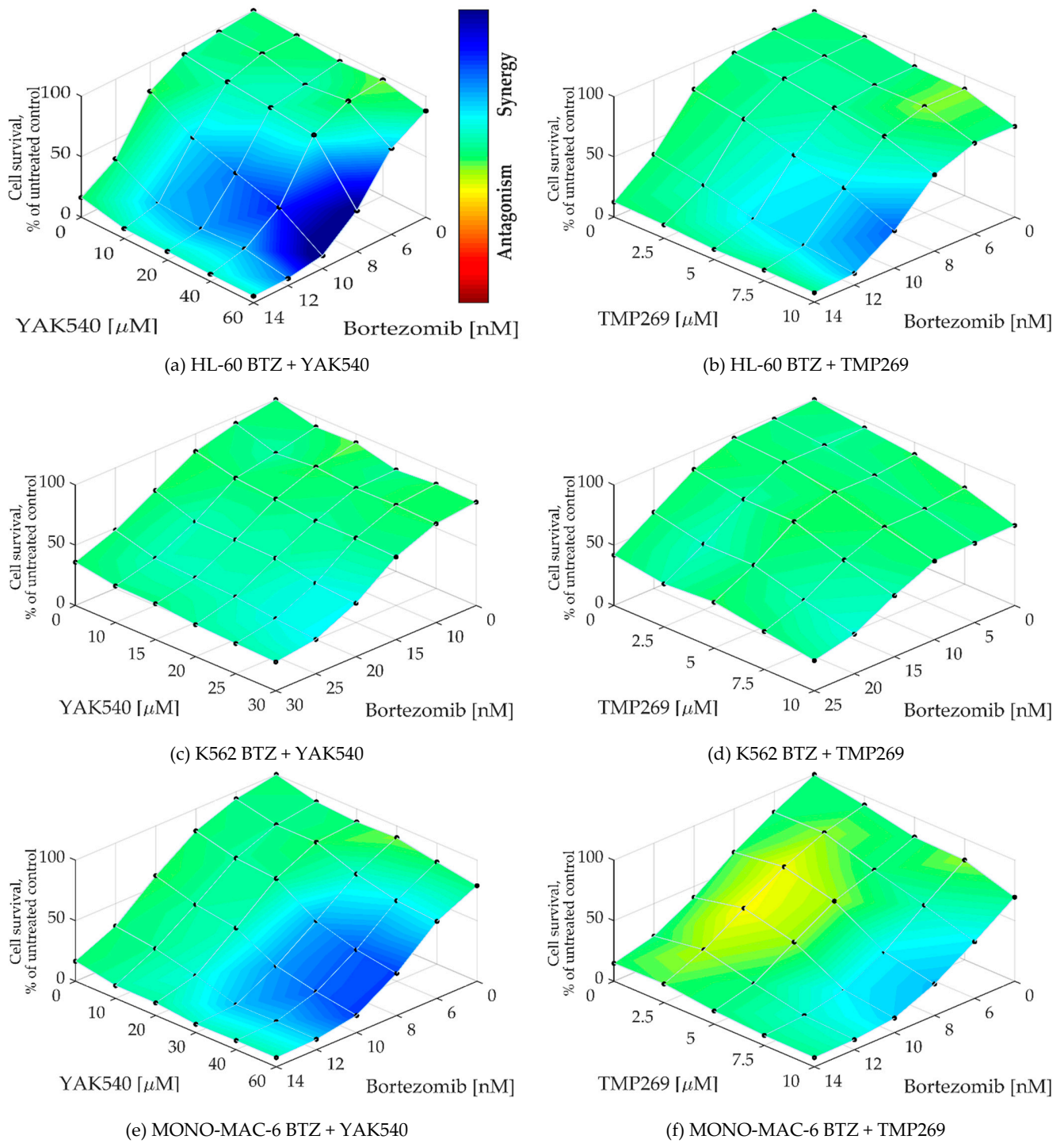


Figure 6. Cont.

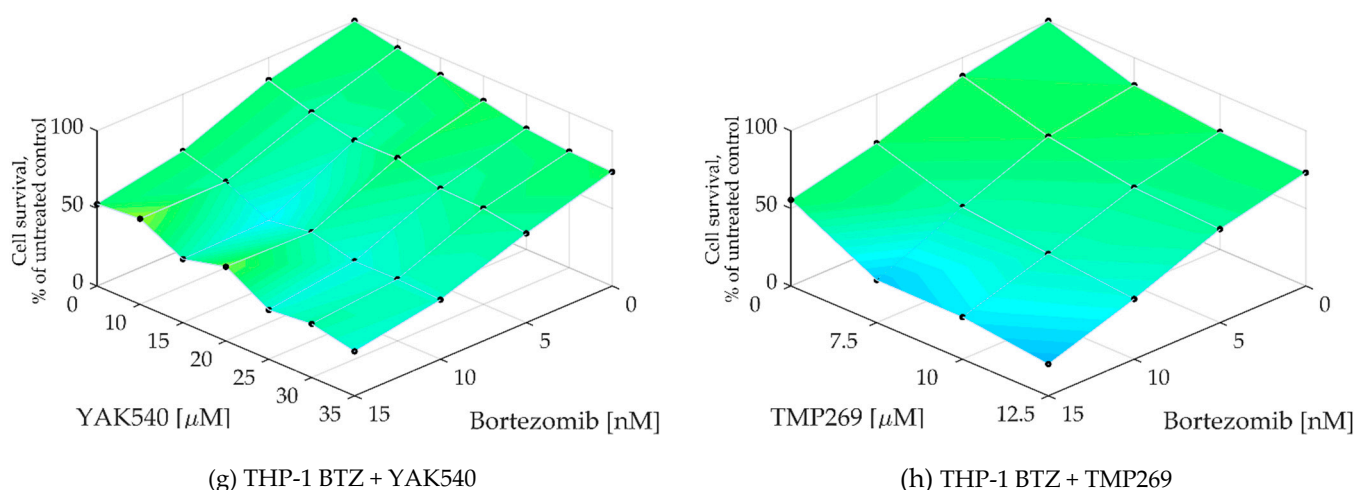


Figure 6. (a–h) Synergy levels calculated with Bliss model [26] in CombeneFit [25], depending on experimental concentration-response surface of combinations of BTZ and class IIa HDACIs YAK540 and TMP269 for 72 h in all leukemia cell lines. Data shown are from at least two independent experiments with each carried out at least in duplicates.

2.5. Mechanistic Study of Class IIa Inhibitors in Combination with Proteasome Inhibitors

The next step was the detailed mechanistic investigation of the combination therapy. For this purpose, RT-qPCR was first performed for various proapoptotic and anti-apoptotic genes (Figure 7). In all cell lines, BTZ or combinations of class IIa HDACIs and BTZ affect gene expression estimated by PCR (with few exceptions, such as *Bcl-2* in HL-60). HL-60 cells show a marked increase in *BAD* expression (1.3–1.4 log units) upon BTZ treatment (alone or in combination with class IIa HDACI), whereas this increase is much lower (only up to 0.198 log units) in the other two cell lines. In contrast, *p21* is strongly upregulated in K562 and MONO-MAC-6 upon BTZ single or combination treatment but downregulated in HL-60. However, *p21* is also upregulated in HL-60 upon treatment with class IIa HDACI without BTZ. Expression changes of *survivin* are heterogenous. Decreased expression of *survivin* is observed with the combination of BTZ and YAK540 in HL-60, and in MONO-MAC-6 with the combination of BTZ with TMP269 or YAK540. In contrast, expression of *survivin* increased in K562 upon treatment with BTZ alone or in combination with the class IIa HDACIs. Treatment-induced expression changes were also heterogenous for the antiapoptotic proteins *Bcl-2* and *Bcl-xl*. In HL-60 cells, class IIa HDACIs, BTZ and combinations thereof increased expression of *Bcl-2* whereas expression of *Bcl-xl* was decreased, particularly by BTZ and combinations of BTZ and class IIa HDACIs. In K562 cells, bortezomib-containing treatments decreased expression of both, *Bcl-2* and *Bcl-xl*. In MONO-MAC-6, treatments increased *Bcl-xl* and decreased *Bcl-2* expression. Expression of *HDAC 4* was not markedly influenced by any treatment, showing no counter regulation by treatment with class IIa HDACI. Treatment-induced gene expression changes for *PTEN*, *BIM*, *BAK*, *APAF-1*, *MCL-1*, *XIAP*, *p53*, *FOXO1*, and *FOXO3a* were heterogeneous in the 3 cell lines. In summary, PCR data show that treatment with BTZ and class IIa HDACI and combinations thereof have a strong influence on the expression of pro- and antiapoptotic genes, however with no clear tendency towards pro- or anti-apoptosis.

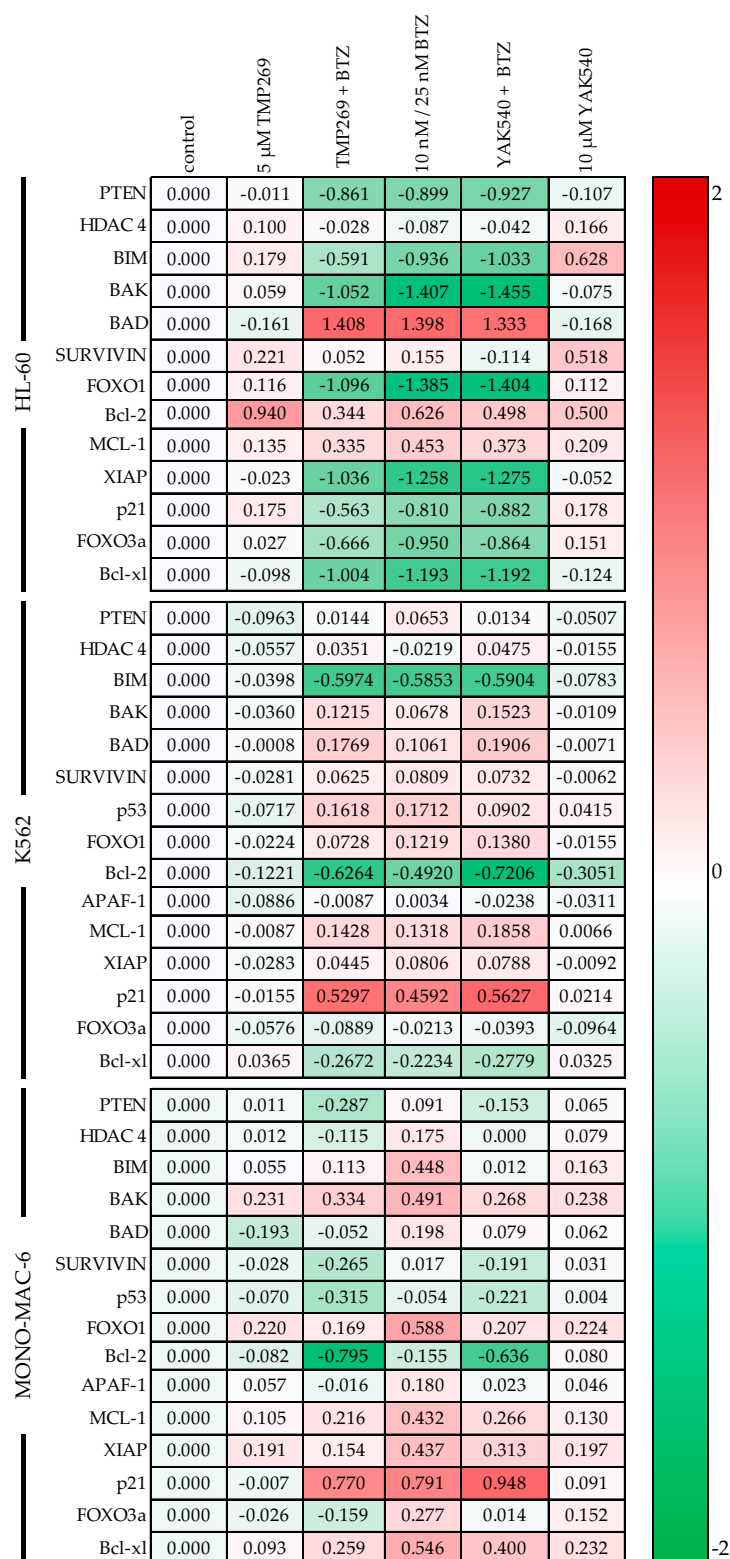


Figure 7. Gene expression was examined by RT-qPCR. Cells were incubated for 48 h with the inhibitors. HL-60 and MONO-MAC-6 were treated with 10 nM BTZ and K562 was treated with 25 nM BTZ. The analysis was performed according to Vandesompele [27]. Shown is the decadic logarithm of the values normalized to the expression of the control genes GUSB (beta-glucuronidase), TBP (TATA binding protein) and HPRT1 (hypoxanthine-guanine phosphoribosyltransferase) of the respective cell line. Negative values are green and indicate lower expression of the gene. A red marking indicates positive values and thus a higher expression of the gene compared to the cell-specific control.

Thus, western blot was used to trace the expression of key proteins in apoptosis, survival, or cell cycle, and to monitor treatment-dependent changes of HDAC 4 expression, induction of PARP cleavage and H2AX phosphorylation (γ -H2AX) (Figure 8).

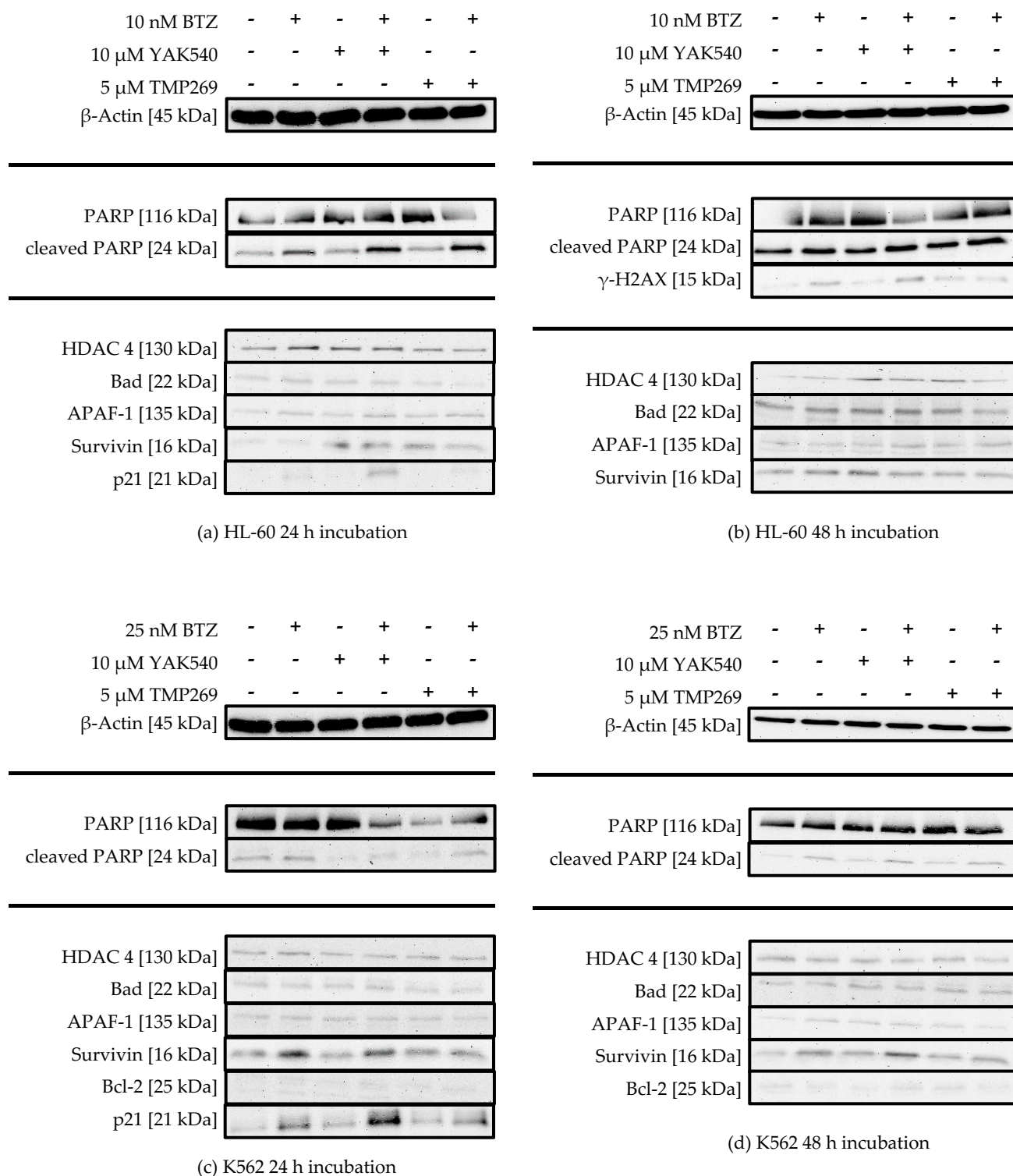


Figure 8. Cont.

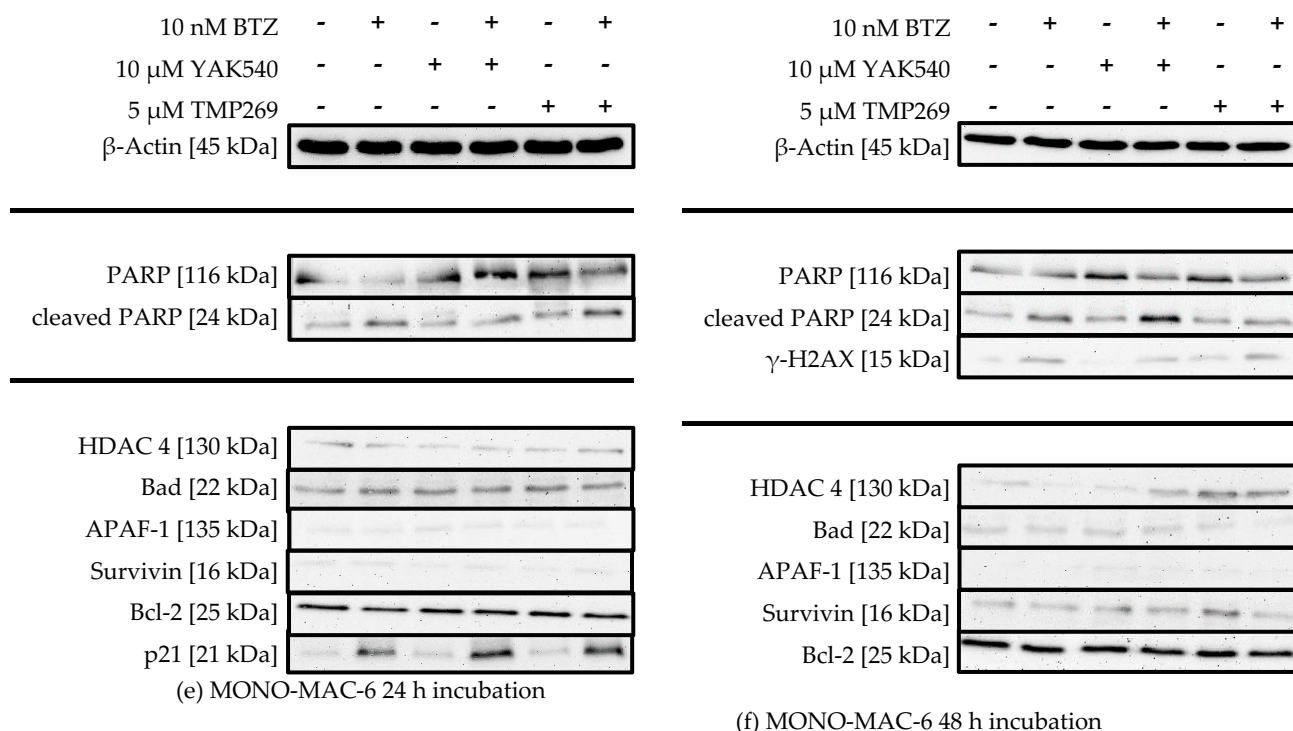


Figure 8. (a–f) Western Blot analysis of HL-60, K562 and MONO-MAC-6 after treatment with BTZ and YAK540 or TMP269 for 24 h or 48 h incubation. The data presents a representative blot. For the following blots a representative blot of two blots in total is shown: HL-60 24 h (β -Actin, PARP, Bad, APAF-1, Survivin, p21), HL-60 48 h (γ -H2AX), K562 24 h (p21, Survivin), MONO-MAC-6 24 h (β -Actin, APAF-1, p21), MONO-MAC-6 48 h (β -Actin, PARP, γ -H2AX, Survivin). The uncropped blots are presented in the supplement (Figures S2–S22).

There is no significant change in HDAC 4 expression upon treatment with class IIa HDACIs, suggesting there is no counter-regulation of HDAC 4 activity upon inhibition by class IIa HDACIs. PARP cleavage can be observed in all cell lines, sometimes more pronounced after 24 h sometimes more pronounced after 48 h, and to a much lesser extent in K562 cells (Figure 8c,d). HL-60 cells show increased PARP cleavage upon combination of YAK540 or TMP269 with BTZ compared to BTZ alone after 24 h (Figure 7a). After 48 h, PARP cleavage was observed under all conditions (Figure 7b). K562 cells show faint PARP cleavage bands after BTZ and BTZ/class IIa HDACI treatment after 48 h (Figure 7d). In MONO-MAC-6 cells after 48 h, PARP cleavage is clearly stronger after combination treatment YAK540 plus BTZ compared to BTZ alone. Interestingly, TMP269 plus BTZ demonstrates no stronger band than BTZ alone (Figure 7f). Phosphorylation of H2AX (γ -H2AX) indicating DNA double strand breaks was only weakly detectable in HL-60 and MONO-MAC-6 cells after 48 h in treatments containing BTZ (Figure 7b,f). In the K562 cells, γ -H2AX was not observed. Proapoptotic proteins BAD and APAF-1 are mostly weakly expressed in all three cell lines with basically no difference between the various treatments. Survivin expression was increased by BTZ in K562 cells (Figure 7c,d) and slightly increased by class IIa HDACIs in HL-60 and MONO-MAC-6 (Figure 7a,b,f). Bcl-2 expression was only observed in MONO-MAC-6 cells and similar under all treatment conditions (Figure 7e,f).

Most prominent changes were observed in the expression of p21 after 24 h treatment (Figure 7a,c,e). BTZ induced a small increase in p21 expression which was markedly enhanced by class IIa HDACIs. Interestingly, in HL-60 and K-562 cells, only YAK540 but not TMP269 increased p21 expression compared to BTZ.

2.6. Selectivity of Class IIa HDACI in Combination with BTZ for Leukemia vs. Non-cancer Cell

IC₅₀ values of YAK540, TMP269, and BTZ for the non-cancer cell line HEK293 are displayed in Table 4. Corresponding concentration-response-curves are presented in Figure S1.

Table 4. Cytotoxic activity (MTT, 72 h) of BTZ, YAK540, TMP269. Data shown are normalized to vehicle control and the mean of at least three independent experiments each carried out in triplicates.

Cell Line	Bortezomib		YAK540		TMP269	
	IC ₅₀	pIC ₅₀ ± SEM [nM]	IC ₅₀	pIC ₅₀ ± SEM [μM]	IC ₅₀	pIC ₅₀ ± SEM [μM]
HEK293	11.8	7.93 ± 0.014	> 100		32.8	4.49 ± 0.025

Then, the combination of class IIa HDACIs and BTZ was tested at HEK293 and for comparison at HL-60 cells. Results are displayed in Figure 9.

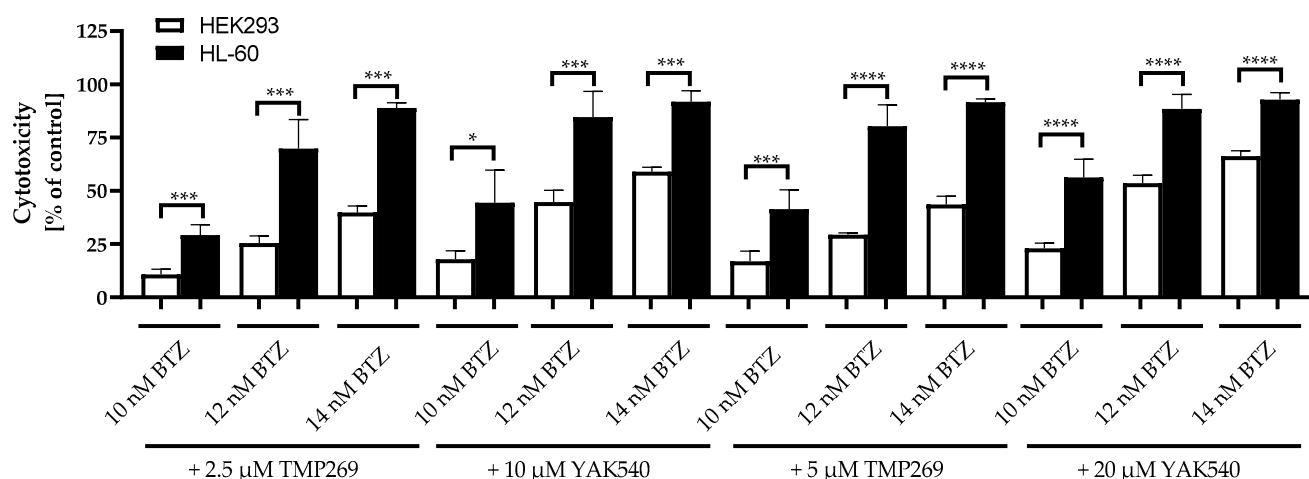


Figure 9. Cytotoxicity of combination treatment for 72 h incubation of Class IIa HDACIs and BTZ. Control is untreated cells and corresponds to 0% cytotoxicity. Data shown for HL-60 and HEK293 cells. Presented data is the mean ± SD, n ≥ 3. Statistical analysis was conducted by t-test with significance level: ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$).

Equitoxic concentrations of TMP269 and YAK540 were used based on the IC₅₀ values at HEK293 (Table 4). Since the IC₅₀ of YAK540 is > 100 μM, the IC₅₀ of YAK540 is at least 3–4-fold higher than the IC₅₀ of TMP269 which was estimated as 32.8 μM (Table 4). Thus, concentrations used were: 2.5 and 5 μM for TMP269 and 10 and 20 μM for YAK540. The combination of class IIa HDACIs with BTZ was significantly less toxic in the non-cancer cell line HEK293 than in HL-60 for all concentrations tested. As an example, the cytotoxicity of 12 nM BTZ plus 10 μM YAK540 was 44.7% in HEK293 compared to 84.7% in HL-60. Thus, roughly a 2-fold selectivity for leukemia versus HEK293 was observed for the combination treatment.

3. Discussion

Sufficient treatment of AML is still an unmet medical need. Combination trials of BTZ with the standard therapy consisting of cytarabine, daunorubicin and etoposide in newly diagnosed pediatric AML patients showed the greatest beneficial effect in a patientsubgroup with reduced phosphorylation of heat shock factor 1 at serine³²⁶ (low HSF1-pSer³²⁶) as well as low expression of RelA-pSer⁵³⁶. A smaller beneficial effect was demonstrated in subgroups with reduced expression of RelA-pSer⁵³⁶ only or reduced expression of HSF1-pSer³²⁶ only [28,29]. In a subsequent work, the findings regarding HSF1-pSer³²⁶ were confirmed using in vitro data [29]. No improvement in overall survival and event-free survival was detected considering the entire cohort of the study indicating

that innovative strategies are urgently needed for all leukemias, especially AML patients. Novel strategies have included HDACI as combination partners of various cytostatic agents, among them proteasome inhibitors such as BTZ [30]. Whereas so far mainly pan-HDACI such as vorinostat have been used as combination partners, class IIa HDACIs have less been investigated [30]. BTZ is approved for the treatment of multiple myeloma, its use is however limited by severe side effects [5,8,9]. Therefore, strategies to increase the efficacy of proteasome inhibitors and thus to reduce their required dose and consequently their toxic side effects are highly desirable. Switching to ixazomib could reduce toxicity through an improved safety profile, however ixazomib is significantly less potent than BTZ, thus reducing the efficacy of the therapy [10–13]. Therefore, we dedicated our study to the combination of HDACI with BTZ to investigate whether a synergistic effect would allow a dose reduction of BTZ maintaining the efficacy against leukemias but reducing toxic side effects. Since pan-HDACI and class I HDACI also have severe side effects, we focused in this paper on class IIa HDACI known for their reduced toxicity compared to pan-HDACI [20]. Previous studies have investigated carfilzomib and TMP269, one of the first TFMO-based class IIa HDACI (Figure 1) [5,14,21]. TMP269 has however the problem of limited water solubility [17,31]. We have thus developed YAK540, an improved TFMO-based class IIa HDACI, accessible with a simple straightforward synthesis (Schemes 1 and 2) and improved water solubility compared to TMP269. We then compared the anti-leukemia activity of YAK540 in combination with BTZ in comparison to TMP269. Most interestingly, YAK540 has superior effects over TMP269 although inhibition data at recombinant HDAC enzymes are comparable for these two compounds: IC_{50} at HDAC4 is 114 nM for YAK540 and 157 nM for TMP269 (Table 1). Superior effects of YAK540 include an almost 200-fold higher cellular HDAC activity compared to TMP269 and a high selectivity for class IIa HDAC enzymes over class I/IIb enzymes (108fold, Figure 2). Furthermore, YAK540 is 4–7.7 fold less cytotoxic at leukemia and non-cancer cells (Tables 2 and 4) but still increases caspase activation and apoptosis (measured as subG1 fraction) in a synergistic manner at basically the same concentration as used for TMP269 (5 μ M TMP269, 5.7 μ M YAK540; Figures 4 and 5). YAK540 seems more efficacious than TMP269 as YAK540 is synergistic even at low BTZ concentrations (3.8 nM) at which TMP269 shows no over-additive effects (Figures 4 and 5). The toxicity mechanism of caspase3/7-mediated apoptosis induction is in accordance to literature data where co-incubation of carfilzomib and TMP269 was studied [14].

Chou-Talalay analysis, the gold standard of synergy analysis, was performed in 4 leukemia cell lines using MTT assay. YAK540 and TMP269 were highly synergistic as noticed by CI values below 0.5 (Table 3) and graphical display as blue surface using the Bliss model in Figure 6. Synergy was stronger in 2 of the 4 cell lines (HL-60, MONO-MAC-6; Figure 6a,b,e,f) for YAK540 than for TMP269. Strong synergy for YAK540 was already detected at 10 μ M, a concentration that still retains class IIa HDAC preference as seen in enzyme assays and cellular HDAC assays (Table 1, Figure 2). Considering the clearly lower cellular toxicity of YAK540 compared to TMP269 (Tables 2 and 4), YAK540 treatment could result in fewer side effects than would be expected from the more toxic TMP269.

Toxicity of class IIa HDACI is rather low [20]. YAK540 showed IC_{50} values > 100 μ M (except MONO-MAC-6, where an IC_{50} of 91.4 μ M was estimated) in leukemia and non-cancer HEK293 cells (Tables 2 and 4) and is thus clearly less toxic than TMP269. Still, TMP269 was 1.4–2.5 less toxic in HEK293 cells than in the leukemia cells (Tables 2 and 4), confirming generally low toxicity of class IIa HDACIs. Further, the combination of class IIa HDACI with BTZ was clearly less toxic in non-cancer HEK293 cells than in HL-60 as proven over a wide concentration range (Figure 9) although IC_{50} values of BTZ were similar in leukemia and HEK293 cells. YAK540 is thus clearly advantageous due to lower toxicity than TMP269 and selective toxicity in combination with BTZ for leukemia over non-cancer cells.

A further advantage of YAK540 is the induction of p21 in combination with BTZ compared to the combination of TMP269 and BTZ (Figure 8, except MONO-MAC-6 cells). Our results also supported an advantage for YAK540 over TMP269 due to stronger apoptosis

and caspase activation (Figures 4 and 5) which may be based on the detected increased p21 induction upon YAK540/BTZ compared to TMP269/BTZ leading to cell cycle arrest and subsequent apoptosis. Data on PARP cleavage (Figure 8) confirmed these results.

A similar effect on p21 expression has been described for the pan-HDAC inhibitor vorinostat [32]. Expression of p21 was similar in our PCR and western blot data. For other genes/proteins, such correlation between mRNA and protein data could not be confirmed. Still, class IIa HDACI treatment leads to major PCR-detectable changes in expression of pro- and antiapoptotic genes (Figures 7 and 8). Still, p21 seems to be the key regulator for the synergistic effect of YAK540 and BTZ in caspase-mediated apoptosis.

In summary, we have introduced the improved class IIa HDACI YAK540 with advantages over the first TFMO-based class IIa HDACI TMP269. Improved activity (synergy) of YAK540 and selective toxicity for leukemia versus non-cancer cells may allow to reduce the therapeutic dose of BTZ in combination with YAK540 (class IIa HDACI) retaining or yet improving therapeutic efficacy while reducing severe patient-associated toxicity of BTZ. Clinical data have shown a c_{max} value of 580 nM for BTZ after intravenous administration [33]. Regarding IC_{50} values of BTZ estimated in this study (up to 21 nM, Table 2), a dose-reduction of BTZ while maintaining therapeutic efficacy through combination with low toxic YAK540 (class IIa HDACI) is a hypothesis worth being tested in further studies, including animal and clinical studies, to improve multiple myeloma, leukemia and especially AML treatment. Our study reveals synergism between class IIa HDACI (here YAK540) and proteasome inhibitors (here BTZ). This has the potential to improve future treatment of multiple myeloma and/or leukemias like AML using proteasome inhibitors in combination with novel low toxic class IIa HDACI (e.g., YAK540) whereas today the use of proteasome inhibitors often has to be stopped due to severe side effects. Future animal and later clinical studies are needed to investigate the therapeutic benefit of class IIa HDACI in combination with proteasome inhibitors, clinical synergy, and possible dose reduction of proteasome inhibitors due to synergy to reduce severe side effects.

4. Materials and Methods

4.1. Materials

The culture medium RPMI 1640 and DMEM, Penicillin/streptomycin (pen/strep) (10,000 U/mL; 10 mg/mL) and trypsin-EDTA (0.05% Trypsin, 0.02% EDTA in Phosphate Buffer Saline) were related from PAN-Biotech (Aidenbach, Germany). Bortezomib and TMP269 were ordered from AdooQ (AdooQ Bioscience, Irvine, California, USA). Both compounds were dissolved in DMSO at 10 mM. Further dilutions were made with the appropriate culture medium. A maximum concentration of 1% DMSO was used in all experiments.

4.2. Cell Lines and Cell culture

The human promyeloblast leukemia cell line HL-60, the human lymphoblast leukemia Cell line K562 and the monocytic leukemia Cell line MONO-MAC-6 and the human monocytic leukemia Cell line THP-1 were kindly provided by Helmut Hanenberg, Department of Otorhinolaryngology and Head/Neck Surgery, Heinrich Heine University, 40,225 Düsseldorf, Germany. All leukemia cell lines were cultured with RPMI 1640 spiked with 120 IU/mL penicillin and 120 µg/mL streptomycin and 10% heat inactivated fetal calf serum at 37 °C and 5% CO₂. The non-cancer cell line HEK293 was acquired by DSMZ (Braunschweig, Germany). The cells were cultured with DMEM mixed with 120 IU/mL penicillin and 120 µg/mL streptomycin and 10% heat inactivated fetal calf serum at 37 °C and 5% CO₂.

4.3. MTT Cell Viability Assay

Cell viability during treatment with BTZ and the class IIa inhibitors YAK540 and TMP269, respectively, and concentration-response curves were determined by MTT assay with minor modifications as previously described [34,35]. Briefly, cells were incubated with the compounds for 72 h. The following cell numbers were plated: HL-60 25.000 c/w,

K562 35.000 c/w, MONO-MAC-6 30.000 c/w, and THP-1 15.000 c/w. After 72 h, 25 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Serva, Heidelberg, Germany) solution (5 mg/mL in phosphate buffer saline) was added. The formed formazan was dissolved by pipetting up and down after addition of 150 µL isopropanol/concentrated hydrochloric acid (50 mL/165 µL). Subsequently, the absorbance option was measured at 595 nm and 690 nm on the microplate reader ThermoFisher Multiskan FC Microplate Photometer (Thermo Scientific, Wesel, Germany).

4.4. Enzyme HDAC Inhibition Assay

The human recombinant enzymes were ordered from Reaction Biology Corp (Malvern, PA, USA). To determine the inhibitory activity of the enzymes HDAC 2 (cat. no. KDA-21-277), HDAC 4 (cat. nr. KDA-21-279), HDAC 6 (cat. no. KDA-21-213), and HDAC 8 (cat. no. KDA-21-481), 20 ng each of HDAC 2 and 8, 17.5 ng of HDAC 6, and 2 ng of HDAC 4 were used per well. After pipetting 10 µL of increasing concentrations of YAK540 and TMP269 to a 96-well plate (Corning, Kaiserslautern, Germany), recombinant enzymes diluted in assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM KCl, 1 mM MgCl₂, and 1 mg/mL BSA) were added. After five minutes of incubation, the reaction was started with 10 µL of a 30 µM solution of Boc-Lys(Ac)-AMC (Bachem, Bubendorf, Switzerland) for HDAC 2 or 15 µM solution of Boc-Lys(Ac)-AMC for HDAC 6 or a 10 µM solution of Boc-Lys(Tfa)-AMC (Bachem, Bubendorf, Switzerland) for HDAC 4 or a 6 µM solution of Boc-Lys(Tfa)-AMC for HDAC 8. After 90 min, the reaction was stopped by adding 100 µL of stop buffer (16 mg/mL trypsin and 4 µM vorinostat for HDAC 2, 2 µM CHDI00390576 for HDAC 4, 4 µM tubastatin A for HDAC 6 and 4 µM panobinostat for HDAC 8 in 50 mM Tris-HCl, pH 8.0 and 100 mM NaCl). After an additional 15 min, fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm in the NOVOstar microplate reader (BMG LabTech, Offenburg, Germany).

4.5. Whole-Cell HDAC Inhibition Assay

The cellular HDAC assay is based on the publications by Heltweg and Jung [36], Ciossek et al. [37] and Bonfils et al. [38] Minor modifications were still made as described in [39]. In 96-well plates (Corning, Kaiserslautern, Germany), 25,000 c/w THP-1 were seeded in 90 µL culture medium. After 24 h, increasing concentrations of YAK540 and TMP269 were added and incubated for an additional 18 h. Then, the reaction was started by adding 10 µL of 3 mM Boc-Lys(Ac)-AMC or 1 mM Boc-Lys(Tfa)-AMC (Bachem, Bubendorf, Switzerland) to achieve a final concentration of 0.3 mM Boc-Lys(Ac)-AMC or 0.1 mM Boc-Lys(Tfa)-AMC, respectively. After three hours of incubation under culture conditions, 100 µL/well stop buffer (25 mM Tris-HCl, pH 8.0, 127 mM KCl, 1 mM MgCl₂, 1% NP40, 2 mg/mL trypsin, 10 µM panobinostat) was added. After another three hours under culture conditions, fluorescence intensity was measured at an excitation wavelength of 320 nm and an emission wavelength of 520 nm in the NOVOstar microplate reader (BMG LabTech, Offenburg, Germany).

4.6. Measurement of Apoptotic Nuclei

THP-1 cells were seeded at a density of 10.000 c/w in 24-well plates (Sarstedt, Nürnbrecht, Germany). Cells were treated with BTZ and/or HDACI for the indicated time. Supernatant was removed after a centrifugation step and the cells were lysed in 500 µL hypotonic lysis buffer (0.1% TritonX-100, 100 µg/mL propidium iodide) at 4 °C in the dark overnight. Triton X-100 was obtained from AppliChem (Darmstadt, Germany). Propidium iodide was delivered by Santa Cruz Biotechnology (Heidelberg, Germany). The percentage of apoptotic nuclei with DNA content in sub-G1 was analyzed by flow cytometry using the CyFlow instrument (Partec, Norderstedt, Germany).

4.7. Activation of Caspase 3/7

Compound-induced activation of caspase 3/7 was analyzed using the CellEvent Caspase 3/7 green detection reagent (Thermo Scientific, Wesel, Germany) according to the

manufacturer's instructions. Briefly, THP-1 cells were seeded in 96-well-plates (Corning, Kaiserslautern, Germany) at a density of 10,000 c/w. Cells were treated with BTZ and/or HDACI for the indicated time. Then, a solution of CellEvent Caspase 3/7 green detection reagent in cell culture medium supplemented with 5% heat inactivated FCS for a final assay concentration of 10 μ M was added. Cells were incubated for 30 min at 37 °C under cell culture conditions before imaging by using the Thermo Fischer ArrayScan XTI high content screening (HCS) system with a 10 $\times$ magnification (Thermo Scientific, Wesel, Germany). Hoechst 33,342 was used for nuclei staining at a concentration of 10 mg/mL in water (Sigma-Aldrich, Steinheim, Germany).

4.8. Immunoblotting

Protein extraction and Western blot analysis were performed as previously described with minor modifications [34]. Cells were incubated for 24 h or 48 h with the concentrations listed. Cells were centrifuged after the incubation period and the medium was aspirated. Cells were washed with phosphate buffer saline and centrifuged again. After re-aspiration, RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM sodium chloride, 2 mM EDTA, spiked with protease and phosphatase inhibitor (Pierce protease and phosphatase inhibitor mini tablets, Thermo Scientific, Wesel, Germany)) was added and the pellet was lysed for 30 min at 4 °C. Then the protein preparations were boiled at 95 °C with 2x Laemmli buffer containing β -mercaptoethanol for 3 min. Pierce BCA protein assay was used to determine the total protein content in the protein preparations (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (40 μ g, except for mentioned variations) were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany). PageRuler Prestained Ladder, 10 to 180 kDa (Thermo Scientific, Wesel, Germany) was used as a size comparison marker. Blots were incubated with the primary antibodies (supplemental Table S2). The secondary antibodies conjugated to HRP used in the Western blot were supplied by R&D Systems (Minneapolis, MN, USA). Proteins of interest were visualized using Luminol reagent (Santa Cruz Biotechnology, Heidelberg, Germany) on Intas Imager (Intas, Göttingen, Germany).

4.9. RT-PCR

Cells were incubated with the intentional concentrations of BTZ, TMP269, and YAK540 for 48 h. RNA was then isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Subsequently, the RNA was transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Wesel, Germany). For RT-PCR, GoTaq qPCR Master Mix (Promega, Fitchburg, WI, USA) and a CFX96 Real-Time System (BIO-RAD, Hercules, CA, USA) were used. The relative changes in gene expression were normalized to the control genes *GUSB* (beta-glucuronidase), *TBP* (TATA binding protein), and *HPRT1* (hypoxanthine-guanine phosphoribosyltransferase) by Vandesompele method [23]. Primers were designed using Primer-BLAST (NIH, Bethesda, Maryland, USA) and had an efficiency between 80% and 115% (Table 5). Efficiency was determined using an isolated template of HeLa cells.

Table 5. Primer sequences for RT-PCR.

Gene	Primer Forward	Primer Reverse
<i>APAF1</i>	AGTGAATAACTTCGTATGTAAGGA	AAACAACCTGGCCTCTGTGGT
<i>BAD</i>	TTGTGGACTCCTTTAAGAAGG	CACCAGGACTGGAAGACTCG
<i>BAK</i>	TCATCGGGGACGACATCAAC	CAAACAGGCTGGTGGCAATC
<i>BCL2</i>	GATAACGGAGGCTGGGATGC	TCACTTGTGGCCCAGATAGG
<i>Survivin</i>	TGAGAACGAGCCAGACTTGG	TGTTCCCTCTATGGGGTCGTCA
<i>FOXO1</i>	ATGTGTTGCCCAACCAAAGC	AGTGTAACCTGCTCACTAACCC
<i>FOXO3a</i>	AAGGATCACTGAGGAAGGG	GTGTCAGTTTGAGGGTCTGC
<i>GUSB</i>	ACCTCCAAGTATCCCAAGGGT	GTCTTGCTCCACGCTGGT
<i>HDAC4</i>	TTGGATGTCACAGACTCCGC	CCTTCTCGTGCCACAAGTCT
<i>HPRT1</i>	CCTGGCGTCGTGATTAGTGA	CGAGCAAGACGTTTCAGTCCT
<i>MCL1</i>	GTAAACAAAGAGGCTGGGATGG	AGCAGCACATTCCTGATGCC
<i>PTEN</i>	ACTTGCAATCCTCAGTTTGTGG	TCGTGTGGGTCCTGAATTGG
<i>TBP</i>	GTGACCCAGCATCACTGTTTC	GAGCATCTCCAGCACACTCT
<i>p53</i>	AGTCAGATCCTAGCGTCG	TCAGGAAGTAGTTTCCATAGG
<i>Bcl-xL</i>	CAGTAAAGCAAGCGCTGAGG	CCACAAAAGTATCCTGTTCAAAGC
<i>Bim</i>	AGACAGAGCCACAAGCTTCC	CAATACGCCGCAACTCTTGG
<i>p21</i>	TGCCGAAGTCAGTTTCTTGT	GTCTTGACATGGCGCTCC
<i>XIAP</i>	TTGGAAGCCCAGTGAAGACC	CAGATATTTGCACCCTGGATACC

5. Conclusions

We have developed the improved TFMO-based class IIa selective HDACI YAK540 and tested this inhibitor in combination with BTZ for synergy in comparison to the first generation TFMO class IIa HDACI TMP269 to investigate the basis for improved treatment of leukemia, particularly AML. Advantages of YAK540 over TMP269 are simple, straight-forward synthesis; improved solubility; 4-fold less toxic in non-cancer HEK293 cells; 200-fold stronger cellular HDAC activity but yet similar HDAC enzyme activity and class IIa selectivity; more efficacious in inducing caspase-mediated apoptosis; stronger synergy in 2 of 4 examined leukemia cell lines; stronger induction of p21; similar cytotoxic selectivity for leukemia versus HEK293 cells in combination with BTZ. The strong synergy of YAK540 with BTZ may allow reducing currently clinically used doses of BTZ limiting the use of proteasome inhibitors. Synergistic combination treatment of a class IIa HDACI and a proteasome inhibitor would allow reducing severe toxicity of proteasome inhibitors but retaining clinical anticancer efficacy. Based on our promising results, we suggest further investigation of YAK540 in combination with proteasome inhibitors in leukemia, particularly AML.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms232113398/s1>, Figure S1: Concentration-response-curves HEK293; The uncropped western blots are presented in the supplement (Figures S2–S22); Figure S23: Caspase 3/7 fluorescent imaging pictures Table S1: The solvent gradient table for analytic HPLC analysis; Table S2: Listing of primary antibodies for the western blot

Author Contributions: Conceptualization, M.U.K.; formal analysis, L.M.B. and A.J.S.; investigation, L.M.B.; A.J.S.; Y.A.; N.H.; resources, M.U.K.; H.H.; T.K.; writing—original draft preparation, L.M.B.; writing—review and editing, M.U.K. and A.H.; visualization, L.M.B.; supervision, M.U.K. and A.H. and T.K.; project administration, M.U.K.; funding acquisition, M.U.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Deutsche Forschungsgemeinschaft (DFG): grant numbers KA 1942/1-1 (M.U.K.), INST208/690-1 (M.U.K., ThermoFisher Arrayscan XTI High Content Platform), KU 1577/2-1 (T.K.), GRK 2158 (DFG research training group, T.K., M.U.K.).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in this article and the corresponding supplemental information.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. National Cancer Institute. Available online: <https://seer.cancer.gov/statfacts/html/leuks.html> (accessed on 13 June 2022).
2. National Cancer Institute. Available online: <https://seer.cancer.gov/statfacts/html/amyl.html> (accessed on 13 June 2022).
3. National Cancer Institute. Available online: <https://seer.cancer.gov/statfacts/html/cmly.html> (accessed on 13 June 2022).
4. Howlader, N.; Noone, A.M.; Krapcho, M.; Miller, D.; Brest, A.; Yu, M.; Ruhl, J.; Tatalovich, Z.; Mariotto, A.; Lewis, D.R.; et al. (Eds.) *SEER Cancer Statistics Review 1975–2018*; National Cancer Institute: Bethesda, MD, USA, 2021; Available online: https://seer.cancer.gov/csr/1975_2018/ (accessed on 13 June 2022).
5. European Medicine Agency. Available online: https://www.ema.europa.eu/en/documents/product-information/velcade-epar-product-information_de.pdf (accessed on 13 June 2022).
6. Csizmar, C.M.; Kim, D.-H.; Sachs, Z. The Role of the Proteasome in AML. *Blood Cancer J.* **2016**, *6*, e503. [CrossRef]
7. Accardi, F.; Toscani, D.; Bolzoni, M.; Dalla Palma, B.; Aversa, F.; Giuliani, N. Mechanism of Action of Bortezomib and the New Proteasome Inhibitors on Myeloma Cells and the Bone Microenvironment: Impact on Myeloma-Induced Alterations of Bone Remodeling. *BioMed Res. Int.* **2015**, *2015*, 172458. [CrossRef] [PubMed]
8. Meregalli, C. An Overview of Bortezomib-Induced Neurotoxicity. *Toxics* **2015**, *3*, 294–303. [CrossRef] [PubMed]
9. Robak, P.; Robak, T. Bortezomib for the Treatment of Hematologic Malignancies: 15 Years Later. *Drugs R&D* **2019**, *19*, 73–92. [CrossRef]
10. Roeten, M.S.F.; van Meerloo, J.; Kwidama, Z.J.; Ter Huizen, G.; Segerink, W.H.; Zweegman, S.; Kaspers, G.J.L.; Jansen, G.; Cloos, J. Pre-Clinical Evaluation of the Proteasome Inhibitor Ixazomib against Bortezomib-Resistant Leukemia Cells and Primary Acute Leukemia Cells. *Cells* **2021**, *10*, 665. [CrossRef] [PubMed]
11. Kupperman, E.; Lee, E.C.; Cao, Y.; Bannerman, B.; Fitzgerald, M.; Berger, A.; Yu, J.; Yang, Y.; Hales, P.; Bruzzese, F.; et al. Evaluation of the Proteasome Inhibitor MLN9708 in Preclinical Models of Human Cancer. *Cancer Res.* **2010**, *70*, 1970–1980. [CrossRef]
12. Richardson, P.G.; Baz, R.; Wang, M.; Jakubowiak, A.J.; Laubach, J.P.; Harvey, R.D.; Talpaz, M.; Berg, D.; Liu, G.; Yu, J.; et al. Phase 1 study of twice-weekly ixazomib, an oral proteasome inhibitor, in relapsed/refractory multiple myeloma patients. *Blood* **2014**, *124*, 1038–1047. [CrossRef]
13. Moreau, P.; Karamanesh, I.I.; Domnikova, N.; Kyselyova, M.Y.; Vilchevska, K.V.; Doronin, V.A.; Schmidt, A.; Hulin, C.; Leleu, X.; Esseltine, D.L.; et al. Pharmacokinetic, pharmacodynamic and covariate analysis of subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma. *Clin. Pharmacokinet.* **2012**, *51*, 823–829. [CrossRef]
14. Kikuchi, S.; Suzuki, R.; Ohguchi, H.; Yoshida, Y.; Lu, D.; Cottini, F.; Jakubikova, J.; Bianchi, G.; Harada, T.; Gorgun, G.; et al. Class IIa HDAC Inhibition Enhances ER Stress-Mediated Cell Death in Multiple Myeloma. *Leukemia* **2015**, *29*, 1918–1927. [CrossRef]
15. Eckschlager, T.; Plch, J.; Stiborova, M.; Hrabeta, J. Histone Deacetylase Inhibitors as Anticancer Drugs. *Int. J. Mol. Sci.* **2017**, *18*, 1414. [CrossRef]
16. Harada, T.; Hideshima, T.; Anderson, K.C. Histone deacetylase inhibitors in multiple myeloma: From bench to bedside. *Int. J. Hematol.* **2016**, *104*, 300–309. [CrossRef] [PubMed]
17. AdooQ Bioscience. Available online: <https://www.adooq.com/datasheet?A14128> (accessed on 5 July 2022).
18. Turkman, N.; Liu, D.; Pirola, I. Design, synthesis, biochemical evaluation, radiolabeling and in vivo imaging with high affinity class-IIa histone deacetylase inhibitor for molecular imaging and targeted therapy. *Eur. J. Med. Chem.* **2021**, *228*, 114011. [CrossRef] [PubMed]
19. Stott, A.J.; Maillard, M.C.; Beaumont, V.; Allcock, D.; Aziz, O.; Borchers, A.H.; Blackaby, W.; Breccia, P.; Creighton-Gutteridge, G.; Haughan, A.F.; et al. Evaluation of 5-(Trifluoromethyl)-1,2,4-oxadiazole-Based Class IIa HDAC Inhibitors for Huntington’s Disease. *ACS Med. Chem. Lett.* **2021**, *12*, 380–388. [CrossRef] [PubMed]
20. Asfaha, Y.; Schrenk, C.; Alves Avelar, L.A.; Hamacher, A.; Pflieger, M.; Kassack, M.U.; Kurz, T. Recent advances in class IIa histone deacetylases research. *Bioorganic Med. Chem.* **2019**, *27*, 115087. [CrossRef]
21. Lobera, M.; Madauss, K.P.; Pohlhaus, D.T.; Wright, Q.; Trocha, M.; Schmidt, D.R.; Baloglu, E.; Trump, R.P.; Head, M.S.; A Hofmann, G.; et al. Selective class IIa histone deacetylase inhibition via a nonchelating zinc-binding group. *Nat. Chem. Biol.* **2013**, *9*, 319–325. [CrossRef]
22. Di Giorgio, E.; Gagliostro, E.; Brancolini, C. Selective class IIa HDAC inhibitors: Myth or reality. *Cell. Mol. Life Sci.* **2015**, *72*, 73–86. [CrossRef]
23. Bradner, J.E.; West, N.; Grachan, M.L.; Greenberg, E.F.; Haggarty, S.J.; Warnow, T.; Mazitschek, R. Chemical phylogenetics of histone deacetylases. *Nat. Chem. Biol.* **2010**, *6*, 238–243. [CrossRef]
24. Chou, T.-C.; Talalay, P. Quantitative Analysis of Dose-Effect Relationships: The Combined Effects of Multiple Drugs or Enzyme Inhibitors. *Adv. Enzym. Regul.* **1984**, *22*, 27–55. [CrossRef]
25. Di Veroli, G.Y.; Fornari, C.; Wang, D.; Mollard, S.; Bramhall, J.L.; Richards, F.M.; Jodrell, D.I. CombeneFit: An interactive platform for the analysis and visualisation of drug combinations. *Bioinformatics* **2016**, *32*, 2866–2868. [CrossRef]
26. Zhao, W.; Sachsenmeier, K.; Zhang, L.; Sult, E.; Hollingsworth, R.E.; Yang, H. A New Bliss Independence Model to Analyze Drug Combination Data. *J. Biomol. Screen.* **2014**, *19*, 817–821. [CrossRef]

27. Vandesompele, J.; De Preter, K.; Pattyn, F.; Poppe, B.; Van Roy, N.; De Paepe, A.; Speleman, F. Accurate Normalization of Real-Time Quantitative RT-PCR Data by Geometric Averaging of Multiple Internal Control Genes. *Genome Biol.* **2002**, *3*, RESEARCH0034. [CrossRef] [PubMed]
28. van Dijk, A.D.; Hoff, F.W.; Qiu, Y.; Gerbing, R.B.; Gamis, A.S.; Aplenc, R.; Kolb, E.A.; Alonzo, T.A.; Meshinchi, S.; Jenkins, G.N.; et al. Bortezomib is significantly beneficial for de novo pediatric AML patients with low phosphorylation of the NF- κ B subunit RelA. *Proteom. Clin. Appl.* **2022**, *16*, 2100072. [CrossRef] [PubMed]
29. Hoff, F.W.; van Dijk, A.D.; Qiu, Y.H.; Ruvolo, P.P.; Gerbing, R.B.; Leonti, A.R.; Jenkins, G.N.; Gamis, A.S.; Aplenc, R.; Kolb, E.A.; et al. Heat shock factor 1 (HSF1-pSer326) predicts response to bortezomib-containing chemotherapy in pediatric AML: A COG report. *Blood* **2021**, *137*, 1050–1060. [CrossRef]
30. San José-Enériz, E.; Gimenez-Camino, N.; Agirre, X.; Prosper, F. HDAC Inhibitors in Acute Myeloid Leukemia. *Cancers* **2019**, *11*, 1794. [CrossRef]
31. Asfaha, Y. Development of Class IIa Histone Deacetylase Inhibitors. Ph.D. Thesis, Universitäts- und Landesbibliothek, Düsseldorf, Germany, 2020.
32. Gui, C.Y.; Ngo, L.; Xu, W.S.; Richon, V.M.; Marks, P.A. Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 1241–1246. [CrossRef]
33. Journal Onkologie. Available online: https://www.journalonko.de/downloads/herunterladen/fi_velcade_jan2014 (accessed on 8 July 2022).
34. Wang, C.; Hamacher, A.; Petzsch, P.; Köhrer, K.; Niegisch, G.; Hoffmann, M.J.; Schulz, W.A.; Kassack, M.U. Combination of Decitabine and Entinostat Synergistically Inhibits Urothelial Bladder Cancer Cells via Activation of FoxO1. *Cancers* **2020**, *12*, 337. [CrossRef]
35. Engelke, L.H.; Hamacher, A.; Proksch, P.; Kassack, M.U. Ellagic Acid and Resveratrol Prevent the Development of Cisplatin Resistance in the Epithelial Ovarian Cancer Cell Line A2780. *J. Cancer* **2016**, *7*, 353–363. [CrossRef]
36. Heltweg, B.; Jung, M. A Microplate Reader-Based Nonisotopic Histone Deacetylase Activity Assay. *Anal. Biochem.* **2002**, *302*, 175–183. [CrossRef] [PubMed]
37. Ciossek, T.; Julius, H.; Wieland, H.; Maier, T.; Beckers, T. A Homogeneous Cellular Histone Deacetylase Assay Suitable for Compound Profiling and Robotic Screening. *Anal. Biochem.* **2008**, *372*, 72–81. [CrossRef]
38. Bonfils, C.; Kalita, A.; Dubay, M.; Siu, L.L.; Carducci, M.A.; Reid, G.; Martell, R.E.; Besterman, J.M.; Li, Z. Evaluation of the Pharmacodynamic Effects of MGCD0103 from Preclinical Models to Human Using a Novel HDAC Enzyme Assay. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **2008**, *14*, 3441–3449. [CrossRef]
39. Marek, L.; Hamacher, A.; Hansen, F.K.; Kuna, K.; Gohlke, H.; Kassack, M.U.; Kurz, T. Histone Deacetylase (HDAC) Inhibitors with a Novel Connecting Unit Linker Region Reveal a Selectivity Profile for HDAC4 and HDAC5 with Improved Activity against Chemoresistant Cancer Cells. *J. Med. Chem.* **2013**, *56*, 427–436. [CrossRef] [PubMed]

The novel Class IIa Selective Histone Deacetylase Inhibitor YAK540 is synergistic with Bortezomib in Leukemia Cell Lines

Supplemental Information

Experimental data

General methods: All chemicals and solvents were purchased from commercial suppliers (Sigma Aldrich, Alfa Aesar, Fluorochem, TCI, abcr and Acros Organics) and used without further purification. All anhydrous reactions were carried out in flame-dried Schlenk-flasks and under argon atmosphere. Dry solvents were used directly from Seal® bottles from Acros Organics. Analytic Thin Layer Chromatography (TLC) was carried out with Macherey Nagel precoated silica gel plates (ALUGRAM® Xtra SIL G/UV₂₅₄). Detection was achieved with ultraviolet irradiation (254 nm) and/or staining with potassium permanganate solution (9 g KMnO₄, 60 g K₂CO₃, 15 mL of a 5% aqueous NaOH-solution, and 900 mL demineralised water). Flash column chromatography was performed with CombiFlashRf200 (TeleDyneIsco) with the solvent mixtures specified in the corresponding procedure. **Physical data:** Proton (¹H) and carbon (¹³C) NMR spectra were recorded on Bruker Avance III – 300, Bruker Avance DRX – 500 or Bruker Avance III – 600. Spectra were referenced to the residual non-deuterated solvent signal (¹H-NMR: DMSO-*d*₆ (2.50 ppm), ¹³C-NMR: DMSO-*d*₆ (39.52 ppm); ¹H-NMR: CDCl₃ (7.26 ppm), ¹³C-NMR: CDCl₃ (77.16 ppm)). Chemical shifts are quoted in parts per million (ppm). Signal patterns are indicated as: singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). Coupling constants, *J*, are measured in Hz. Proton (¹H) and carbon (¹³C) NMR spectra were recorded by the NMR-Divisions of the Department of Chemistry (Heinrich Heine University Duesseldorf). Electrospray Ionisation (ESI) mass spectra were carried out by the Mass spectrometry-Division of the Heinrich Heine University Duesseldorf, using Bruker Daltonics UHR-QTOF maXis 4G (Bruker Daltonics). APCI-MS was carried out using Advion expression¹ CMS spectrometer. Melting points (mp.) were determined using a Büchi M-565 melting point apparatus and are uncorrected. Analytical HPLC analysis were carried out on a Knauer HPLC system comprising an Azura P 6.1L pump, an Optimas 800 autosampler, a Fast Scanning Spectro-Photometer K-2600 and a Knauer Reversed Phase column (SN: FK36). UV absorption was detected at 254 nm. The solvent gradient table is shown below (Table S1). The purity of all final compounds was 95% or higher.

Table S1. The solvent gradient table for analytic HPLC analysis.

Time / min	Water + 0.1% TFA	ACN + 0.1% TFA
Initial	90	10
0.50	90	10
20.0	0	100
30.0	0	100
31.0	90	10
40.0	90	10

Synthesis of 2-(2-(piperidin-1-yl)ethoxy)isoindoline-1,3-dione (2)

Following the procedure of Asfaha et al.,^[3] 1.00 eq of the respective halide derivative **1** and 1.20 eq *N*-hydroxyphthalimide (NHPI) were dissolved in acetonitrile (4 mL/mmol). After the addition of 2.00 eq triethylamine, the resulting solution was refluxed for 12 h. The solvent was evaporated under reduced pressure and the residue was diluted with EtOAc, washed with saturated NaHCO₃ solution (7x 50 mL),

dried over Na₂SO₄ and filtered. The crude product was purified by flash chromatography (n hexane/EtOAc/0.1% NEt₃) to afford **2**. Yellow solid, yield: 52%, mp: 91.1 °C. ¹H NMR (300 MHz, DMSO-d₆) δ = 1.18 – 1.31 (m, 6H), 2.27 (d, J = 4.6 Hz, 4H), 2.57 – 2.68 (m, 2H), 4.19 – 4.28 (m, 2H), 7.86 (s, 4H) ppm. ¹³C NMR (75 MHz, DMSO-d₆) δ = 23.67, 25.23, 53.90, 57.03, 73.48, 123.01, 128.66, 134.56, 163.00 ppm.

HPLC analysis: Rt = 6.291 min, 92%. HRMS (ESI+) = calcd. for C₁₆H₂₃N₂O₄ [M+H]⁺ = 307.1652, found: 307.1653.

Synthesis of O-(2-(piperidin-1-yl)ethyl)hydroxylamine (**3**)

To a solution of the respective NHPI-derivative **2** (1.00 eq) in CH₂Cl₂ (20 mL/mmol), 2.00 eq hydrazine monohydrate was added dropwise. The reaction mixture was stirred overnight at rt. The resulting precipitate was filtered, and the organic layer was washed with saturated NaHCO₃ solution (3x 10 mL). After drying over Na₂SO₄ and filtration, the solvent was removed *in vacuo* providing the hydroxylamine **3**. The hydroxylamine **3** was then used directly for the next step without any further purification.

Synthesis of 4-(N'-hydroxycarbamimidoyl)benzoic acid: Following the procedure of Lobera et al.,^[1] 1.00 eq 4-cyanobenzoic acid and 0.4 mol% 8-hydroxyquinoline were dissolved in EtOH (10 mL/mmol). To this reaction mixture 2.00 eq hydroxylamine hydrochloride in water (2.0 mL/mmol) and 1.60 eq potassium carbonate in water (1 mL/mmol) were added. The mixture was stirred at reflux for 6 h. The solvent was then removed under reduced pressure. The residue was diluted with water and the aqueous phase was acidified to pH = 3. The formed precipitate was then filtered, washed with water and acetone to furnish the corresponding amidoxime. White solid, yield: 86%. All spectroscopic data were in agreement with the literature.^[1]

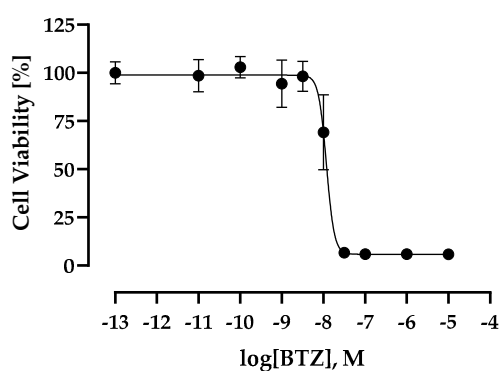
Synthesis of 4-(5-(Trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (4**):** A solution of corresponding amidoxime (1.00 eq) in anhydrous toluene (1 mmol/mL) was cooled to 0 °C and 3.00 eq trifluoroacetic anhydride was added dropwise. The reaction mixture was slowly allowed to warm to rt and then refluxed further for 8 h. The solvent was removed *in vacuo*, and the crude solid was diluted with ethyl acetate. The organic layer was washed with brine (3x25 mL), dried over Na₂SO₄, filtered and the solvent was then removed under reduced pressure. The crude product was purified by recrystallisation (ethyl acetate/ *n*-hexane) to yield the desired product **4**. White solid, yield: 60%, mp: 251 °C. ¹H NMR (300 MHz, DMSO-d₆) δ = 8.08 – 8.24 (m, 4H), 13.38 (s, 1H) ppm. ¹³C NMR (75 MHz, DMSO) δ = 115.65 (q), 127.56, 128.04, 130.20, 134.20, 165.47, 165.24 (q), 167.86 ppm. HPLC: t_R = 12.483 min, AUC = 95.8%.

Synthesis of YAK 540

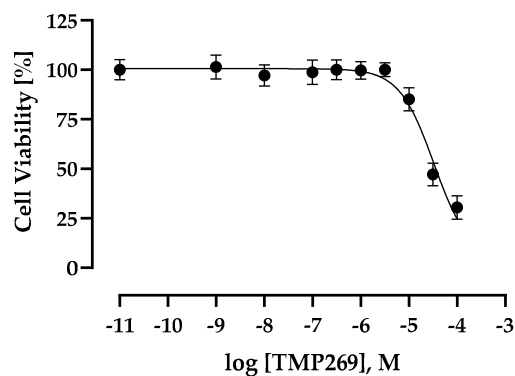
For the synthesis of **YAK 540**, 1.00 eq of the corresponding carboxylic acid **4** was dissolved in dry DMF (0.1 mmol/mL) and 1.00 eq HATU and 2.00 eq DIPEA were added. After stirring for 15 min at rt, the respective hydroxylamine **3** (1.00 eq) was then added and the resulting mixture was stirred for further 16h at rt. The solvent was removed *in vacuo* and the residue was diluted with EtOAc. The organic layer was washed with saturated NaHCO₃ (3x 50 mL), brine (1x 50 mL), dried over Na₂SO₄ and filtered. After removing the solvent, the crude product was purified as stated. The crude product was purified by flash chromatography (CH₂Cl₂/30% MeOH in CH₂Cl₂/ 0.1% NEt₃) to furnish **YAK 540**. Yellowish solid. yield: 54%, mp.: 131 °C.

¹H NMR (300 MHz, DMSO-d₆) δ = 1.41 (q, J = 6.0 Hz, 2H), 1.55 (p, J = 5.5 Hz, 4H), 2.57 (t, J = 5.3 Hz, 4H), 2.71 (t, J = 5.5 Hz, 2H), 4.06 (t, J = 5.5 Hz, 2H), 7.95 – 8.01 (m, 2H), 8.12 – 8.20 (m, 2H) ppm. ¹³C NMR (75 MHz, DMSO-d₆) δ = 23.39, 24.95, 53.82, 56.02, 72.27, 115.70 (d, J = 273.2 Hz), 126.91, 127.47, 128.18, 135.88, 163.10, 165.18 (d, J = 43.7 Hz), 167.86 ppm. HPLC analysis: Rt = 10.321 min, 96.2%. HRMS (ESI+) = calcd. for C₁₇H₂₀F₃N₄O₃ [M+H]⁺ = 385.1482, found: 385.1482.

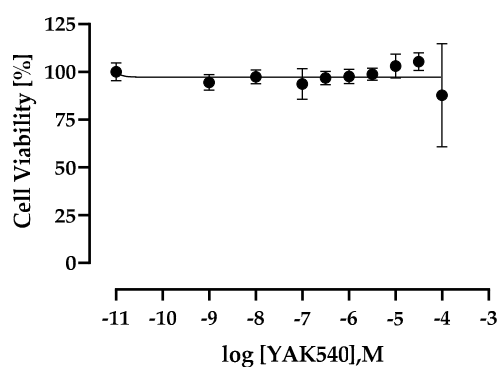
Concentration-response-curves HEK293



(a)



(b)



(c)

Figure S1. (a)–(c) Concentration-response-curves of HEK293. The data shown are from an $n \geq 8$ from at least 3 independent experiments.

Western Blot:

Table S2. Listing of primary antibodies for the western blot

Protein of interest	expected size (kDa)	Primary antibody (company, Catalog nr.)
Survivin	16	R&D systems, AF886
APAF-1	130-140	R&D systems, MAB868
PARP	116	R&D systems, AF-600-NA
HDAC 4	140	R&D systems, AF6205
Beta Aktin	45	Cell Signaling Technologie, 13E5
BAD	22	R&D systems, AF819
p21	18	Cell Signaling Technology #2947
Phospho-Histone H2AX	15	R&D systems, AF2288
Bcl-2	25	R&D systems, AF810

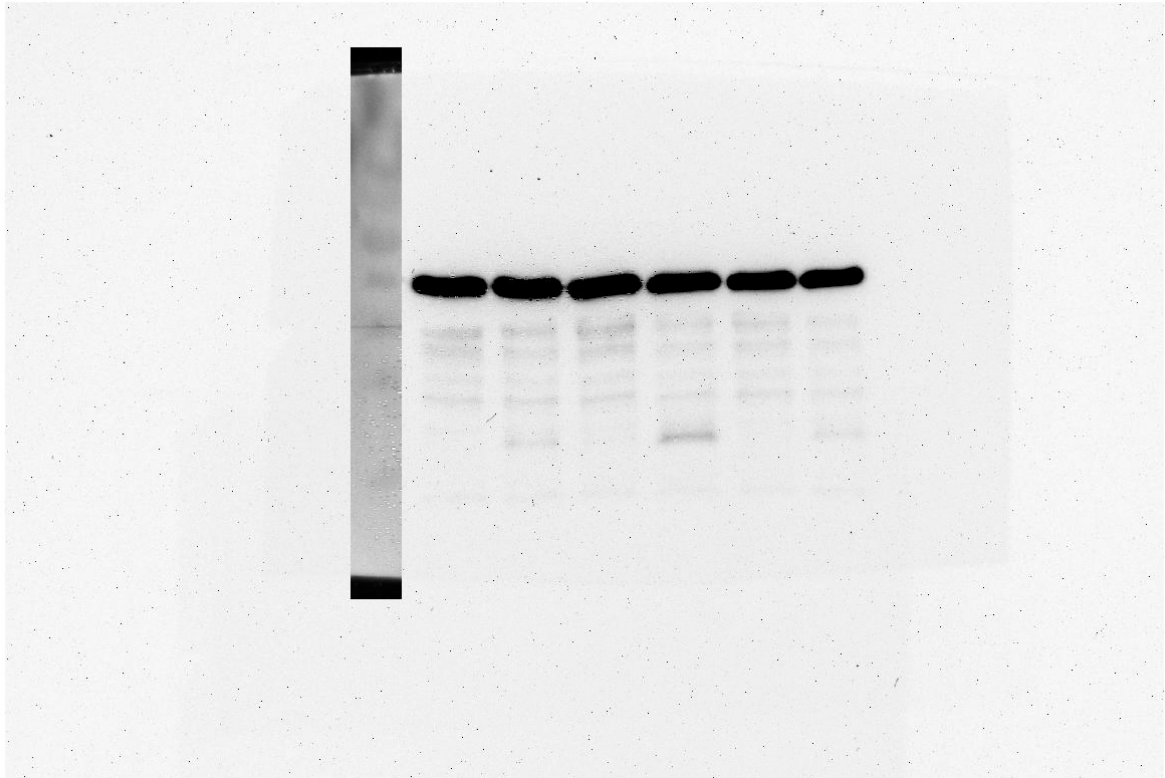
Uncropped western blots:

HL-60 General Order of samples (24 h, 48 h incubation):

1. control
2. 10 nM bortezomib
3. 10 μ M YAK540
4. 10 nM bortezomib + 10 μ M YAK540
5. 5 μ M TMP269
6. 10 nM bortezomib + 5 μ M TMP269

8 min mit leiter.tif

24-MAR-2022



Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2022-03-24 12:15:59

Palette: inverted Grayscale
Range: 1342 - 2556, Gamma: 1.000

Figure S2. Uncropped western blot, HL-60, blot shows β -Aktin and p21, 24 h incubation.

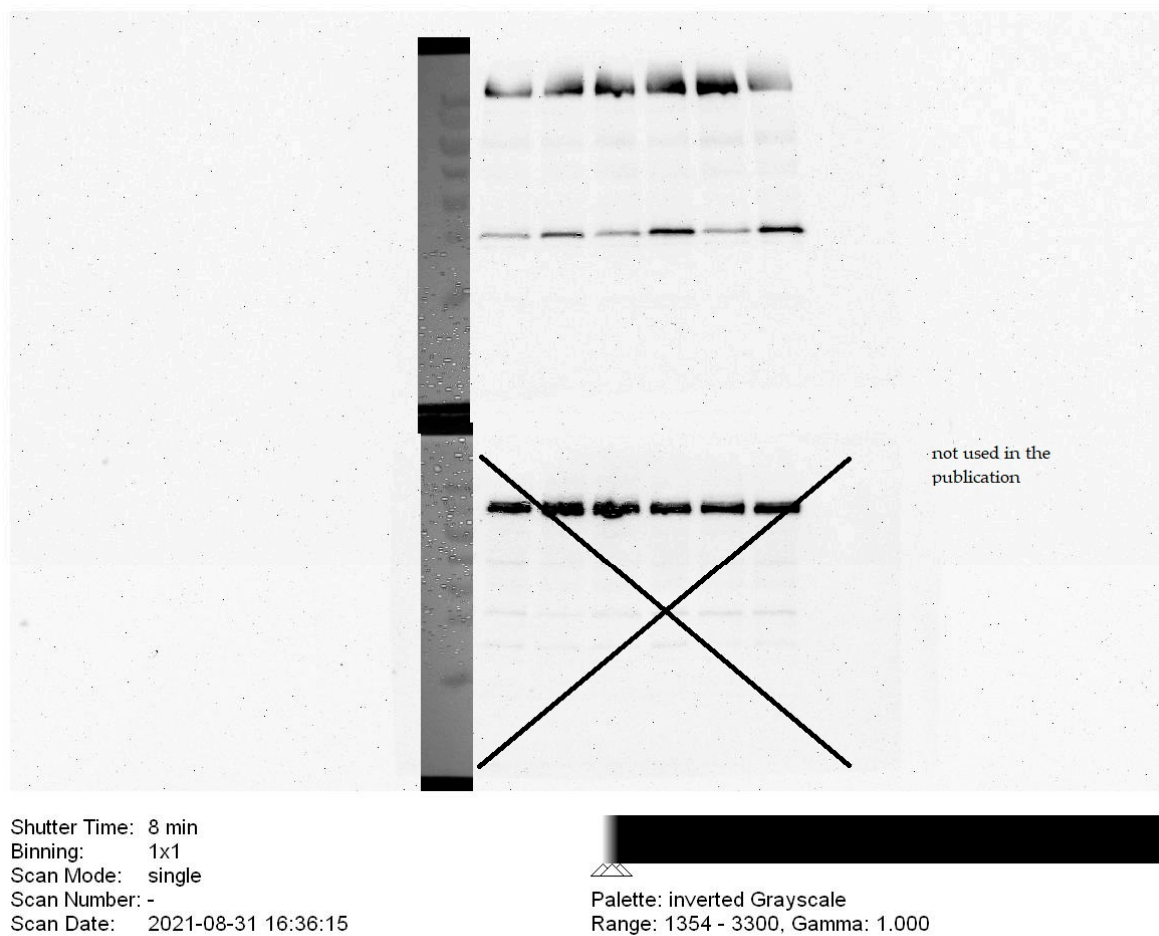
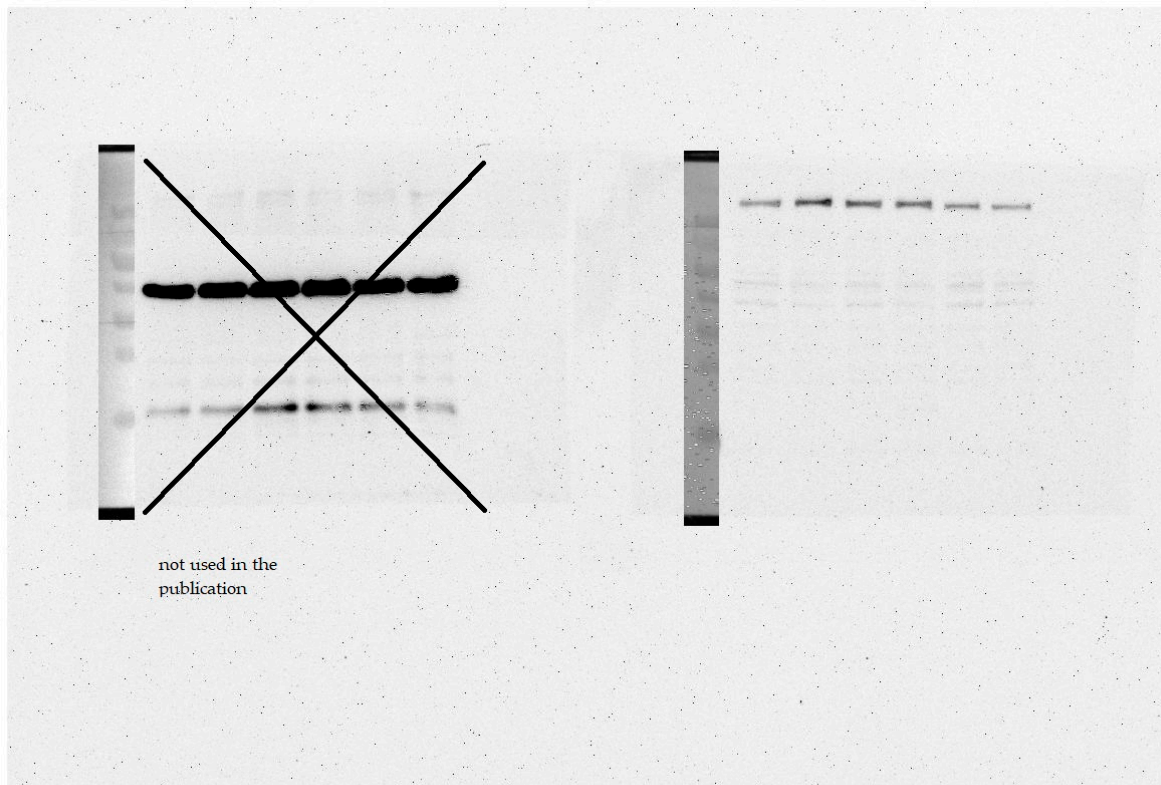


Figure S3. Uncropped western blot HL-60 blot at top shwos PARP, 24 h incubation.



Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2021-08-31 15:15:49

Palette: inverted Grayscale
Range: 1358 - 2269, Gamma: 1.000

Figure S4. Uncropped western blot, HL-60, right blot shows at top HDAC 4, 24 h incubation.

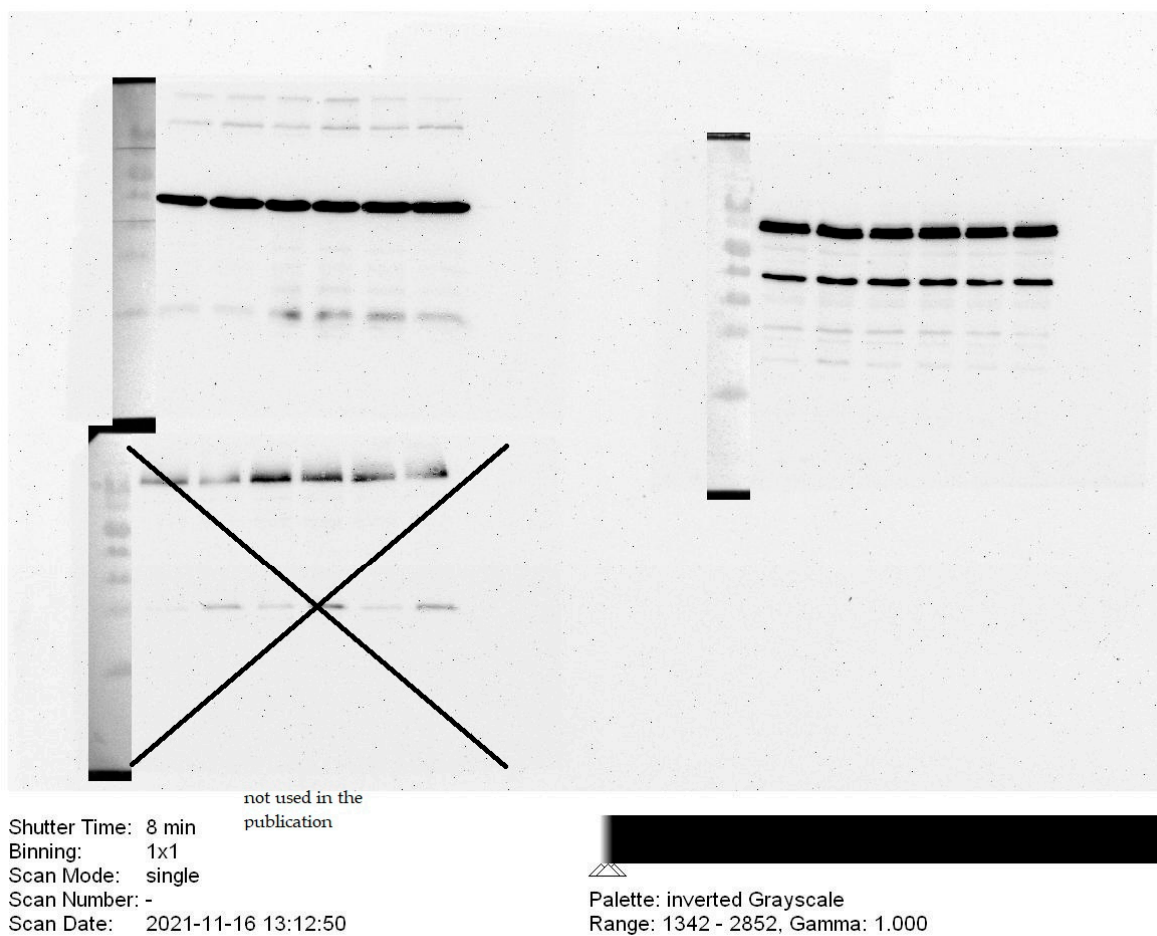


Figure S5. Uncropped western blot, HL-60, blot top left shows APAF-1 and Survivin, right blot illustrate Bad, 24 h incubation.

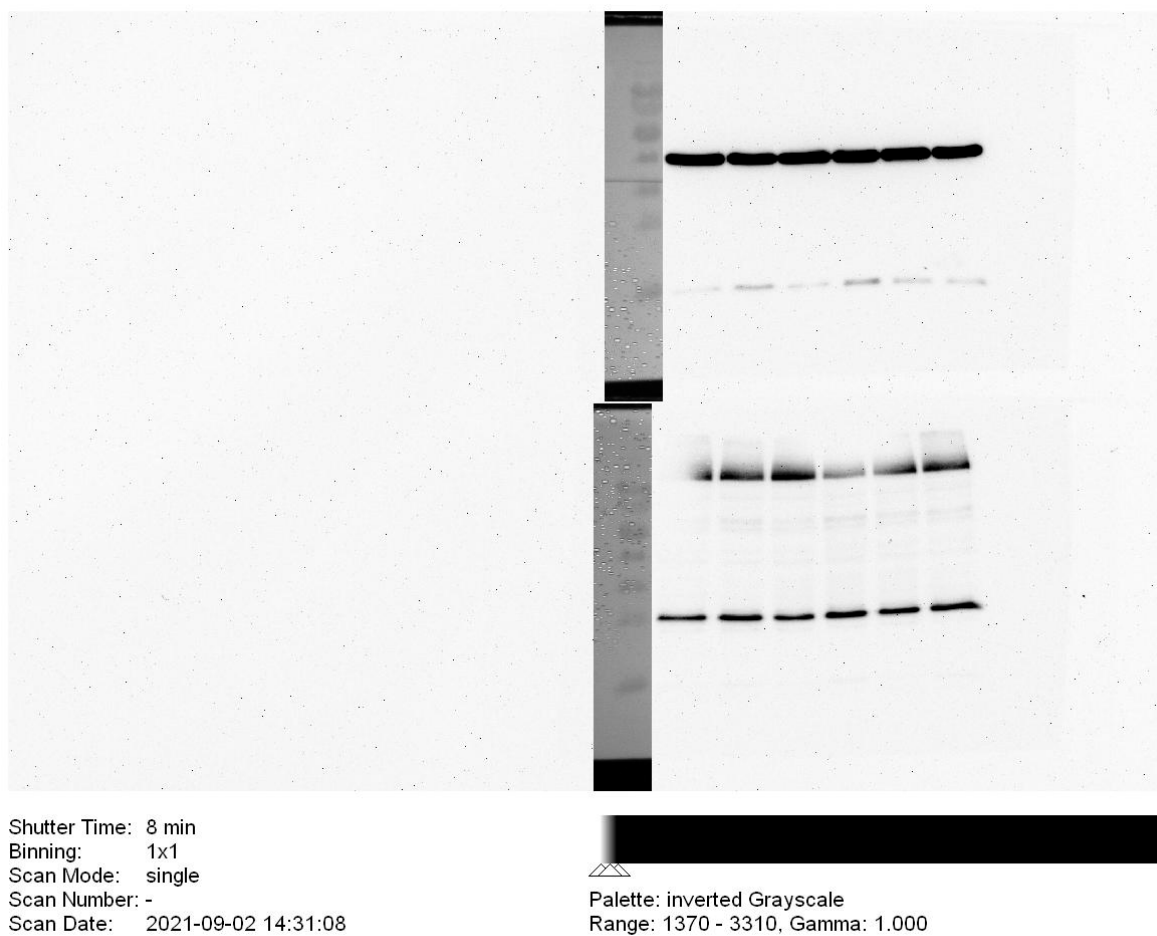
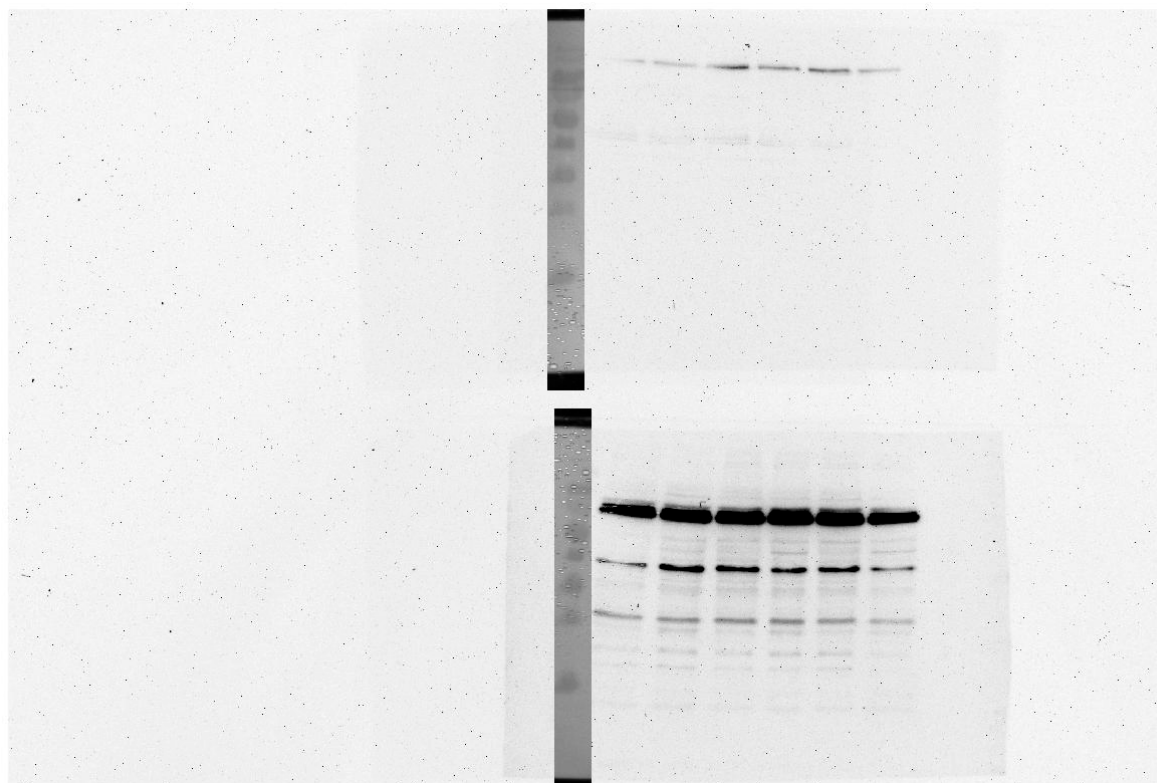


Figure S6. Uncropped western blot, HL-60, blot at top shows β -Actin and γ -H2AX, the blot below illustrate PARP, 48 h incubation.



Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2021-09-01 15:40:25

Palette: inverted Grayscale
Range: 1342 - 2419, Gamma: 1.000

Figure S7. Uncropped western blot, HL-60, blot at top shows HDAC 4, blot below illustrate Bad, 48 h incubation.

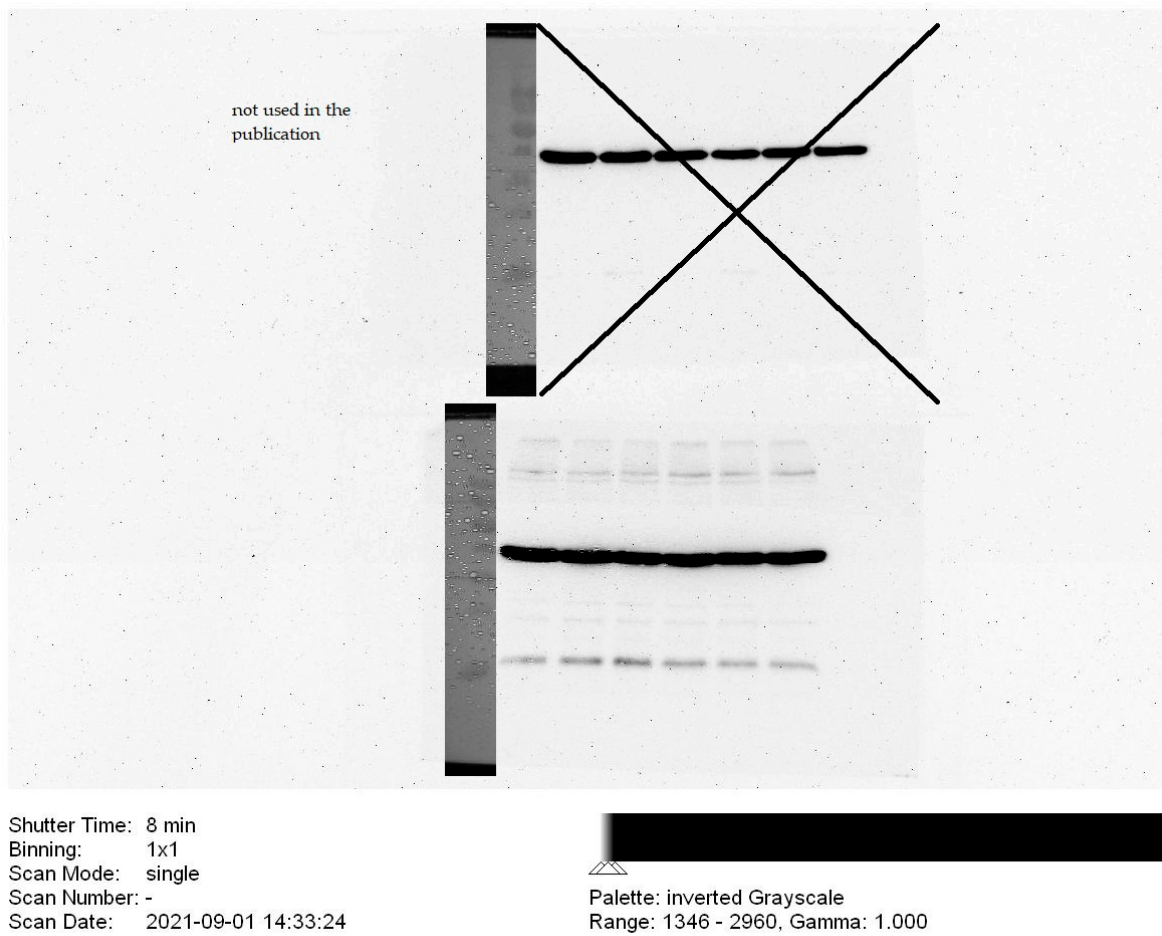
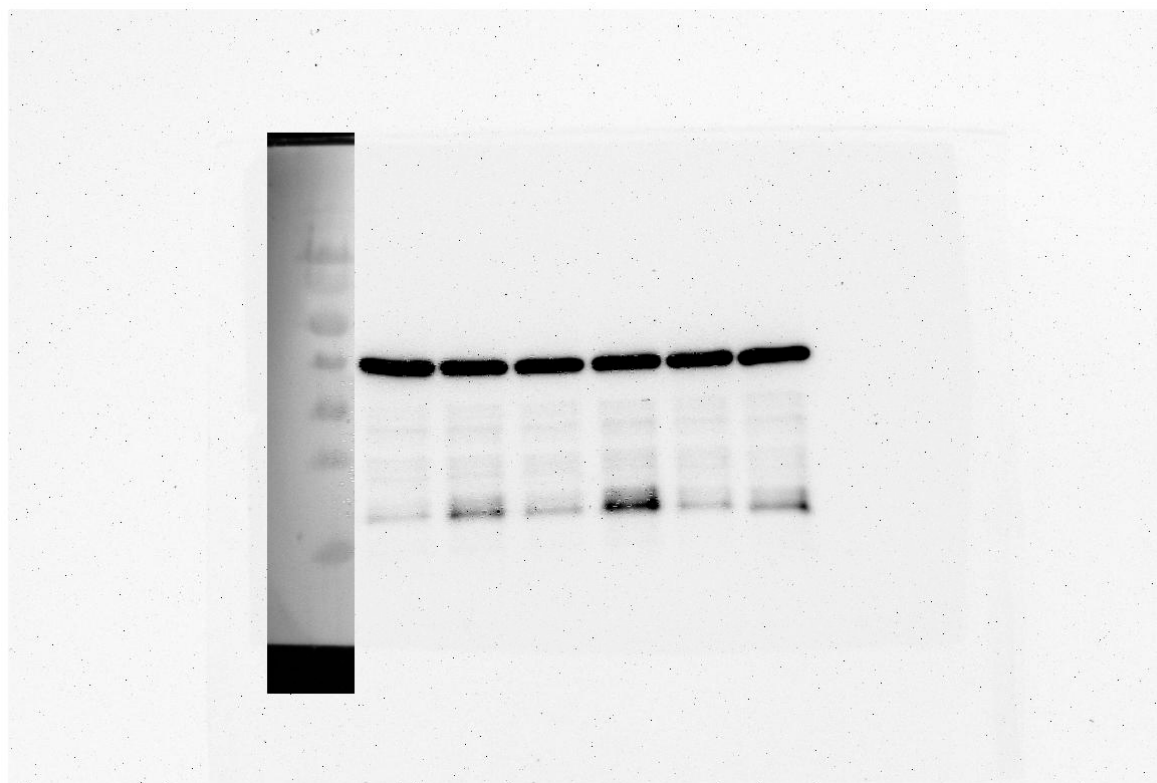


Figure S8. Uncropped western blot, HL-60 lower blot shows APAF-1 and Survivin, 48 h incubation.

K562 General Order of samples (24 h, 48 h incubation):

1. Control
2. 25 nM bortezomib
3. 10 μ M YAK540
4. 10 μ M YAK540 + 10 nM bortezomib
5. 5 μ M TMP269
6. 25 nM bortezomib + 5 μ M TMP269

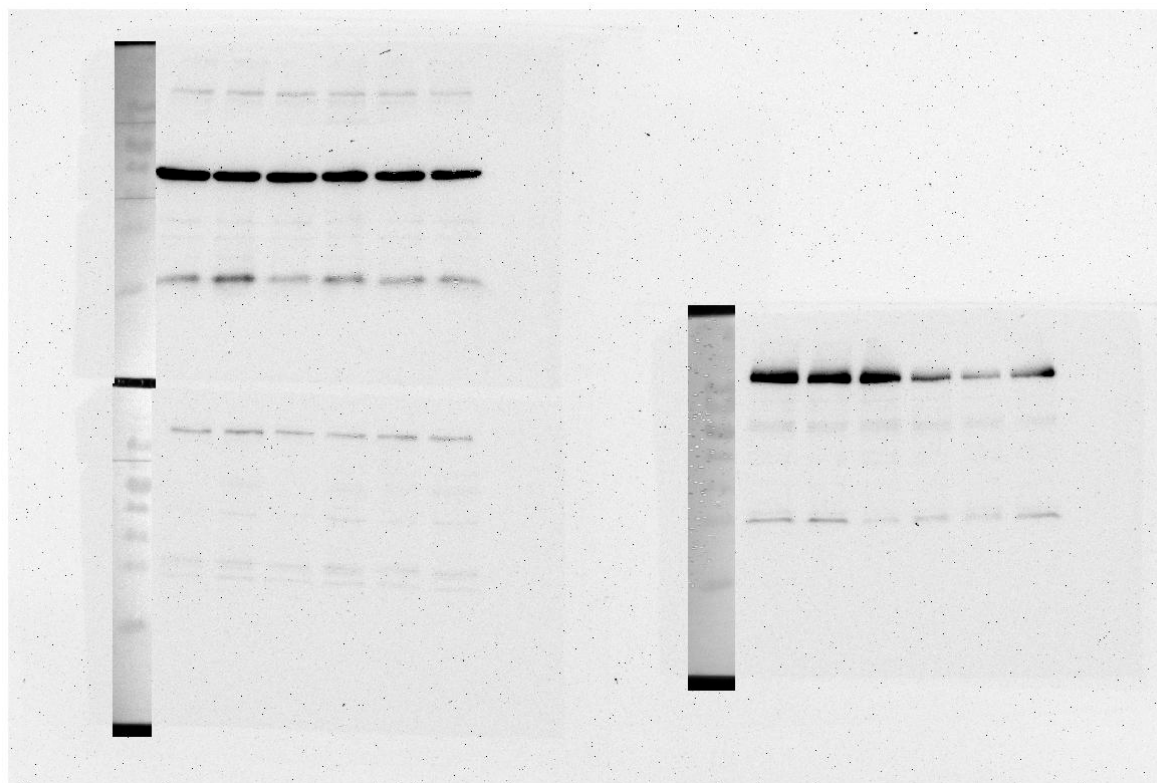


Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2022-03-15 15:09:56



Palette: inverted Grayscale
Range: 1353 - 2922, Gamma: 1.000

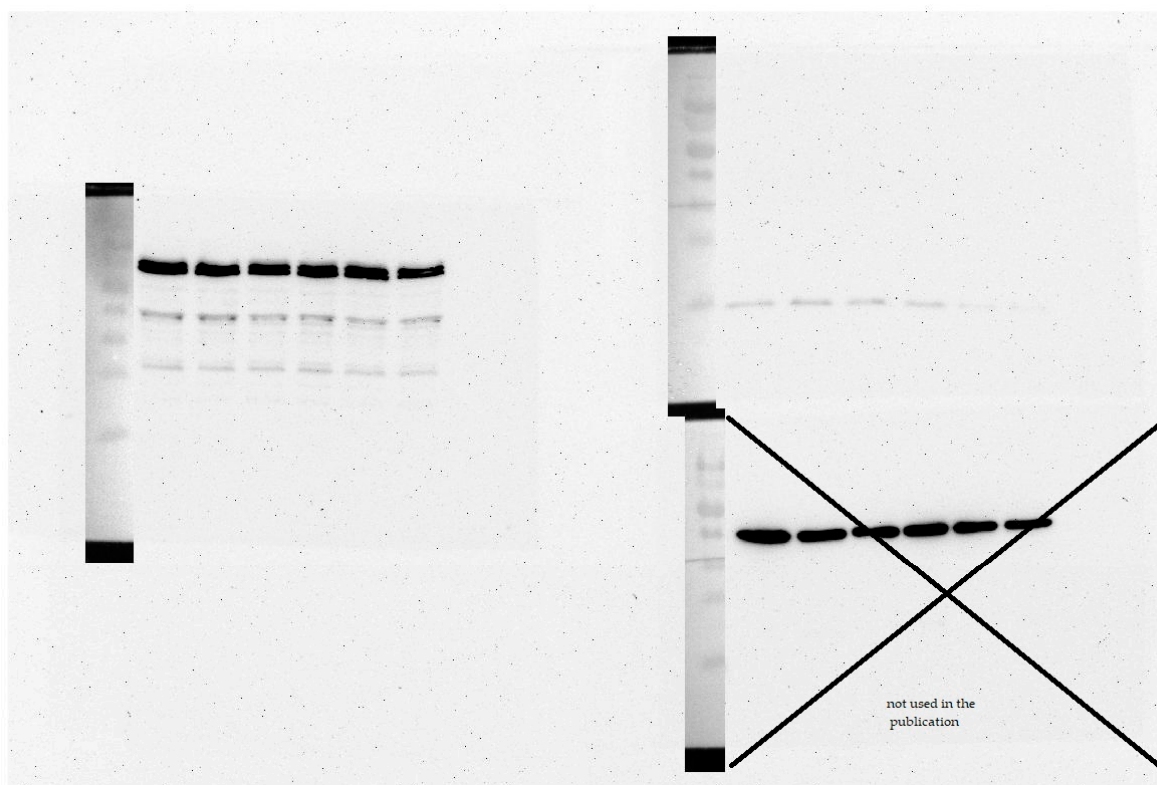
Figure S9. Uncropped western blot, K562, the blot shows β -Actin and p21, 24 h incubation.



Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2021-10-05 13:04:07

Palette: inverted Grayscale
Range: 1349 - 2270, Gamma: 1.000

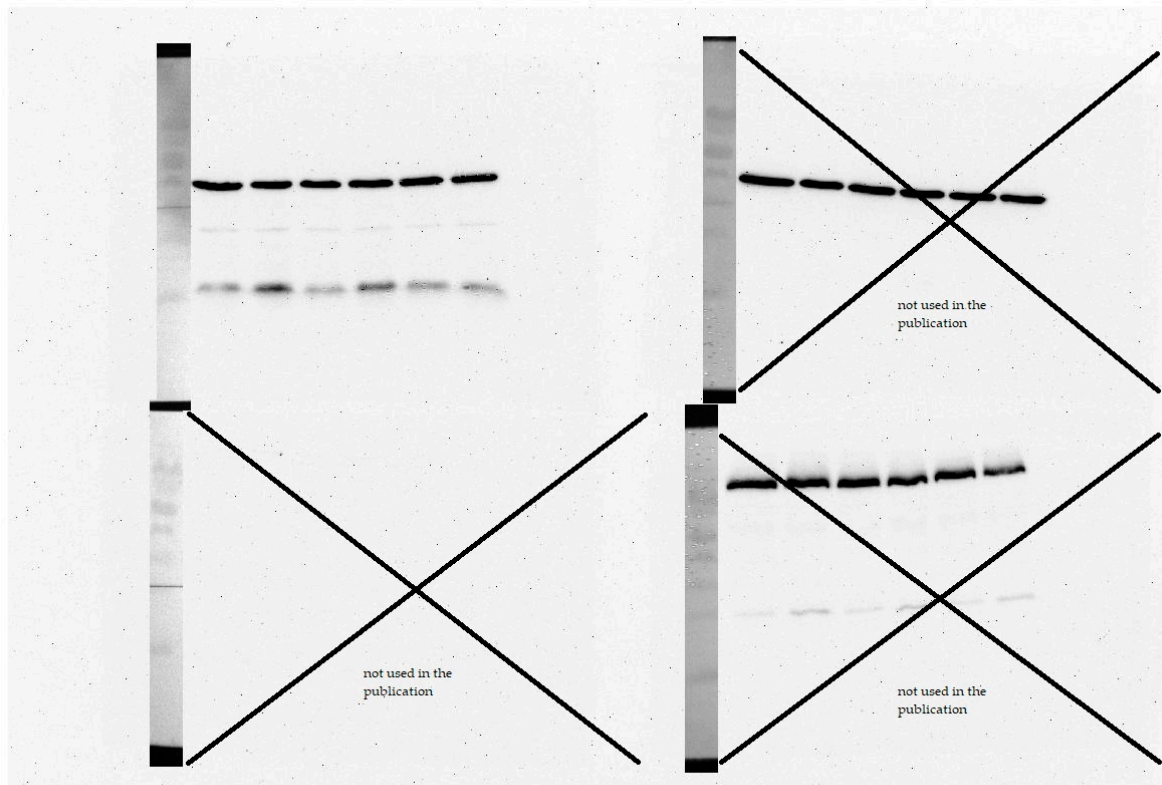
Figure S10. Uncropped western blot, K562 blot on the top left shows APAF-1, the blot below illustrate HDAC 4 and Bcl-2, the blot on the right side displays PARP, 24 h incubation.



Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2021-10-05 14:16:21

Palette: inverted Grayscale
Range: 1359 - 2614, Gamma: 1.000

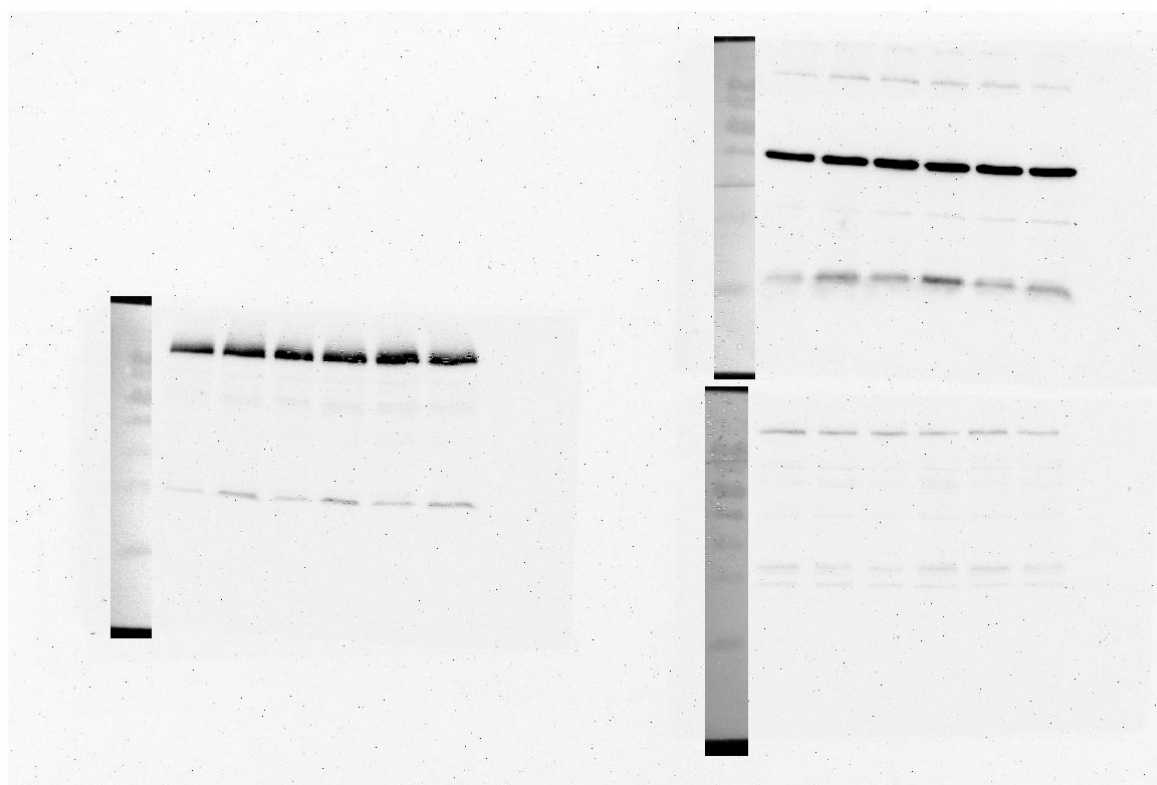
Figure S11. Uncropped western blot, K562, left blot shows Bad, the blot on top right illustrate γ -H2AX, 24 h incubation.



Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2021-11-18 14:08:22

Palette: inverted Grayscale
Range: 1366 - 3044, Gamma: 1.000

Figure S12. Uncropped western blot, K562, blot top left shows Survivin, 24 h incubation.



Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2021-10-06 16:32:45



Palette: inverted Grayscale
Range: 1350 - 3016, Gamma: 1.000

Figure S13. Uncropped western blot, K562, blot on the left side shows PARP, blot top right illustrate APAF-1 and β -Actin and Survivin, the blot below displays HDAC 4 and Bcl-2, 48 h incubation.

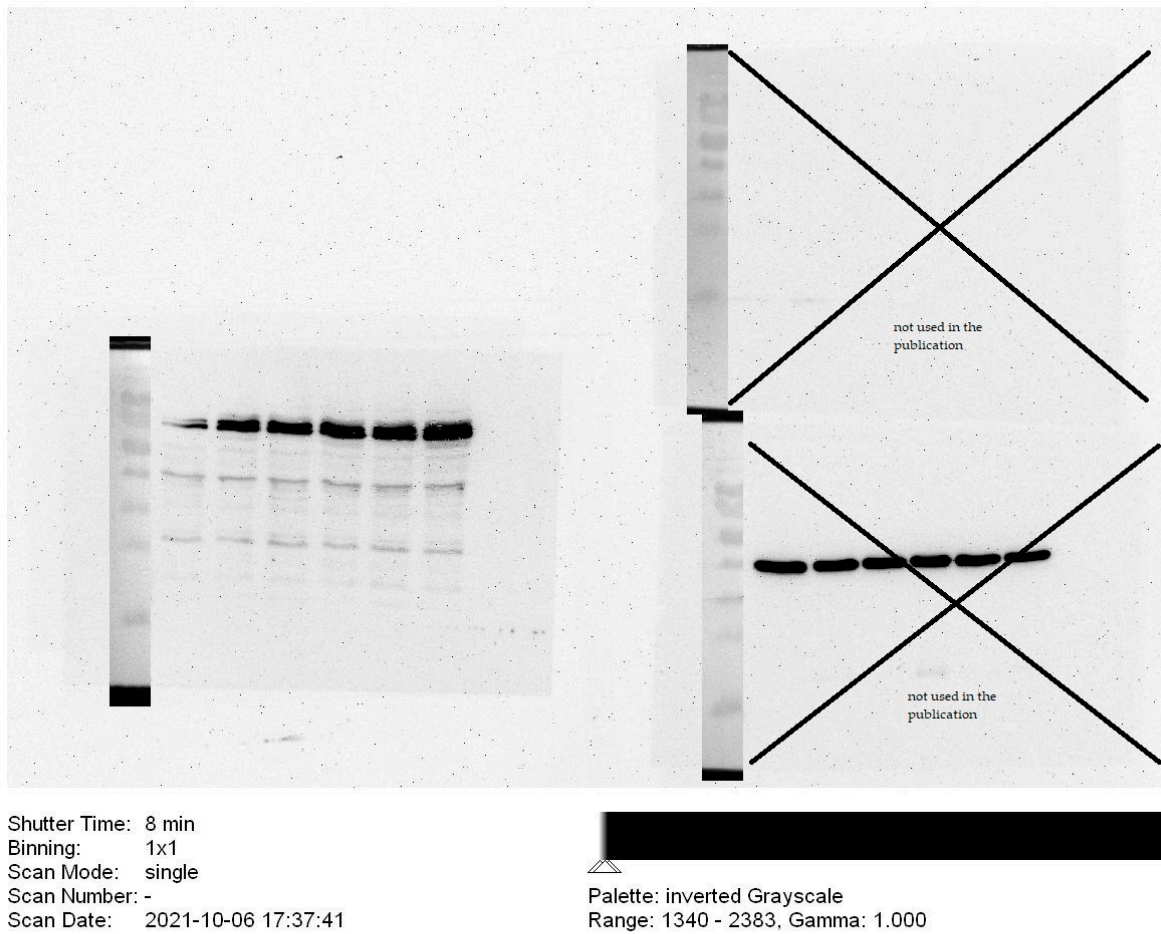
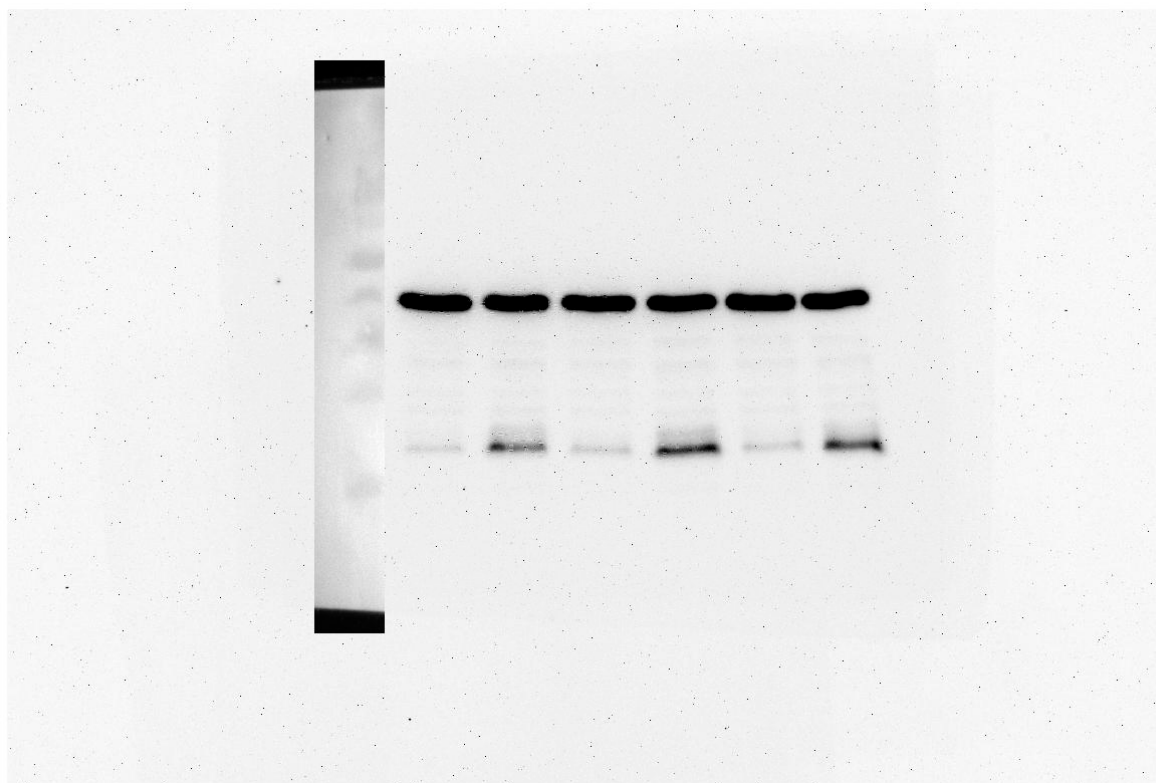


Figure S14. Uncropped western blot, K562, blot left side shows Bad, 48 h incubation.

MONO-MAC-6 General Order of samples (24 h, 48 h coincubation):

1. Control
2. 10 nM bortezomib
3. 10 μ M Yak540
4. 10 nM bortezomib + 10 μ M YAK540
5. 5 μ M TMP269
6. 10 nM bortezomib + 5 μ M TMP269



Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2022-03-22 12:29:26



Palette: inverted Grayscale
Range: 1346 - 2714, Gamma: 1.000

Figure S15. Uncropped western blot, MONO-MAC-6, blot shows β -Actin and p21, 24 h incubation.

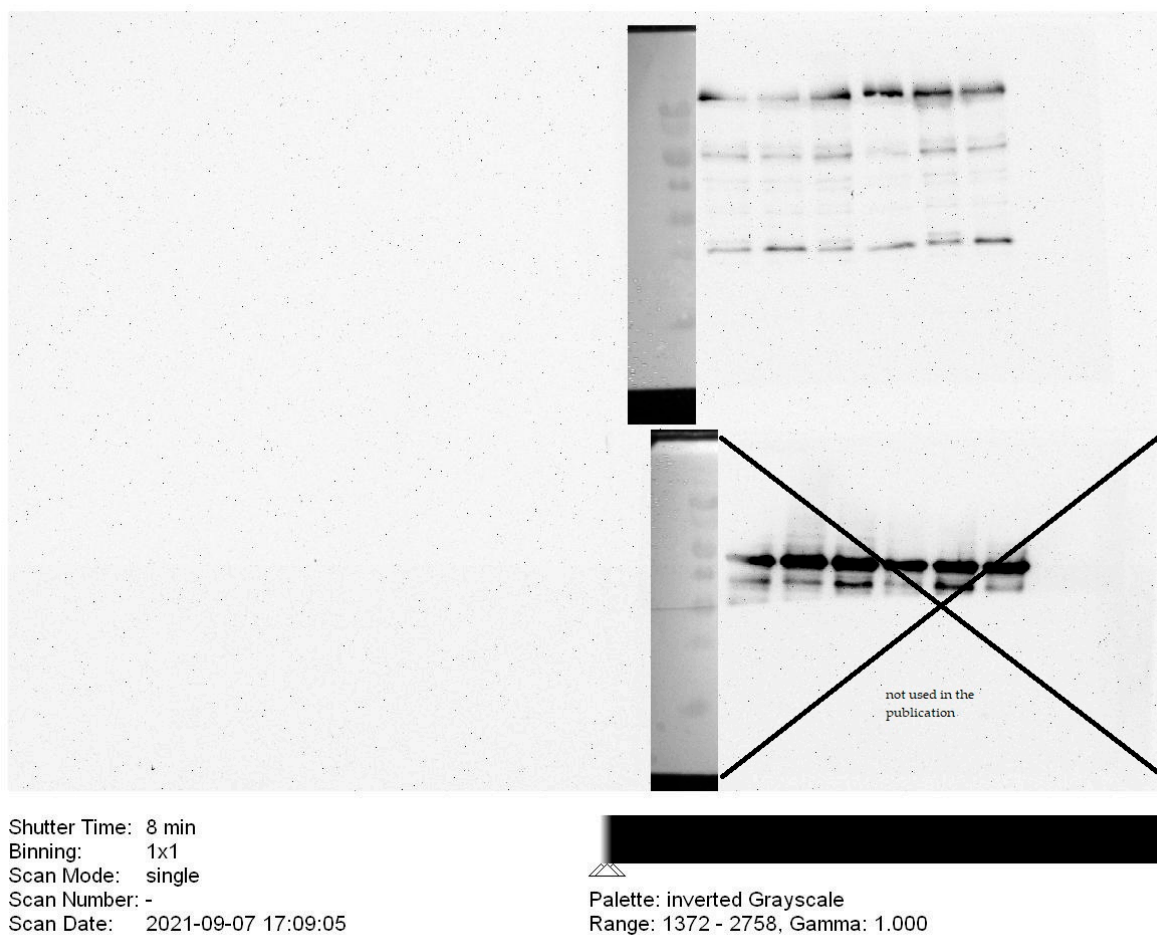


Figure S16. Uncropped western blot, MONO-MAC-6, blot at top shows PARP, 24 h incubation.

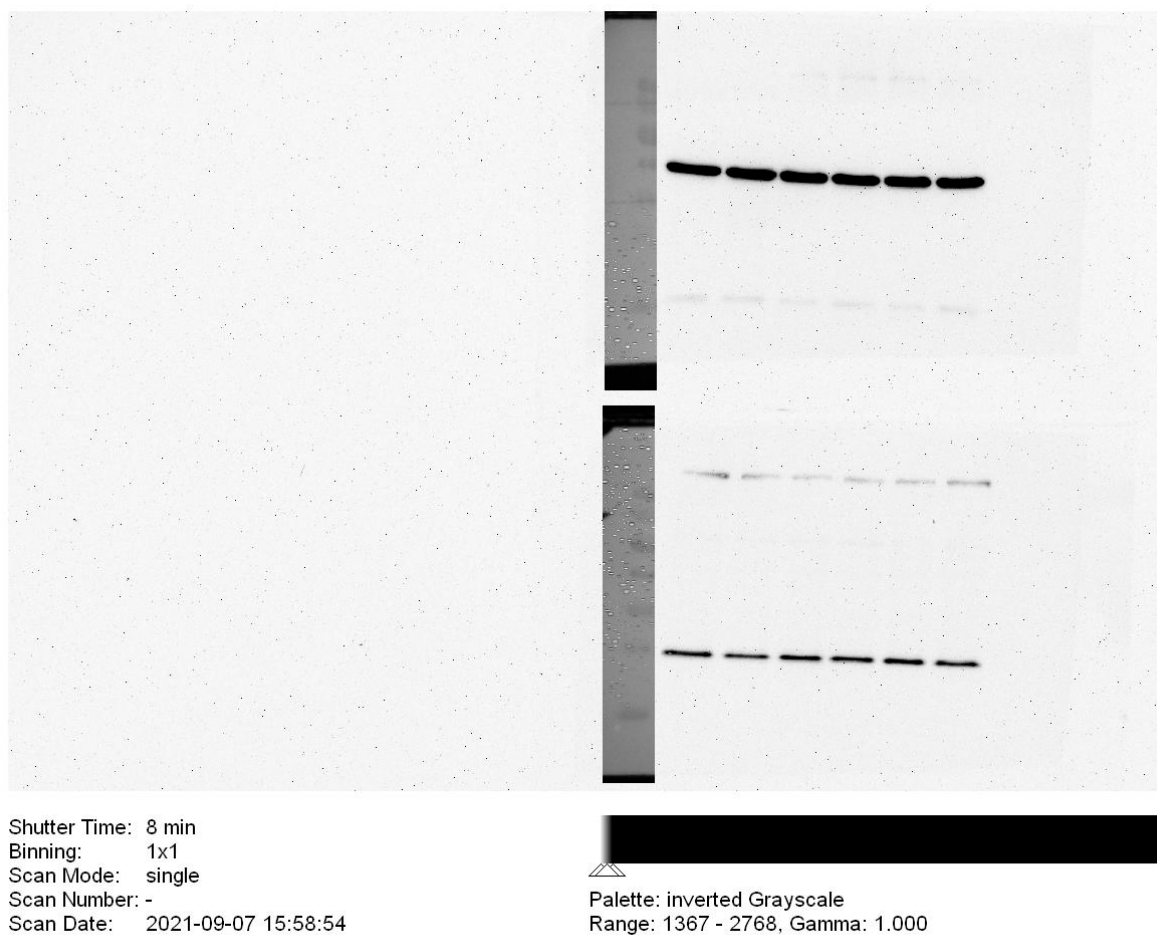


Figure S17. Uncropped western blot, MONO-MAC-6, blot at top shows Survivin, blot below illustrate HDAC 4 and Bcl-2, 24 h incubation.

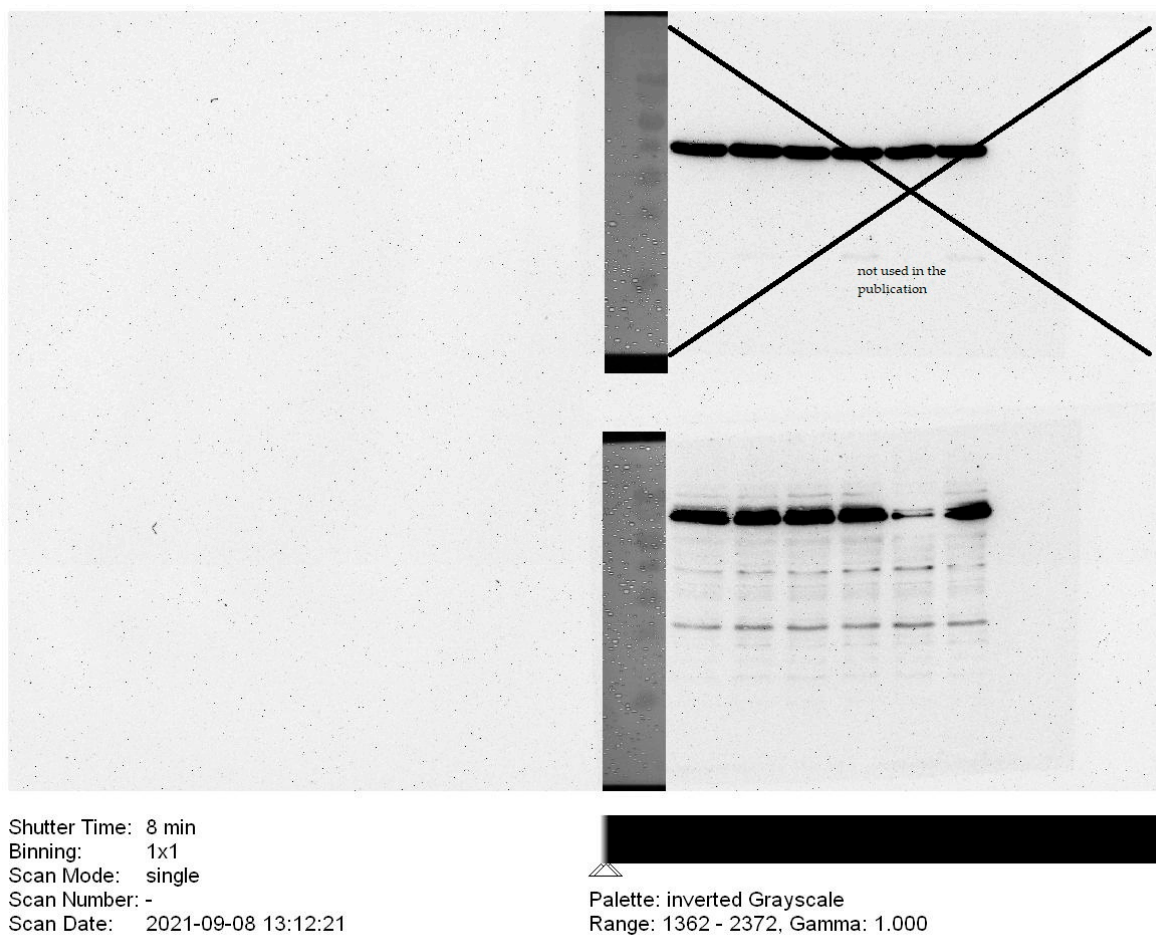
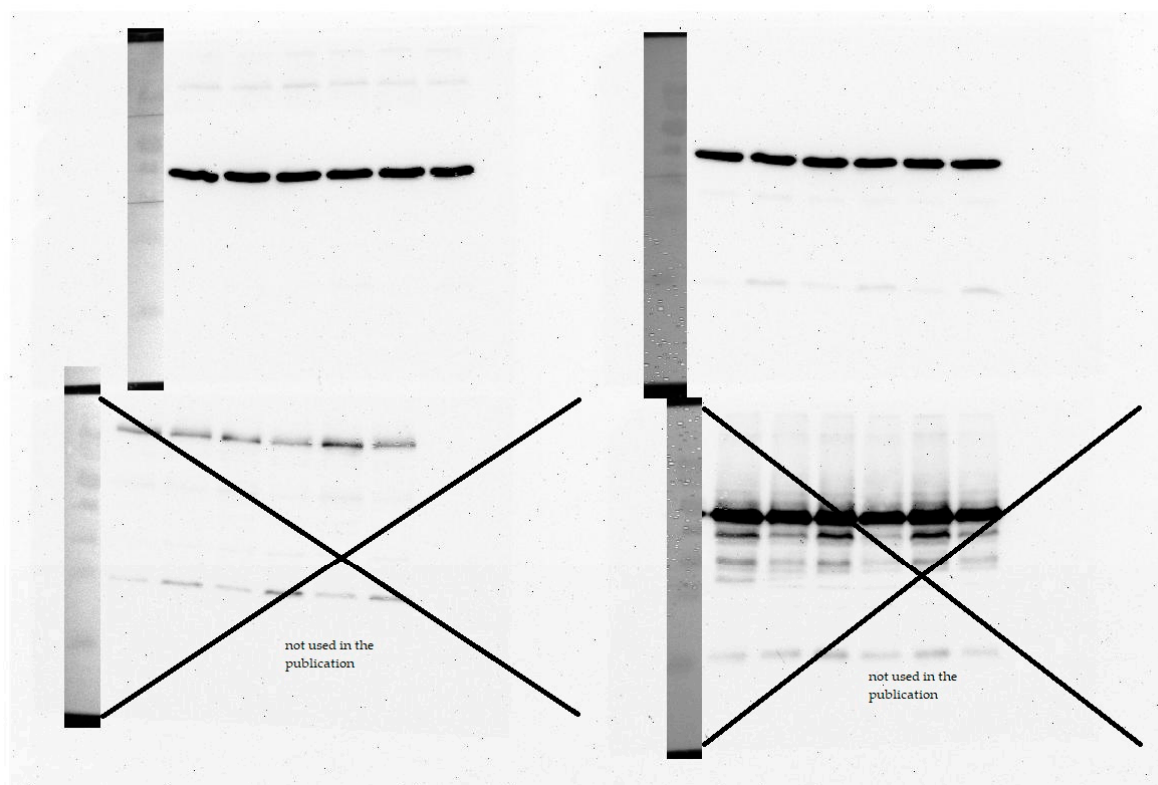


Figure S18. Uncropped western blot, MONO-MAC-6, lower blot shows Bad, 24 h incubation.

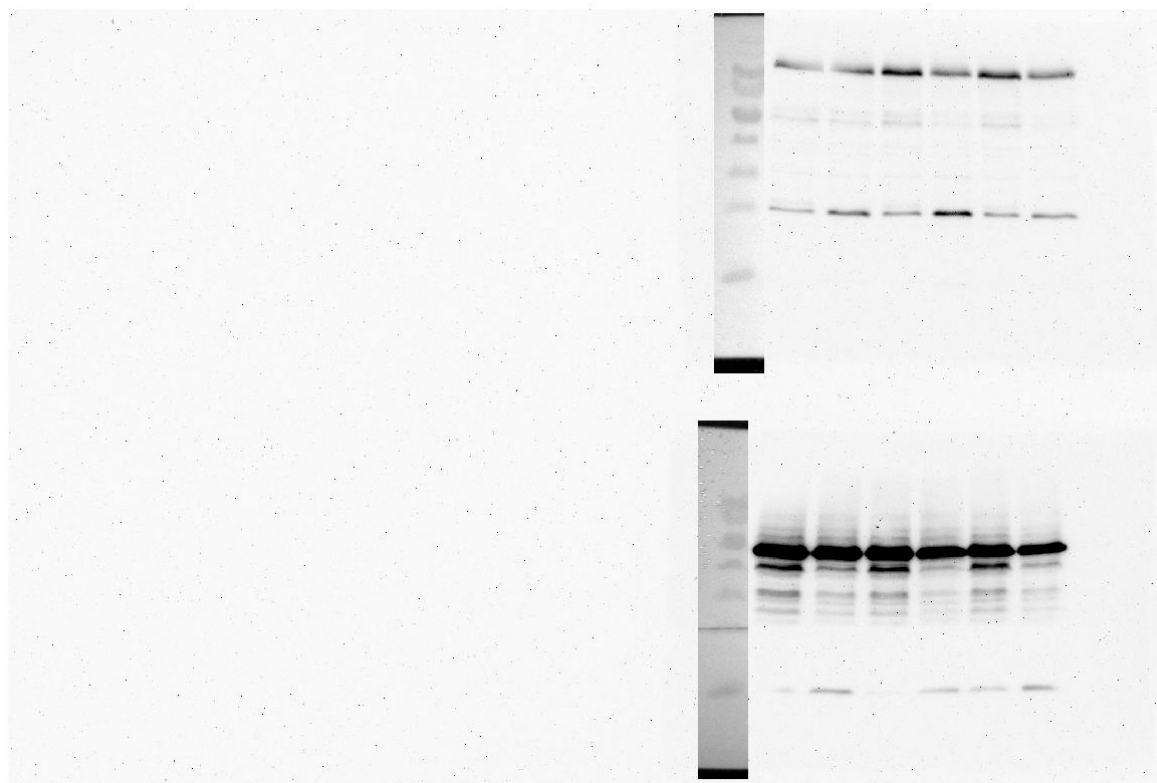


Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2021-11-17 14:15:12



Palette: inverted Grayscale
Range: 1352 - 3568, Gamma: 1.000

Figure S19. Uncropped western blot, MONO-MAC-6, blot top left shows APAF-1, 24 h incubation; blot top right illustrate β -Actin, 48 h incubation.

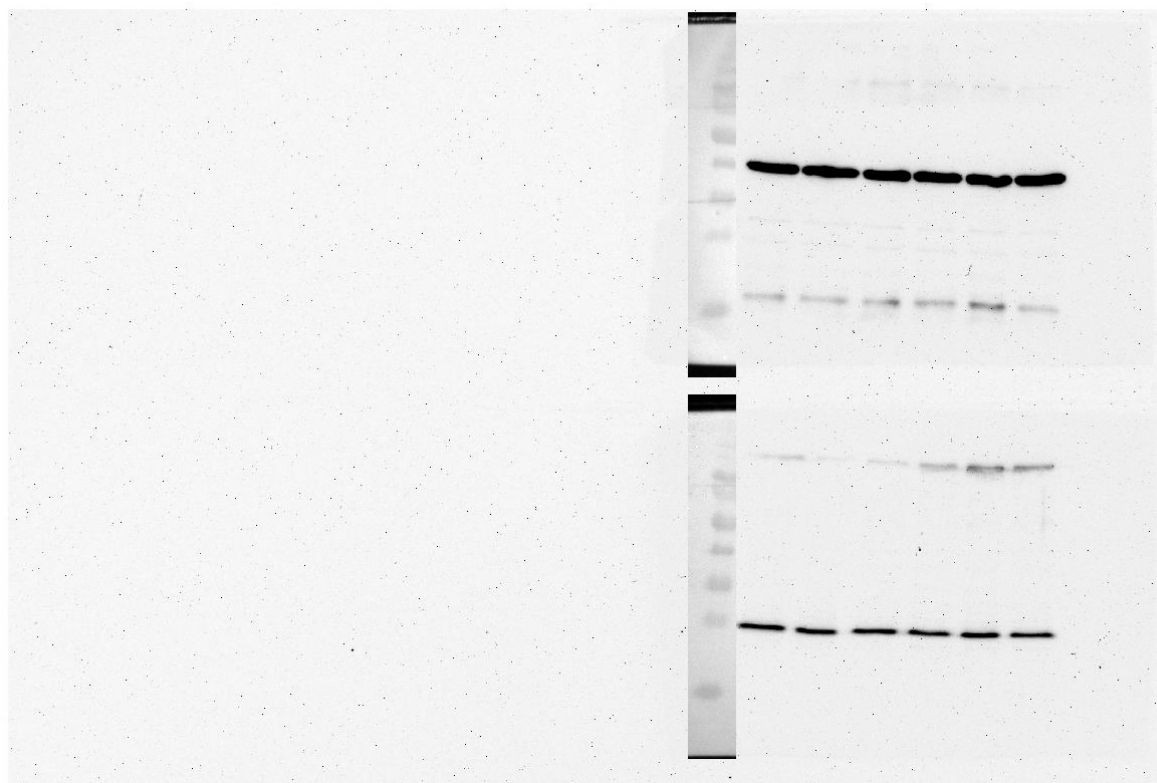


Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2021-09-09 15:52:15



Palette: inverted Grayscale
Range: 1358 - 3381, Gamma: 1.000

Figure S20. Uncropped western blot, MONO-MAC-6, blot at top shows PARP, blot below illustrate γ -H2AX, 48 h incubation.

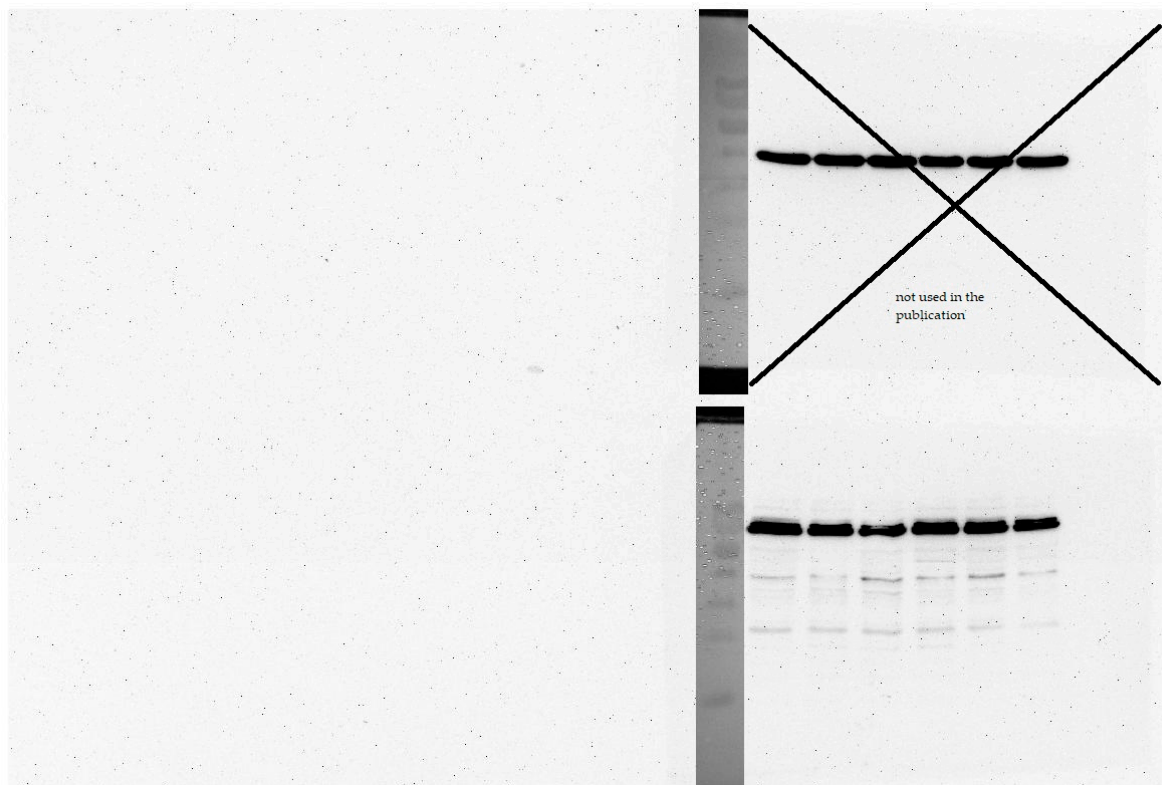


Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2021-09-08 14:16:51



Palette: inverted Grayscale
Range: 1359 - 2678, Gamma: 1.000

Figure S21. Uncropped western blot, MONO-MAC-6, blot at top shows APAF-1 and Survivin, blot below displays HDAC 4 and Bcl-2, 48 h incubation.



Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2021-09-09 14:38:14



Palette: inverted Grayscale
Range: 1366 - 2834, Gamma: 1.000

Figure S22. Uncropped western blot, MONO-MAC-6, blot below shows Bad, 48 h incubation.

Caspase 3/7 fluorescent imaging pictures

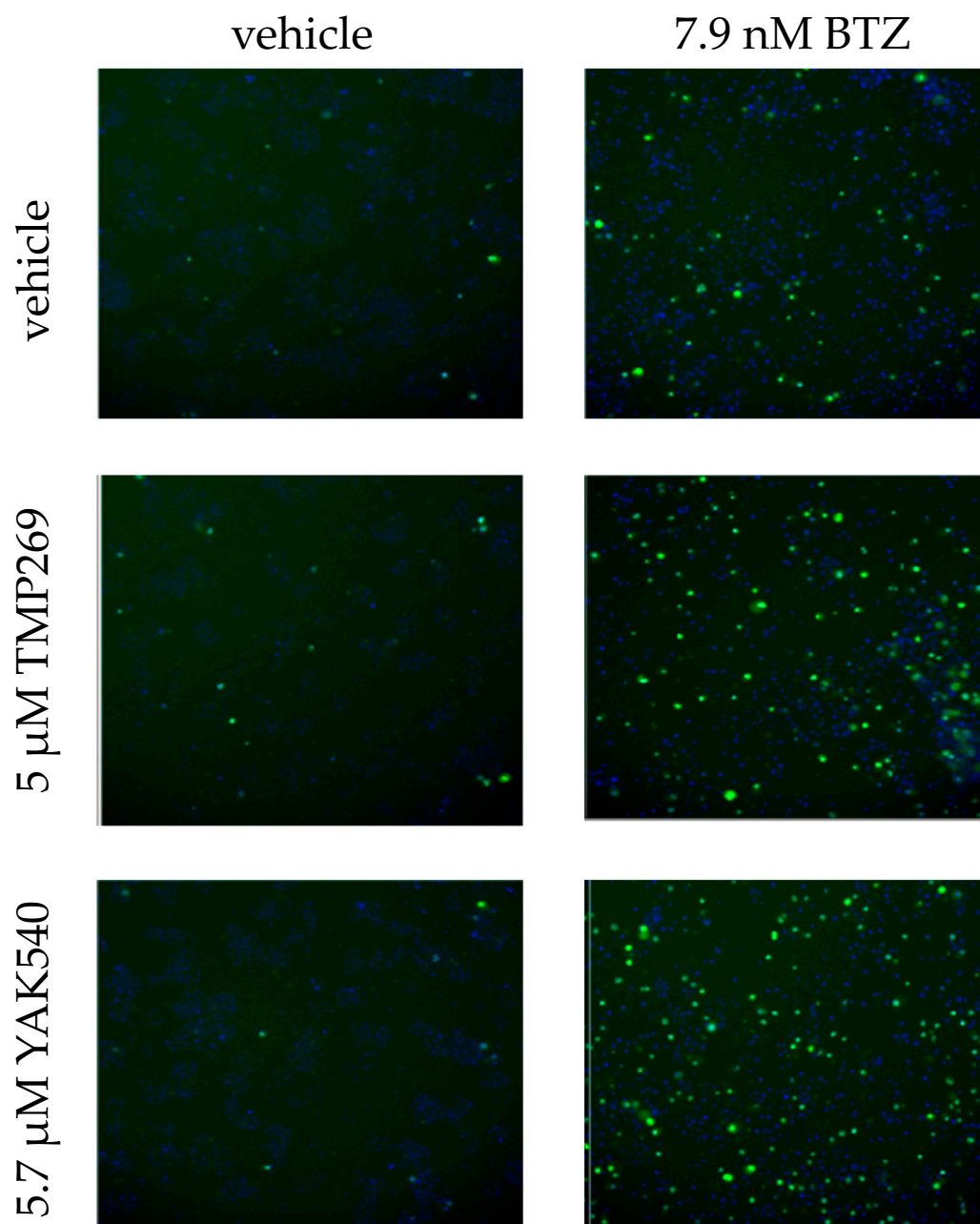


Figure S23. Representative fluorescent imaging pictures (Thermofisher Arrayscan XTI) are shown for vehicle control, treatment with 7.9 nM BTZ, 5 μ M TMP269, 5.7 μ M YAK540, respectively, and combination treatments of BTZ with TMP269 or YAK540 in THP-1 cells. Nuclei were stained using Hoechst 33342 (blue), cells showing caspase 3/7 activation are shown in green.

3.2 Publication II

5-(Trifluoromethyl)-1,2,4-oxadiazole (TFMO)-based highly selective class IIa HDAC inhibitors exhibit synergistic anticancer activity in combination with bortezomib

Yodita Asfaha^{a,1}, Lukas M. Bollmann^{a,1}, Alexander J. Skerhut^{a,1}, Fabian Fischer^a, Nadine Horstick^a, Dennis Roth^b, Maria Wecker^b, Christian Mammen^c, Sander H.J. Smits^{c,d}, Georg Fluegen^b, Matthias U. Kassack^{a,2}, Thomas Kurz^{a,2}

^a Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225, Düsseldorf, Germany

^b Department of Surgery (A), Medical Faculty, University Hospital of the Heinrich Heine University Düsseldorf, Moorenstr. 5, 40225, Düsseldorf, Germany

^c Institute of Biochemistry I, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225, Düsseldorf, Germany

^d Center for Structural Studies, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225, Düsseldorf, Germany

published in: European Journal of Medicinal Chemistry, 10. November 2023

DOI: 10.1016/j.ejmech.2023.115907.

The following content is derived from the aforementioned publication, entitled "5-(Trifluoromethyl)-1,2,4-oxadiazole (TFMO)-based highly selective class IIa HDAC inhibitors exhibit synergistic anticancer activity in combination with bortezomib." It is important to note that word-by-word citations are not explicitly highlighted in this section.

Authors Contribution

First authorship was shared by Y.A., L.M.B., and A.J.S. Y.A., L.M.B., A.J.S., N.H., F.F., D.R., M.W., and C.M. performed the experiments or synthesized the compounds. Y.A. and L.M.B. were responsible for writing the original draft. Visualization of the data was performed by L.M.B. M.U.K. and T.K. revised and edited the manuscript and also carried out the supervision. M.U.K., T.K., and G.F. provided resources. Funding acquisition was conducted by M.U.K., T.K., S.S., and G.F.

Background

This subsequent study was conducted building upon the findings of the earlier investigation of YAK540 [210]. The investigation entailed the synthesis and characterization of a series of 5-(trifluoromethyl)-1,2,4-oxadiazole (TFMO)-based amides and alkoxyamides. Anticancer activity was evaluated using the THP-1 cell line.

3. Publications

Further mechanistic studies were performed with the combination of the most promising drug FFK24 and BTZ.

Results

The most active class IIa inhibitor FFK24 showed nanomolar inhibition of the class IIa enzymes 4, 5, 7 (IC₅₀ HDAC4: 12 nM) and high selectivity (selectivity index >318 for HDAC4) over non-class IIa HDACs (Figure 20a). The inhibitory effect of FFK24 on cellular HDAC activity was observed to occur within the double-digit nanomolar range.

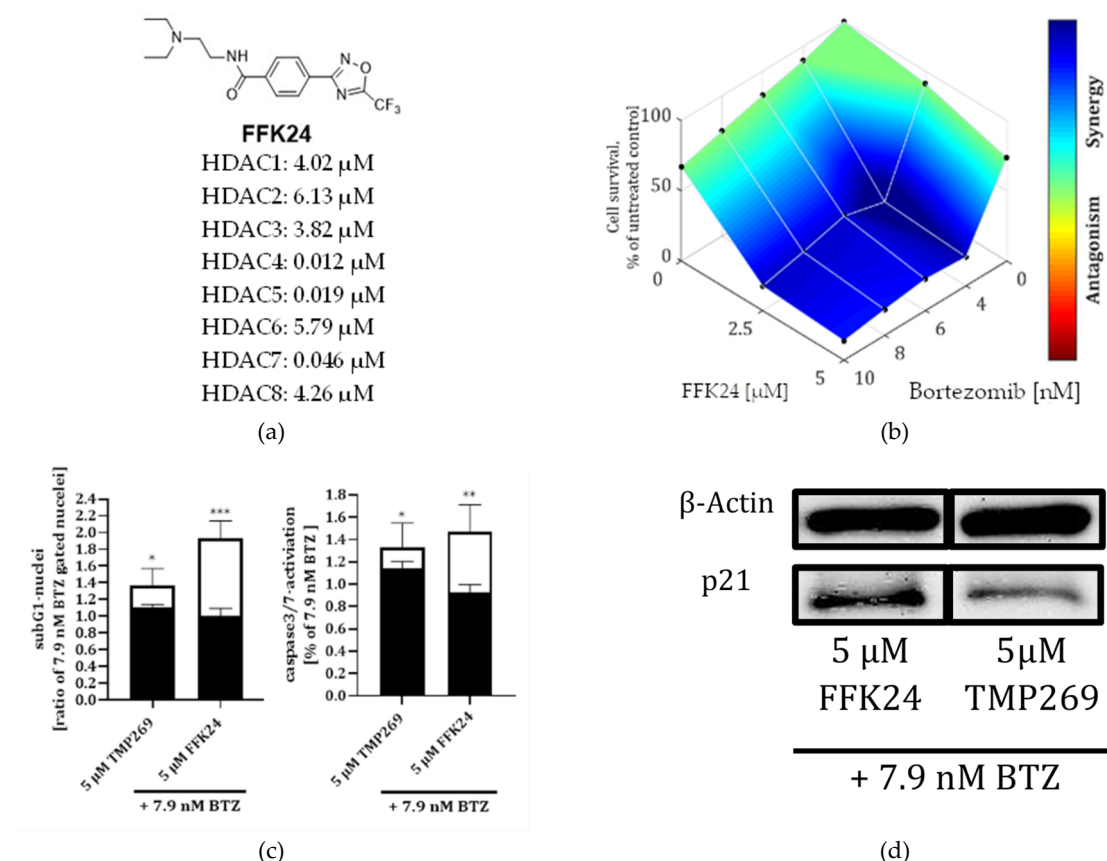


Figure 20. The most meaningful results of the combination of FFK24 and BTZ in THP-1 cells. **(a)** Structure of FFK24 and its HDAC enzyme inhibition activity. **(b)** Synergy levels calculated applying the Bliss model. **(c)** Effects of FFK24 and BTZ on caspase-mediated apoptosis. **(d)** p21 expression analyzed by Western blot. Figures taken and modified from [211].

Applying the Chou-Talalay-method and Bliss model, we found an increased synergistic cytotoxic effect of FFK24 in combination with BTZ in THP1 cells (Figure 15b). Further, we observed an increased caspase-induced apoptosis (Figure 20c). As demonstrated in Figure 20d, the combination of FFK24 and BTZ exhibited a stronger p21 expression than the reference compound TMP269. In the in vivo chorioallantoic membrane model, FFK24 significantly increased the growth inhibition of the head-neck cancer cell line Cal27 with HDAC4 overexpression.

Conclusion

The class IIa HDACi FFK24 outperforms the previously described class IIa HDACi YAK540 in terms of anti-cancer activity. The following observations were made:

- FFK24 is highly selective for class IIa HDACs (at least 142-fold)
- FFK24 shows a strong synergistic effect in combination with BTZ in the leukemia cell line THP-1
- FFK24 outperforms the class IIa HDACi TMP269 and YAK540 by displaying increased caspase-mediated apoptosis and enhanced p21 expression in combination with BTZ in THP-1 compared to TMP269 and YAK540

Thus, FFK24 may represent a novel better option compared to pan-, class I HDACi or YAK540 in anticancer combination treatments in leukemia. Further studies are warranted to prove the superior anticancer effects of FFK24 in combination with BTZ.



Research paper

5-(Trifluoromethyl)-1,2,4-oxadiazole (TFMO)-based highly selective class IIa HDAC inhibitors exhibit synergistic anticancer activity in combination with bortezomib

Yodita Asfaha^{a,1}, Lukas M. Bollmann^{a,1}, Alexander J. Skerhut^{a,1}, Fabian Fischer^a, Nadine Horstick^a, Dennis Roth^b, Maria Wecker^b, Christian Mammen^c, Sander H.J. Smits^{c,d}, Georg Fluegen^b, Matthias U. Kassack^{a,2}, Thomas Kurz^{a,*,2}

^a Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225, Düsseldorf, Germany

^b Department of Surgery (A), Medical Faculty, University Hospital of the Heinrich Heine University Düsseldorf, Moorenstr. 5, 40225, Düsseldorf, Germany

^c Institute of Biochemistry I, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225, Düsseldorf, Germany

^d Center for Structural Studies, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225, Düsseldorf, Germany

ARTICLE INFO

Keywords:

Selective class IIa HDAC inhibitors

Proteasome inhibitors

Bortezomib

Synergistic anti-cancer activity

Combination therapy

ABSTRACT

Clinically used pan and class I HDACi cause severe side effects, whereas class IIa HDACi are less cytotoxic. Here, we present the synthesis and anticancer effects of a series of 5-(trifluoromethyl)-1,2,4-oxadiazole (TFMO)-based amides and alkoxyamides derived from the previously reported class IIa HDACi YAK540. The most active class IIa inhibitor **1a** showed nanomolar inhibition of the class IIa enzymes 4, 5, 7 (IC₅₀ HDAC4: 12 nM) and high selectivity (selectivity index >318 for HDAC4) over non-class IIa HDACs. Instead of a hydroxamic acid group, **1a** has a trifluoromethyloxadiazolyl (TFMO) moiety as a non-chelating Zinc-binding group (ZBG). Applying the Chou-Talalay-method we found an increased synergistic cytotoxic effect of **1a** in combination with bortezomib in THP1 cells. **1a** in combination with bortezomib enhanced expression of p21 leading to increased caspase-induced apoptosis. Eventually, growth inhibition by **1a** of the head-neck cancer cell line Cal27 was increased upon HDAC4 overexpression in Cal27 in cell culture and using the *in vivo* chorioallantoic membrane model. The class IIa HDACi **1a** outperforms previously described HDAC class IIa inhibitor YAK540 regarding anticancer effects and may constitute a novel option compared to pan and class I HDACi in anticancer combination treatments.

1. Introduction

The histone deacetylase (HDAC) protein family acts as eraser of epigenetic marks, thereby regulating transcription. The 11 human zinc-dependent HDACs are subdivided into three classes based their sequence homologies to yeast deacetylases: class I (HDACs 1–3, 8), class II (IIa: HDACs 4, 5, 7, 9; IIb: HDACs 6, 10), and class IV (HDAC 11) [1–3].

An important difference compared to class I HDACs and HDAC6 is the weak deacetylation activity of class IIa HDACs due to the replacement of the catalytically essential tyrosine residue by a histidine residue in the active site of class IIa HDACs. In contrast to class I HDAC enzymes, class IIa HDACs are significantly larger (120–135 kDa) enzymes. Furthermore, no natural substrate of the class IIa HDAC enzymes has

been conclusively identified [2–5]. Possibly the “catalytic domain” serves as a recognition domain for binding to N-ε-acetyl lysine residues of proteins to regulate protein-protein interactions and protein signaling. The class IIa enzymes shuttle between the nucleus and the cytoplasm. In the nucleus, they are associated in multi enzyme complexes with other transcription factors and class I HDACs [4]. Within these complexes they use the deacetylase activity of other HDAC enzymes to modulate epigenetic effects [6]. To date, several potent and selective class IIa HDAC inhibitors (HDACis) have been described [4, 7–11]. First, the CHDI foundation described benzhydryl- and cyclopropane-based hydroxamic acids as HDAC4 inhibitors occupying the lower selectivity pocket of class IIa HDACs with their phenyl-linker moieties leading to IC₅₀ values for HDAC 4 inhibition as low as 10 nM

* Corresponding author.

E-mail address: thomas.kurz@hhu.de (T. Kurz).

¹ These authors contributed equally to this work (YA; LMB; AJS).

² These authors share corresponding authorship (MUK, TK).

<https://doi.org/10.1016/j.ejmech.2023.115907>

Received 2 September 2023; Received in revised form 18 October 2023; Accepted 21 October 2023

Available online 10 November 2023

0223-5234/© 2023 Published by Elsevier Masson SAS.

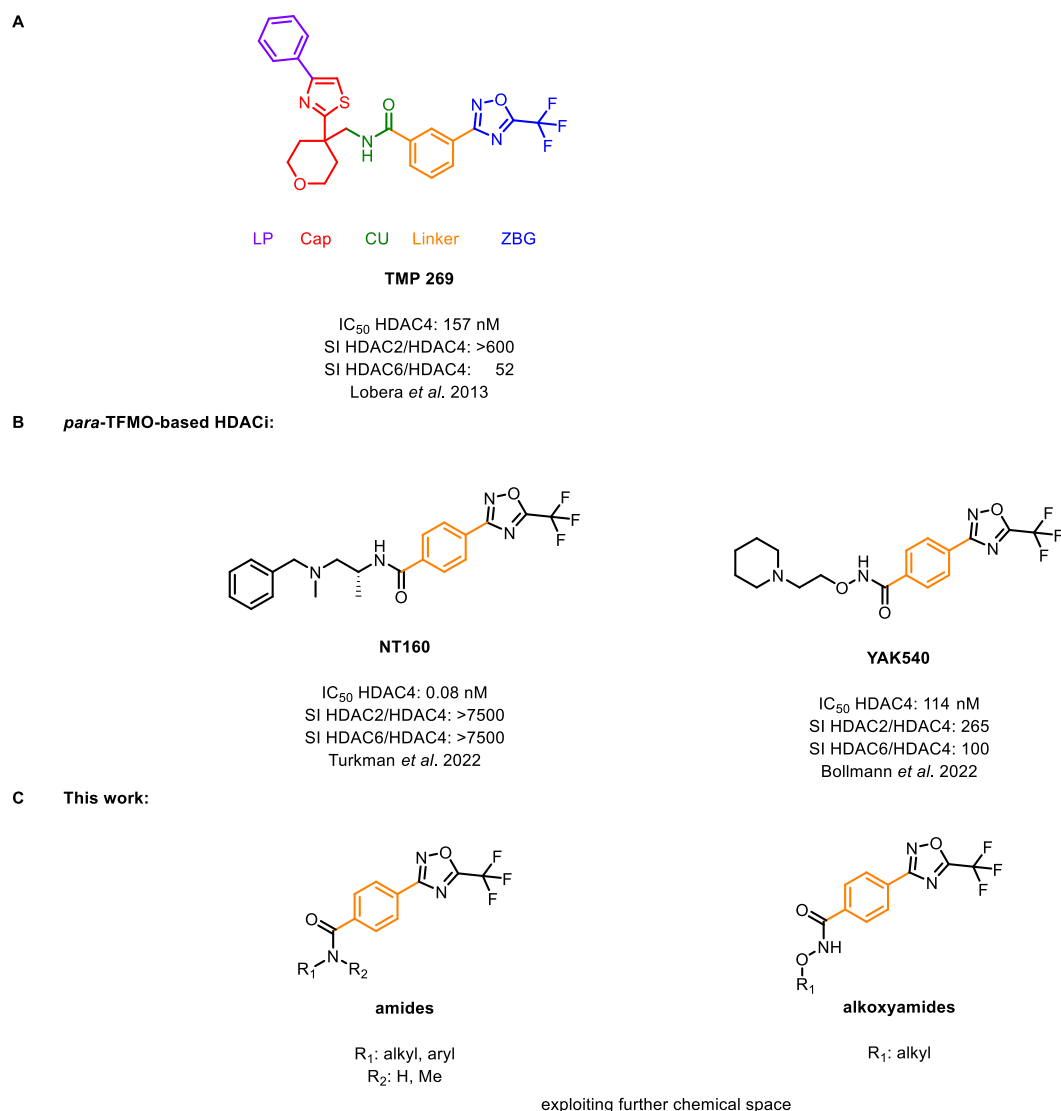


Fig. 1. A: Chemical structure of **TMP 269** and pharmacophore model for class IIa HDAC inhibitors (HDACis). B: Chemical structures of selective class IIa HDACis. C: Chemical structures of the *para*-substituted TFMO-inhibitors described in this publication. LP = lower pocket targeting group, CU = connecting unit, ZBG = zinc-binding group.

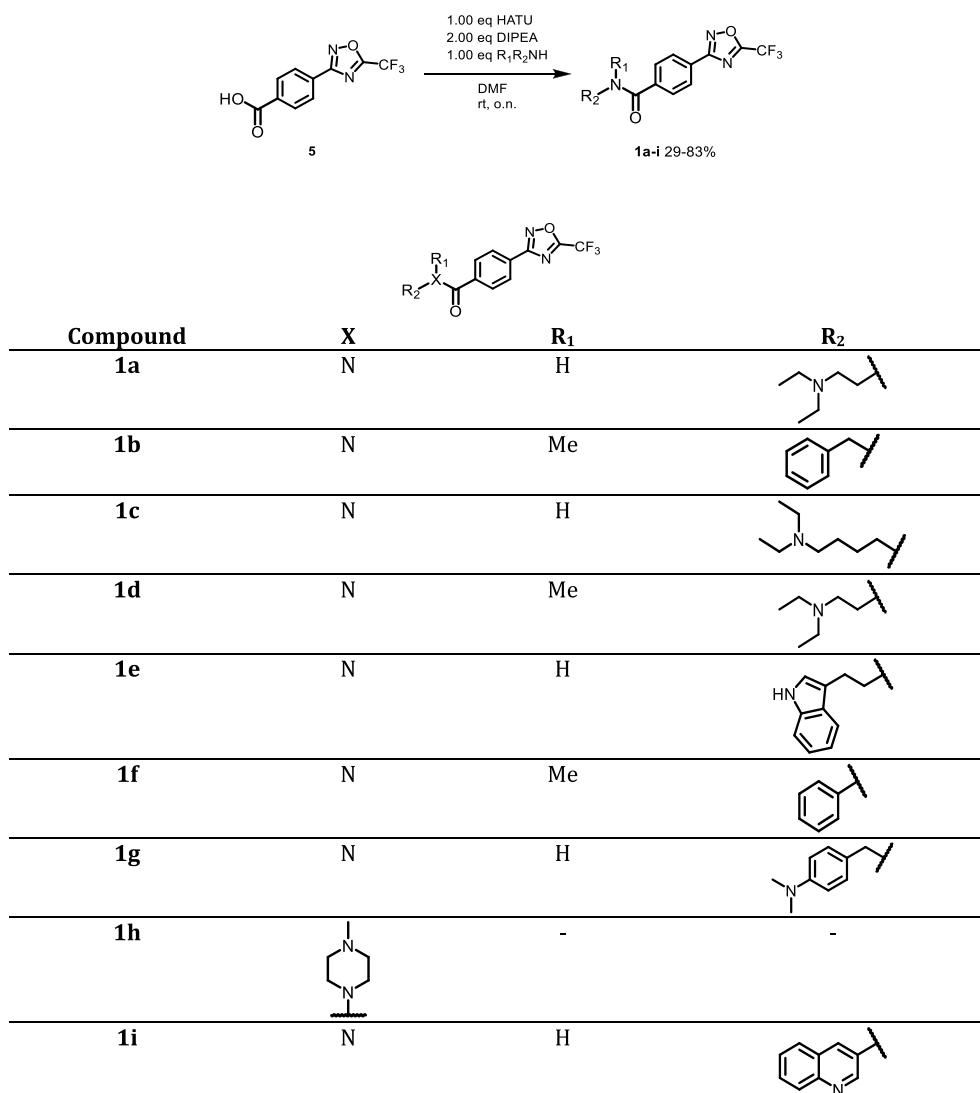
[10,11]. These inhibitors still comprise the in certain cases disadvantageous hydroxamic acid group. In 2013, Lobera et al. described the 5-(trifluoromethyl)-1,2,4-oxadiazole (TFMO)-group as a novel selectivity directing zinc-binding group (ZBG) for the development of selective class IIa HDACi (Fig. 1) [7,12].

A crystallographic analysis demonstrated that the TFMO group serves as a non-chelating ZBG via interactions of one fluorine atom and the 1,2,4-oxadiazole oxygen with the zinc ion in the catalytic center of HDAC7 [7]. Compound TMP269 in this series, showed an IC₅₀ for HDAC4 of 157 nM (Fig. 1.) and had a rather poor solubility. Further optimizations by other groups led to compound 12 (IC₅₀ for HDAC 4: 10 nM; [9]) and to NT160 (IC₅₀ HDAC 4: 0.08 nM; [8]) with improved pharmacokinetics and solubility due to the tertiary amine structures. Several TFMO-based inhibitors were evaluated for diagnosis and treatment in Huntington's disease and have not been tested as anticancer agents. However, there is still scope for further exploitation of TFMO derivatives as class IIa HDACis, particularly for the development as anticancer agents in combination therapies.

In many cases cancer development, progression and chemoresistance are associated with epigenetic dysregulation, particularly with an imbalance of histone acetyl transferases and histone deacetylases.

Ongoing clinical studies are investigating the chemosensitizing effects of HDACi in combination with different types of anti-cancer drugs [13]. Currently approved HDACis are the broad (pan)-HDACi vorinostat, belinostat, panobinostat, and romidepsin for the treatment of hematological malignancies. Tucidinostat (chidamide, class I HDACi) is only approved in China for the treatment of hematological malignancies and breast cancer [5,14–19]. All approved HDACi cause significant adverse effects such as diarrhea, nausea, anorexia, cardiac toxicity and thrombocytopenia [20].

In contrast to pan and class I HDACi, class IIa HDACi are less cytotoxic and may therefore be advantageous, particularly in combination with highly cytotoxic anti-cancer drugs. A promising novel approach is the combination of class IIa HDACis with proteasome inhibitors (PIs) [21]. Class IIa HDACi enhanced cell death in multiple myeloma and induced cell cycle arrest in pancreatic cancer in combination with PIs [22,23]. Further, we recently demonstrated synergism between the novel TFMO-based class IIa HDACi YAK540 and bortezomib (BTZ) in leukemia cell lines [21]. This is of particular interest since a synergism of highly toxic BTZ and low-toxic class IIa HDACi may allow to reduce the dose of BTZ and thus reduce its severe side effects such as neuropathy and hematotoxicity [24,25]. In addition, we recently showed that the



Scheme 1. Synthesis of para-substituted TFMO-based HDACi **1a-i**.

combination of BTZ and CHDI0039, a selective and potent hydroxamate class IIa HDACi [26], is also synergistic in head and neck squamous cell cancer (HNSCC), thus allowing an alternative treatment option if HNSCC has acquired resistance against platinum compounds [27]. In this work, we describe the development of a series of TFMO-based class IIa HDACi and their chemosensitizing effects in combination with the PI BTZ in the human monocytic leukemia cell line THP-1 and HNSCC cell line Cal27.

2. Results and discussion

2.1. Synthesis of para-substituted TFMO-based HDACi

New para-substituted TFMO-based HDACis were designed using structural features from TMP269 and YAK540 as shown in Fig. 1. The para-substituted TFMO-derivatives **1a-b** and **1f-i** were firstly reported by Hebach et al. and Wieja et al. [12,28]. The para-substituted TFMO-based amides **1a-i** were prepared via an optimized HATU-mediated acylation of commercially available amines with carboxylic acid **5**, in moderate to good yields (Scheme 1).

The TFMO-based alkoxyamides **2a-c** were synthesized according to Bollmann et al. [21], via a HATU-mediated coupling of carboxylic acid **5** with the *O*-substituted hydroxylamines **12a-c** (see SI), in moderate to

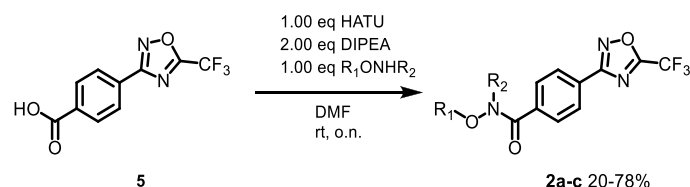
good yields yields (Scheme 2).

2.2. Investigation of HDAC inhibitory effect

First, the IC₅₀ values of the compounds at HDAC enzymes 2, 4, 6, and 8 were determined. HDAC2 and 8 are representatives of class I HDACs, HDAC4 is a representative of class IIa, and HDAC6 is a representative of class IIb (Table 1).

Compound **1a** showed an IC₅₀ of 12 nM against HDAC4 and the best overall selectivity among the TFMO-based amides and reference compound **2b** (YAK540) for HDAC 4 over HDAC 2 (524-fold), HDAC 6 (495-fold), or HDAC 8 (364-fold). **1b** exhibited an IC₅₀ value of 24.5 nM against HDAC 4 together with high selectivity over HDAC 2 (332-fold) and HDAC 8 (>4081-fold), but clearly less over HDAC 6 (83-fold). The alkoxyamides **2a-c** were slightly less active and inhibited HDAC 4 with IC₅₀ values from 114 to 141 nM. In the alkoxyamide series, **2a** revealed high selectivity for HDAC 4 (>847-fold) over HDAC 2, 6, and 8, respectively. **2c** had a similar selectivity for HDAC 4 over HDAC 2, 6, and 8 as the reference compound **2b** (YAK540).

Elongation of the alkyl chain (**1c**) as well as methylation of the amide moiety (**1d**) diminished the HDAC 4 enzyme inhibitory potency as well as the selectivity indices towards the other investigated HDAC enzymes. In general, the introduction of more bulky aromatic cap groups (**1e**, **1i**)



Compound	R ₁	R ₂
2a	H	
2b	H	
2c	H	

Scheme 2. Synthesis of para-substituted TFMO-based HDACi **2a-c** [21].

Table 1

HDAC enzyme inhibitory activity. Shown are IC₅₀ values in μM ± SD and selectivity indices for HDAC4.

	Cpd.	HDAC2 IC ₅₀	HDAC2 selectivity Index	HDAC4 IC ₅₀	HDAC6 IC ₅₀	HDAC6 selectivity Index	HDAC8 IC ₅₀	HDAC8 selectivity Index
amides	1a	6.13 ± 1.14	524	0.012 ± 0.001	5.79 ± 1.20	495	4.26 ± 0.39	364
	1b	8.13 ± 0.56	332	0.025 ± 0.002	2.03 ± 0.19	83	>100	>4081
	1c	25.4 ± 1.7	183	0.139 ± 0.010	5.61 ± 0.91	40	6.72 ± 0.62	48
	1d	34.8 ± 2.9	250	0.198 ± 0.018	16.9 ± 3.6	85	10.8 ± 1.0	55
	1e	>100	>139	0.690 ± 0.067	>100	>139	>100	>139
	1f	>100	>158	0.750 ± 0.026	19.6 ± 2.7	31	71.8 ± 15.1	114
	1g	>100	>108	0.940 ± 0.086	>100	>108	>100	>108
	1h	80.0 ± 5.5	68	1.18 ± 0.03	55.4 ± 14.1	47	16.1 ± 1.1	14
	1i	>100	18	5.54 ± 2.78	>100	18	>100	18
Alkoxy-amides	2a	>100	>847	0.118 ± 0.011	>100	>847	>100	>847
	2c	9.77 ± 2.73	86	0.141 ± 0.010	18.1 ± 3.0	128	7.13 ± 1.81	51
ref.	#2b	30.2	265	0.114	11.4	100	9.39	82

Data shown are at least two independent experiments, each carried out in duplicates. Selectivity indices were calculated from the IC₅₀ values for the respective HDAC enzyme and the IC₅₀ value for HDAC4. # Data of **2b** (YAK540) is taken from literature [21].

and the exchange of the amide moiety through an alkoxyamide moiety yielded less active but still preferential HDAC 4 inhibitors.

Subsequently, cellular HDAC inhibition was investigated to also prove the cellular activity of the compounds. (Table 2). Two specific substrates allow the differentiation between class IIa HDACs/HDAC 8 (substrate Boc-Lys-(TfA)-AMC) and class I HDACs/IIb HDACs (substrate Boc-Lys-(Ac)-AMC) [29,30].

The series of amides showed nanomolar to micromolar inhibition of cellular class IIa HDACs except for **1i**, whereas inhibition of cellular class I/IIb HDACs was at least 10-fold lower for most compounds. Compound **1a** showed the highest cellular class IIa HDAC inhibition (IC₅₀: 0.077 μM) and a cellular selectivity for class IIa of 142-fold. Replacing the diethylamino group in **1a** by a phenyl group in **1b** resulted in a similar HDAC enzyme inhibition profile (Table 1) and a moderately (7-fold) reduced cellular class IIa HDAC inhibition (IC₅₀: 0.54 μM) with similar selectivity for class IIa over class I/IIb HDACs as **1a** (selectivity factor: 53). Alkoxyamide derivatives **2a** and **2c** revealed no cellular activity at class I/IIb HDACs (IC₅₀ > 100 μM) and poor (**2a**) or sub micromolar inhibition (**2c**) at class IIa HDACs/HDAC 8. **2c** is comparable in cellular

potency to the reference **2b** (YAK540).

Fig. 2 summarizes the concentration-response-curves of **1a**, **2b** (YAK540) and vorinostat of the cellular HDAC assays for class IIa HDACs/HDAC 8 (grey lines) and class I/class IIb HDACs (blue curves).

These concentration-response-curves visualize that **1a** and **2b** (YAK540) have a higher cellular activity for class IIa HDACs/HDAC 8 than for class I HDAC enzymes. The broad (pan) HDACi vorinostat, which presents a significantly lower cellular activity than **1a** and **2b** (YAK540) for class IIa HDACs/HDAC8, serves as a comparison.

Based on selectivity for HDAC 4, potency and cellular activity, compounds **1a**, **1b**, **2a**, and **2c** were selected for more comprehensive HDAC enzyme profiling. The results of the HDAC enzyme assays are presented in Table 3.

The IC₅₀ values obtained for the inhibition of the HDAC isoenzymes 5 and 7 by the most active HDACis (**1a**, **1b**, **2a-c**) confirmed the excellent potency and selectivity towards IIa enzymes. (Tables 1 and 3). Structural requirements for the highly effective inhibition of class IIa HDACs are a TFMO ZBG, an amide CU in the para position of the phenyl linker, and a basic tertiary amine moiety in the cap group. An alkoxyamide CU leads

Table 2
Cellular HDAC inhibitory effect.

	Cpd.	Inhibition of class I/IIb HDACs		Inhibition of class IIa HDACs and HDAC 8	
		% Inhibition by 100 μ M	IC ₅₀ [μ M] $\pm$ SD	% Inhibition by 100 μ M	IC ₅₀ [μ M] $\pm$ SD
amides	1a	93	10.9 $\pm$ 1.4	100	0.077 $\pm$ 0.005
	1b	78	29.1 $\pm$ 4.7	117	0.540 $\pm$ 0.025
	1c	73	13.7 $\pm$ 1.6	102	1.17 $\pm$ 0.14
	1d	65	14.0 $\pm$ 4.9	98	1.27 $\pm$ 0.17
	1e	64	ND	97	14.5 $\pm$ 2.3
	1f	31	>100	118	6.39 $\pm$ 1.23
	1g	25	>100	103	5.51 $\pm$ 0.99
	1h	23	>100	103	5.51 $\pm$ 0.98
	1i	0	>100	18	>100
	2a	0	>100	76	38.1 $\pm$ 14.3
Alkoxy-amides	2c	41	>100	100	0.429 $\pm$ 0.059
	ref.	#2b	70	64.2 $\pm$ 8.0	0.590 $\pm$ 0.066

Data shown are normalized to vehicle control and the mean of at least two independent experiments each carried out in duplicates. # The values of **2b** (YAK540) were taken from literature [21]. ND: not determined.

to a slight reduction in class IIa HDAC inhibition. The optimal distance between the amide and the amine nitrogen is two methylene groups. Among all investigated compounds, the selective class IIa inhibitor **1a** is the most active HDAC 4, 5, 7 inhibitor of this study. **1a** exhibits IC₅₀ values in the low nanomolar range along with exquisite selectivity over almost all other HDACs and the most pronounced cellular activity.

The catalytic domain of HDAC 4 was recombinantly expressed and purified. This HDAC version lacks the GST-tag present within the commercially obtained HDAC 4 protein. The IC₅₀ of the frontrunner **1a** was 0.017 μ M (HDAC 4 enzyme from our groups, Fig. 3) which is almost identical to the results with commercial HDAC 4 enzyme (0.012 μ M, Table 1), thus assuming that the GST tag does not interfere with the catalytic core of HDAC 4.

2.3. Investigation of cytotoxic effect and synergism with bortezomib on THP-1 cell line

Cytotoxicity was tested at the human acute monocytic leukemia cell line THP-1. The results are presented in Table 4.

Most compounds exhibited very low cytotoxicity ($\sim$ 100 μ M or > 100 μ M, Table 4). Compared to pan and class I inhibitors, the selective class IIa inhibitor **1a** revealed relatively low cytotoxicity against THP-1 cells (IC₅₀) consistent with the cytotoxicity of TMP269.

Notably, the cytotoxicity of **2b** (YAK540) is lower than the cytotoxicity of **1a** and TMP269. **1a** is an about 10-fold more potent inhibitor of HDAC 4, 5-fold more potent at HDAC 5 and equal at HDAC 7 (Table 3) compared to TMP269 but has similar cytotoxic effects (Table 4).

Next, **1a** was investigated in combination with BTZ. BTZ displayed a strong cytotoxic effect (IC₅₀ of 6.28 nM) at THP-1 cells (Fig. S1). Synergism of **1a** in combination with BTZ was studied by Chou-Talalay analysis [32]. THP-1 cells were co-incubated with BTZ and **1a** or **2b** (YAK540) and combination index values were calculated (Table 5). A graphical illustration of the synergy of **1a** or **2b** (YAK540) with BTZ based on the Bliss model is presented in Fig. 4.

1a had a strong synergistic effect in combination with BTZ as can be seen by CI values even lower than 0.5. The synergistic effect observed with **1a** occurs already at lower concentrations of BTZ than with **2b** (YAK540). Overall, the CI values for the combination of **1a** and BTZ are lower than those of **2b** (YAK540) with BTZ. The stronger synergistic effect of **1a** with BTZ compared to **2b** (YAK540) with BTZ is also directly evident in Fig. 4 and supports previous results.

2.4. Investigation of apoptosis induction and caspase activation

Next, we studied whether the synergistic cytotoxic effect of **1a** and BTZ was mediated by caspase-induced apoptosis. THP-1 cells were treated with HDACis (**1a**, **2b** (YAK540), TMP269) and BTZ for 48 h and then analyzed for subG1 fraction by flow cytometry. The results of the subG1 analysis are shown in Fig. 5.

HDACi alone induce little subG1 nuclei in contrast to BTZ alone (Fig. 5). HDACi in combination with BTZ however induced significantly increased subG1 compared to BTZ alone. **1a** had the highest increase of subG1 fraction in combination with BTZ and a significant increase of subG1 fraction in comparison with the combination of **2b** (YAK540)/TMP269 and BTZ. Fig. 6 illustrates the synergistic effect. Black bars represent the sum of the single treatments, i.e. HDACi alone plus BTZ alone. White bars show the effects of the combination treatments, i.e. effect of HDACi plus BTZ.

All HDACi in combination with BTZ have a significant synergistic effect on the induction of subG1 nuclei. Notably, the synergistic effect of **1a** plus BTZ is much larger than for TMP269 or **2b** (YAK540). In conclusion, the synergistic cytotoxicity of the combination of the PI BTZ and class IIa HDACi induces caspase-mediated apoptosis.

Then, we investigated compound-mediated caspase activation (Fig. 7).

Single treatments with HDACi showed no or very little caspase activation in contrast to BTZ alone. Upon combination of BTZ with **1a**, **2b** (YAK540), or TMP269, caspase 3/7 activation increased significantly compared to BTZ alone (Fig. 7).

BTZ in combination with **1a**, **2b** (YAK540), or TMP269, respectively, displays significant synergistic caspase activation as shown in Fig. 8. Black bars represent the sum of the single treatments, i.e. effect of HDACi alone plus effect of BTZ alone. White bars display the effects of the combination treatments, i.e. effect of HDACi plus BTZ. All combination treatments show significantly more caspase activation than the sum of the single treatments (Fig. 8).

It should be noted that **1a** has the greatest synergistic effect of the HDACi investigated, indicating that **1a** is superior to **2b** (YAK540) and TMP269 in activating caspase 3/7 at similar concentrations in combination with BTZ. In summary, **1a** is the most potent compound of our series of class IIa HDACi that in combination with BTZ leads to synergistically enhanced caspase 3/7-mediated apoptosis in THP-1 cells.

2.5. Investigation of **1a** in Cal27 cells with high HDAC4 expression in vitro and in vivo

A genetically engineered Cal27 clone with increased HDAC 4 expression, named Cal27_HDAC4, was found to be more susceptible to the class IIa HDACi CHDI0039 than the corresponding vector control Cal27_VC [13]. Here, we wanted to test if the same holds true for our TFMO-based frontrunner **1a**. Fig. 9 shows the cytotoxic effect of **1a**, **2b** (YAK540) and BTZ, respectively, at Cal27_HDAC4 (blue graph) and Cal27_VC (black graph). IC₅₀-values and statistical comparison are given in Table 6.

BTZ, although mainly used for the treatment of haematological tumors, has a high potency in the single digit nanomolar range in Cal27 cells (Table 6) and is slightly (1.6-fold) but significantly more potent in Cal27_HDAC4 than in Cal27_VC. The IC₅₀ values for **2b** (YAK540) are around 70 μ M in both cell lines. Probably due to the low cytotoxic effect of **2b** (YAK540), there are no differences observable between

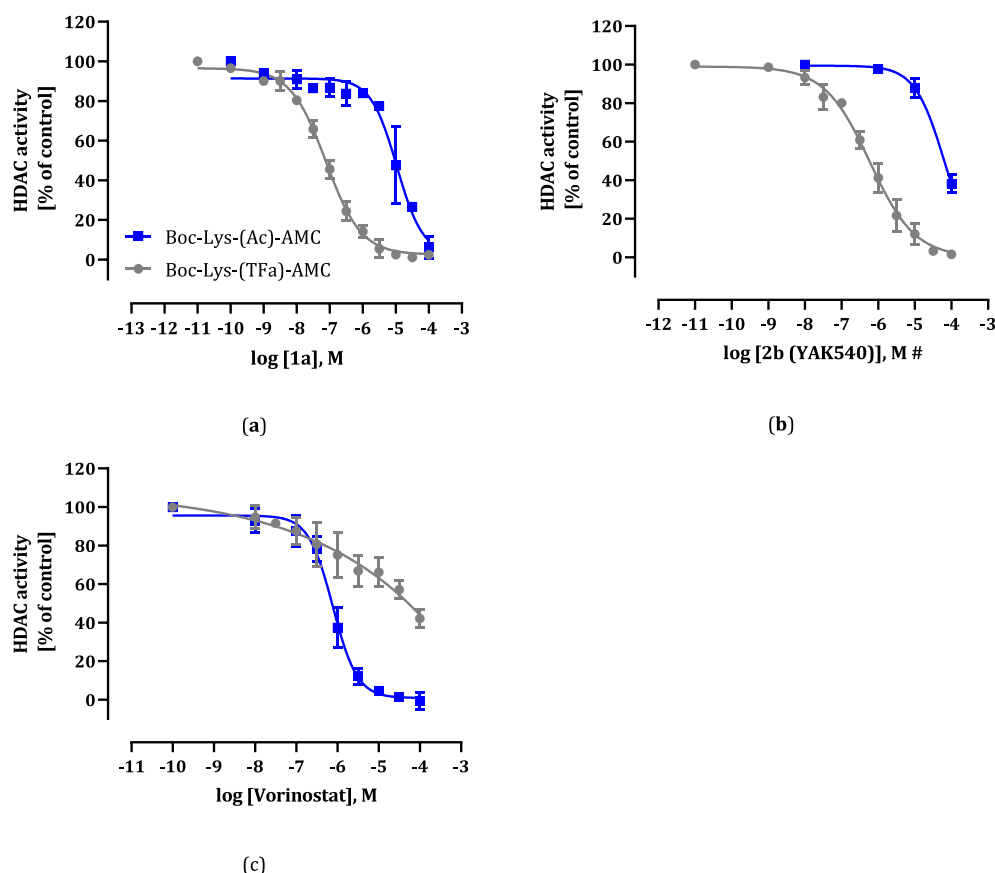


Fig. 2. Cellular histone deacetylase (HDAC) activity assay of (a) **1a**, (b) **2b** (YAK540) and (c) vorinostat. Cells were treated with increasing concentrations for 24 h. Data shown are normalized to vehicle control and the mean of at least two independent experiments each carried out in at least duplicates. # Data were taken from Ref. [21].

Table 3

HDAC enzyme inhibitory activity profile of frontrunner compounds.

	IC ₅₀ [μM]								
	Class I HDACs				Class IIa HDACs		Class IIb HDAC	Class IV HDAC	
Cpd.	1	2	3	8	4	5	7	6	11
1a	4.02	6.13	3.82	4.26	0.012	0.019	0.046	5.79	66.0
1b	14.6	8.13	6.86	>100	0.025	0.154	0.096	2.03	>100
2a	>100	>100	>100	>100	0.118	1.09	1.97	>100	>100
#2b	20.7	30.2	15.0	9.39	0.114	0.268	0.284	11.4	>100
2c	2.21	9.77	2.36	7.13	0.141	0.151	0.263	18.1	>100
TMP269	>100	>100#	>100	4.2#	0.157#	0.097	0.043	8.2#	>100
#vorino-stat	0.111	0.146	0.01	11.9	42.2	ND	ND	0.102	ND

Data presented are the mean of at least two experiments performed in duplicates. SD is <15 % of the mean. # Some data of **2b** (YAK540) and TMP269 is taken from Refs. [21,30]; vorinostat data is taken from Ref. [31], ND is not determined.

Cal27_HDAC4 and Cal27_VC. **1a** is clearly more potent than **2b** (YAK540) and displays 2.6-fold higher potency in Cal27_HDAC4 compared to Cal27_VC (Table 6). Next, synergism studies of **1a** with BTZ in Cal27_HDAC4 and Cal27_VC according to Chou-Talalay were performed [32]. Combination indices (CI) were calculated and are displayed in Table 7.

Results demonstrate a synergistic effect for **1a** in combination with BTZ in Cal27_HDAC4 but not in Cal27_VC cells in the used concentration range. High HDAC 4 expression thus seems necessary to obtain a synergistic effect of **1a** with BTZ. This was not the case with CHDI0039 [35]. The reason for the discrepancy of **1a** and CHDI0039 could be the differences in their behaviour to inhibit cell growth in Cal27_HDAC4 and Cal27_VC cells. Whereas CHDI0039 was almost equipotent in VC and

HDAC 4 clones of Cal27 (9.69 μM versus 12.8 μM; [35]), **1a** was 2.6-fold more potent in HDAC 4 versus VC clones (Table 6). To assess the *in vivo* potency and to confirm increased potency of **1a** in cells with increased HDAC4 expression, **1a** was investigated in Cal27_VC and Cal27_HDAC4 cells in the chorio-allantoic membrane assay (CAM-assay) (Fig. 10).

In this *in-vivo* model, where the Cal27_HDAC4 cells were added to the extraembryonic membrane of the developing chicken embryo, proliferation from the VC and HDAC 4 clones was comparable. Treatment with 5 μM **1a** reduced proliferation in both clones but to a larger extent and significantly only in the HDAC 4 clone. CAM data thus confirm that **1a** is more effective in reducing proliferation in cells with increased HDAC 4 expression. These data are in agreement with data from Schrenk et al. found with CHDI0039 [35].

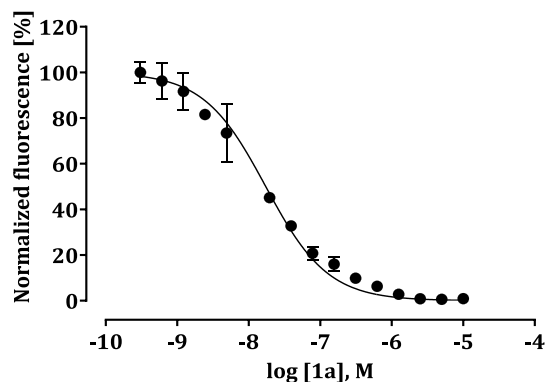


Fig. 3. Concentration-response-curves of **1a** at recombinant HDAC4 enzyme. Data shown one representative experiment with $n \geq 2$.

Table 4
Cytotoxic effect of the compounds.

	Compound	Relative cytotoxicity at 100 μ M compound	IC ₅₀ [μ M] $\pm$ SD
amides	1a	80	9.24 $\pm$ 0.841
	1b	34	>100
	1c	47	~100
	1d	31	>100
	1e	49	~100
	1f	33	>100
	1g	49	~100
	1h	39	>100
	1i	63	47.3 $\pm$ 7.57
	2a	47	~100
Alkoxy-amides	2c	47	~100
	2b	45	~100
references	#TMP269	100	13.0 $\pm$ 1.04
	vorinostat	97	0.18 $\pm$ 0.013
	panobinostat	96	0.001 $\pm$ 0.00002

Data shown are the mean of pooled data from at least two experiments on THP-1 cells, each carried out in at least duplicates. If the cytotoxic effect was >50 %, IC₅₀ values were obtained. # Data taken from Ref. [21].

2.6. Effects of **1a** on the expression of *p21*

Eventually, we investigated if caspase-mediated apoptosis and cytotoxicity by **1a** and BTZ occurred in parallel with increased expression of *p21*, the master protein inducing cell cycle arrest often down-regulated in chemoresistant cancers. QPCR and western blot were performed to test for expression of *p21* in THP-1 cells after treatment with **1a** or TMP269 in the absence or presence of BTZ. The results are presented in Figs. 11 and 12.

The highest increase in *p21* expression is found for the combination of **1a** with BTZ (14-fold), followed by the combination of TMP269 plus BTZ and the single treatment with **1a** (both 7-fold). Single treatments

Table 5
Synergism studies on THP-1 cell line.

		1a [μ M]				2b (YAK540) # [μ M]				
		2.5	5			15	20	25	30	35
BTZ [nM]	4	0.291	0.349	BTZ [nM]	5	1.42	1.74	1.35	1.47	1.34
	6	0.498	0.456		10	0.62	0.81	0.67	0.73	0.73
	8	0.441	0.431		15	0.79	1.11	0.61	0.73	0.59
	10	0.377	0.371							

72 h MTT-assay co-incubation of **1a** or **2b** (YAK540) and bortezomib (BTZ) in THP-1. Data presented are combination indices (CI) calculated by CompuSyn 1.0 based on Chou-Talalay method [32]. CI > 1.1 indicates antagonism, 0.9 < CI < 1.1 indicates an additive effect and CI < 0.9 indicates synergism. Values of **1a** were calculated from $n \geq 8$. Data were obtained from 72h co-incubated MTT assay. # CI - values of **2b** (YAK540) were taken from literature [21].

with BTZ or TMP269 increased *p21* expression to a lower extent: BTZ 2.5-fold, TMP269 4.3-fold. This suggests that the combination of class IIa HDACi and BTZ increases *p21* gene expression to a higher extent than the class IIa HDACi alone. Further, **1a** is more potent in raising *p21* gene expression than TMP269. The Western blot analysis confirmed the qPCR results (Fig. 12).

Strongest expression of *p21* occurred for the combination of **1a** and BTZ. The combination of TMP269 and BTZ gave a *p21* expression similar to treatment with **1a** only, and TMP269 alone showed only a slight increase in *p21* expression. The stronger induction of *p21* expression by **1a** plus BTZ compared to TMP269 plus BTZ may explain the strong synergistic effect of **1a** plus BTZ observed in caspase-mediated apoptosis (Figs. 7 and 8) and in Chou-Talalay analyses (Table 5 in comparison to literature [21]). In summary, we could demonstrate that the consecutive chain of *p21* expression, caspase activation, apoptosis induction and eventually increased cell death (cytotoxicity) is increased at all stages by the combination of BTZ and the class IIa HDACi **1a** compared to single treatments or the sum of single treatments accounting for synergism between BTZ and **1a**.

3. Conclusion

We have synthesized and biologically characterized a series of new non-hydroxamate, TFMO-based selective class IIa HDACi. Compared to pan and class I inhibitors, the selective class IIa inhibitor **1a** exhibited low cytotoxicity against THP-1 cells (IC₅₀: 9.24 μ M). Compound **1a** exhibits high class IIa inhibitory potency (IC₅₀ HDAC 4: 12 nM), significant cellular HDAC inhibition (IC₅₀: 77 nM for class IIa HDACs), high selectivity for class IIa HDACs (e.g. selectivity factor >318 for HDAC 4 over class I HDACs), strong synergy in combination with the PI BTZ (Chou-Talalay analysis) in the acute myeloid leukemia cell line THP-1, and exceeds the potency and synergy of the recently published compound **2b** (YAK540) [21]. In addition, particularly in combination with BTZ, **1a** increases expression of *p21* and leads in a synergistic manner to caspase-mediated apoptosis. Notably, **1a** also inhibits proliferation of the solid cancer cell line Cal27_HDAC4 with increased HDAC 4 expression in the CAM model and is synergistic with BTZ in Cal27_HDAC4 cells. The potent and selective class IIa HDACi **1a** may thus extend the therapeutic potential of PIs to solid cancers as novel combination therapy.

4. Materials and methods

4.1. Materials

The culture medium RPMI 1640 and DMEM, Penicillin/streptomycin (pen/strep) (10,000 U/mL; 10 mg/mL) and trypsin-EDTA (0.05% Trypsin, 0.02% EDTA in Phosphate Buffer Saline) were delivered by PAN-Biotech (Aidenbach, Germany). TMP269 and bortezomib were ordered from AdooQ (AdooQ Bioscience, Irvine, California, USA). All compounds were dissolved in DMSO at 10 mM. Further dilutions were made with the appropriate culture medium. A maximum concentration of 1% DMSO was used in all experiments.

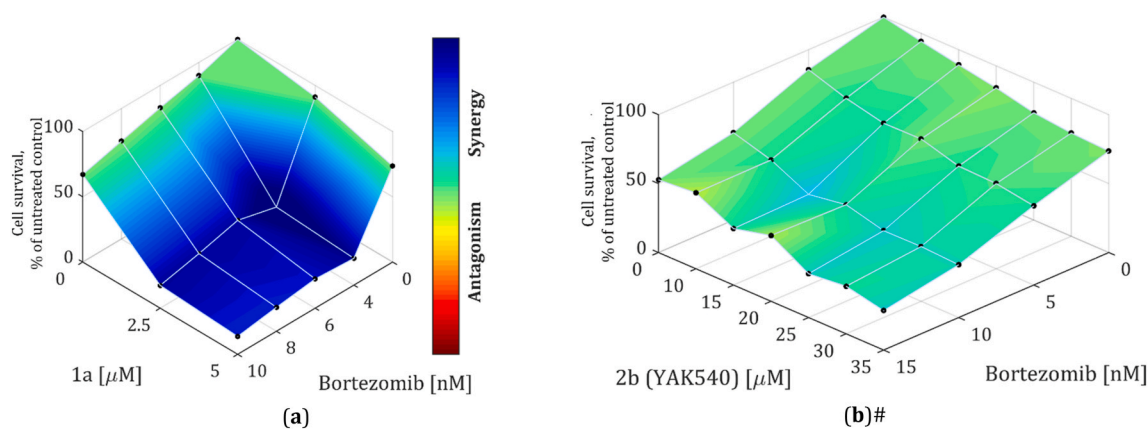


Fig. 4. Synergy levels calculated with Bliss model [33] in Combeneft [34], depending on experimental concentration-response surface of combinations of BTZ and class IIa HDACs (a) **1a** and (b) **2b** (YAK540) for 72 h in THP-1 cell line. Data shown are from at least two independent experiments each carried out at least in duplicates. # Data taken from Ref. [21].

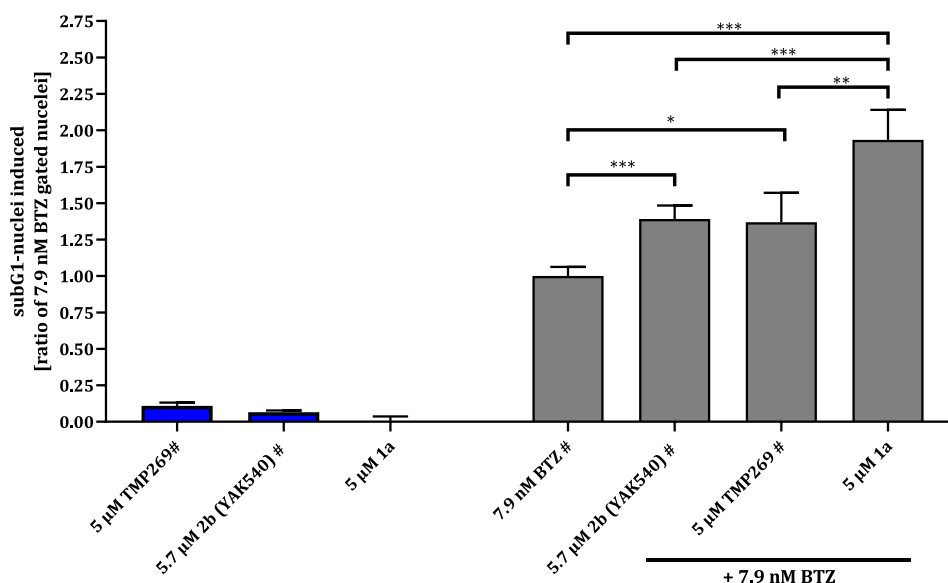


Fig. 5. Apoptosis induction by combination treatment of **1a**, **2b** (YAK540) or TMP269 and BTZ. THP-1 cells were co-incubated for the 48 h. The fraction of apoptotic cells was analyzed by measuring the subG1-nuclei and the cell count by flow cytometry analysis. The presented data are the mean $\pm$ SD calculated as the ratio of the highest BTZ concentration of each treatment and $n \geq 2$ with at least three replicates. Statistical analysis was performed using *t*-test. Level of significance: * ($p \leq 0,05$); ** ($p \leq 0,01$); *** ($p \leq 0,001$). # Some data were taken from Ref. [21].

4.2. Cell lines and cell culture

The human monocytic leukemia Cell line THP-1 was kindly provided by Helmut Hanenberg, Department of Otorhinolaryngology and Head/Neck Surgery, Heinrich Heine University, 40225 Düsseldorf, Germany. The cell line Cal27 was delivered by German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The corresponding cisplatin-resistant (CisR) cell line Cal27 cisR was generated as previously described [37]. THP-1 cells were cultured with RPMI 1640 and the Cal27 cells were cultured with DMEM, both culture medias spiked with 120 IU/mL penicillin and 120 μ g/mL streptomycin and 10% heat inactivated fetal calf serum at 37 °C and 5% CO₂.

4.3. MTT cell viability assay

Cell viability during the compound screening, the treatment with the BTZ and the class IIa HDACs in the synergism studies and

concentration-response-curves were determined by MTT-assay with minor modifications as previously described [38,39]. Briefly, cells were incubated with the compounds for 72 h. After 72 h, 25 μ L MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Serva, Heidelberg, Germany) solution (5 mg/mL in phosphate buffer saline) was added. After formazan formation in THP-1 cells, 150 μ L isopropanol/concentrated hydrochloric acid (50 mL/165 μ L) were added per well and formazan was dissolved by pipetting up and down. Subsequently, absorbance was measured at 595 nm and 690 nm with a ThermoFisher Multiskan FC Microplate Photometer (Thermo Scientific, Wesel, Germany). After formazan formation in Cal27 cells, medium was removed and formazan was dissolved by adding 75 μ L DMSO per well and absorbance was measured at 540 nm and 690 nm (ThermoFisher Multiskan FC Microplate Photometer, Thermo Scientific, Wesel, Germany). Cytotoxic effects were calculated as follows: absorption of samples treated with 100 μ M compound were divided by the absorption of untreated cells. The resulting ratio was subtracted from 1 and shown

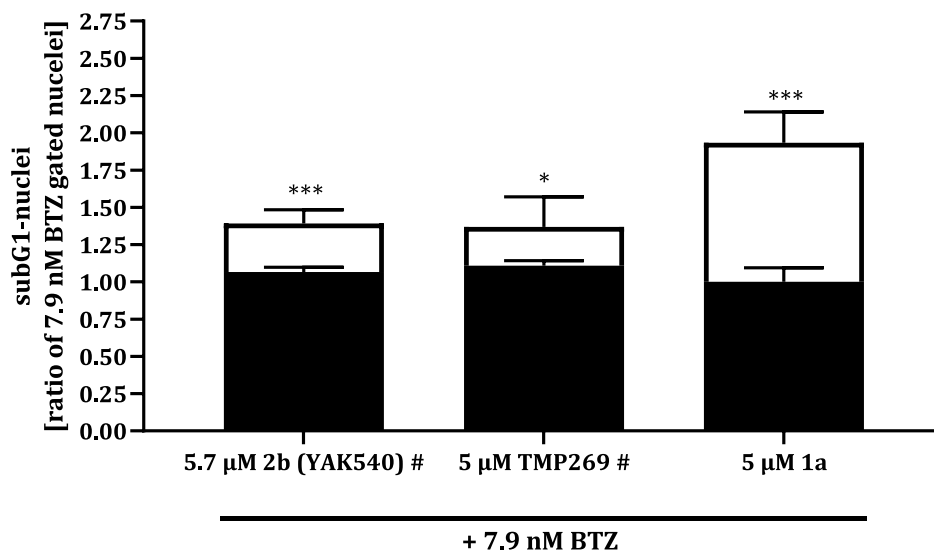


Fig. 6. Apoptosis induction by combination treatment of **1a** or TMP269 or **2b** (YAK540) plus BTZ. THP-1 cells were co-incubated for 48 h. The fraction of apoptotic cells was analyzed by measuring the subG1-nuclei and the cell count by flow cytometry analysis. The presented data are the mean $\pm$ SD calculated as the ratio of the highest BTZ concentration of each treatment. The sum of the single treatment effects is shown as black bars. The measured combination effects are shown as white bars. Statistical analysis was performed using *t*-test. Level of significance: * ($p \leq 0,05$); *** ($p \leq 0,001$); # Data were taken from Ref. [21].

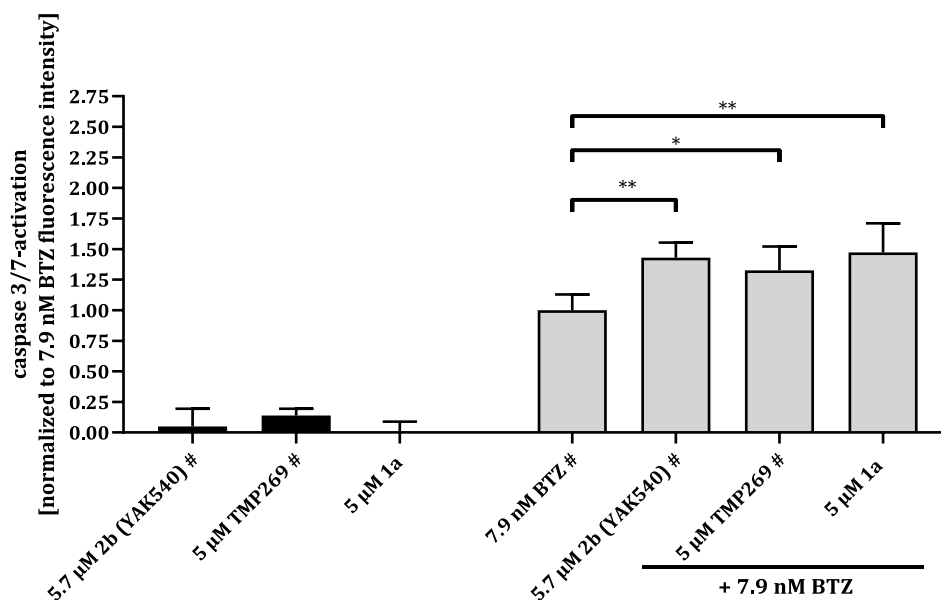


Fig. 7. Caspase3/7-activation by single and combination treatment of **1a**, **2b** (YAK540) or TMP269 and BTZ. THP-1 cells were co-incubated for 48 h. The presented data are the mean $\pm$ SD calculated as the ratio of the highest BTZ concentration (7.9 nM) of each treatment and $n \geq 2$ each with at least 3 replicates. Statistical analysis was performed using *t*-test. Level of significance: * ($p \leq 0,05$); ** ($p \leq 0,01$); # Data taken from Ref. [21].

as percent cell viability.

4.4. Enzyme HDAC inhibition assay

The human recombinant enzymes were ordered from Reaction Biology Corp (Malvern, PA, USA). To determine the inhibitory activity of the enzymes HDAC 2 (cat. no. KDA-21-277), HDAC 4 (cat. nr. KDA-21-279), HDAC 6 (cat. no. KDA-21-213), and HDAC 8 (cat. no. KDA-21-481), 20 ng each of HDAC 2 and 8, 17.5 ng of HDAC 6, and 2 ng of HDAC 4 were used per well. After pipetting 10 μ L of increasing concentrations of the compounds to a 96-well plate (Corning, Kaiserslautern, Germany), recombinant enzymes diluted in assay buffer (50 mM

Tris-HCl, pH 8.0, 137 mM KCl, 1 mM MgCl₂, and 1 mg/mL BSA) were added. After 5 min of incubation, the reaction was started with 10 μ L of a 30 μ M solution of Boc-Lys(Ac)-AMC (Bachem, Bubendorf, Switzerland) for HDAC 2 or 15 μ M solution of Boc-Lys(Ac)-AMC for HDAC 6 or a 10 μ M solution of Boc-Lys(Tfa)-AMC (Bachem, Bubendorf, Switzerland) for HDAC 4 or a 6 μ M solution of Boc-Lys(Tfa)-AMC for HDAC 8. After 90 min, the reaction was stopped by adding 100 μ L of stop buffer (16 mg/mL trypsin and 4 μ M vorinostat for HDAC 2, 2 μ M CHDI00390576 for HDAC 4, 4 μ M tubastatin A for HDAC 6 and 4 μ M panobinostat for HDAC 8 in 50 mM Tris-HCl, pH 8.0 and 100 mM NaCl). After an additional 15 min, fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm in the

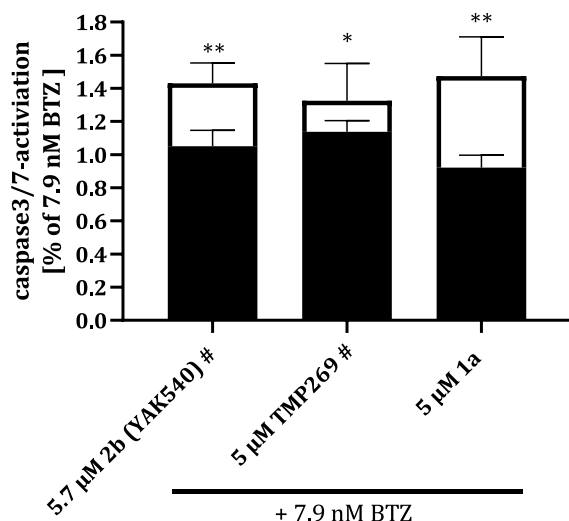


Fig. 8. Synergistic effects in caspase3/7-activation of combination treatment of **1a**, **2b** (YAK540), or TMP269 and BTZ. The sum of single treatments (HDACi alone plus BTZ alone) are shown in black bars. White bars represent the effect of the combination treatment (HDACi plus BTZ). Data are the mean $\pm$ SD calculated as the ratio of the highest BTZ concentration (7.9 nM) of each treatment and $n \geq 2$ with at least 3 replicates. Statistical analysis was performed using *t*-test. Level of significance: ns ($p > 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); # Data taken from Ref. [21].

NOVostar microplate reader (BMG LabTech, Offenburg, Germany).

4.5. Whole-cell HDAC inhibition assay

The cellular HDAC assay is based on the publications by Heltweg and Jung [40], Ciossek et al. [41] and Bonfils et al. [42]. Minor modifications were still made as described in Ref. [43]. In 96-well plates (Corning, Kaiserslautern, Germany), 25,000 c/w THP-1 were seeded in 90 μ L culture medium. After 24 h, increasing concentrations of test compounds and buffer (for untreated control) were added and incubated for an additional 18 h. Then, the reaction was started by adding 10 μ L of 3 mM Boc-Lys(Ac)-AMC or 1 mM Boc-Lys(Tfa)-AMC (Bachem, Bubendorf, Switzerland) to achieve a final concentration of 0.3 mM Boc-Lys(Ac)-AMC or 0.1 mM Boc-Lys(Tfa)-AMC, respectively. After 3 h of incubation under culture conditions, 100 μ L/well stop buffer (25 mM Tris-HCl, pH 8.0, 127 mM KCl, 1 mM MgCl₂, 1% NP40, 2 mg/mL trypsin, 10 μ M panobinostat or 1 μ M vorinostat) was added. After

another 3 h under culture conditions, fluorescence intensity was measured at an excitation wavelength of 320 nm and an emission wavelength of 520 nm in the NOVostar microplate reader (BMG Lab-Tech, Offenburg, Germany).

4.6. Enzyme HDAC inhibition assay with recombinant HDAC 4 enzym

The catalytical domain of HDAC 4 (amino acids 648–1057) was expressed in *E. coli* (DE3) Rosetta cells with the plasmid pET21-HDAC4-TEV-8xHis in LB media supplemented with 100 μ g/mL ampicillin. Cultures were inoculated to an OD₆₀₀ of 0.05 and cultivated at 37 °C and 180 rpm. Protein expression was induced with 20 μ M ZnCl₂ and 0.5 mM IPTG after cultures reached an OD₆₀₀ of 0.8–1. Subsequently, the temperature was lowered to 22 °C and cells were harvested after overnight cultivation at 5,000 $\times$ g for 15 min at 4 °C. Next, cells were resuspended in Buffer A (200 mM NaCl, 50 mM Tris pH 8, 1 mM DTT) supplemented with EDTA free protease inhibitor tablets (Roche) and lysed by cell disruption at 1.5 kbar (Constant Systems). Membranes were removed by ultracentrifugation at 150,000 $\times$ g for 1 h at 4 °C. Afterwards, the supernatant was used for IMAC (HiTrap IMAC HP 1 mL column, Cytiva). Binding was performed with Buffer A containing 5 mM imidazole, while the column was washed with 20 column volumes Buffer A containing 100 mM imidazole. Lastly the catalytical domain of HDAC 4 was eluted with Buffer A containing 300 mM imidazole, prior to centrifugation at 100,000 $\times$ g for 15 min at 4 °C. Next, the centrifuged sample was subjected to size-exclusion-chromatography (Superdex 200 increase 10/300 GL, Cytiva) preequilibrated with Buffer A. The fractions containing the catalytical domain of HDAC 4 were pooled and used for deacetylase assays. In contrast to the described assay, the protein amount was increased to 200 ng and 10 μ L of a 10 μ M Boc-Lys-(tfa)-AMC solution were used. Additionally, the buffer contained 137 mM NaCl instead of KCl and the stopping reaction was performed without the specific inhibitor CHDI00390576.

Table 6

Cytotoxic activity of **1a**, **2b** (YAK540) and BTZ.

	1a	2b (YAK540)	BTZ
	IC ₅₀ $\pm$ SD [μ M]	IC ₅₀ $\pm$ SD [μ M]	IC ₅₀ $\pm$ SD [nM]
Cal27_HDAC4	8.63 $\pm$ 0.803	69.8 $\pm$ 4.78	4.73 $\pm$ 0.540
Cal27_VC	22.5 $\pm$ 1.03	77.7 $\pm$ 3.58	7.60 $\pm$ 0.349
SF	2.61 (****)	1.11 (ns)	1.61 (***)

Data shown are the mean of $n \geq 2$. Shift-factors (SF) were calculated by dividing the IC₅₀ values of Cal27_VC by the IC₅₀ values of Cal27_HDAC4 for each compound. Statistical analysis was performed using *t*-test. Level of significance: ns ($p > 0.05$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$).

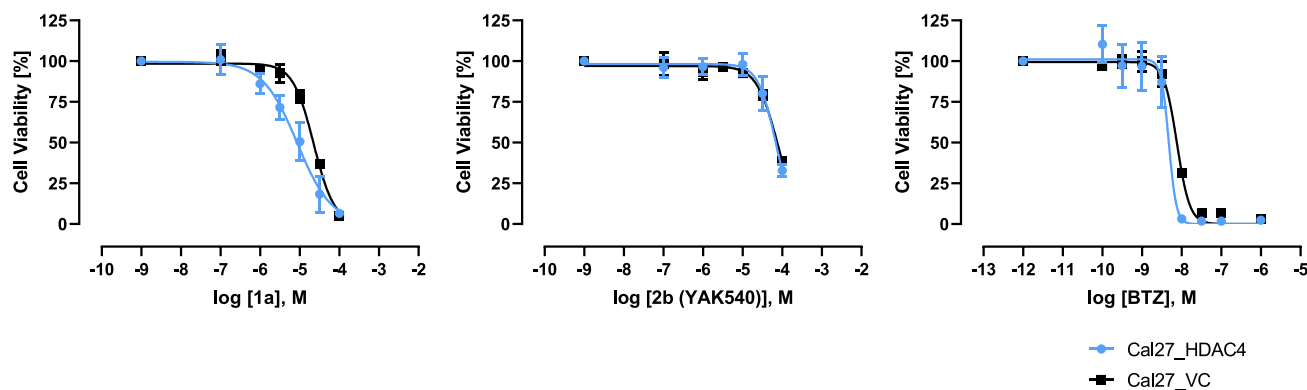


Fig. 9. Concentration-response-curves determined by 72 h MTT of **1a**, **2b** (YAK540) and BTZ in Cal27_HDAC4 (blue curves) and Cal27_VC (black curves). Data shown are the mean of $n \geq 2$.

Table 7
Synergism studies on Cal27_HDAC4 and Cal27_VC.

		1a [μ M]							
		Cal27_HDAC4				Cal27_VC			
		1	2.5	5	7.5	1	2.5	5	7.5
BTZ [nM]	4	1,02	1,62	1,26	0,72	1,60	1,64	1,21	1,05
	6	0,29	0,35	0,29	0,31	1,38	1,30	1,13	1,00
	8	0,18	0,19	0,20	0,21	1,46	1,62	1,42	1,22
	10	0,18	0,20	0,20	0,22	1,44	1,34	1,20	1,09

Data presented are combination indices (CI) calculated by Compusyn 1.0 based on Chou-Talalay method. $CI < 0.9$ indicates synergism. Concentrations were chosen to achieve a fraction affected between 0.1 and 0.9 in the Chou-Talalay-analysis. Data shown are the mean of $n \geq 2$. SD is less than 0.15. Data were obtained from 72 h co-incubations and MTT assay. Red labeled values have a $CI \geq 0.9$. Green labeled values have a $CI < 0.9$.

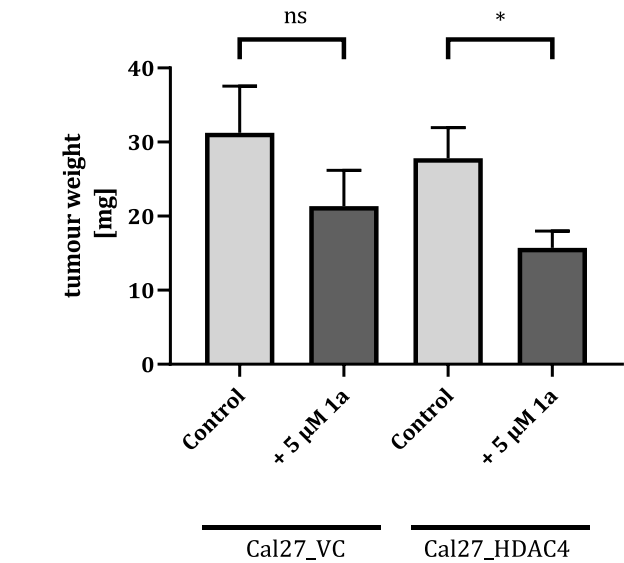


Fig. 10. Chorio-allantoic membrane assay of **1a** in Cal27_VC and Cal27_HDAC4 cells. Data shown are mean tumour weight in mg with $n \geq 5$. Statistical analysis was performed using *t*-test. Level of significance: ns ($p > 0,05$); * ($p \leq 0.05$).

4.7. Measurement of apoptotic nuclei

THP-1 cells were seeded at a density of 10.000 c/w in 24-well plates (Sarstedt, Nürnbrecht, Germany). Cells were treated with BTZ and/or HDACi for the indicated time. Supernatant was removed after a centrifugation step and the cells were lysed in 500 μ l hypotonic lysis buffer (0.1% TritonX-100, 100 μ g/mL propidium iodide) at 4 $^{\circ}$ C in the dark overnight. Triton X-100 was obtained from AppliChem (Darmstadt, Germany). Propidium iodide was delivered by Santa Cruz Biotechnology (Heidelberg, Germany). The percentage of apoptotic nuclei with DNA content in sub-G1 was analyzed by flow cytometry using the CyFlow instrument (Partec, Norderstedt, Germany).

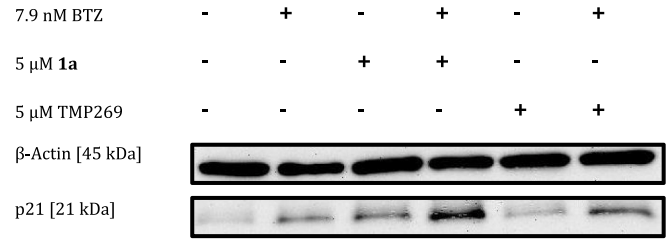


Fig. 12. Western Blot analysis of p21 in THP-1 cells after treatment with 5 μ M **1a** or 5 μ M TMP269 alone or in combination with 7.9 nM BTZ for 24 h. Data presented is a representative blot out of two. The uncropped blot is presented in the supplement (Fig. S2).

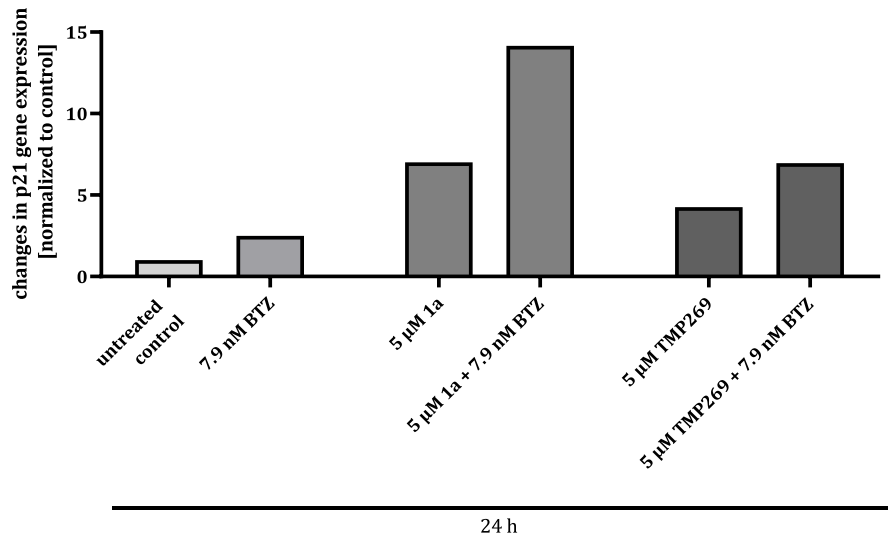


Fig. 11. Changes in *p21* gene expression by combination treatment of **1a** or TMP269 plus BTZ in THP-1 cells. Data were obtained by RT-PCR and analyzed according to Vandesompele [36]. Values are normalized to untreated control gene expression of HPRT1 (hypoxanthine-guanine phosphoribosyltransferase), TBP (TATA binding protein), and GUSB (beta-glucuronidase) and scaled to control. $N = 1$ with duplicates.

4.8. Activation of caspase 3/7

Compound-induced activation of caspase3/7 was analyzed using the CellEvent Caspase-3/7 green detection reagent (Thermo Scientific, Wesel, Germany) according to the manufacturer's instructions. Briefly, THP-1 cells were seeded in 96-well-plates (Corning, Kaiserslautern, Germany) at a density of 10.000 c/w. Cells were treated with BTZ and/or HDACi for the indicated time. Then, a solution of CellEvent Caspase 3/7 green detection reagent in cell culture medium supplemented with 5% heat inactivated FCS for a final assay concentration of 10 μ M was added. Cells were incubated for 30 min at 37 °C under cell culture conditions before imaging by using the Thermo Fisher ArrayScan XTI high content screening (HCS) system with a 10x magnification (Thermo Scientific, Wesel, Germany). Hoechst 33342 was used for nuclei staining at a concentration of 10 mg/mL in water (Sigma-Aldrich, Steinheim, Germany).

4.9. Chicken chorioallantoic membrane (CAM) model

CAM xenografts were performed as previously described [44]. For the CAM xenografts, specific-pathogen-free chicken eggs supplied by VALO BioMedia GmbH, Osterholz-Scharmbeck, Germany, were used. Briefly, cell lines Cal27_VC and Cal27_HDAC4 were washed with PBS following trypsinization. Cells were then resuspended in PBS, counted and placed on ice until used. One million cells of either Cal27_VC or Cal27_HDAC4 were seeded on E10 CAMs and tumors were grown for 7 days. At days 2 and 4, **1a** was injected below the CAM.

4.10. Immunoblotting

Protein extraction and western blot analysis were performed as previously described with minor modifications [39]. Cells were incubated for 24 h with the compounds and concentrations listed. Cells were centrifuged after the incubation period and the medium was aspirated. Cells were washed with phosphate buffer saline and centrifuged again. After re-aspiration, RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM sodium chloride, 2 mM EDTA, spiked with protease and phosphatase inhibitor (Pierce protease and phosphatase inhibitor mini tablets, Thermo Scientific, Wesel, Germany)) was added and the pellet was lysed for 30 min at 4 °C. Then the protein preparations were boiled at 95 °C with 2x Laemmli buffer containing β -mercaptoethanol for 3 min. Pierce BCA protein assay was used to determine the total protein content in the protein preparations (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (40 μ g) were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany). PageRuler Prestained Ladder, 10–180 kDa (Thermo Scientific, Wesel, Germany) was used as a size comparison marker. Blots were incubated with the primary antibodies (Supplemental Table S1). The secondary antibodies conjugated to HRP used in the Western blot were supplied by R&D Systems (Minneapolis, Minnesota, USA). Proteins of interest were visualized using Luminol reagent (Santa Cruz Biotechnology, Heidelberg, Germany) on Intas Imager (Intas, Göttingen, Germany).

4.11. RT-PCR

Cells were incubated with the intentional concentrations of BTZ, TMP269, and **1a** for 24 h. RNA was then isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Subsequently, the RNA was transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Wesel, Germany). For RT-PCR, GoTaq qPCR Master Mix (Promega, Fitchburg, Wisconsin, USA) and a CFX96 Real-Time System (BIO-RAD, Hercules, California, USA) were used. The relative changes in gene expression were normalized to the control genes *GUSB* (beta-glucuronidase), *TBP* (TATA binding protein), and *HPRT1* (hypoxanthine-guanine phosphoribosyltransferase) by Vandesompele method

Table 8

Primer sequences for RT-PCR.

Gene	Primer Forward	Primer Reverse
<i>GUSB</i>	ACCTCCAAGTATCCCAAGGGT	GTCTTGCTCCACGCTGGT
<i>HPRT1</i>	CCTGGCGTCGTGATTAGTGA	CGAGCAAGACGTTTCAGTCCT
<i>TBP</i>	GTGACCCAGCATCACTGTTTC	GAGCATCTCCAGCACACTCT
<i>p21</i>	TGCCGAAGTCAGTTCCTGT	GTTCTGACATGGCGCCTCC

[36]. Primers were designed using Primer-BLAST (NIH, Bethesda, Maryland, USA) and had an efficiency between 80% and 115% (Table 8). Efficiency was determined using an isolated template of HeLa cells.

4.12. Data analysis

Concentration-response-curves were constructed using Prism 7 (GraphPad, San Diego, CA, USA) by fitting data to the four parameter logistic equation. Statistical analysis was performed using *t*-test.

Author contributions

Conceptualization, M.U.K.; T.K.; formal analysis, Y.A., L.M.B., M.U.K., A.J.S.; investigation, Y.A., L.M.B.; A.J.S.; N.H.; F.F., D.R., M.W.; CM; resources, M.U.K.; T.K.; S.S.; G.F.; writing—original draft preparation, Y.A., L.M.B.; writing—review and editing, M.U.K.; T.K.; visualization, L.M.B.; supervision, M.U.K.; T.K.; project administration, M.U.K.; T.K.; funding acquisition, M.U.K., T.K., S.S., G.F. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by Deutsche Forschungsgemeinschaft (DFG) grant numbers KU 1577/2-1, KA 1942/1-1 and INST208/690-1 (ThermoFisher Arrayscan XTI High Content Platform). The Center for Structural Studies is funded by the Deutsche Forschungsgemeinschaft (DFG Grant number 417919780)

This study was additionally funded in part by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 270650915 (Research Training Group GRK 2158: SS, TK, MUK and DFG grant number FL 865/2-1 (GF).

Institutional review board statement

Not applicable.

Informed consent statement

Not applicable.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Matthias U. Kassack, Thomas Kurz reports financial support was provided by Deutsche Forschungsgemeinschaft. Yodita Asfaha, Lukas M. Bollmann, Alexander J. Skerhut, Fabian Fischer reports financial support was provided by Deutsche Forschungsgemeinschaft (DFG).

Data availability

Data are published in manuscript.

Acknowledgments

Not applicable.

Chemistry

Experimental part

Chemistry: All chemicals and solvents were purchased from commercial suppliers (Sigma Aldrich, Alfa Aesar, Fluorochem, TCI, abcr and Acros Organics) and used without further purification. Dry solvents were used directly from Seal® bottles from Acros Organics. Analytic Thin Layer Chromatography (TLC) was carried out with Macherey Nagel precoated silica gel plates (ALUGRAM® Xtra SIL G/UV₂₅₄). Detection was achieved with ultraviolet irradiation (254 nm) and/or staining with potassium permanganate solution (9 g KMnO₄, 60 g K₂CO₃, 15 mL of a 5% aqueous NaOH-solution, and 900 mL demineralised water). Flash column chromatography was performed with CombiFlashRf200 (TeleDyne) with the solvent mixtures specified in the corresponding procedure. Proton (¹H) and carbon (¹³C) NMR spectra were recorded on Bruker Avance III – 300, Bruker Avance DRX – 500 or Bruker Avance III – 600. Spectra were referenced to the residual non-deuterated solvent signal (¹H NMR: DMSO-*d*₆ (2.50 ppm), ¹³C NMR: DMSO-*d*₆ (39.52 ppm); ¹H NMR: CDCl₃ (7.26 ppm), ¹³C NMR: CDCl₃ (77.16 ppm)). Chemical shifts are quoted in parts per million (ppm). Signal patterns are indicated as: singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). Coupling constants, *J*, are measured in Hz. Proton (¹H) and carbon (¹³C) NMR spectra were recorded by the Center for Molecular and Structural Analytics of the Heinrich Heine University Duesseldorf (<https://www.cemsa.hhu.de/>). Electrospray Ionisation (ESI) mass spectra were carried out by the Center for Molecular and Structural Analytics of the Heinrich Heine University Duesseldorf, using Bruker Daltonics UHR-QTOF maXis 4G (Bruker Daltonics). APCI-MS was carried out using Advion expression¹ CMS spectrometer. Melting points (mp.) were determined using a Büchi M – 565 melting point apparatus and are uncorrected. Analytical HPLC analysis were carried out on a Knauer HPLC system comprising an Azura P 6.1L pump, an Optimas 800 autosampler, a Fast Scanning Spectrophotometer K-2600 and a Knauer Reversed Phase column (SN: FK36). UV absorption was detected at 254 nm. The solvent gradient table is shown below. The purity of all final compounds was 95% or higher.

Table 9
The solvent gradient table for analytic HPLC analysis.

Time/min	Water +0.1% TFA	ACN +0.1% TFA
Initial	90	10
0.50	90	10
20.0	0	100
30.0	0	100
31.0	90	10
40.0	90	10

General procedure 1: HATU-coupling: For the synthesis of the TFMO-based amides **1a-i**, 1.00 eq of the respective carboxylic acid **5** was dissolved in DMF (0.1 mmol/mL) and 1.00 eq HATU as well as 2.00 eq DIPEA were added. After stirring for 15 min at rt, the corresponding amine (1.00 eq) was then added and the resulting mixture was stirred for further 16 h. The solvent was removed under reduced pressure and the residue was diluted with EtOAc. The organic layer was washed with saturated NaHCO₃ (3x 50 mL), brine (1x 50 mL), dried over Na₂SO₄ and filtered. After removing the solvent, the crude product was purified as described.

Synthesis of *N*-(2-(diethylamino)ethyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benz-amide (1a**):** The crude product was purified by flash chromatography with *n*-hexane/EtOAc as eluent. White solid, yield: 60%, mp.: 68.6 °C. ¹H NMR (300 MHz, CDCl₃) δ = 1.06 (t, *J* = 7.1 Hz, 7H), 2.60 (q, *J* = 7.1 Hz, 4H), 2.70 (t, *J* = 5.9 Hz, 2H), 3.41–3.60 (m, 2H), 7.10 (s, 1H), 7.87–7.97 (m, 2H), 8.15–8.24 (m, 2H) ppm. ¹³C NMR (75 MHz, DMSO-*d*₆) δ = 11.85, 37.68, 46.74, 51.36, 110.22, 115.65 (q, *J* = 273.3 Hz), 121.08, 126.46, 127.29, 128.10, 138.05, 164.78, 165.02, 165.36, 167.93 ppm. HPLC: *t*_R = 9.683 min, AUC = 99%. HRMS (ESI+) = calcd. for C₁₆H₂₀F₃N₄O₂ [M+H]⁺ = 357.1533, found: 357.1535. All spectroscopic data were in agreement with All spectroscopic data were in agreement with published data [12].

Synthesis of *N*-benzyl-*N*-methyl-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (1b**):** The crude product was purified by flash chromatography with *n*-hexane/EtOAc as eluent. White solid, yield: 72%, mp.: 89.8 °C. ¹H NMR (600 MHz, DMSO-*d*₆) δ = 2.89 (d, *J* = 53.4 Hz, 3H), 4.48 (s, 1H), 4.71 (s, 1H), 7.10–7.46 (m, 4H), 7.68 (dd, *J* = 26.6, 7.9 Hz, 2H), 8.12 (dd, *J* = 27.3, 7.9 Hz, 2H) ppm. ¹³C NMR (151 MHz, DMSO-*d*₆) δ = 34.73 (d, *J* = 608.2 Hz), 51.94 (d, *J* = 630.0 Hz), 115.74 (q, *J* = 273.0 Hz), 125.20, 126.76, 127.27, 127.46, 127.56, 127.60, 127.67, 127.99, 128.64, 128.77, 136.63, 137.12, 140.09, 140.19, 165.16 (q, *J* = 43.9 Hz), 167.97, 169.29, 169.87 ppm. HPLC: *t*_R = 15.150 min, AUC = 99%. HRMS (ESI+) = calcd. for C₁₈H₁₅F₃N₃O₂ [M+H]⁺ = 362.1111, found: 362.113. All spectroscopic data are in agreement with published data [28].

Synthesis of *N*-(4-(diethylamino)butyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (1c**):** The crude product was purified by flash chromatography with CH₂Cl₂/30% MeOH and CH₂Cl₂/0.1% NEt₃ as eluent. White solid, yield: 83%, mp.: 84.4 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ = 0.97 (t, *J* = 7.1 Hz, 6H), 1.39–1.62 (m, 4H), 2.40–2.60 (m, 6H), 3.29 (q, *J* = 6.5 Hz, 2H), 8.01–8.08 (m, 2H), 8.12–8.18 (m, 2H), 8.70 (t, *J* = 5.6 Hz, 1H) ppm. ¹³C NMR (75 MHz, DMSO-*d*₆) δ = 11.22, 23.75, 26.92, 46.30, 51.79, 115.75 (d, *J* = 273.2 Hz), 126.55, 127.38, 128.28, 138.17, 164.91, 165.18, 165.49, 167.99 ppm. HPLC: *t*_R = 9.919 min, AUC >99%. HRMS (ESI+) = calcd. for C₁₈H₂₄F₃N₄O₂ [M+H]⁺ = 385.1846, found: 385.1848.

Synthesis of *N*-(2-(diethylamino)ethyl)-*N*-methyl-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (1d**):** The crude product was purified by flash chromatography with CH₂Cl₂/30% MeOH and CH₂Cl₂/0.1% NEt₃ as eluent. Yellowish oil, yield: 72%. ¹H NMR (600 MHz, DMSO-*d*₆) δ = 0.79 (t, *J* = 7.1 Hz, 3H), 0.99 (t, *J* = 7.1 Hz, 3H), 2.26 (q, *J* = 7.1 Hz, 2H), 2.46 (t, *J* = 6.8 Hz, 1H), 2.51–2.56 (m, 2H), 2.63 (t, *J* = 6.9 Hz, 1H), 2.96 (d, *J* = 46.7 Hz, 3H), 3.24 (t, *J* = 6.8 Hz, 1H), 3.52 (t, *J* = 6.8 Hz, 1H), 7.61 (dd, *J* = 16.6, 7.8 Hz, 2H), 8.12 (t, *J* = 6.2 Hz, 2H) ppm. ¹³C NMR (151 MHz, DMSO-*d*₆) δ = 11.59, 12.10, 32.81, 37.76, 40.06, 45.10, 46.59, 46.74, 49.05, 49.54, 50.16, 115.75 (q, *J* = 273.0 Hz), 124.81, 124.95, 127.32, 127.52, 127.78, 127.93, 140.77, 165.15 (q, *J* = 43.6 Hz), 167.98, 168.81, 169.75 ppm. HPLC: *t*_R = 9.719 min, AUC >99%. HRMS (ESI+) = calcd. for C₁₇H₂₂F₃N₄O₂ [M+H]⁺ = 371.1689, found: 371.1686.

Synthesis of *N*-(2-(1*H*-indol-3-yl)ethyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (1e**):** The crude product was purified by flash chromatography with *n*-hexane/EtOAc as eluent and then further purified by recrystallisation (*n*-hexane/EtOAc). Beige solid, yield: 45%, mp.: 167.0 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ = 2.98 (t, *J* = 7.5 Hz, 2H), 3.58 (td, *J* = 7.4, 5.6 Hz, 2H), 7.03 (dddd, *J* = 25.8, 8.0, 7.0, 1.2 Hz, 2H), 7.20 (d, *J* = 2.3 Hz, 1H), 7.35 (dt, *J* = 8.1, 0.9 Hz, 1H), 7.59 (dd, *J* = 7.6, 1.1 Hz, 1H), 8.02–8.21 (m, 4H), 8.85 (t, *J* = 5.6 Hz, 1H), 10.82 (s, 1H) ppm. ¹³C NMR

(151 MHz, DMSO- d_6) δ = 25.08, 40.35, 111.38, 111.80, 115.75 (q, J = 273.1 Hz), 118.23, 118.26, 120.92, 122.63, 126.58, 127.27, 127.39, 128.30, 136.25, 138.14, 165.13, 165.18 (q, J = 43.7 Hz), 167.97 ppm. HPLC: t_R = 14.467 min, AUC >99%. HRMS (ESI+) = calcd. for $C_{19}H_{18}F_3N_4O_2$ [M+H]⁺ = 391.1376, found: 391.1378.

Synthesis of *N*-methyl-*N*-phenyl-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (1f): The crude product was purified by flash chromatography with *n*-hexane/EtOAc as eluent. White solid, yield: 74%, mp.: 89.7 °C. ¹H NMR (300 MHz, DMSO- d_6) δ = 3.40 (s, 3H), 7.06–7.35 (m, 5H), 7.47 (d, J = 8.0 Hz, 2H), 7.89 (d, J = 8.2 Hz, 2H) ppm. ¹³C NMR (151 MHz, DMSO- d_6) δ = 37.69, 115.70 (q, J = 273.2 Hz), 124.88, 126.77, 126.84, 127.18, 129.12, 129.17, 140.26, 143.97, 165.04 (q, J = 43.6 Hz), 167.80, 168.48 ppm. HPLC: t_R = 14.717 min, AUC = 98.7%. HRMS (ESI+) = calcd. for $C_{17}H_{13}F_3N_3O_2$ [M+H]⁺ = 348.0954, found: 348.0954. All spectroscopic data were in agreement with published data [28].

Synthesis of *N*-(4-(dimethylamino)benzyl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (1g): The crude product was purified by flash chromatography with CH_2Cl_2 /30% MeOH and CH_2Cl_2 /0.1% NEt_3 as eluent and further purified by recrystallisation (*n*-hexane/EtOAc). Green solid, yield: 36%, mp.: 182.6 °C. ¹H NMR (300 MHz, DMSO- d_6) δ = 2.85 (s, 6H), 4.38 (d, J = 5.9 Hz, 2H), 6.66–6.74 (m, 2H), 7.12–7.22 (m, 2H), 8.06–8.12 (m, 2H), 8.13–8.21 (m, 2H), 9.13 (t, J = 5.9 Hz, 1H) ppm. ¹³C NMR (75 MHz, DMSO- d_6) δ = 40.29, 42.36, 112.38, 115.75 (d, J = 273.1 Hz), 126.63, 126.89, 127.40, 128.39, 138.03, 149.64, 164.90, 164.99, 165.48, 167.96 ppm. HPLC: t_R = 10.103 min, AUC = 99%. HRMS (ESI+) = calcd. for $C_{19}H_{18}F_3N_4O_2$ [M+H]⁺ = 391.1376, found: 391.1376. All spectroscopic data were in agreement with published data [28].

Synthesis of 4-methylpiperazin-1-yl(4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)phenyl)-methanone (1h): The crude product was purified by flash chromatography with CH_2Cl_2 /30% MeOH and CH_2Cl_2 /0.1% NEt_3 as eluent. Yellow solid, yield: 75%, mp.: 77.8 °C. ¹H NMR (300 MHz, DMSO- d_6) δ = 2.23 (s, 3H), 2.37 (d, J = 19.8 Hz, 5H), 3.64 (s, 2H), 7.53–7.70 (m, 2H), 8.04–8.26 (m, 2H) ppm. ¹³C NMR (75 MHz, DMSO- d_6) δ = 45.29, 54.21, 115.64 (d, J = 273.1 Hz), 125.12, 127.48, 127.86, 139.66, 164.76, 165.34, 167.74, 167.93 ppm. HPLC: t_R = 8.552 min, AUC = 96.8%. HRMS (ESI+) = calcd. for $C_{15}H_{16}F_3N_4O_2$ [M+H]⁺ = 341.1220, found: 341.1221. All spectroscopic data were in agreement with published data [12].

Synthesis of *N*-(quinolin-3-yl)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (1i): The crude product was purified by flash chromatography with CH_2Cl_2 /0.1% NEt_3 and 30% MeOH in CH_2Cl_2 /0.1% NEt_3 as eluent. White solid, yield: 29%, mp.: 240.1 °C. ¹H NMR (600 MHz, DMSO- d_6) δ = 7.60 (ddd, J = 8.1, 6.9, 1.1 Hz, 1H), 7.68 (tq, J = 7.9, 1.1 Hz, 1H), 7.95–8.02 (m, 2H), 8.26 (t, J = 1.0 Hz, 4H), 8.84–8.89 (m, 1H), 9.16 (dd, J = 2.4, 1.2 Hz, 1H), 10.92 (s, 1H) ppm. ¹³C NMR (151 MHz, DMSO- d_6) δ = 115.76 (d, J = 273.1 Hz), 123.64, 127.10, 127.34, 127.57, 127.70, 127.86, 128.19, 128.58, 128.96, 132.66, 137.68, 144.50, 145.43, 165.15, 165.27 (d, J = 43.9 Hz), 167.90 ppm. HPLC: t_R = 11.786 min, AUC = 98.4%. HRMS (ESI+) = calcd. for $C_{19}H_{12}F_3N_4O_2$ [M+H]⁺ = 385.0907, found: 385.0904. All spectroscopic data were in agreement with published data [12].

General procedure 2: HATU-coupling

For the synthesis of the TFMO-based alkoxyamides **2a-c**, 1.00 eq of the carboxylic acid **5** was dissolved in DMF (0.1 mmol/mL) and 1.00 eq HATU and 2.00 eq DIPEA were added. After stirring for 15 min at rt, the respective hydroxylamine (1.00 eq) was then added and the resulting mixture was stirred for further 16h. After removing the solvent, the residue was diluted with EtOAc. The organic layer was washed with saturated $NaHCO_3$ (3x 50 mL), brine (1x 50 mL) dried over Na_2SO_4 and filtered. After removing the solvent, the crude product was purified as stated.

Synthesis of *N*-((2-phenyloxazol-5-yl)methoxy)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (2a): The crude product was purified by flash chromatography with *n*-hexane and EtOAc as eluent. White solid, yield: 20%, mp.: 199 °C. ¹H NMR (300 MHz, DMSO- d_6) δ = 4.95 (s, 2H), 7.55 (p, J = 3.8, 3.4 Hz, 3H), 7.87–8.08 (m, 4H), 8.15 (d, J = 8.2 Hz, 2H), 8.34 (s, 1H), 12.00 (s, 1H) ppm. ¹³C NMR (151 MHz, DMSO- d_6) δ = 68.26, 116.65 (q), 125.95, 126.77, 127.13, 127.52, 128.40, 129.21, 130.78, 135.78, 136.68, 139.46, 160.84, 163.45, 165.23 (d, J = 43.7 Hz), 167.88 ppm. HPLC analysis: R_t = 14.050 min, 99.6%. HRMS (ESI+) = calcd. for $C_{20}H_{14}F_3N_4O_4$ [M+H]⁺ = 431.0962, found: 431.0958.

Synthesis of *N*-(2-(piperidin-1-yl)ethoxy)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (2b): The crude product was purified by flash chromatography with CH_2Cl_2 /30% MeOH/0.1% NEt_3 and CH_2Cl_2 /0.1% NEt_3 as eluent. Yellowish solid, yield: 54%, mp.: 131 °C. ¹H NMR (300 MHz, DMSO- d_6) δ = 1.41 (q, J = 6.0 Hz, 2H), 1.55 (p, J = 5.5 Hz, 4H), 2.57 (t, J = 5.3 Hz, 4H), 2.71 (t, J = 5.5 Hz, 2H), 4.06 (t, J = 5.5 Hz, 2H), 7.95–8.01 (m, 2H), 8.12–8.20 (m, 2H) ppm. ¹³C NMR (75 MHz, DMSO- d_6) δ = 23.39, 24.95, 53.82, 56.02, 72.27, 115.70 (d, J = 273.2 Hz), 126.91, 127.47, 128.18, 135.88, 163.10, 165.18 (d, J = 43.7 Hz), 167.86 ppm. HPLC analysis: R_t = 10.321 min, 96.2%. HRMS (ESI+) = calcd. for $C_{17}H_{20}F_3N_4O_3$ [M+H]⁺ = 385.1482, found: 385.1482. All spectroscopic data were in agreement with published data [21].

Synthesis of *N*-(2-morpholinoethoxy)-4-(5-(trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzamide (2c): The crude product was purified by flash chromatography with CH_2Cl_2 /30% MeOH/0.1% NEt_3 and CH_2Cl_2 /0.1% NEt_3 as eluent. Yellowish solid, yield: 72%, mp.: 75 °C. ¹H NMR (300 MHz, DMSO- d_6) δ = 2.55 (t, J = 4.4 Hz, 4H), 2.69 (t, J = 5.3 Hz, 2H), 3.60 (t, J = 4.7 Hz, 4H), 4.06 (t, J = 5.4 Hz, 2H), 7.94–8.00 (m, 2H), 8.14–8.20 (m, 2H) ppm. ¹³C NMR (126 MHz, DMSO- d_6) δ = 3.26, 55.99, 65.89, 72.67, 115.70 (q, J = 273.2 Hz), 127.02, 127.51, 128.17, 135.68, 162.90, 165.19 (q, J = 44.0 Hz), 167.88 ppm. HPLC analysis: R_t = 15.637 min, 95.2%. HRMS (ESI+) = calcd. for $C_{16}H_{18}F_3N_4O_4$ [M+H]⁺ = 387.1275, found: 387.1279.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmech.2023.115907>.

References

- [1] X.-J. Yang, E. Seto, The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men, *Nat. Rev. Mol. Cell Biol.* 9 (2008) 206–218, <https://doi.org/10.1038/nrm2346>.
- [2] M. Haberland, R.L. Montgomery, E.N. Olson, The many roles of histone deacetylases in development and physiology: implications for disease and therapy, *Nat. Rev. Genet.* 10 (2009) 32–42, <https://doi.org/10.1038/nrg2485>.
- [3] C.M. Grozinger, C.A. Hassig, S.L. Schreiber, Three proteins define a class of human histone deacetylases related to yeast Hda1p, *Proc. Natl. Acad. Sci. U.S.A.* 96 (1999) 4868–4873, <https://doi.org/10.1073/pnas.96.9.4868>.
- [4] Y. Asfaha, C. Schrenk, L.A. Alves Avelar, A. Hamacher, M. Pflieger, M.U. Kassack, T. Kurz, Recent advances in class IIa histone deacetylases research, *Bioorg. Med. Chem.* 27 (2019), 115087, <https://doi.org/10.1016/j.bmc.2019.115087>.
- [5] T.C.S. Ho, A.H.Y. Chan, A. Ganesan, Thirty years of HDAC inhibitors: 2020 insight and hindsight, *J. Med. Chem.* 63 (2020) 12460–12484, <https://doi.org/10.1021/acs.jmedchem.0c00830>.
- [6] S.-Y. Park, G.S. Kim, H.-J. Hwang, T.-H. Nam, H.-S. Park, J. Song, T.-H. Jang, Y. C. Lee, J.-S. Kim, Structural basis of the specific interaction of SMRT corepressor with histone deacetylase 4, *Nucleic Acids Res.* 46 (2018) 11776–11788, <https://doi.org/10.1093/nar/gky926>.
- [7] M. Lobera, K.P. Madauss, D.T. Pohlhaus, Q.G. Wright, M. Trocha, D.R. Schmidt, E. Baloglu, R.P. Trump, M.S. Head, G.A. Hofmann, M. Murray-Thompson, B. Schwartz, S. Chakravorty, Z. Wu, P.K. Mander, L. Kruidenier, R.A. Reid, W. Burkhardt, B.J. Turunen, J.X. Rong, C. Wagner, M.B. Moyer, C. Wells, X. Hong, J. T. Moore, J.D. Williams, D. Soler, S. Ghosh, M.A. Nolan, Selective class IIa histone deacetylase inhibition via a nonchelating zinc-binding group, *Nat. Chem. Biol.* 9 (2013) 319–325, <https://doi.org/10.1038/nchembio.1223>.

- [8] N. Turkman, D. Liu, I. Pirola, Design, synthesis, biochemical evaluation, radiolabeling and in vivo imaging with high affinity class-IIa histone deacetylase inhibitor for molecular imaging and targeted therapy, *Eur. J. Med. Chem.* 228 (2022), 114011, <https://doi.org/10.1016/j.ejmech.2021.114011>.
- [9] A.J. Stott, M.C. Maillard, V. Beaumont, D. Allcock, O. Aziz, A.H. Borchers, W. Blackaby, P. Breccia, G. Creighton-Gutteridge, A.F. Haughan, R.E. Jarvis, C. A. Luckhurst, K.L. Matthews, G. McAllister, S. Pollack, E. Saville-Stones, A.J. van de Poël, H.D. Vater, J. Vann, R. Williams, D. Yates, I. Muñoz-Sanjuán, C. Dominguez, Evaluation of 5-(Trifluoromethyl)-1,2,4-oxadiazole-Based class IIa HDAC inhibitors for huntington's disease, *ACS Med. Chem. Lett.* 12 (2021) 380–388, <https://doi.org/10.1021/acsmchemlett.0c00532>.
- [10] R.W. Bürlü, C.A. Luckhurst, O. Aziz, K.L. Matthews, D. Yates, K.A. Lyons, M. Beconi, G. McAllister, P. Breccia, A.J. Stott, S.D. Penrose, M. Wall, M. Lamers, P. Leonard, I. Müller, C.M. Richardson, R. Jarvis, L. Stones, S. Hughes, G. Wishart, A. F. Haughan, C. O'Connell, T. Mead, H. McNeil, J. Vann, J. Mangette, M. Maillard, V. Beaumont, I. Muñoz-Sanjuán, C. Dominguez, Design, synthesis, and biological evaluation of potent and selective class IIa histone deacetylase (HDAC) inhibitors as a potential therapy for Huntington's disease, *J. Med. Chem.* 56 (2013) 9934–9954, <https://doi.org/10.1021/jm4011884>.
- [11] C.A. Luckhurst, P. Breccia, A.J. Stott, O. Aziz, H.L. Birch, R.W. Bürlü, S.J. Hughes, R.E. Jarvis, M. Lamers, P.M. Leonard, K.L. Matthews, G. McAllister, S. Pollack, E. Saville-Stones, G. Wishart, D. Yates, C. Dominguez, Potent, selective, and CNS-penetrant tetrasubstituted cyclopropane class IIa histone deacetylase (HDAC) inhibitors, *ACS Med. Chem. Lett.* 7 (2016) 34–39, <https://doi.org/10.1021/acsmchemlett.5b00302>.
- [12] A.F. Abdel-Magid, Histone deacetylase 4 (HDAC4) inhibitors: a promising treatment for huntington's disease, *ACS Med. Chem. Lett.* 4 (2013) 692–693, <https://doi.org/10.1021/ml4002216>.
- [13] D. Morel, D. Jeffery, S. Aspeslagh, G. Almouzni, S. Postel-Vinay, Combining epigenetic drugs with other therapies for solid tumours - past lessons and future promise, *Nat. Rev. Clin. Oncol.* 17 (2020) 91–107, <https://doi.org/10.1038/s41571-019-0267-4>.
- [14] M. Duvic, R. Talpur, X. Ni, C. Zhang, P. Hazarika, K. Kelly, J.H. Chiao, J.F. Reilly, J. L. Ricker, V.M. Richon, S.R. Frankel, Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL), *Blood* 109 (2007) 31–39, <https://doi.org/10.1182/blood-2006-06-025999>.
- [15] P. Atadja, Development of the pan-DAC inhibitor panobinostat (LBH589): successes and challenges, *Cancer Lett.* 280 (2009) 233–241, <https://doi.org/10.1016/j.canlet.2009.02.019>.
- [16] P.A. Marks, Histone deacetylase inhibitors, in: E.P. Gelmann, C.L. Sawyers, I. J. Rauscher (Eds.), *Molecular Oncology*, Cambridge University Press, 2015, pp. 912–920.
- [17] S. Minucci, P.G. Pelicci, Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer, *Nat. Rev. Cancer* 6 (2006) 38–51, <https://doi.org/10.1038/nrc1779>.
- [18] V.M. Richon, S. Emiliani, E. Verdin, Y. Webb, R. Breslow, R.A. Rifkind, P.A. Marks, A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases, *Proc. Natl. Acad. Sci. U.S.A.* 95 (1998) 3003–3007, <https://doi.org/10.1073/PNAS.95.6.3003>.
- [19] A. Monks, C.D. Hose, P. Pezzoli, S. Kondapaka, G. Vansant, K.D. Petersen, M. Sehested, J. Monforte, R.H. Shoemaker, Gene expression-signature of belinostat in cell lines is specific for histone deacetylase inhibitor treatment, with a corresponding signature in xenografts, *Anti Cancer Drugs* 20 (2009) 682–692, <https://doi.org/10.1097/cad.0b013e32832e14e1>.
- [20] S. Shen, A.P. Kozikowski, Why hydroxamates may not be the best histone deacetylase inhibitors—what some may have forgotten or would rather forget? *ChemMedChem* 11 (2016) 15–21, <https://doi.org/10.1002/cmdc.201500486>.
- [21] L.M. Bollmann, A.J. Skerhut, Y. Asfaha, N. Horstick, H. Hanenberg, A. Hamacher, T. Kurz, M.U. Kassack, The novel class IIa selective histone deacetylase inhibitor YAK540 is synergistic with bortezomib in leukemia cell lines, *Int. J. Mol. Sci.* 23 (2022), <https://doi.org/10.3390/ijms232113398>.
- [22] S. Kikuchi, R. Suzuki, H. Ohguchi, Y. Yoshida, D. Lu, F. Cottini, J. Jakubikova, G. Bianchi, T. Harada, G. Gorgun, Y.-T. Tai, P.G. Richardson, T. Hideshima, K. C. Anderson, Class IIa HDAC inhibition enhances ER stress-mediated cell death in multiple myeloma, *Leukemia* 29 (2015) 1918–1927, <https://doi.org/10.1038/leu.2015.83>.
- [23] M. Usami, S. Kikuchi, K. Takada, M. Ono, Y. Sugama, Y. Arihara, N. Hayasaka, H. Nakamura, Y. Ikeda, M. Hirakawa, M. Yoshida, K. Miyashita, M. Kobune, J. Kato, FOXO3a activation by HDAC class IIa inhibition induces cell cycle arrest in pancreatic cancer cells, *Pancreas* 49 (2020) 135–142, <https://doi.org/10.1007/MPA.0000000000001462>.
- [24] C. Meregalli, An overview of bortezomib-induced neurotoxicity, *Toxics* 3 (2015) 294–303, <https://doi.org/10.3390/toxics3030294>.
- [25] P. Robak, T. Robak, Bortezomib for the treatment of hematologic malignancies: 15 Years later, *Drugs R* 19 (2019) 73–92, <https://doi.org/10.1007/s40268-019-0269-9>.
- [26] C.A. Luckhurst, O. Aziz, V. Beaumont, R.W. Bürlü, P. Breccia, M.C. Maillard, A. F. Haughan, M. Lamers, P. Leonard, K.L. Matthews, G. Raphy, A.J. Stott, I. Muñoz-Sanjuán, B. Thomas, M. Wall, G. Wishart, D. Yates, C. Dominguez, Development and characterization of a CNS-penetrant benzhydryl hydroxamic acid class IIa histone deacetylase inhibitor, *Bioorg. Med. Chem. Lett.* 29 (2019) 83–88, <https://doi.org/10.1016/j.bmcl.2018.11.009>.
- [27] C. Schrenk, L.M. Bollmann, C. Haist, A. Bister, C. Wiek, M. Wecker, D. Roth, P. Petzsch, K. Köhrer, A. Hamacher, H. Hanenberg, G. Fluegen, M.U. Kassack, Synergistic interaction of the class IIa HDAC inhibitor CHDI0039 with bortezomib in head and neck cancer cells, *Indian J. Manag. Sci.* 24 (2023) 5553, <https://doi.org/10.3390/ijms24065553>.
- [28] W.I.E.J.A. Andy, Christian Winter, Claudia Rosenbaum, Doris Kremzow-Graw, Franz Roehl, Joachim Rheinheimer, Manojkumar Poonoth, Violeta Terteryan, Egon Haden, Ana Escribano Cuesta, Janosch Harald Achenbach, Tobias Mentzel, Christine WIEBE WO 2015/185485 A1, 2015.
- [29] J.E. Bradner, N. West, M.L. Grachan, E.F. Greenberg, S.J. Haggarty, T. Warnow, R. Mazitschek, Chemical phylogenetics of histone deacetylases, *Nat. Chem. Biol.* 6 (2010) 238–243, <https://doi.org/10.1038/nchembio.313>.
- [30] E. Di Giorgio, E. Gagliostro, C. Brancolini, Selective class IIa HDAC inhibitors: myth or reality, *Cell. Mol. Life Sci.* 72 (2015) 73–86, <https://doi.org/10.1007/s00018-014-1727-8>.
- [31] V. Krieger, A. Hamacher, F. Cao, K. Stenzel, C.G.W. Gertzen, L. Schäker-Hübner, T. Kurz, H. Gohlke, F.J. Dekker, M.U. Kassack, F.K. Hansen, Synthesis of peptoid-based class I-selective histone deacetylase inhibitors with chemosensitizing properties, *J. Med. Chem.* 62 (2019) 11260–11279, <https://doi.org/10.1021/acs.jmedchem.9b01489>.
- [32] T.C. Chou, P. Talalay, Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors, *Adv. Enzym. Regul.* 22 (1984) 27–55, [https://doi.org/10.1016/0065-2571\(84\)90007-4](https://doi.org/10.1016/0065-2571(84)90007-4).
- [33] W. Zhao, K. Sachsenmeier, L. Zhang, E. Sult, R.E. Hollingsworth, H. Yang, A new Bliss independence model to analyze drug combination data, *J. Biomol. Screen* 19 (2014) 817–821, <https://doi.org/10.1177/1087057114521867>.
- [34] G.Y. Di Veroli, C. Fornari, D. Wang, S. Mollard, J.L. Bramhall, F.M. Richards, D. I. Jodrell, Combeneft: an interactive platform for the analysis and visualization of drug combinations, *Bioinformatics* 32 (2016) 2866–2868, <https://doi.org/10.1093/bioinformatics/btw230>.
- [35] C. Schrenk, L.M. Bollmann, C. Haist, A. Bister, C. Wiek, M. Wecker, D. Roth, P. Petzsch, K. Köhrer, A. Hamacher, H. Hanenberg, G. Fluegen, M.U. Kassack, Synergistic interaction of the class IIa HDAC inhibitor CHDI0039 with bortezomib in head and neck cancer cells, *Indian J. Manag. Sci.* 24 (2023), <https://doi.org/10.3390/ijms24065553>.
- [36] J. Vandesompele, K. de Preter, F. Pattyn, B. Poppe, N. van Roy, A. de Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, *Genome Biol.* 3 (2002), <https://doi.org/10.1186/gb-2002-3-7-research0034>. RESEARCH0034.
- [37] E.M. Gosepath, N. Eckstein, A. Hamacher, K. Servan, G. von Jonquieres, H. Lage, B. Györfy, H.D. Royer, M.U. Kassack, Acquired cisplatin resistance in the head-neck cancer cell line Cal27 is associated with decreased DKK1 expression and can partially be reversed by overexpression of DKK1, *Int. J. Cancer* 123 (2008) 2013–2019, <https://doi.org/10.1002/ijc.23721>.
- [38] L.H. Engelke, A. Hamacher, P. Proksch, M.U. Kassack, Ellagic acid and resveratrol prevent the development of cisplatin resistance in the epithelial ovarian cancer cell line A2780, *J. Cancer* 7 (2016) 353–363, <https://doi.org/10.7150/jca.13754>.
- [39] C. Wang, A. Hamacher, P. Petzsch, K. Köhrer, G. Niegisch, M.J. Hoffmann, W. A. Schulz, M.U. Kassack, Combination of decitabine and entinostat synergistically inhibits urothelial bladder cancer cells via activation of FoxO1, *Cancers* 12 (2020), <https://doi.org/10.3390/cancers12020337>.
- [40] B. Heltweg, M. Jung, A microplate reader-based nonisotopic histone deacetylase activity assay, *Anal. Biochem.* 302 (2002) 175–183, <https://doi.org/10.1006/abio.2001.5542>.
- [41] T. Ciossek, H. Julius, H. Wieland, T. Maier, T. Beckers, A homogeneous cellular histone deacetylase assay suitable for compound profiling and robotic screening, *Anal. Biochem.* 372 (2008) 72–81, <https://doi.org/10.1016/j.ab.2007.07.024>.
- [42] C. Bonfils, A. Kalita, M. Dubay, L.L. Siu, M.A. Carducci, G. Reid, R.E. Martell, J. M. Besterman, Z. Li, Evaluation of the pharmacodynamic effects of MGCD0103 from preclinical models to human using a novel HDAC enzyme assay, *Clin. Cancer Res.* 14 (2008) 3441–3449, <https://doi.org/10.1158/1078-0432.CCR-07-4427>.
- [43] L. Marek, A. Hamacher, F.K. Hansen, K. Kuna, H. Gohlke, M.U. Kassack, T. Kurz, Histone deacetylase (HDAC) inhibitors with a novel connecting unit linker region reveal a selectivity profile for HDAC4 and HDAC5 with improved activity against chemoresistant cancer cells, *J. Med. Chem.* 56 (2013) 427–436, <https://doi.org/10.1021/jm301254q>.
- [44] G. Fluegen, A. Avivar-Valderas, Y. Wang, M.R. Padgen, J.K. Williams, A.R. Nobre, V. Calvo, J.F. Cheung, J.J. Bravo-Cordero, D. Entenberg, J. Castracane, V. Verkhusha, P.J. Keely, J. Condeelis, J.A. Aguirre-Ghisio, Phenotypic heterogeneity of disseminated tumour cells is preset by primary tumour hypoxic microenvironments, *Nat. Cell Biol.* 19 (2017) 120–132, <https://doi.org/10.1038/ncb3465>.

5-(Trifluoromethyl)-1,2,4-oxadiazole (TFMO)-based highly selective class IIa HDAC inhibitors exhibit synergistic anticancer activity in combination with bortezomib

Yodita Asfaha^{1‡}, Lukas M. Bollmann^{1‡}, Alexander J. Skerhut^{1‡}, Fabian Fischer¹, Nadine Horstick¹, Dennis Roth², Maria Wecker², Christian Mammen³, Sander H.J. Smits^{3,4}, Georg Fluegen², Matthias U. Kassack^{1*}, Thomas Kurz^{1*}

¹ Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany.

² Klinik für Allgemein-, Viszeral- und Kinderchirurgie, University Hospital of the Heinrich Heine University Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany.

³ Institute of Biochemistry I, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany.

⁴ Center for Structural Studies, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany.

‡These authors contributed equally to this paper (YA, LMB; AJS).

*These authors share senior authorship (MUK, TK).

KEYWORDS: Selective class IIa HDAC inhibitors, proteasome inhibitors, bortezomib, synergistic anti-cancer activity, combination therapy

Table of contents

Supplementary figures and tables	3
Overview of antibodies.....	4
Synthetic procedures	4
^1H and ^{13}C NMR spectra of 1c and 2a.....	11

Supplementary figures and tables

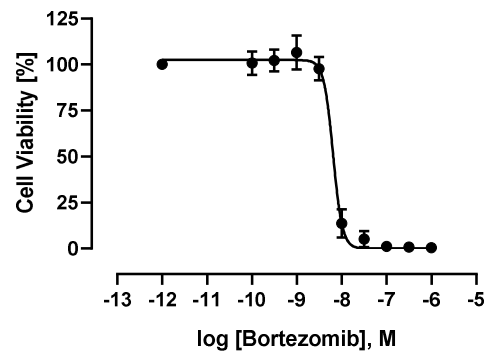


Figure S1. Concentration-response-curves of bortezomib (BTZ) determined by 72 h MTT-Assay on THP-1 cells. Data show at least two independent experiments carried out in triplicates.

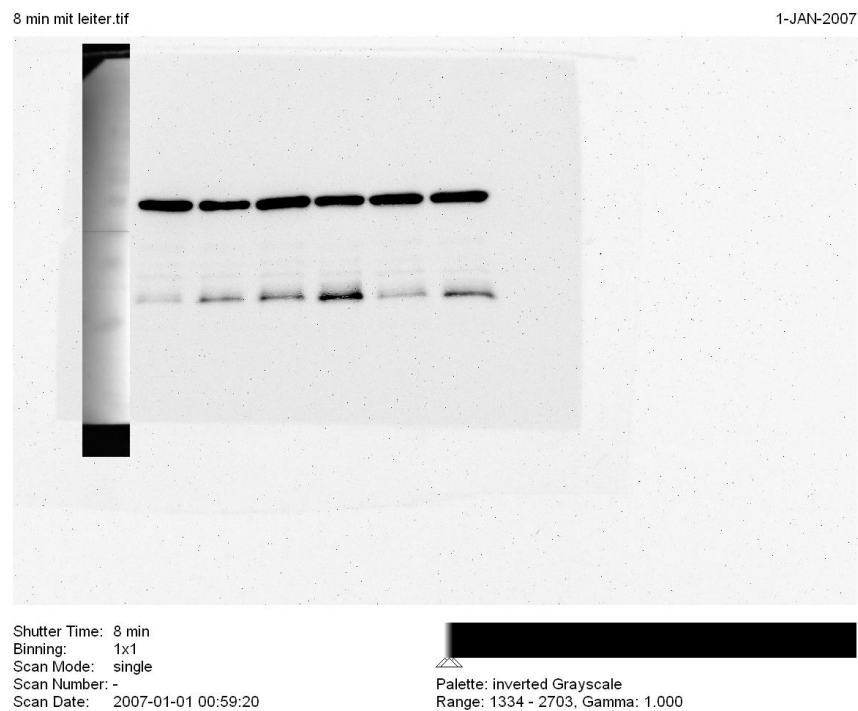


Figure S2. Uncropped Western Blot of beta Actin and p21 after 24 h incubation in THP-1 cells. The bands are corresponding to (from left to right): control; 7.9 nM bortezomib; 5 μ M 1a; 7.9 nM bortezomib + 5 μ M 1a; 5 μ M TMP269; 7.9 nM bortezomib + 5 μ M TMP269

Overview of antibodies

Table S1. Listing of primary antibodies for the western blot

Protein of interest	expected size (kDa)	Primary antibody (company, Catalog nr.)
Beta Aktin	45	Cell Signaling Technology, 13E5
p21	21	Cell Signaling Technology #2947

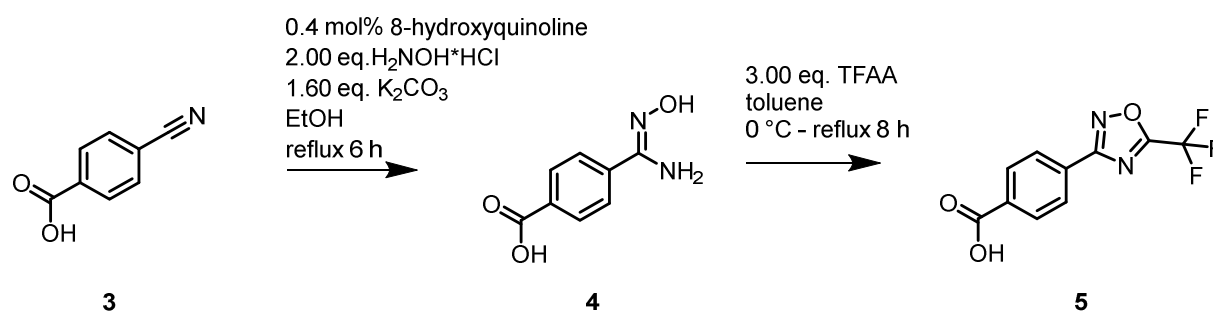
Synthetic procedures

Chemistry: All chemicals and solvents were purchased from commercial suppliers (Sigma Aldrich, Alfa Aesar, Fluorochem, TCI, abcr and Acros Organics) and used without further purification. Dry solvents were used directly from Seal® bottles from Acros Organics. Analytic Thin Layer Chromatography (TLC) was carried out with Macherey Nagel precoated silica gel plates (ALUGRAM® Xtra SIL G/UV₂₅₄). Detection was achieved with ultraviolet irradiation (254 nm) and/or staining with potassium permanganate solution (9 g KMnO₄, 60 g K₂CO₃, 15 mL of a 5% aqueous NaOH-solution, and 900 mL demineralised water). Flash column chromatography was performed with CombiFlashRf200 (TeleDyneIsco) with the solvent mixtures specified in the corresponding procedure. Proton (¹H) and carbon (¹³C) NMR spectra were recorded on Bruker Avance III – 300, Bruker Avance DRX – 500 or Bruker Avance III – 600. Spectra were referenced to the residual non-deuterated solvent signal (¹H-NMR: DMSO-*d*₆ (2.50 ppm), ¹³C-NMR: DMSO-*d*₆ (39.52 ppm); ¹H-NMR: CDCl₃ (7.26 ppm), ¹³C-NMR: CDCl₃ (77.16 ppm)). Chemical shifts are quoted in parts per million (ppm). Signal patterns are indicated as: singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). Coupling constants, *J*, are measured in Hz. Proton (¹H) and carbon (¹³C) NMR spectra were recorded by the Center for Molecular and Structural Analytics of the Heinrich Heine University Duesseldorf (<https://www.cemsa.hhu.de/>). Electrospray Ionisation (ESI) mass spectra were carried out by the Center for Molecular and Structural Analytics of the Heinrich Heine University Duesseldorf, using Bruker Daltonics UHR-QTOF maXis 4G (Bruker Daltonics). APCI-MS was carried out using Advion expression^L CMS spectrometer. Melting points (mp.) were determined using a Büchi M-565 melting point apparatus and are uncorrected. Analytical HPLC analysis were carried out on a Knauer HPLC system comprising an Azura P 6.1L pump, an Optimas 800 autosampler, a Fast Scanning Spectro-Photometer K-

2600 and a Knauer Reversed Phase column (SN: FK36). UV absorption was detected at 254 nm. The solvent gradient table is shown below. The purity of all final compounds was 95% or higher.

Table S2. The solvent gradient table for analytic HPLC analysis.

Time / min	Water + 0.1% TFA	ACN + 0.1% TFA
Initial	90	10
0.50	90	10
20.0	0	100
30.0	0	100
31.0	90	10
40.0	90	10

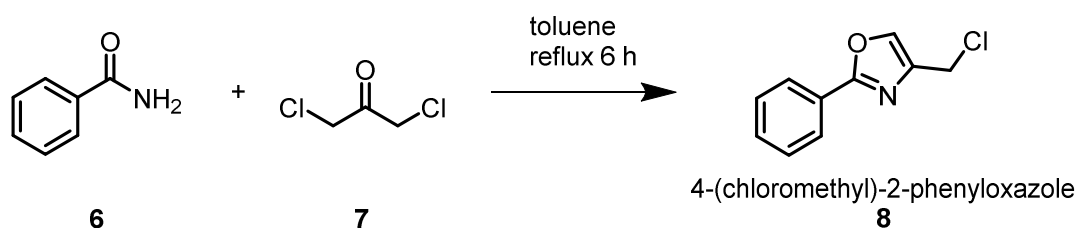


Synthesis of 4-(N'-hydroxycarbamimidoyl)benzoic acid (**4**):

Following the procedure of Lobera et al., (Lobera et al., 2013b) 1.00 eq of 4-cyanobenzoic acid **3** and 0.4 mol% 8-hydroxyquinoline were dissolved in EtOH (10 mL/mmol). To this reaction mixture 2.00 eq hydroxylamine hydrochloride in water (2.0 mL/mmol) and 1.60 eq potassium carbonate in water (1 mL/mmol) were added. The reaction mixture was stirred under reflux for 6 hours and then the solvent removed under reduced pressure. The remaining residue was diluted with water and the aqueous phase was acidified to pH = 3. The formed precipitate was then filtered, washed with water and acetone to provide the

corresponding amidoxime **4**. White solid, yield: 87%. All spectroscopic data were in agreement with the published data. (Lobera et al., 2013b)

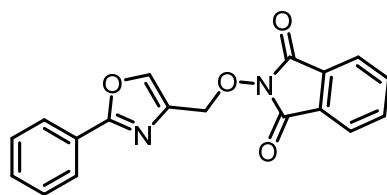
Synthesis of 4-(5-(Trifluoromethyl)-1,2,4-oxadiazol-3-yl)benzoic acid (5**):** A solution of 4-(*N'*-hydroxycarbamimidoyl)benzoic acid **4** (1.00 eq) in anhydrous toluene (1 mmol/mL) was cooled to 0 °C and 3.00 eq trifluoroacetic anhydride was added dropwise. The reaction mixture was slowly allowed to warm to rt and then refluxed further for 8 h. The solvent was removed under reduced pressure, and the crude solid was diluted with ethyl acetate. The organic layer was washed with brine (3x25 mL), dried over Na₂SO₄, filtered and the solvent was then removed under reduced pressure. The crude product was purified by recrystallisation from ethyl acetate/ *n*-hexane to yield **5**. White solid, yield: 60%, mp: 251 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ = 8.08 – 8.24 (m, 4H), 13.38 (s, 1H) ppm. ¹³C NMR (75 MHz, DMSO) δ = 115.65 (q), 127.56, 128.04, 130.20, 134.20, 165.47, 165.24 (q), 167.86 ppm. HPLC: t_R = 12.483 min, 95.8%.



Synthesis of 4-(chloromethyl)-2-phenyloxazole (8**):** A mixture of benzamide (**6**) (1.82 g, 15.0 mmol, 1.00 eq) and 1,3-dichloroacetone (**7**) (3.81 g, 30.0 mmol, 2.00 eq) in toluene (80 mL) was refluxed for 6 h. The solvent was evaporated under reduced pressure and the crude product was purified by flash column chromatography with *n*-hexane/ethyl acetate as eluent. White solid. yield: 53%. mp.: 55 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ = 4.75 (d, *J* = 0.8 Hz, 2H), 7.51 – 7.59 (m, 3H), 7.95 – 8.03 (m, 2H), 8.27 – 8.28 (m, 1H) ppm. ¹³C NMR (75 MHz, DMSO-*d*₆) δ = 37.13, 125.93, 126.44, 129.07, 130.79, 137.80, 138.26, 161.02 ppm. HPLC: t_R = 12.467 min, 99.9%. HRMS (ESI+) = calcd. for C₁₀H₉ClNO [M+H]⁺ = 194.0367, found: 194.0371.

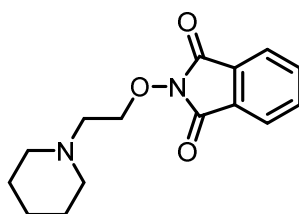
General procedure for the synthesis of *O*-phthaloyl protected hydroxyamines (11a-c**)**

Following the procedure of Asfaha et al.,[3] 1.00 eq of the respective halide derivative and 1.20 eq *N*-hydroxyphthalimide (NHPI) were dissolved in acetonitrile (4 mL/mmol). After addition of 2.00 eq triethylamine, the resulting red solution was refluxed for 12 h. The solvent was evaporated under reduced pressure and the residue was diluted with EtOAc, washed with saturated NaHCO₃ solution (7x 50 mL) and dried over Na₂SO₄. After filtration, the solvent was removed under reduced pressure to yield the crude product which was purified as stated.



2-((2-phenyloxazol-4-yl)methoxy)isoindoline-1,3-dione
11a

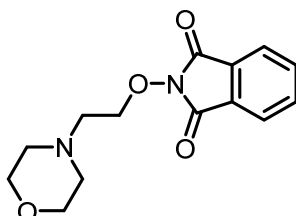
Synthesis of 2-((2-phenyloxazol-4-yl)methoxy)isoindoline-1,3-dione (11a): The crude product was purified by flash chromatography with *n*-hexane/EtOAc as eluent. White solid. yield: 59%. mp.: 152 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ = 5.15 (s, 2H), 7.47 – 7.58 (m, 3H), 7.82 – 7.93 (m, 6H), 8.40 (s, 1H) ppm. ¹³C NMR (75 MHz, DMSO-*d*₆) δ = 69.66, 123.14, 125.85, 126.46, 128.45, 129.02, 130.72, 134.68, 135.56, 140.17, 160.85, 162.97 ppm. HPLC analysis: R_t = 13.833 min, 99.2%. HRMS (ESI+) = calcd. for C₁₈H₁₃N₂O₄ [M+H]⁺ = 321.0870, found: 321.0869.



2-(2-(piperidin-1-yl)ethoxy)isoindoline-1,3-dione
11b

Synthesis of 2-(2-(piperidin-1-yl)ethoxy)isoindoline-1,3-dione (11b): The crude product was purified by flash chromatography with with *n*-hexane/0.1% NEt₃ and EtOAc/0.1% NEt₃ as eluent. Yellow solid. yield: 52%. mp.: 91.1 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ = 1.18 – 1.31 (m, 6H), 2.27 (d, *J* = 4.6 Hz, 4H), 2.57 – 2.68 (m, 2H), 4.19 – 4.28 (m, 2H),

7.86 (s, 4H) ppm. ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ = 23.67, 25.23, 53.90, 57.03, 73.48, 123.01, 128.66, 134.56, 163.00 ppm. HPLC analysis: R_t = 6.291 min, 92%. HRMS (ESI+) = calcd. for $\text{C}_{16}\text{H}_{23}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ = 307.1652, found: 307.1653. All spectroscopic data are in agreement with published data. (Bollmann et al., 2022)

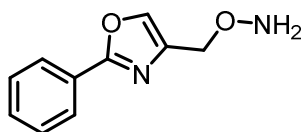


2-(2-morpholinoethoxy)isoindoline-1,3-dione
11c

Synthesis of 2-(2-morpholinoethoxy)isoindoline-1,3-dione (11c): The crude product was purified by flash chromatography with *n*-hexane/0.1% NEt_3 and EtOAc/0.1% NEt_3 as eluent. White solid. yield: 47%. mp.: 98.2 °C. ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ = 2.36 (dd, J = 5.7, 3.6 Hz, 4H), 2.63 – 2.71 (m, 2H), 3.34 – 3.39 (m, 5H), 4.22 – 4.28 (m, 2H), 7.87 (s, 4H) ppm. ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ = 53.13, 56.53, 65.89, 73.56, 123.06, 128.61, 134.62, 163.01 ppm. HPLC analysis: R_t = 5.223 min, 98%. HRMS (ESI+) = calcd. for $\text{C}_{14}\text{H}_{17}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ = 277.1183, found: 277.1181.

General procedure 4: Formation of *O*-hydroxylamines

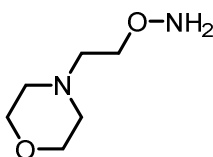
To a solution of the respective NHPI-derivative **11a-c** (1.00 eq) in CH₂Cl₂ (20 mL/mmol), 2.00 eq hydrazine monohydrate was added dropwise. The reaction mixture was stirred overnight at rt. The resulting precipitate was filtered and the organic layer was washed with saturated NaHCO₃ solution (3x 10 mL). After drying over Na₂SO₄ and filtration, the solvent was removed under reduced pressure yielding the hydroxylamines **12a-c**.



O-((2-phenylthiazol-4-yl)methyl)hydroxylamine
12a

Synthesis of *O*-((2-phenylthiazol-4-yl)methyl)hydroxylamine (**12a**):

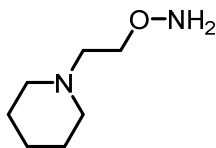
2-((2-Phenyloxazol-4-yl)methoxy)isoindoline-1,3-dione (**11a**) (442 mg, 1.38 mmol, 1.00 eq) was subjected to general procedure 4. The hydroxylamine **12a** (218 mg, 1.15 mmol, 83%) was used directly for the next step.



O-(2-morpholinoethyl)hydroxylamine
12b

Synthesis of *O*-(2-morpholinoethyl)hydroxylamine (**12b**):

2-(2-Morpholinoethoxy)isoindoline-1,3-dione (**11b**) (553 mg, 2.00 mmol, 1.00 eq) was subjected to general procedure 4. The hydroxylamine **12b** (240 mg, 1.64 mmol, 82%) was then used without any further purification.

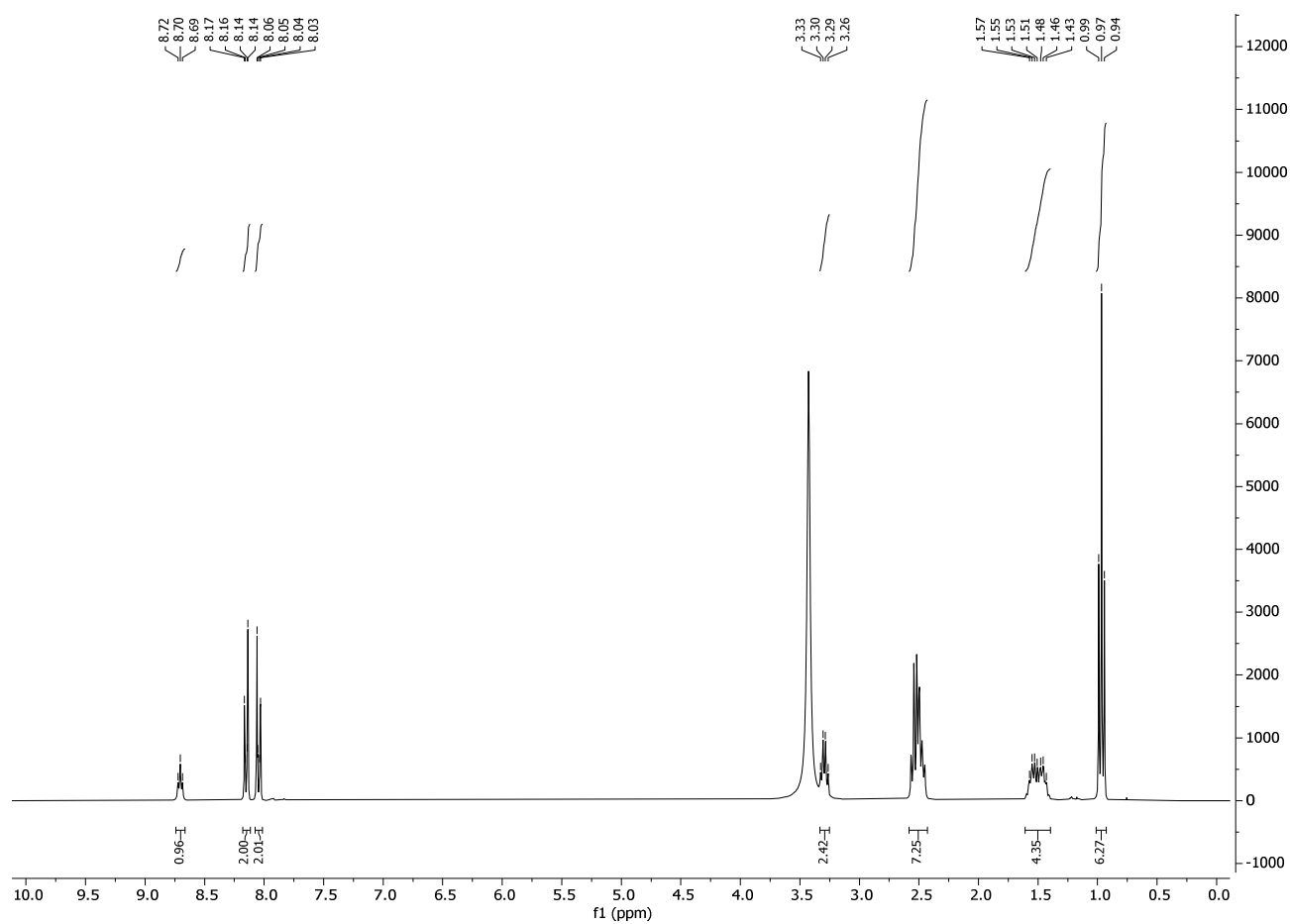
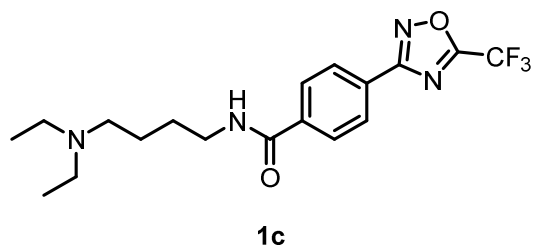


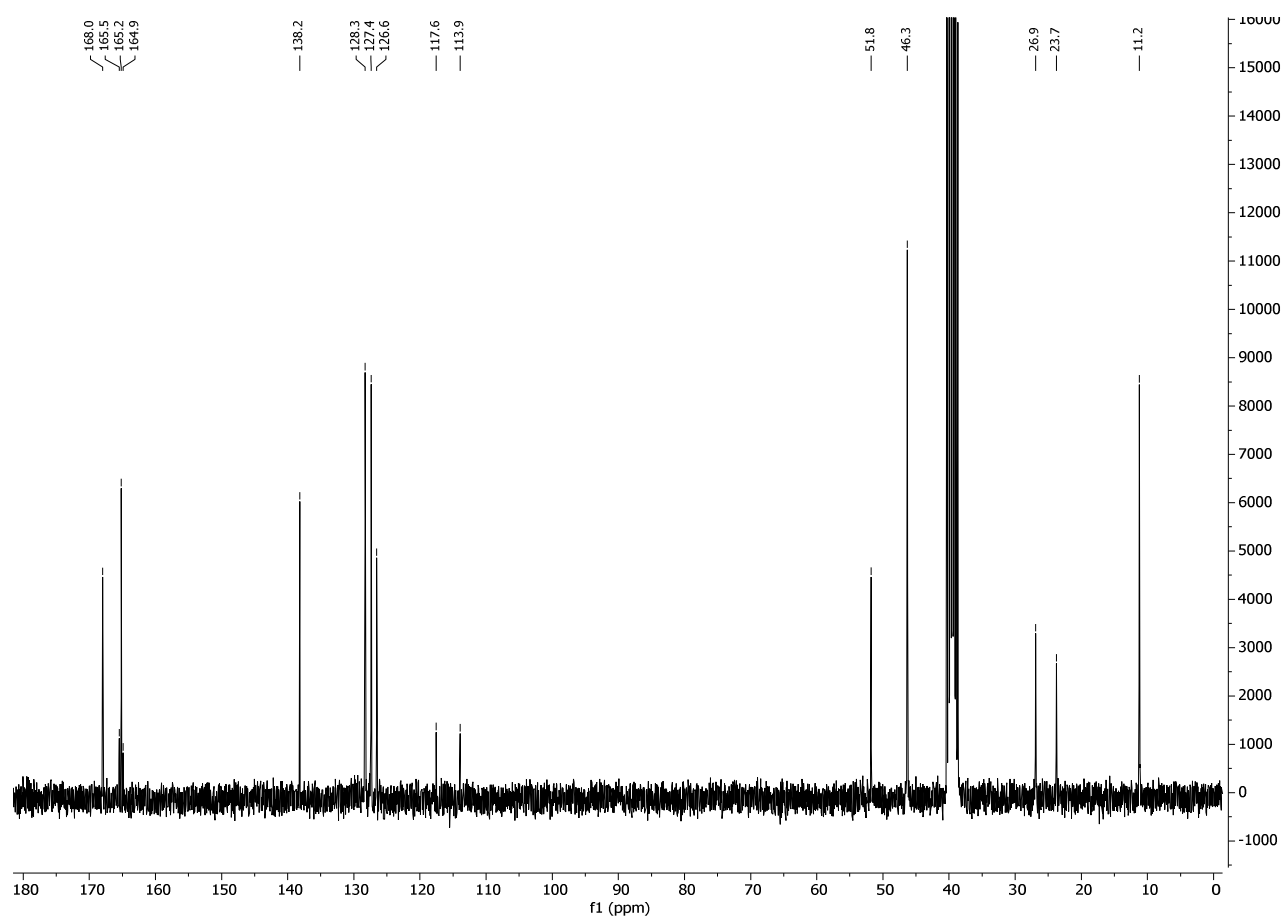
O-(2-(piperidin-1-yl)ethyl)hydroxylamine
12c

Synthesis of O-(2-(piperidin-1-yl)ethyl)hydroxylamine (12c): 2-(2-(Piperidin-1-yl)ethoxy)isoindoline-1,3-dione (**11c**) (686 mg, 2.50 mmol, 1.00 eq) was subjected to general procedure 4. The hydroxylamine **12c** (190 mg, 1.32 mmol, 53%) was then used without any further purification.

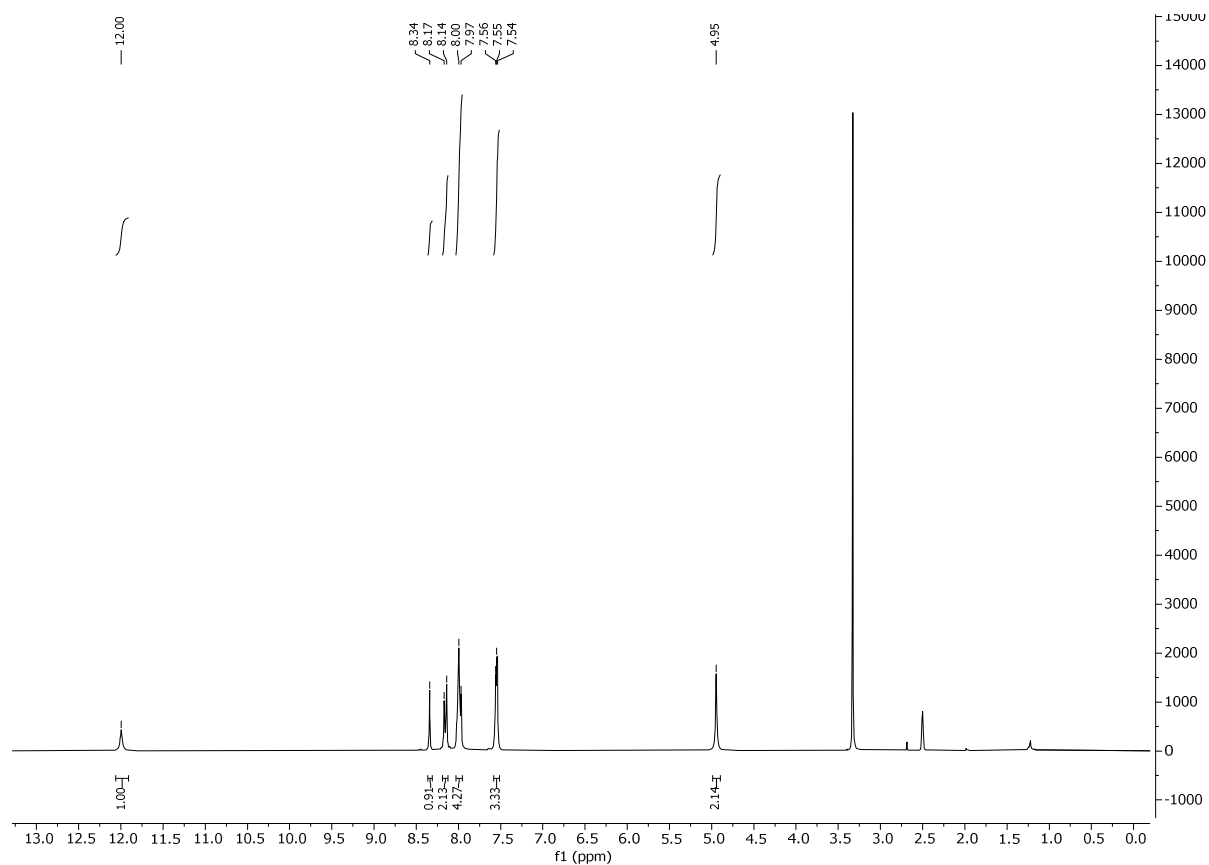
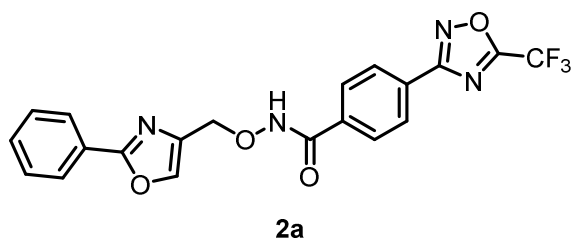
^1H and ^{13}C NMR spectra of **1c** and **2a**

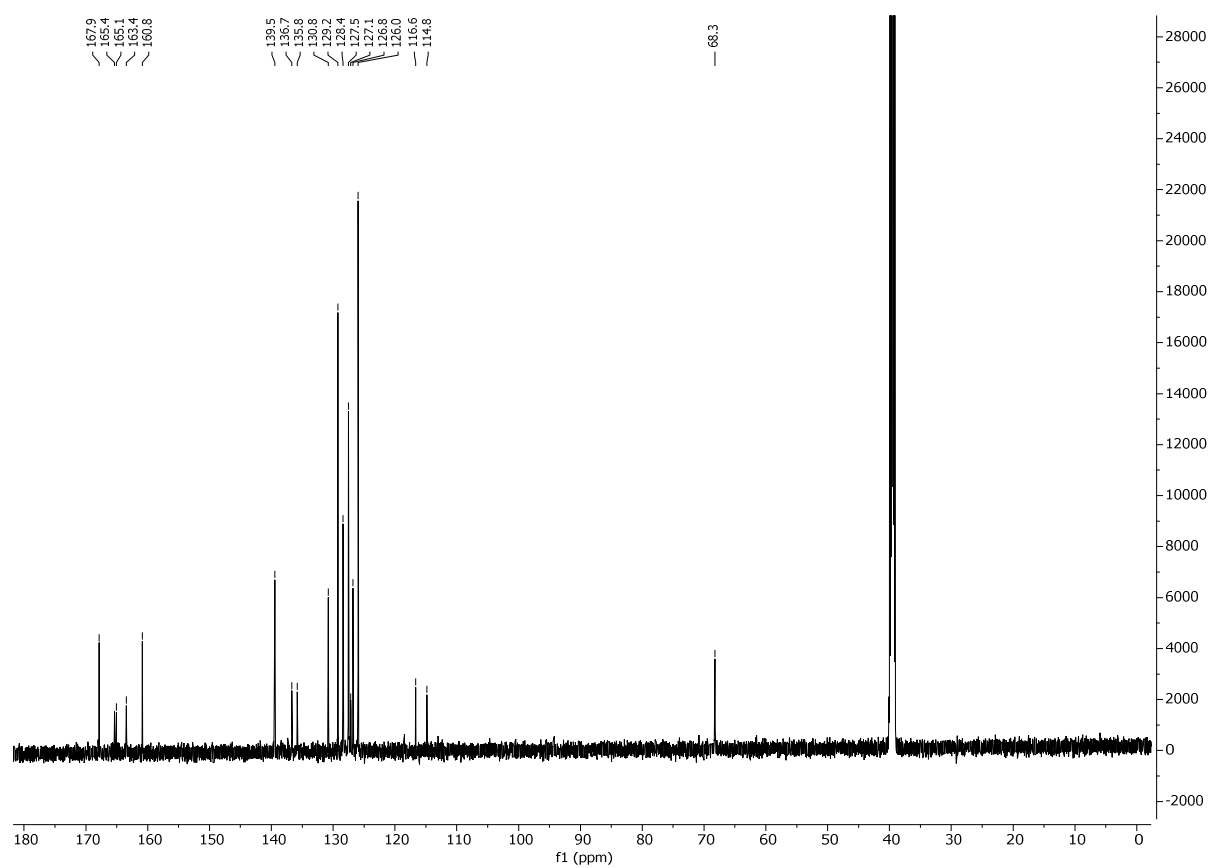
Compound **1c** ^1H NMR (DMSO- d_6 , 300 MHz) and ^{13}C NMR (DMSO- d_6 , 75 MHz) spectra





Compound **2a** ^1H NMR (DMSO- d_6 , 300 MHz) and ^{13}C NMR (DMSO- d_6 , 151 MHz) spectra





3.3 Publication III

Triple Combination of Entinostat, a Bromodomain Inhibitor, and Cisplatin Is a Promising Treatment Option for Bladder Cancer

Lukas M Bollmann¹, Friedrich Lange¹, Alexandra Hamacher¹, Lukas Biermann¹, Linda Schäker-Hübner², Finn K Hansen², Matthias U Kassack¹

¹ Institute of Pharmaceutical and Medicinal Chemistry, Heinrich-Heine-University Duesseldorf, 40225 Duesseldorf, Germany.

² Department of Pharmaceutical and Cell Biological Chemistry, Pharmaceutical Institute, University of Bonn, An der Immenburg 4, 53121 Bonn, Germany.

published in: Cancers, 02. October 2024

DOI: 10.3390/cancers16193374.

The following content is derived from the aforementioned publication, entitled "Triple Combination of Entinostat, a Bromodomain Inhibitor, and Cisplatin Is a Promising Treatment Option for Bladder Cancer." It is important to note that word-by-word citations are not explicitly highlighted in this section.

Authors Contribution

First authorship by L.M.B. L.M.B. was responsible for conducting all experiments, except of enzyme HDAC inhibition assay which were performed by L.B. Investigation was performed by L.M.B., F.L., L.B., and L.S.-H. Evaluation of data were carried out by L.M.B. and M.U.K. Original draft preparation and visualization was performed by L.M.B. M.U.K. and A.H. revised and edited the manuscript and also carried out the supervision.

Background

Cisplatin-based combination therapies play an important role in treating advanced urothelial carcinoma. Cisplatin resistance is a major problem but may be overcome by combination treatments such as targeting epigenetic aberrances. Here, we investigated the effect of the class I HDACi entinostat and bromodomain inhibitors (BETis) on the potency of cisplatin in two pairs of sensitive and cisplatin-resistant bladder cancer cell lines. Cisplatin-resistant J82 cisR and T24 LTT were 3.8- and 24-fold more resistant to cisplatin compared to the native cell lines J82 and T24. In addition, the hybrid **compound 20** comprising structural features of an HDACi and a BETi was investigated.

Results

The combination of entinostat and JQ1 resulted in a decrease in the IC₅₀ values of cisplatin across all four cell lines. Figure 21a shows the results for J82 and J82 cisR cells.

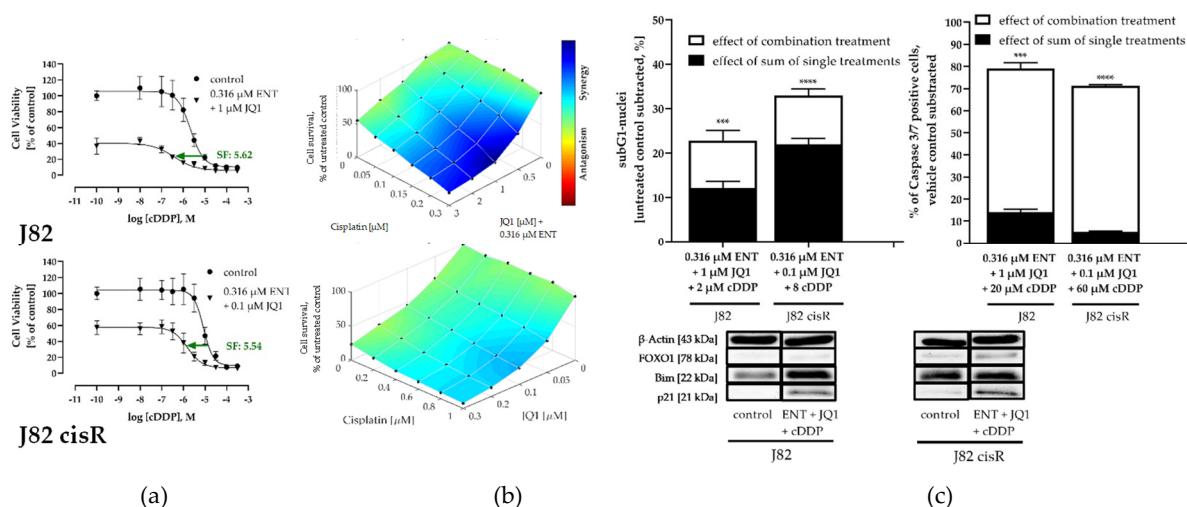


Figure 21. The most meaningful results of the triple combination (entinostat, JQ1 and cisplatin) are shown for J82 and J82 cisR. (a) The decrease in cisplatin IC₅₀ value by the combination of entinostat and JQ1. (b) Calculated synergy levels (Bliss model) of the triple combination. (c) Mechanistic studies involving caspase-mediated apoptosis and protein expression analysis by western blot. Figures taken and modified from [212].

We found complete (J82cisR) or partial (T24 LTT) reversal of chemoresistance upon combination treatment with entinostat, JQ1, and cisplatin. The same was found for the BETi JQ35 and OTX015, both were in clinical trials, and for **compound 20** (dual HDACi and BETi). The combinations were highly synergistic according to Chou Talalay analysis and Bliss model in all four cell lines (Figure 21b). We identified an increased caspase-mediated apoptosis accompanied by enhanced expression of p21, Bim, and FOXO1 (Figure 21c). The triple combination induced the most double-strand breaks (identified as co-stained foci of γ -H2AX and 53BP1) in J82 and J82 cisR. Notably, the combinations were at least 4-fold less toxic in non-cancer cell lines HBLAK and HEK293 than in bladder cancer cells.

Conclusion

The following conclusions can be drawn from this study:

- The triple combination of entinostat, a BETi, and cisplatin is highly synergistic, notably, including in cisplatin-resistant bladder cancer cell lines
- The triple combination has been shown to induce caspase-mediated apoptosis through enhanced expression of p21, Bim, and FOXO1
- The triple combination has the potential to be less toxic in non-cancerous cell lines than in bladder cancer cell lines

The encouraging results of this study suggest the potential for further investigation of the triple combination treatment in subsequent studies. This approach may offer a

novel therapeutic strategy for the treatment of bladder cancer or for addressing cisplatin resistance.

Article

Triple Combination of Entinostat, a Bromodomain Inhibitor, and Cisplatin Is a Promising Treatment Option for Bladder Cancer

Lukas M. Bollmann ¹, Friedrich Lange ¹, Alexandra Hamacher ¹, Lukas Biermann ¹ , Linda Schäker-Hübner ² , Finn K. Hansen ²  and Matthias U. Kassack ^{1,*} 

¹ Institute of Pharmaceutical and Medicinal Chemistry, Heinrich-Heine-University Duesseldorf, 40225 Duesseldorf, Germany; lukas.bollmann@hhu.de (L.M.B.)

² Department of Pharmaceutical and Cell Biological Chemistry, Pharmaceutical Institute, University of Bonn, An der Immenburg 4, 53121 Bonn, Germany; finn.hansen@uni-bonn.de (F.K.H.)

* Correspondence: matthias.kassack@uni-duesseldorf.de

Simple Summary: The treatment of bladder cancer is still a challenge. New treatment options are required for patients not responding to established chemotherapy (e.g., cisplatin) due to primary or acquired chemoresistance. Bladder cancer cells are known for disturbed epigenetics contributing to resistance. This study aimed to investigate the combination of cisplatin, the class I histone deacetylase inhibitor (HDACi) entinostat, and bromodomain inhibitors (BETis) in two urothelial grade 3 bladder carcinoma cell pairs J82, cisplatin-resistant J82 cisR, T24, and cisplatin-resistant T24 LTT. Our results indicate that treatment of bladder cancer cells with entinostat and a BETi prior to cisplatin can completely reverse cisplatin resistance. This is of particular interest since entinostat and OTX015 (one of the used BETi) are already in clinical trials in other cancers. Thus, this study encourages further preclinical and eventually clinical trials with epigenetic inhibitors in combination with cisplatin to re-establish cisplatin efficacy in bladder cancer.



Citation: Bollmann, L.M.; Lange, F.; Hamacher, A.; Biermann, L.; Schäker-Hübner, L.; Hansen, F.K.; Kassack, M.U. Triple Combination of Entinostat, a Bromodomain Inhibitor, and Cisplatin Is a Promising Treatment Option for Bladder Cancer. *Cancers* **2024**, *16*, 3374. <https://doi.org/10.3390/cancers16193374>

Academic Editors: Pierfrancesco Bassi and Giuseppe Palermo

Received: 30 August 2024

Revised: 16 September 2024

Accepted: 30 September 2024

Published: 2 October 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Background/Objectives. Cisplatin is part of the first-line treatment of advanced urothelial carcinoma. Cisplatin resistance is a major problem but may be overcome by combination treatments such as targeting epigenetic aberrances. Here, we investigated the effect of the class I HDACi entinostat and bromodomain inhibitors (BETis) on the potency of cisplatin in two pairs of sensitive and cisplatin-resistant bladder cancer cell lines. Cisplatin-resistant J82cisR and T24 LTT were 3.8- and 24-fold more resistant to cisplatin compared to the native cell lines J82 and T24. In addition, a hybrid compound (compound **20**) comprising structural features of an HDACi and a BETi was investigated. Results. We found complete (J82cisR) or partial (T24 LTT) reversal of chemoresistance upon combination of entinostat, JQ1, and cisplatin. The same was found for the BETis JQ35 and OTX015, both in clinical trials, and for compound **20**. The combinations were highly synergistic (Chou Talalay analysis) and increased caspase-mediated apoptosis accompanied by enhanced expression of p21, Bim, and FOXO1. Notably, the combinations were at least 4-fold less toxic in non-cancer cell lines HBLAK and HEK293. Conclusions. The triple combination of entinostat, a BETi, and cisplatin is highly synergistic, reverses cisplatin resistance, and may thus serve as a novel therapeutic approach for bladder cancer.

Keywords: bladder cancer; resistance; epigenetics; class I HDACi; bromodomain inhibitor; entinostat; OTX015; JQ1; histone deacetylase (HDAC) inhibitor; cisplatin

1. Introduction

Bladder cancer is the tenth most common cancer worldwide in both sexes, with men more commonly affected [1]. Further, bladder cancer is the most common malignant disease of the urinary tract [2]. Between 2020 and 2025, the incidence of bladder cancer is expected to increase across Europe through demographic change [3]. Cisplatin is

used as standard therapy for urothelial carcinoma. For patients with advanced urothelial carcinoma, first-line treatment comprises cis- or carboplatin-based chemotherapy. For patients with muscle-invasive bladder cancer, neoadjuvant (chemotherapy before surgery) cisplatin-based chemotherapy is preferred over adjuvant treatment [4]. The development of cisplatin resistance is a common challenge [5,6]. Combination therapy is one way to overcome chemoresistance. For example, in advanced bladder cancer, a combination of cisplatin, gemcitabine, and paclitaxel has been studied. The combination therapy resulted in a prolonged survival of approximately 3 months, which was not statistically significant [7]. New effective combination therapies are thus urgently needed for the treatment of chemoresistant cancer cells to increase the effectiveness of chemotherapy and delay the problem of chemoresistance.

Bladder cancer shows epigenetic aberrances [8]. In addition, it is well known that aberrant histone and non-histone protein acetylation is a key determinant of acquired chemoresistance and, consequently, cisplatin resistance in cancer [9,10]. Epigenetic modulation, which describes reversible inhibition or activation of epigenetic writers, erasers, or readers, is one way of facing the chemoresistance problem [10]. Deacetylation by histone deacetylases (HDACs) of histones results in a condensed chromatin structure (heterochromatin), which suppresses gene expression. Histone deacetylase inhibitors (HDACis) can conversely be used to favor the open euchromatin structure and facilitate gene expression [11–13]. Epigenetic modulation of cancer cells by class I HDACi or pan-HDACi, followed by cisplatin treatment, demonstrated promising results in solid tumor cells [14–17]. Treatment with HDACi resulted in an increased expression of pro-apoptotic genes such as p21, APAF1, PUMA, and BAK1 and repression of anti-apoptotic genes such as survivin [14,18]. However, the sole use of class I HDACi or pan-HDACi is not a universal solution to the problem of chemoresistance because using a single epigenetic modifier such as an HDACi may not completely reverse cisplatin resistance, and additionally, cancer cells can also develop resistance to HDACi [17,19]. Therefore, further combination therapies need to be explored to overcome chemoresistance.

The bromodomain and extra-terminal domain inhibitors (BETis) containing bromodomains (BRDs) as an additional epigenetic target offer new potential for combination therapies [20]. BRD proteins can be divided into eight larger families, of which the BET proteins represent one family [21]. BET proteins orchestrate gene transcription by interacting with acetylated histones or transcription factors. In addition, BET proteins influence the JAK/STAT pathway as well as the cell cycle by activating oncogenes such as MYC, JUNB, CCND1, and CCNA1 [22–25]. As a result, BET proteins play a key role in cell survival and homeostasis. BET inhibitors (BETis) preferentially inhibit interaction with oncogenes that are essential for the survival of cancer cells [26–28]. A high sensitivity to BETi was discovered in chemoresistant ovarian tumors and patient-derived xenograft models with high MYC amplification. It was demonstrated that BETi had a synergistic effect in combination with cisplatin in ovarian cancer, even in cisplatin-resistant cells. Different explanations were given, including suppression of ALDH and downregulation of anti-apoptotic proteins BCL-2 and survivin. In addition, BETi sensitized ovarian cancer cells, which had a functional homologous recombination DNA repair pathway, to DNA damage by decreased expression of BRCA1 and RAD51 [18,25,29]. Modification of these signaling pathways by BETi may also lead to increased sensitivity to cisplatin in bladder cancer. Moreover, studies showed that the combination of the BET inhibitor JQ1 with the MAP kinase inhibitor trametinib in thyroid tumors resulted in a strong inhibition of tumor growth [30]. Furthermore, the BETi JQ1 suppressed cell proliferation by cell-cycle arrest and induction of apoptosis in acute lymphocytic leukemia cells [31]. Synergistic antiproliferative effects of BETi and cisplatin were demonstrated in malignant pleural mesothelioma cells via downregulation of MYC [32].

OTX015 is a BETi derived from JQ1. OTX015 has—in contrast to JQ1—an extended half-life and better oral bioavailability [27,33]. OTX015 revealed antitumor effects *in vitro* and *in vivo* against glioblastoma cells and non-small cell lung carcinoma cell lines [34,35].

Further, OTX015 has already been tested in clinical trials in hematopoietic and solid tumors. Dose-limiting toxicities in patients were anemia, thrombocytopenia, fatigue, and hyperbilirubinemia [27,36–39]. A clinical trial of OTX015 in solid tumors, such as midline carcinoma, was discontinued because part of the study indicated limited efficacy [40]. JQ35, another BETi, has also been tested in first clinical trials in acute myeloid leukemia and various solid tumors [41,42]. Further testing as a monotherapy for acute myeloid leukemia was discontinued due to a lack of efficacy [41].

Both HDACis and BETis influence gene expression. Therefore, a combination of BETis with HDACis may be useful to maximize the alteration of gene expression and for overcoming chemoresistance. The combination of HDACis and the BETi JQ1 has already been studied in acute myeloid leukemia, pancreatic ductal adenocarcinoma, glioblastoma, small-cell lung cancer, and bladder carcinoma, demonstrating synergistic anti-proliferative effects [28,43–48]. Furthermore, studies demonstrated that both HDACis and BETis down-regulated the transcription of oncogenes and anti-apoptotic factors such as MYC or BCL-2 [28,48].

The aim of this study was to investigate a triple combination of HDACi, BETi and cisplatin in bladder cancer cells of different cisplatin sensitivity to reverse cisplatin resistance/increase cisplatin sensitivity and to increase the efficacy of treatment by exploiting synergistic effects of the combination. We investigated the combination treatment of the class I HDACi entinostat plus a BETi (JQ1 or JQ35 or OTX015) plus cisplatin in two bladder cancer cell pairs: native J82, cisplatin-resistant J82 cisR and native T24, and cisplatin-resistant T24 LTT (long-term treated). J82 cisR and T24 LTT were both established in laboratories of the University of Duesseldorf by intermittent treatment of the native cell lines with cisplatin as described in the literature [19,49,50]. J82 and T24 are muscle-invasive bladder cancer cell lines, both classified as grade 3 [49]. Muscle-invasive bladder cancer is treated with cisplatin-based combination chemotherapy in addition to radical removal of the bladder [2,31].

JQ1 was used as a model compound for BETis but has the disadvantage of a very short half-life in vivo and limited bioavailability [27,33]. Thus, we included OTX015 and JQ35 in our study, two BETis which are already used in a clinical trials (Figure 1) [27]. Additionally, we pursued a dual inhibitor approach to analyze the efficacy of combining an HDACi and a BETi in one molecule. Compound 20 combines structural features of and acts as an HDACi (class I/IIb) and BETi (Figure 1) [51].

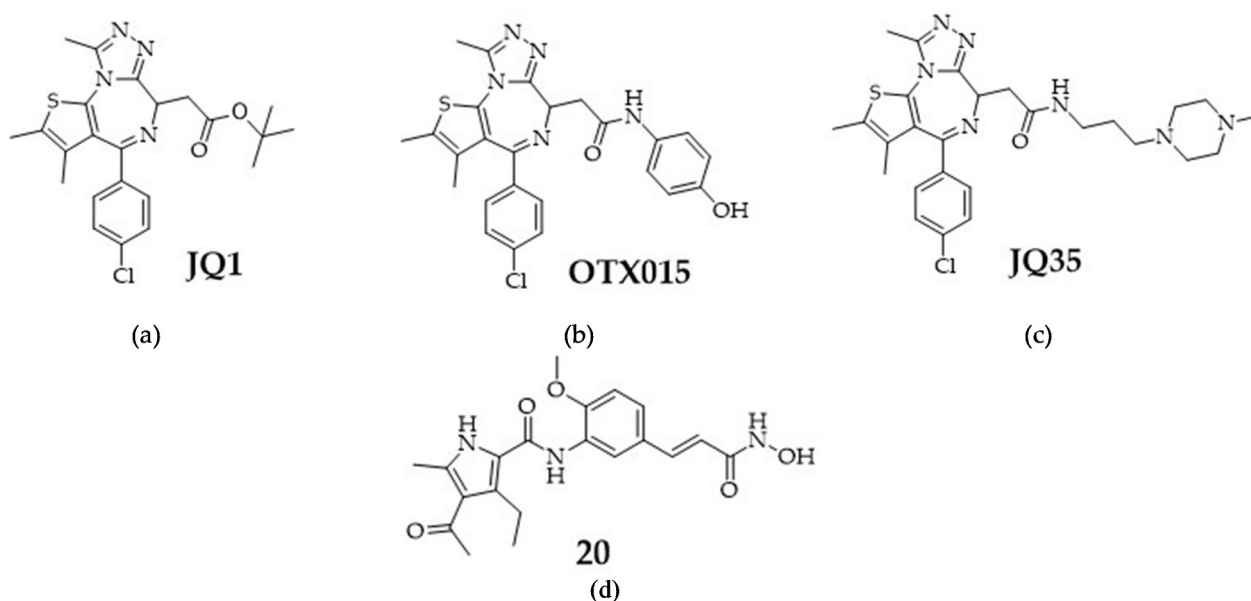


Figure 1. Structures of BETis: (a) JQ1, (b) OTX015, and (c) JQ35; (d) structure of dual HDACi and BETi 20 [51].

Dual inhibitors have the advantage to simplify therapeutic application and may lead to higher efficacy by simultaneously inhibiting two targets by one molecule. We could demonstrate that triple combinations of entinostat, a BETi, and cisplatin act highly synergistically, and that entinostat and a BETi or compound **20** reversed cisplatin resistance while showing at least 4-fold selectivity for the bladder cancer over the non-cancer cell lines HBLAK and HEK293.

2. Materials and Methods

2.1. Materials

DMEM, penicillin/streptomycin (pen/strep) (10,000 U/mL; 10 mg/mL), fetal calf serum (FCS), and trypsin-EDTA (0.05% trypsin, 0.02% EDTA in phosphate-buffered saline) were received from PAN-Biotech (Aidenbach, Germany). Cnt-Prime Medium was obtained from CELLnTEC (Bern, Switzerland). Entinostat was ordered from Selleckchem (Houston, TX, USA). The JQ1 was purchased from Tocris (Bristol, UK). The OTX015 and JQ35 were delivered by AdooQ (AdooQ Bioscience, Irvine, CA, USA). The compound **20** was synthesized by Dr. Finn Hansen, Institute of Pharmaceutical and Cell Biological Chemistry, University of Bonn. The cisplatin was supplied by Sigma-Aldrich (Steinheim, Germany) and dissolved in 0.9% sodium chloride solution and further diluted with 0.9% sodium chloride. All other compounds were dissolved in the DMSO to a concentration of 10 mM. All further dilutions were prepared with the appropriate culture medium. A maximum amount of 1% DMSO was used in all experiments. Triton X-100 was delivered by AppliChem (Darmstadt, Germany), and propidium iodide was purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

2.2. Cell Lines and Cell Culture

The human urothelial bladder carcinoma cell line J82 and the non-cancer cell line HEK293 were purchased from DSMZ (Braunschweig, Germany). The cisplatin-resistant subclone J82 cisR was created by repetitive treatment with cisplatin, as previously described [19]. The primary epithelial cell line HBLAK [52], T24, and T24 LLT cell line were kindly provided by Dr. Michèle Hoffmann, Department of Urology, Heinrich-Heine-university, Duesseldorf. The cell lines J82, J82 cisR, T24, T24 LTT, and HEK293 were cultured with DMEM, where 120 IU/mL penicillin and 120 µg/mL streptomycin and 10% heat-inactivated fetal calf serum were added. For cultivation of the HBLAK cells, the culture medium Cnt-Prime was used without further additives. All cells were incubated at 37 °C, 5% CO₂ under humidified air. The J82 cisR and T24 LTT showed stable cisplatin resistance and did not require permanent treatment with cisplatin to maintain the cisplatin resistance.

2.3. MTT Cell Viability Assay

The cell viability for determination of IC₅₀ values and synergism studies was analyzed by MTT assay as previously described [19,53]. Briefly, cells were incubated with the indicated compounds for 72 h or 48 h pre-incubated plus 72 h in combination with cisplatin. For preincubation experiments, 1600 c/w (cells per well) for J82 and 1200 c/w for J82 cisR were used. After the incubation time, 25 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Serva, Heidelberg, Germany) solution (5 mg/mL in phosphate-buffered saline) was added. The formazan was dissolved in 75 µL DMSO, and the absorption was measured at 540 nm and 690 nm with a microplate reader (ThermoFisher Multiskan, Thermo Scientific, Wesel, Germany).

2.4. Measurement of Apoptotic Nuclei

The amount of apoptotic nuclei with DNA content in sub-G1 was measured as described before [19]. Briefly, J82 (75.000 c/w), J82 cisR (60.000 c/w), T24 (45.000 c/w), and T24 LTT (90.000 c/w) cells were seeded in 6-well plates (Sarstedt, Nümbrecht, Germany). Cells were 48 h preincubated with entinostat and JQ1 or OTX015. Then, the culture medium was removed, and the cells were incubated additional 24 h with entinostat, JQ1 or OTX015

and cisplatin. After the incubation, the 6-well plates were centrifuged, and the supernatant was removed. Cells were lysed by adding 1 mL hypotonic lysis buffer (0.1% tritonX-100, 100 µg/mL propidium iodide) at 4 °C in the dark overnight. The lysed cells were analyzed by flow cytometry using the CyFlow instrument (Partec, Norderstedt, Germany).

2.5. Activation of Caspase 3/7

Activation of caspase 3/7 was examined using the CellEvent Caspase-3/7 green detection reagent (Thermo Scientific, Wesel, Germany) according to the manufacturer's instructions. The cells were seeded in 96-well-plates (Corning, Kaiserslautern, Germany) and treated with the indicated compounds for 48 h (J82 1600 c/w; J82 cisR 800 c/w; T24 1200 c/w; T24 LTT 3000 c/w). Afterwards, the medium was removed, and fresh medium and compounds including cisplatin were given to the cells for 24 h. The supernatant was aspirated and a solution of CellEvent Caspase 3/7 green detection reagent (PBS, 1% heat inactivated fetal calf serum) was added to the cells for a final concentration of 2 µM. Hoechst 33342 (10 mg/mL in water, Sigma-Aldrich, Steinheim, Germany) was used for nuclei staining. The cells were incubated for 30 min at 37 °C and 5% CO₂ before imaging by using the Thermo Fischer ArrayScan XTI high content screening (HCS) system with a 10 × magnification (Thermo Scientific, Wesel, Germany).

2.6. Immunoblotting

Protein extraction and Western blot analysis were performed as previously described with minor modifications [19]. The cells were incubated for 48 h with the indicated concentrations. The medium was aspirated, and the cells were washed with PBS 1X after the incubation period. Then, a RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM sodium chloride, 2 mM EDTA, spiked with protease and phosphatase inhibitor (Pierce protease and phosphatase inhibitor mini tablets, Thermo Scientific, Wesel, Germany)) was added, and the cells were removed using a cell scraper. The cells were lysed for 30 min at 4 °C with agitation. Then, the protein preparations were boiled at 95 °C with 2x Laemmli buffer containing β-mercaptoethanol for 3 min. Pierce BCA protein assay was used to determine the total amount of protein in the protein preparations (Thermo Scientific, Wesel, Germany). Equal amounts of protein were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany). PageRuler Prestained Ladder, 10 to 180 kDa, or 10 to 250 kDa (Thermo Scientific, Wesel, Germany) were used as a size comparison marker. Blots were incubated with the primary antibodies (Table S1). The secondary antibodies conjugated to HRP used in the Western blot were supplied by bio-technie (Minneapolis, MN, USA). Proteins were visualized using Luminol reagent (Santa Cruz Biotechnology, Heidelberg, Germany) in Intas Imager (Intas, Göttingen, Germany). The images were analyzed with ImageJ 1.54g (<http://imagej.org>).

2.7. RT-PCR

The cells were incubated with the indicated concentrations of entinostat and JQ1 for 48 h. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Subsequently, the RNA was transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Wesel, Germany). For an RT-PCR, GoTaq qPCR Master Mix (Promega, Fitchburg, WI, USA) and a CFX96 Real-Time System (BIO-RAD, Hercules, CA, USA) were used. The relative changes in gene expression were normalized to the control genes *GUSB* (beta-glucuronidase), *TBP* (TATA binding protein), and *HPRT1* (hypoxanthine-guanine phosphoribosyltransferase) by Vandesompele method [54]. Primers were designed using Primer-BLAST (NIH, Bethesda, MD, USA) and had an efficiency between 80% and 115% (Table S2). Efficiency was determined using an isolated template of HeLa cells.

2.8. Analysis of Double Strand Breaks with γ -H2AX and 53BP1

An analysis of double-strand breaks measuring γ -H2AX and 53BP1 foci was performed according to the literature, with minor modifications [55]. The J82 (60.000 c/w) and J82 cisR (48.000 c/w) were seeded on a coverslip (Paul Marienfeld GmbH & Co.KG, Lauda Königshofen, Germany) in a 6-well plate (Sarstedt, Nümbrecht, Germany). The cells were treated for the indicated time. Then, the cells were fixed with 3% formaldehyde for 10 min. The cells were permeabilized with 0.3% Triton X-100 in PBS and then blocked with 5% BSA for one hour. After this step, the primary antibody incubation (anti-phospho-Histone H2A.X Ser139 clone JBW301, 05-636, Merck Millipore, Darmstadt, Germany; 53BP1 #4937, Cell Signaling Technology, Danvers, Massachusetts, USA) was carried out overnight at 4 °C. The next day, the secondary antibody incubation was performed for one hour (AlexaFluor 555 and AlexaFluor 647, Thermo Scientific, Wesel, Germany). The cells on the coverslips were mounted in a DAPI-containing mounting medium (UltraCruz Mounting Medium, Santa Cruz Biotechnology, Heidelberg, Germany) and fixed with nail varnish. The evaluation was carried out on a Leica Thunder microscope (Leica Mikrosystems, Wetzlar, Germany).

2.9. Enzyme HDAC Inhibition Assay

The human recombinant enzymes were ordered from Reaction Biology Corp (Malvern, PA, USA). To determine the inhibitory activity of the compounds, the enzymes HDAC 2 (cat. no. KDA-21-277), HDAC 4 (cat. nr. KDA-21-279), HDAC 6 (cat. no. KDA-21-213), and HDAC 8 (cat. no. KDA-21-481); with 20 ng of HDAC 2 and 8, 17.5 ng of HDAC 6, and 2 ng of HDAC 4 per well were used. After pipetting 10 μ L of increasing concentrations of the inhibitors to a 96-well plate (Corning, Kaiserslautern, Germany), the recombinant enzymes were diluted in assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM KCl, 1 mM MgCl₂, and 1 mg/mL bovine serum albumin (BSA) and added to the wells. After five minutes of incubation, the reaction was started with 10 μ L of a 30 μ M solution of Boc-Lys(Ac)-AMC (Bachem, Bubendorf, Switzerland) for HDAC 2 or a 15 μ M solution for HDAC 6. For the HDAC 4, a 10 μ M solution of Boc-Lys-(Tfa)-AMC (Bachem, Bubendorf, Switzerland) was used, and for the HDAC 8, a 6 μ M solution was used. After 90 min, the reaction was stopped by adding 100 μ L of stop buffer (16 mg/mL trypsin and 4 μ M vorinostat for HDAC 2, 4 μ M panobinostat for HDAC 4 and HDAC 8 and 4 μ M tubastatin A for HAC 6 in 50 mM Tris-HCl, pH 8.0 and 100 mM NaCl). After additional 15 min, fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm in the NOVOstar microplate reader (BMG LabTech, Offenburg, Germany).

2.10. Data Analysis

The concentration–effect curves were created with Prism 8.0.2 (GraphPad, San Diego, CA, USA). The statistical analysis was performed using two-tailed unpaired *t*-test in GraphPad Prism. To determine the synergistic effect, the sum of the single treatments was calculated with standard deviation. This value was compared with the measured value of the combination treatment through an unpaired *t*-test. CI-values were calculated according to Chou-Talalay using compusyn 1.0 software [56,57]. Synergy levels were calculated applying the Bliss Model with combenefit [58,59].

3. Results

3.1. Cytotoxic Activity and Synergism Studies of Entinostat, JQ1, and Cisplatin

First, the IC₅₀ values of entinostat, JQ1, and cisplatin were estimated in all bladder cancer cell lines by an MTT assay. Concentration–effect curves are shown in Figure S1, IC₅₀ values are displayed in Table 1.

Two pairs of cisplatin-sensitive and cisplatin-resistant bladder cancer models were selected: J82, J82 cisR; and T24, T24 LTT. The J82 cisR showed a moderate cisplatin-resistance factor of 3.8. For the T24 LTT, the resistance factor was 24, demonstrating very high cisplatin resistance. Entinostat had similar IC₅₀ values in J82 and J82 cisR, but a 4-fold lower IC₅₀ in T24 LTT than T24. Interestingly, both cisplatin-resistant bladder cancer cell lines were more

sensitive to JQ1 than the parental cell lines. J82 demonstrated an 8.4-fold lower sensitivity towards JQ1 than J82 cisR. The same observation was made for T24 with a 4-fold lower sensitivity than T24 LTT (Table 1). For further investigations, concentrations of entinostat and JQ1 were chosen which are achievable in in vivo studies according to the literature data. Furthermore, entinostat and BETi were used in combination studies in a maximum concentration reflecting their IC₂₅, respectively, thus showing low toxicity when given alone [27,60].

Table 1. IC₅₀ values (MTT, 72 h) of cisplatin, entinostat, and JQ1 in all bladder cancer cell lines. Data shown are the mean ± SD of at least three independent experiments, each carried out in triplicates.

	Cisplatin IC ₅₀ ± SD [μM]	Entinostat IC ₅₀ ± SD [μM]	JQ1 IC ₅₀ ± SD [μM]
J82	1.87 ± 0.06	10.4 ± 0.89	18.8 ± 1.55
J82 cisR	7.16 ± 0.24	7.60 ± 0.59	2.22 ± 0.41
T24	2.99 ± 0.12	5.68 ± 0.51	2.29 ± 0.42
T24 LTT	72.2 ± 3.57	1.38 ± 0.10	0.60 ± 0.08

Then, the mutual effect of the two epigenetic modulators was tested: the effect of a low concentration (maximum IC₂₅) of the BETi JQ1 on the IC₅₀ of entinostat (Figure S2a,c) and the effect of a low concentration (maximum IC₂₅) of the HDACi entinostat on the IC₅₀ of JQ1 (Figure S2b,d) in J82 and J82 cisR. Regardless of a 72 h co-incubation of both inhibitors or a sequential treatment (48 h pre-incubation of the IC₂₅ of one inhibitor followed by addition of the second inhibitor for another 72 h), no significant increase in the potency of either epigenetic modulator was found (Figure S2 and Table S3). Shift factors were calculated by dividing the IC₅₀ value of the control treatment with the IC₅₀ value of the treated cells. All shift factors were below 2 (Table S3).

Next, the effect of each epigenetic modulator alone and of the combination of entinostat plus JQ1 on the IC₅₀ of cisplatin was studied. A 72 h co-incubation of entinostat, JQ1, and cisplatin did not achieve a significant increase in the potency of cisplatin. Shift factors of cisplatin were less than 2, except in J82 cells (Figure S3, Table S4). We then used our proven incubation scheme, applying a 48 h preincubation of the epigenetic inhibitors prior to addition of cisplatin for another 72 h, allowing the epigenetic inhibitors to modulate the cellular epigenetic environment prior to addition of the cytotoxic compound. This incubation scheme has proven successful in several of our previous studies [14,61]. Notably, whereas co-incubation of entinostat, JQ1, and cisplatin did not increase the potency of cisplatin (Figure S3, Table S4), preincubation of entinostat and JQ1 for 48 h followed by the addition of cisplatin for another 72 h increased the potency of cisplatin up to 15.6-fold. The concentration–effect curves and corresponding IC₅₀ values with shift factors are displayed in Figure 2 and Table 2.

The 48 h preincubation with entinostat gave only shift factors (SFs) of <2 for cisplatin, except for T24 LTT cells (SF 2.26) whereas preincubation with JQ1 gave SF > 2, except for 0.075 μM JQ1 in T24 LTT where cisplatin remained almost equipotent with or without JQ1 preincubation (Table 2). Preincubation with both epigenetic modifiers (entinostat plus JQ1) increased cisplatin potency more than with one epigenetic modifier only, except for T24 cells, where JQ1 alone gave an SF of 4.92 and entinostat plus JQ1 gave an SF of 3.52. Cisplatin resistance was completely reversed for the J82 cisR cells. Even in the highly resistant T24 LTT cell line, a 4.29-fold resensitization against cisplatin could be achieved. Since 48 h preincubation of the epigenetic modifiers prior to cisplatin addition was more successful than co-incubation of all three compounds, all following experiments were performed using preincubation conditions.

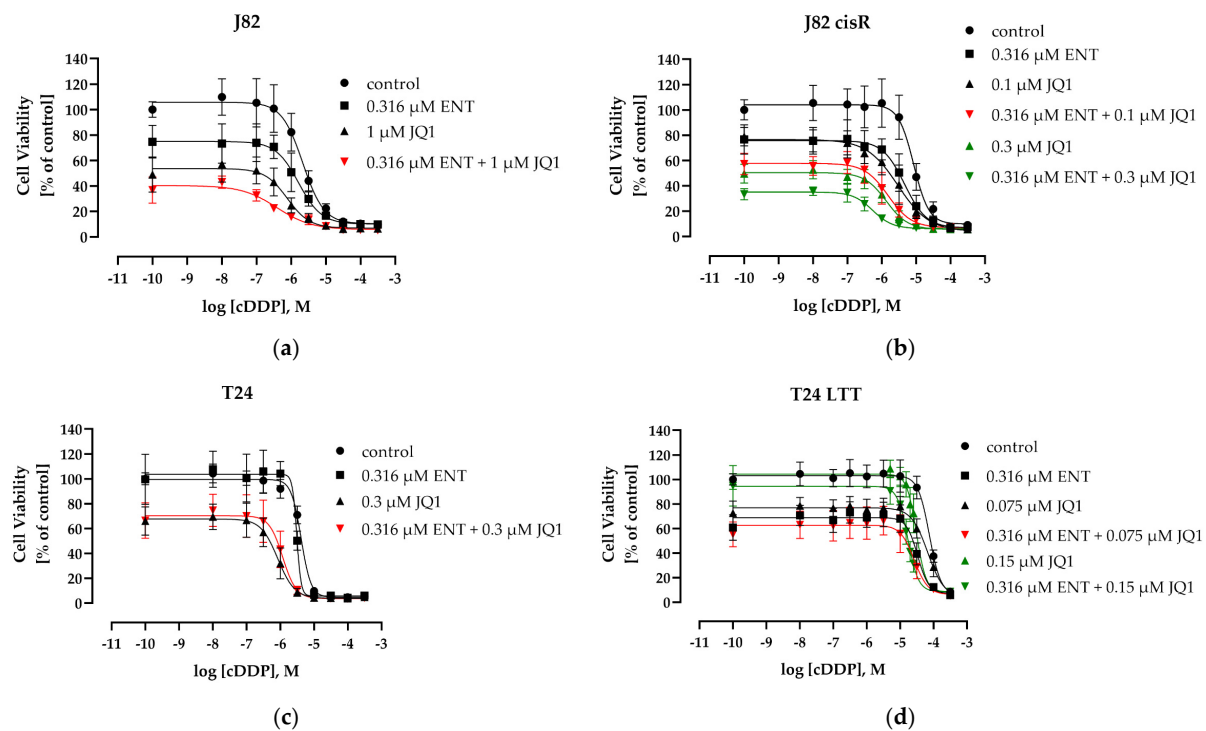


Figure 2. The concentration–effect curves of cisplatin (cDDP) (MTT assay). The cells were preincubated with entinostat (ENT) and/or JQ1 for 48 h followed by the addition of cisplatin for another 72 h. (a) J82, (b) J82 cisR, (c) T24, and (d) T24 LTT. Control means concentration–effect curve of cisplatin in the absence of epigenetic inhibitors. Data shown are the mean $\pm$ SD of at least three independent experiments, each carried out in triplicates.

Table 2. IC₅₀ values (MTT) of cisplatin. Cells were preincubated with entinostat (ENT) and/or JQ1 for 48 h followed by addition of cisplatin for another 72 h. Control was untreated for 48 h followed by addition of cisplatin for another 72 h. Data shown are the mean $\pm$ SD of at least three independent experiments, each carried out in triplicates. All IC₅₀ values of the combination treatments were significantly lower than the IC₅₀ values of the cisplatin alone (control, at least $p < 0.001$).

Cell Line	Control	ENT Plus Cisplatin			JQ1 Plus Cisplatin			ENT Plus JQ1 Plus Cisplatin		
	Cisplatin IC ₅₀ $\pm$ SD [μ M]	ENT [μ M]	Cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	JQ1 [μ M]	Cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	ENT+ JQ1 [μ M]	Cisplatin IC ₅₀ $\pm$ SD [μ M]	SF
J82	2.16 $\pm$ 0.14	0.316	1.63 $\pm$ 0.24	1.33	1	0.77 $\pm$ 0.13	2.81	0.316 + 1	0.38 $\pm$ 0.08	5.62
J82 cisR	8.49 $\pm$ 0.51	0.316	4.62 $\pm$ 0.57	1.84	0.1	2.96 $\pm$ 0.38	2.87	0.316 + 0.1	1.53 $\pm$ 0.22	5.54
					0.3	1.35 $\pm$ 0.15	6.27	0.316 + 0.3	0.54 $\pm$ 0.06	15.6
T24	4.15 $\pm$ 0.12	0.316	3.08 $\pm$ 0.15	1.35	0.3	0.84 $\pm$ 0.09	4.92	0.316 + 0.3	1.18 $\pm$ 0.13	3.52
T24 LTT	75.0 $\pm$ 2.85	0.316	33.2 $\pm$ 3.51	2.26	0.075	67.0 $\pm$ 9.03	1.12	0.316 + 0.075	26.4 $\pm$ 4.28	2.85
					0.15	30.6 $\pm$ 2.32	2.45	0.316 + 0.15	17.5 $\pm$ 1.13	4.29

Next, synergism studies were carried out for all cell lines for dual combinations (JQ1 plus cisplatin; entinostat plus cisplatin) and for triple combinations (48 h preincubation of entinostat plus JQ1 followed by addition of cisplatin for another 72 h). Data were analyzed according to Chou-Talalay [57] (Table 3) and according to the Bliss model [58] (Figure 3). Dual combinations were not or only moderately synergistic (Table S5), particularly with entinostat, as displayed by color coding: red color: non-synergistic, green color: synergistic. The only dual combination displaying strong synergy was JQ1 with cisplatin in T24 cells, showing CI values < 0.9 (indicating synergism) for almost all chosen concentrations (Table S5) which is in accordance with the high SF for cisplatin of 4.92 (Table 2). Further, in T24, the synergy/CI values of the dual combination (JQ1 plus cisplatin) and of the triple combination (entinostat, JQ1, cisplatin) were comparable (Tables 3 and S5).

Table 3. The CI values of the triple combination studies (48 h preincubation with entinostat and JQ1, followed by addition of cisplatin for another 72 h). The CI values were calculated using compusyn from the mean of at least three independent experiments, each carried out in triplicates. A CI < 1.0 indicate a synergistic effect (green), CI = 1 indicate an additive effect, and CI > 1 indicate an antagonistic effect (red); * means fraction affected was less than 0.20 [57].

J82									
0.316 μ M ENT + JQ1 [μ M]					1 μ M JQ1 + ENT [μ M]				
		0.5	1	2	3	0.1	0.316	0.5	0.75
cisplatin [μ M]	0.05	*	0.502	0.413	0.311	*	0.341	0.463	0.216
	0.1	*	0.500	0.327	0.284	*	0.479	0.352	0.193
	0.15	1.40	0.370	0.247	0.215	1.12	0.401	0.269	0.170
	0.2	1.10	0.290	0.190	0.179	1.21	0.287	0.217	0.119
	0.3	0.662	0.163	0.131	0.124	0.786	0.196	0.141	0.087
J82 cisR									
0.316 μ M ENT + JQ1 [μ M]					0.1 μ M JQ1 + ENT [μ M]				
		0.05	0.1	0.2	0.3	0.1	0.316	0.5	0.75
cisplatin [μ M]	0.2	1.10	0.374	0.223	0.100	1.34	0.644	0.480	0.285
	0.4	0.874	0.318	0.141	0.067	1.38	0.481	0.287	0.198
	0.6	0.782	0.243	0.118	0.067	1.07	0.352	0.249	0.167
	0.8	0.689	0.213	0.106	0.064	0.789	0.296	0.194	0.139
	1	0.538	0.192	0.104	0.063	0.795	0.250	0.183	0.133
T24									
0.316 μ M ENT + JQ1 [μ M]					0.3 μ M JQ1 + ENT [μ M]				
		0.1	0.3	0.5	0.7	0.1	0.5	0.75	
cisplatin [μ M]	0.2	*	0.949	0.438	0.349	1.32	0.643	0.509	
	0.4	*	0.655	0.313	0.290	0.844	0.626	0.421	
	0.6	*	0.491	0.294	0.265	0.726	0.431	0.392	
	0.8	*	0.463	0.258	0.247	0.552	0.392	0.322	
	1	*	0.360	0.243	0.235	0.484	0.398	0.287	
T24 LTT									
0.316 μ M ENT + JQ1 [μ M]					0.075 μ M JQ1 + ENT [μ M]				
		0.05	0.075	0.15	0.3	0.1	0.5	0.75	
cisplatin [μ M]	5	*	*	*	1.23	*	*	1.48	
	10	*	*	2.24	0.912	*	1.97	1.08	
	15	*	*	1.04	0.690	*	0.940	0.698	
	20	1.26	0.980	0.668	0.610	*	0.597	0.504	
	25	0.865	0.684	0.522	0.457	*	0.466	0.390	

Triple combinations were analyzed by Chou-Talalay in the following manner: one of the epigenetic modifiers was used at a fixed concentration, whereas the other and cisplatin were used in increasing concentrations. Thus, two data sets were obtained for each cell line (Table 3). In accordance with the previously determined shift factors (Table 2), the strongest synergism was observed in the J82, J82 cisR, and T24 cells (Table 3).

It is remarkable that the preincubation of entinostat plus JQ1 at low-toxic concentrations (0.316 μ M entinostat plus 1 μ M JQ1 in J82 cells; 0.316 μ M entinostat plus 0.1 μ M JQ1 in J82 cisR) already achieved a synergistic effect at 0.05 μ M cisplatin for J82 and at 0.2 μ M cisplatin for J82 cisR (Table 3). Similar data were observed in T24 at a cisplatin concentration of 0.2 μ M and in the highly cisplatin-resistant T24 LTT cell line at 15 μ M cisplatin.

Using combenefit, the synergy levels were calculated according to the Bliss model (Figure 3). Again, a clear synergistic effect was observed for the triple combination in all cancer cell lines.

In addition, the synergy data from MTT assays were further evaluated as a sum of the cytotoxic effects of the respective single compound treatments (black bars) compared to the cytotoxic effect of the combination treatments (white bars), as shown in the supplement (Figure S4). For almost all concentrations in all cell lines, the sum of the single treatment effects (black bars) was significantly lower than the effects of the triple combination treatments (white bars), supporting the synergistic behavior of the combination treatments (Figure S4).

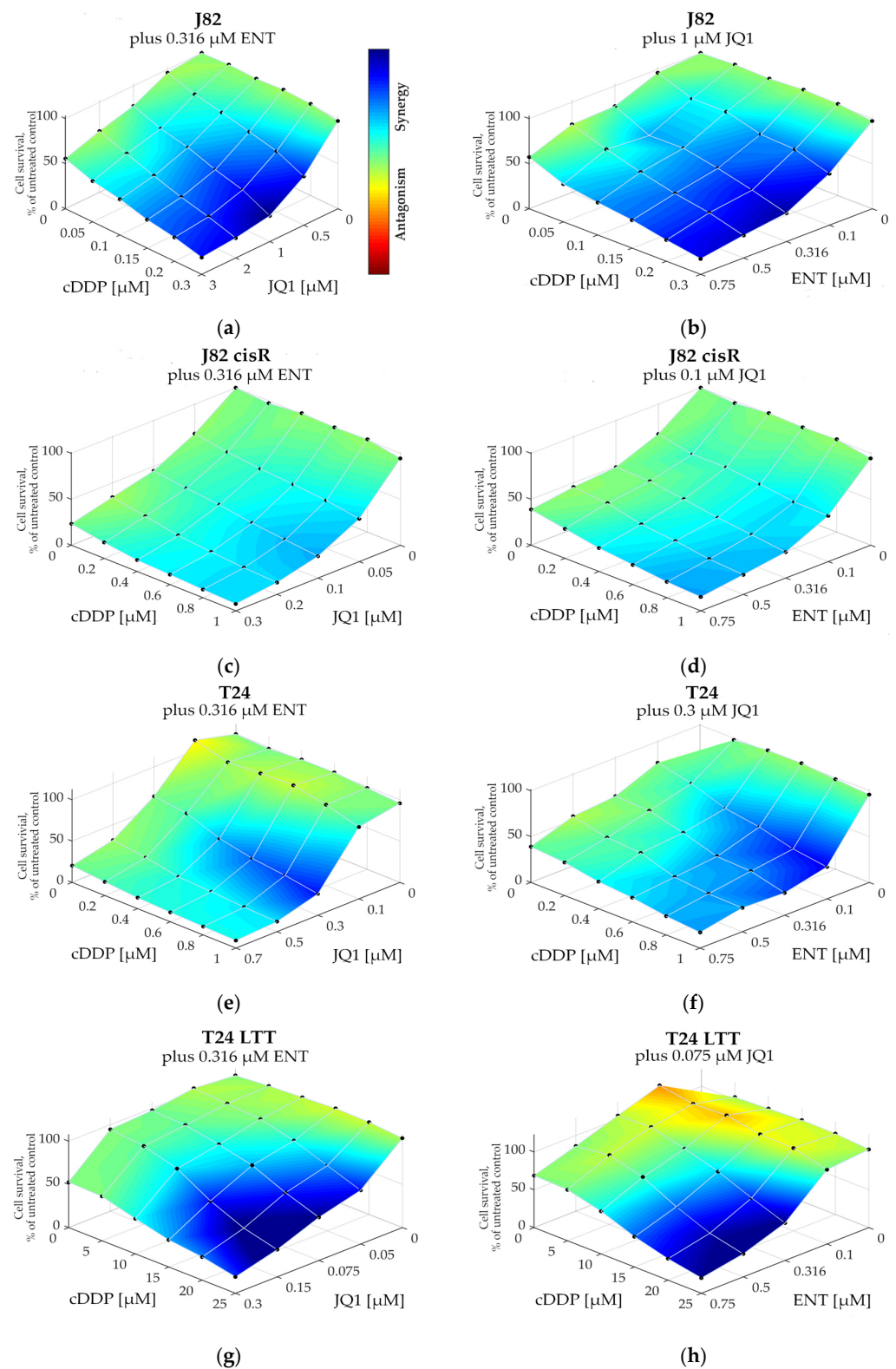


Figure 3. Synergy levels calculated applying the Bliss model using combenefit [58] of the triple combination studies (48 h preincubation with entinostat and JQ1, followed by addition of cisplatin for another 72 h). Entinostat was used at 0.316 μM for all cell lines. JQ1 was used at 1 μM in J82, 0.1 μM in J82 cisR, 0.3 μM in T24, and 0.075 μM in T24 LTT. (a,b) J82, (c,d) J82 cisR, (e,f) T24, and (g,h) T24 LTT.

3.2. Triple Combination Treatment with Entinostat, JQ1, and Cisplatin Enhances Apoptosis Induction Via Caspase-3/7 Activation and Double-Strand Breaks

Subsequently, we investigated whether the synergistic effect of the triple combination was based on an increased induction of caspase-mediated apoptosis. First, the induction of subG1 cell nuclei was measured using flow cytometry (Figure 4). In total, 100 μM of cisplatin was used as positive control for J82, J82 cisR, and T24. In total, 200 μM of cisplatin was chosen as positive control for T24 LTT, based on the high cisplatin resistance of these cells. Representative cytometry flow images are shown in Figure S5.

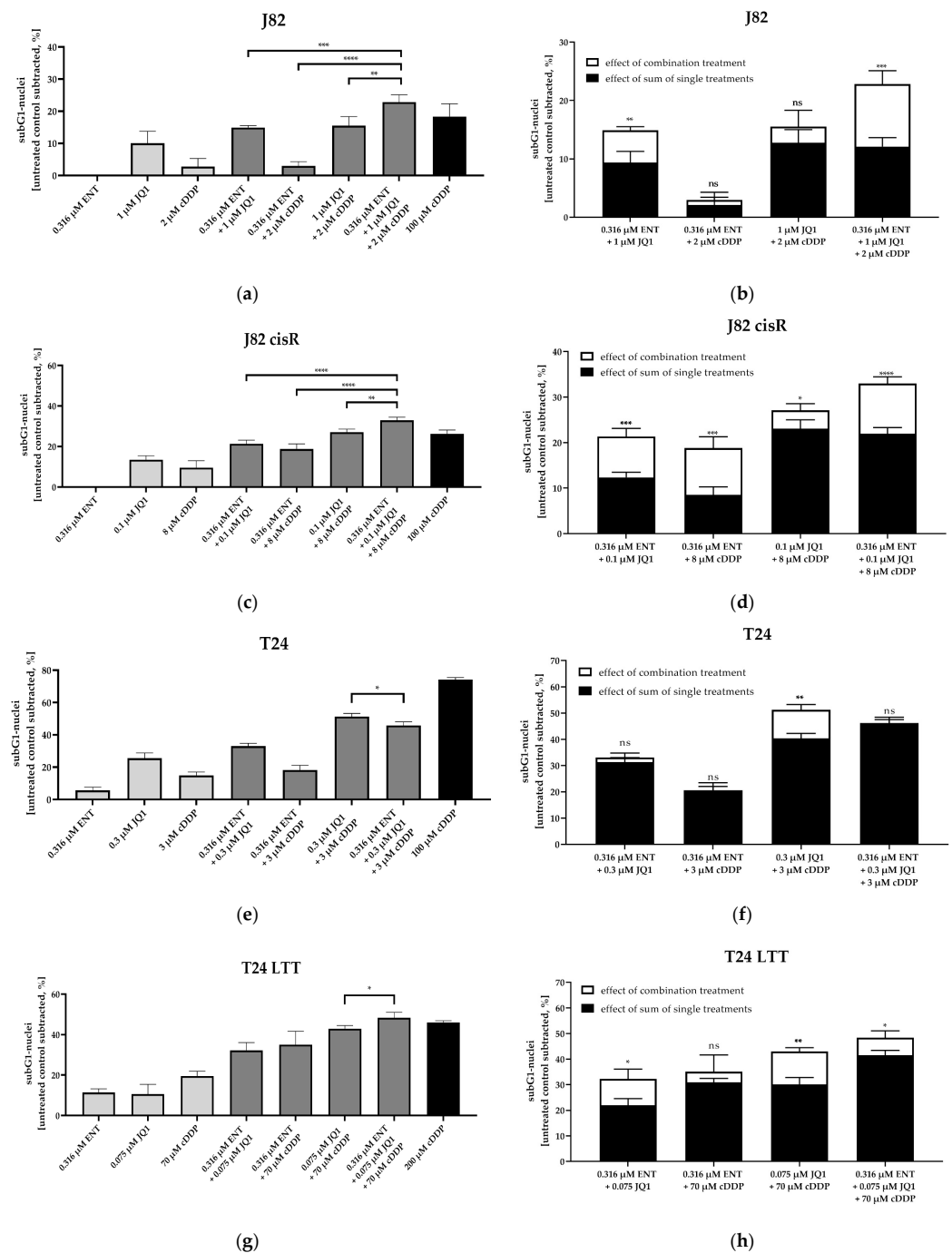


Figure 4. The estimation of apoptosis by measuring induction of the subG1-nuclei. The cells were preincubated with entinostat and JQ1 for 48 h followed by addition of cisplatin for another 24 h. Apoptotic

cells were defined by subG1 fraction (flow cytometry). Data shown are the mean + SD of one representative experiment out of at least two, each carried out at least in duplicates. Untreated control was subtracted. (a,b) J82; (c,d) J82 cisR; (e,f) T24; (g,h) T24 LTT. (a,c,d,e) show subG1 nuclei. (b,d,f,h) show the comparison between the sum of the single compound effects (black bars) versus the effect of the combination treatment (white bars). Statistical analysis was performed using unpaired *t*-test. Level of significance: ns ($p \geq 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

The triple treatment led to the highest induction of subG1 nuclei in J82, J82 cisR, and T24 LTT. In T24, the dual combination consisting of JQ1 and cisplatin had the strongest effect, followed by the triple treatment. This was consistent with the SF in Table 2 (JQ1 and cisplatin: SF 4.92 vs. entinostat, JQ1, and cisplatin: SF 3.52). Statistical evaluation demonstrated that the triple combination (white bars) caused a significantly higher increase in subG1 nuclei than the sum of the single treatments (black bars) in J82, J82 cisR and T24 LTT. This emphasizes the synergistic effect of the triple combination. Again, T24 was outstanding, as the dual combination of JQ1 and cisplatin already had a significant synergistic effect, which was not further enhanced by a triple combination. These results confirmed the previous ones (Section 3.1) and suggest enhanced apoptosis by the triple combination in at least three cell lines.

To examine whether apoptosis was caspase-mediated, caspase 3/7 activation was analyzed (Figure 5). Representative fluorescence images of each cell line are shown in Figure S6. Again, 100 μ M of cisplatin was chosen as positive control for caspase 3/7 activation in J82, J82 cisR, and T24. Due to the high cisplatin resistance of the T24 LTT cells (Table 1), 200 μ M of cisplatin were incubated in the T24 LTT cells for 48 h in contrast to the other cancer cell lines where 100 μ M of cisplatin was used for 24 h.

The triple treatment caused the highest caspase 3/7 activation in J82 and J82 cisR. T24 cells revealed the strongest caspase 3/7 activation with the combination of JQ1 and cisplatin followed by the triple combination. T24 LTT exhibited the greatest caspase 3/7 activation with the combination of entinostat and cisplatin (Figure 5). This is in agreement with a reduction in the IC_{50} value of cisplatin in the dual combination entinostat plus cisplatin, which was in a similar range as the reduction in the IC_{50} value with the triple combination (Table 2). Nevertheless, the triple combination led to a clear increase in caspase 3/7 activation. Further, comparing the sum of the caspase activation of the single treatments (black bars) with the caspase activation of the combination treatments (white bars), we could demonstrate that the triple treatment is synergistic in all cell lines, i.e., the white bars are larger than the black bars. This was also true for the dual combination of JQ1 and cisplatin in the J82, J82 cisR, and T24 cells, and for the dual combination of entinostat and cisplatin in the T24 LTT cells. Overall, these results were in accordance with the apoptosis experiments (Figure 4). Additionally, we could confirm that the increased apoptosis is caspase 3/7-mediated. A further mechanistic investigation is the analysis of compound-induced double-strand breaks as additional readout for the cytotoxic effect. The number of corresponding foci of γ -H2AX and 53BP1 was counted as surrogate parameters for double-strand breaks using fluorescence microscopy (Figure 6) [62–64]. Because γ -H2AX could also be generated by other cellular processes, 53BP1 was used as an additional marker [65,66]. The presence of both proteins at the same position is considered as a specific indication of double-strand breaks [67–69]. Representative fluorescence images of J82 and J82 cisR cells are shown in Figure 7.

Treatment with the triple combination yielded the highest number of co-staining foci of γ -H2AX and 53BP1 in the J82 and J82 cisR cells (Figures 6 and 7). This increase was significant compared to all other treatments underlining the strongest effect for a triple combination treatment. Dual combinations were not more potent in increasing double strand breaks compared to cisplatin treatment only, except for the combination of entinostat or JQ1 and cisplatin in J82 cisR cells. The 100 μ M cisplatin showed only a small number of double-strand breaks in both cell lines, especially in J82 cisR, which might be due to an advanced stage of apoptosis due to the high cisplatin concentration. The total number

of double-strand breaks was lower in J82 cisR than in J82 when treated with cisplatin alone and in combination with JQ1 and cisplatin. Generally, this could be related to an increased DNA repair capacity in J82 cisR contributing to cisplatin resistance. In summary, we could demonstrate synergy for the triple combination in caspase-mediated apoptosis and a significantly stronger effect in inducing double strand breaks.

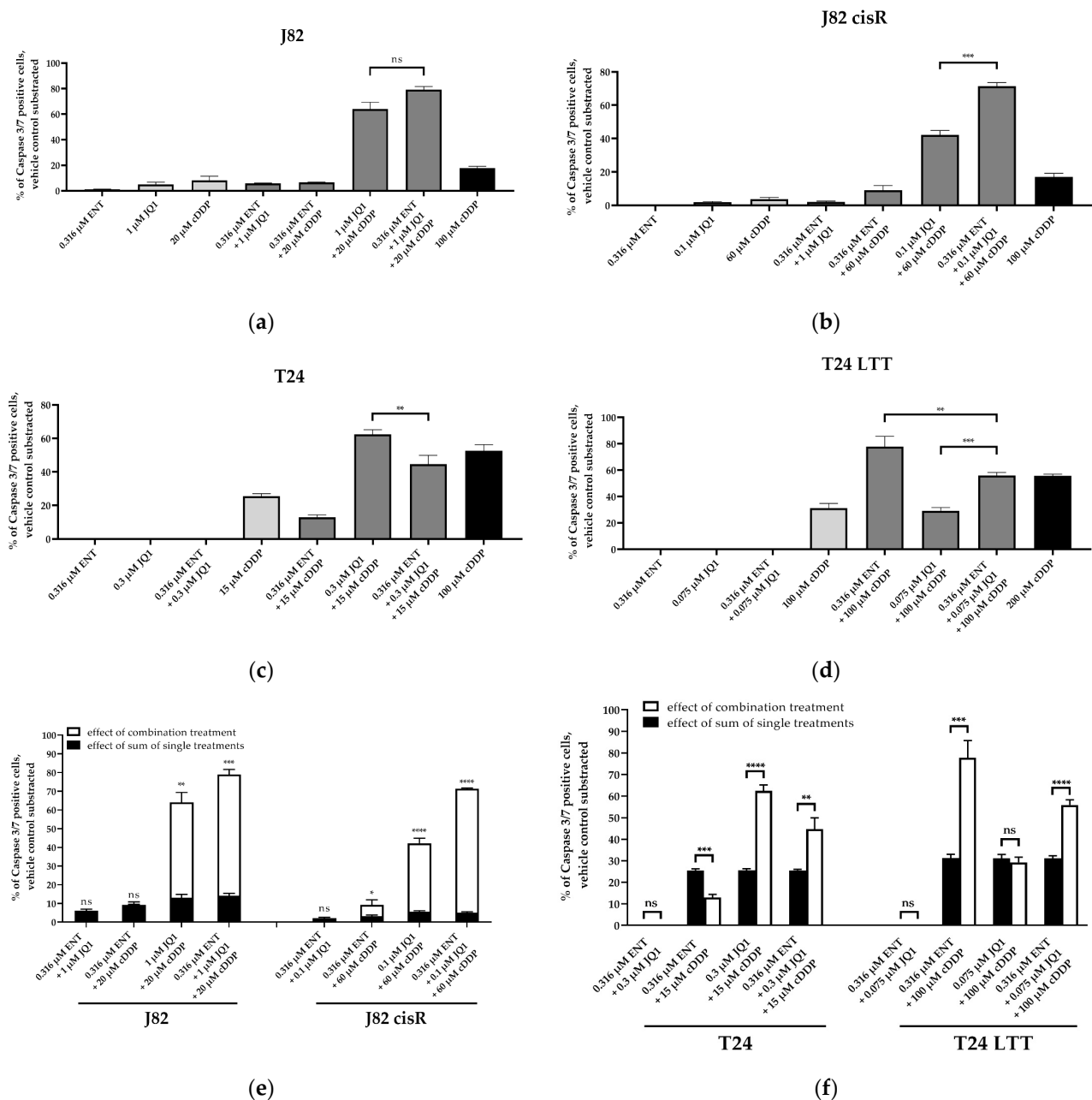


Figure 5. Caspase 3/7 activation in (a) J82, (b) J82 cisR, (c) T24, and (d) T24 LTT. The cells were preincubated with entinostat and JQ1 for 48 h followed by addition of cisplatin for another 24 h (T24 LTT 48 h). (e,f) show the comparison between the sum of the single-compound effects (black bars) versus the effect of the combination treatments (white bars) in J82 and J82 cisR (e) and T24 and T24 LTT (f). Data shown are the mean + SD of one representative experiment out of at least two, each carried out in duplicates. Significance was calculated using unpaired *t*-test. Level of significance: ns ($p \geq 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

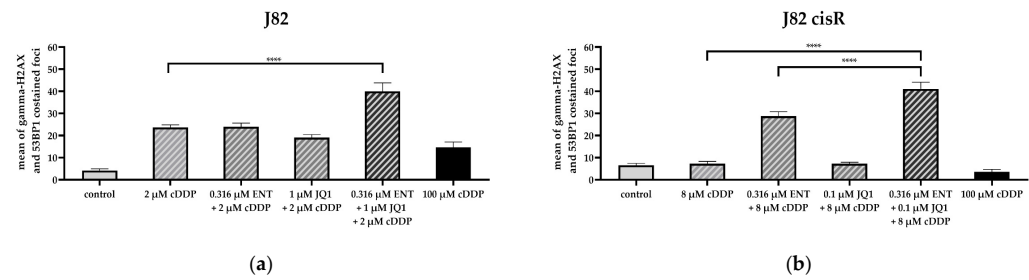


Figure 6. Number of foci stained for γ -H2AX and 53BP1 of (a) J82 and (b) J82 cisR. Cells were preincubated with entinostat and JQ1 for 48 h followed by addition of cisplatin for another 24 h. Foci in at least 19 representative random cells were counted. Data shown are the mean + SEM of counted foci. Significance was calculated using unpaired *t*-test in GraphPad Prism. Level of significance: ns ($p \geq 0.05$); **** ($p < 0.0001$).

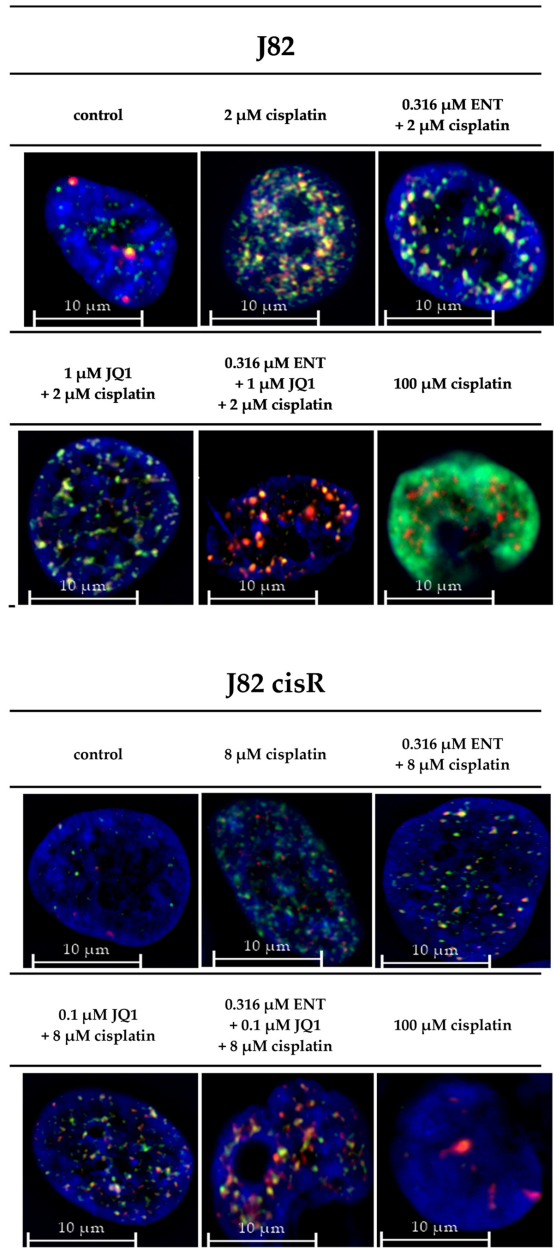


Figure 7. One representative fluorescence image out of five experiments is shown for each treatment condition for J82 and J82 cisR (γ -H2AX (green) and 53BP1 (red); overlay of γ -H2AX and 53BP1 (yellow)).

The nuclei were stained using Hoechst 33342 (blue). The 100 μ M of cisplatin in J82: DAPI staining is overlaid by strong γ -H2AX staining. The cells were preincubated with entinostat and JQ1 for 48 h, followed by addition of cisplatin for another 24 h.

3.3. Effect of Entinostat and JQ1 on Expression of Pro- and Anti-Apoptotic Genes and Proteins

Next, the effects of the epigenetic modulators entinostat and JQ1 on gene and protein expression were analyzed to determine the effect of their preincubation. Therefore, the influence of entinostat, JQ1, and their combination was investigated on the expression of pro- and anti-apoptotic genes in the J82 and J82 cisR cells, showing the most favorable results with the triple combination. The RT-qPCR was performed after 48 h incubation with entinostat and JQ1 and evaluated according to Vandesompele [54]. Genes coding for pro- (Bim, FOXO1, FOXO3a) and anti-apoptotic (survivin) proteins were analyzed. Furthermore, gene expression of proteins that influence the cell cycle (p21, p53) were also examined (Figure 8).

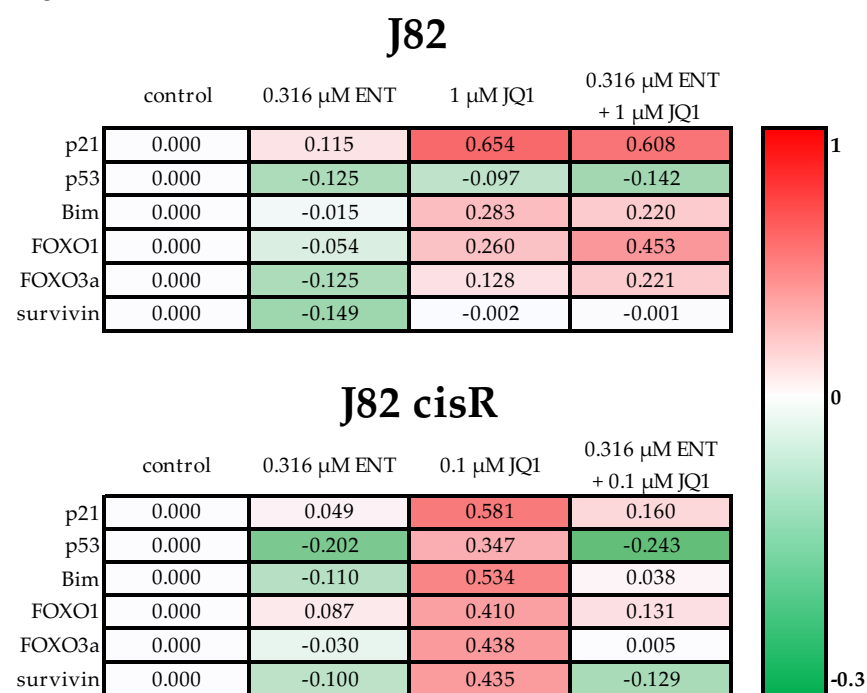


Figure 8. Gene expression was examined by RT-qPCR. Cells were incubated with entinostat and JQ1 for 48 h. The analysis was performed according to Vandesompele [54]. Shown is the decadic logarithm of the expression values normalized to the expression of the control genes GUSB (beta-glucuronidase), TBP (TATA-binding protein), and HPRT1 (hypoxanthine–guanine phosphoribosyltransferase) of the respective cell line. Negative values (green) indicated lower gene expression, and positive values (red) indicated higher gene expression compared to the cell-specific control. Data are shown of one representative experiment carried out in duplicates.

In J82 and J82 cisR, *p21* was only slightly upregulated by treatment with entinostat. In contrast, JQ1 or the combination of entinostat and JQ1 led to a greater upregulation of *p21* in both cell lines. In J82, the increase in *p21* gene expression by the triple treatment was only slightly weaker than by JQ1 alone, whereas the increase in *p21* was 3.6-fold greater by JQ1 than by the combination of entinostat and JQ1 in J82 cisR. *P53* was downregulated by the epigenetic modulators in J82 and J82 cisR, except for JQ1 alone in J82 cisR, where *p53* was upregulated. *Bim*, *FOXO1*, and *FOXO3a* showed an increased gene expression after JQ1 and the dual combination treatment in both cell lines, while *Bim* and *FOXO3a* were only marginally upregulated through the combination treatment in J82 cisR. The gene expression of *survivin* was inconsistent. *Survivin* was less expressed by entinostat in J82. The treatment with JQ1 or the combination of entinostat and JQ1 hardly made any difference to control.

In J82 cisR, *survivin* was strongly upregulated by JQ1, but downregulated by entinostat and the combination treatment.

To verify the results of the RT-qPCR at the protein level, Western blots were performed with the same incubation time. Furthermore, BRD4 and c-Myc were considered as additional target proteins of JQ1. JQ1 has previously been shown to exert its antitumor effects by inhibiting c-Myc expression [70]. The presented blots were analyzed with ImageJ for a more comprehensive analysis. The results are shown in Figure 9 and Figure S7.

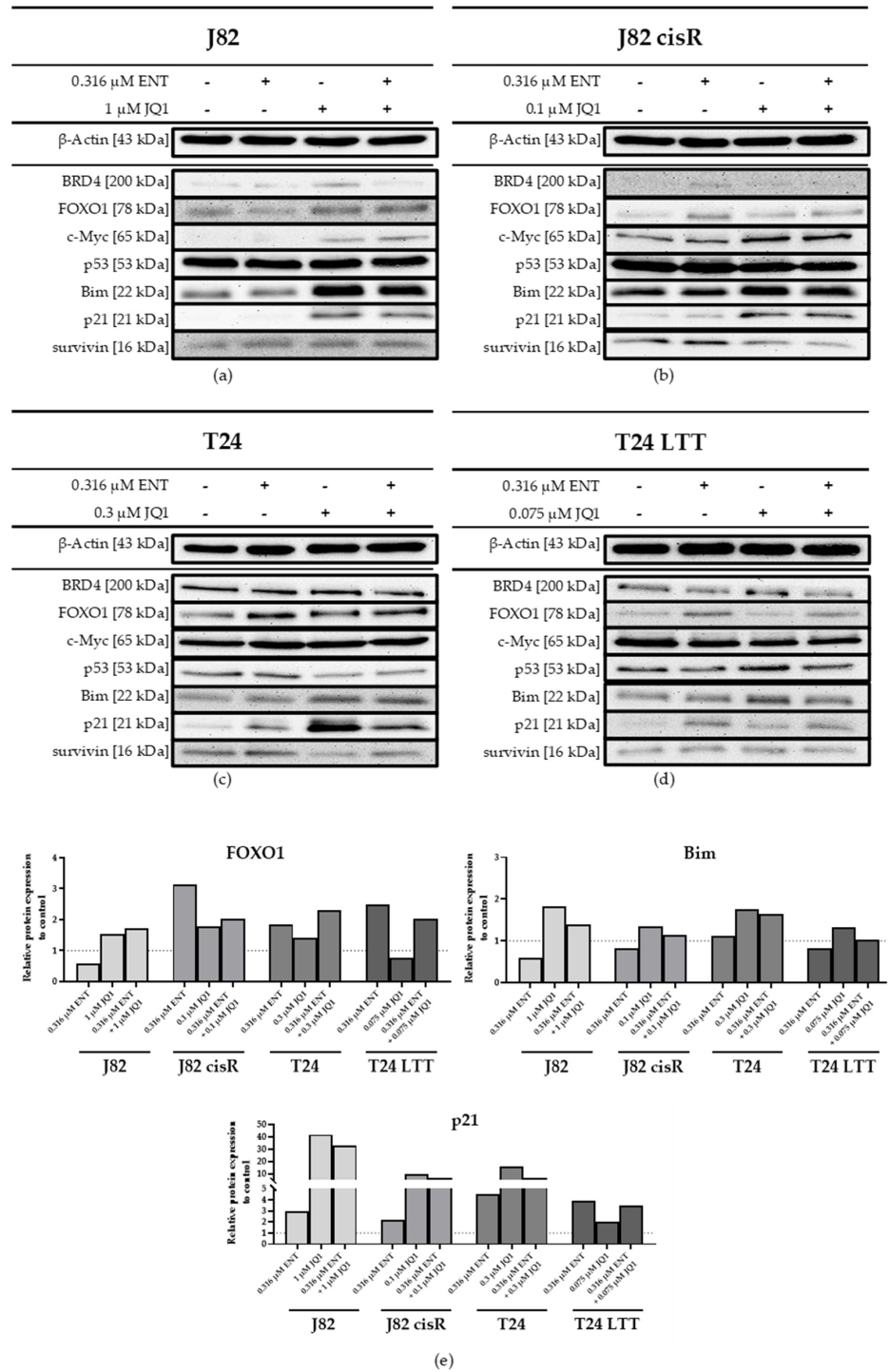


Figure 9. Western blot analysis of (a) J82, (b) J82 cisR, (c) T24, and (d) T24 LTT. The cells were incubated with entinostat and JQ1 for 48 h. The data shown are a representative blot out of at least

two different protein samples. The uncropped blots are presented in the supplement (Figures S8–S14). (e) Relative protein expression of FOXO1, Bim, and p21 related to β -actin as loading control in J82, J82 cisR, T24, and T24 LTT. The ratio of control was set to 1, represented by the dashed line. Shown Western blots were analyzed with ImageJ. The analysis of the remaining proteins is presented in Figure S7.

The combination of entinostat and JQ1 revealed an increased protein expression of FOXO1, Bim, and p21 compared to the control in all four cell lines (Figure 9). The strongest increase in FOXO1 expression was induced by entinostat in J82 cisR and T24 LTT and not by the combination treatment. Interestingly, Bim was most expressed when treated with JQ1 alone, but the combination of entinostat and JQ1 gave similar results. P21 was highly upregulated by JQ1 and the combination treatment in J82, J82 cisR, and T24. In T24 LTT, p21 was also upregulated, but only by entinostat and the combination treatment. A slight decrease in survivin was only evident with JQ1 and the combination treatment in J82 cisR and T24. P53 was downregulated by the combination of entinostat and JQ1 in all four cell lines. C-Myc was detectable in all cell lines, tending towards upregulation in J82 and J82 cisR and downregulation in T24 and T24 LTT (Figure S7) upon treatment. BRD4 was only very slightly expressed in J82 cisR in contrast to the other three cell lines. The present Western blot results were largely consistent with the qPCR results, demonstrating the preincubation with both epigenetic modulators resulted in upregulation of p21, FOXO1, and Bim.

3.4. Investigation of BETi OTX015, JQ35, and Dual Inhibitor 20 in Combination Treatments

JQ1 was used as a model compound for BETi in this study. JQ1 was not investigated clinically due to its short half-time [33]. Advanced BETi as OTX015 and JQ35 were developed and already clinically investigated in B-cell lymphoma, multiple myeloma, castrate-resistant prostate cancer, midline carcinoma, and non-small-cell lung cancer, but to our knowledge, not in bladder cancer [27,36]. Therefore, we investigated the advanced BETi OTX015 and JQ35 in the triple combination (together with cisplatin) as replacement for JQ1 to enable a future potential clinical application perspective for the triple treatment in bladder cancer. The cell lines J82 and J82 cisR were selected for this investigation since the triple treatment was most effective in these cell lines. As additional approach, the dual HDAC- and BET-inhibitor 20 was investigated [51]. Inhibition of two target proteins simultaneously, HDAC and BRD proteins, in one compound could simplify therapy regimes, known in medicinal chemistry as dual-targeting approach. Compound 20 was described in the literature as a compound with sub-micromolar inhibitory activity against HDAC 1-3 and 6 [51]. The IC_{50} values at HDAC4 (207 μ M) and HDAC8 (6.03 μ M) support selectivity towards class I and IIb HDACs (Figure S15, Table S6). The inhibitory activity to BRDs was reported in the single-digit micromolar range [51]. As 20 can inhibit both epigenetic targets, 20 was only tested in dual combination with cisplatin in all four cancer cell lines.

Firstly, the IC_{50} values for all three compounds were determined in J82 and J82 cisR. Compound 20 was additionally tested in T24 and T24 LTT. Results are presented in Figure S16 and Table 4.

Table 4. IC_{50} (MTT, 72 h) of OTX015, JQ35, and 20 in all bladder cancer cell lines. Data shown are the mean $\pm$ SD of at least three independent experiments, each carried out in triplicates. ND means not determined.

	OTX015 $IC_{50} \pm SD$ [μ M]	JQ35 $IC_{50} \pm SD$ [μ M]	20 $IC_{50} \pm SD$ [μ M]
J82	18.9 $\pm$ 2.82	69.5 $\pm$ 7.88	7.17 $\pm$ 0.57
J82 cisR	1.04 $\pm$ 0.13	1.49 $\pm$ 0.34	7.00 $\pm$ 0.49
T24	ND	ND	6.95 $\pm$ 0.54
T24 LTT	ND	ND	4.66 $\pm$ 0.41

The IC_{50} values of OTX015 and JQ35 were comparable to the IC_{50} values of JQ1 in J82 and J82 cisR (Table 1, J82 18.8 μ M, J82 cisR 2.22 μ M), except for JQ35 in J82. JQ35 had a 3.7-fold-increased IC_{50} value in J82 cells compared to JQ1. Compound 20 was approximately equally cytotoxic in the single-digit micromolar range in all four cell lines. Then, the effect of entinostat plus OTX015 or entinostat plus JQ35 or, alternatively, 20 only on the IC_{50} of cisplatin was investigated. The concentration–effect curves of cisplatin are presented in Figures 10 and S17. The IC_{50} values and shift factors of the combination treatments are shown in Table 5 (additional IC_{50} values and shift factors in Tables S7 and S8).

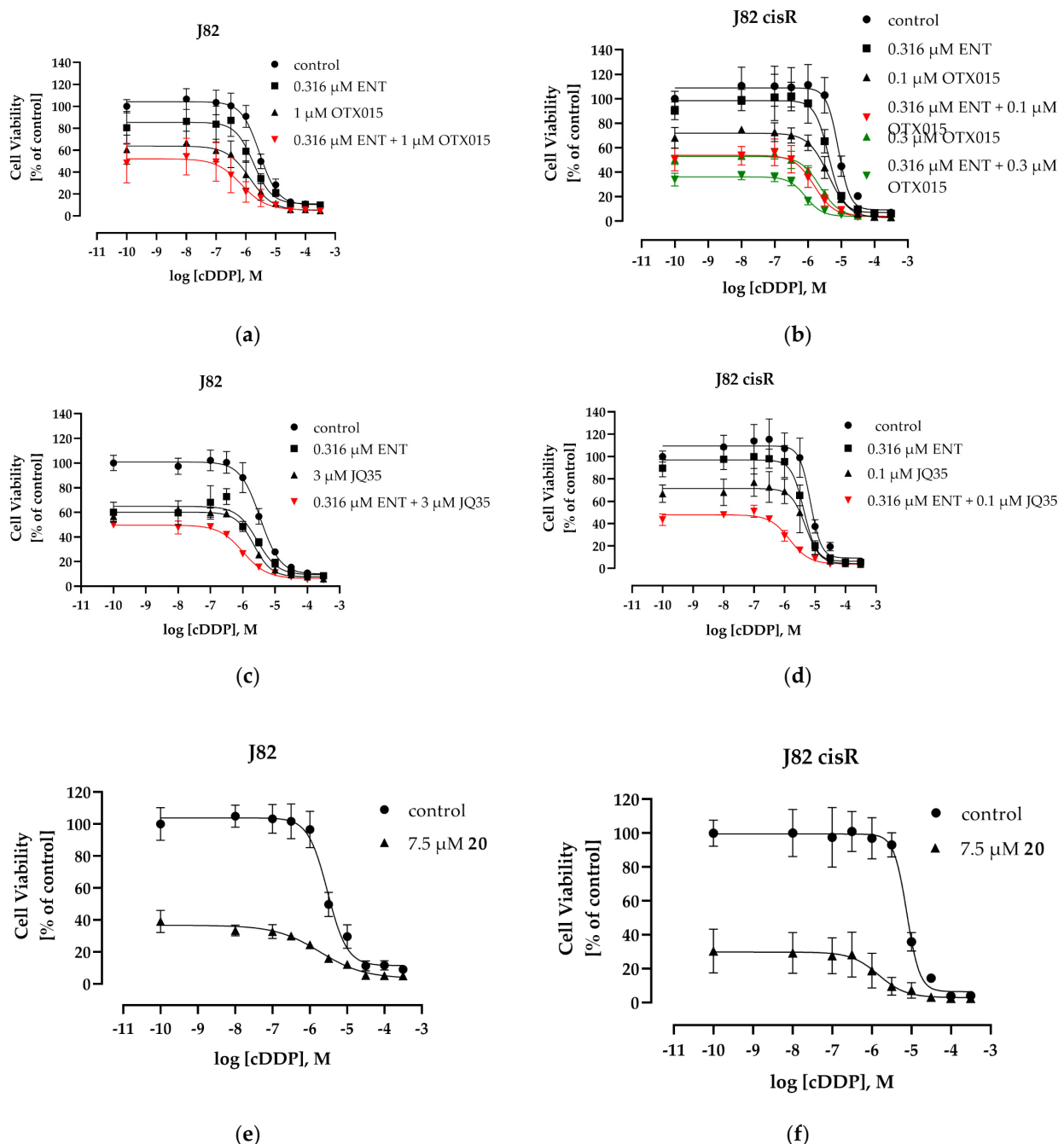


Figure 10. Concentration–effect curves of cisplatin (MTT assay) of (a,c,e) J82 and (b,d,f) J82 cisR. Cells were preincubated with entinostat and/or OTX015 or JQ35 or compound 20 alone for 48 h followed by addition of cisplatin for another 72 h. Control means concentration–effect curve of cisplatin in absence of epigenetic inhibitors. Data shown are the mean $\pm$ SD of at least one experiment, carried out in triplicates.

Table 5. IC₅₀ values (MTT) of cisplatin. Cells were preincubated with entinostat and/or OTX015 or JQ35 or compound **20** alone for 48 h followed by addition of cisplatin for another 72 h. Control was untreated for 48 h followed by addition of cisplatin for another 72 h. Data shown are the mean $\pm$ SD of at least one experiment, carried out in triplicates. All IC₅₀ values of the combination treatments were significantly lower than the IC₅₀ values of cisplatin alone (control, $p < 0.0001$).

Cell Line	Control		+ 0.316 μ M Entinostat							
	Cisplatin IC ₅₀ $\pm$ SD [μ M]	OTX015 [μ M]	Cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	JQ35 [μ M]	Cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	20 [μ M]	Cisplatin IC ₅₀ $\pm$ SD [μ M]	SF
J82	3.02 $\pm$ 0.10	1	0.70 $\pm$ 0.23	4.31	3	1.01 $\pm$ 0.10	2.99	7.5	1.86 $\pm$ 0.56	1.62
J82 cisR	7.80 $\pm$ 0.23	0.1	1.55 $\pm$ 0.20	5.03	0.1	1.47 $\pm$ 0.13	5.31	7.5	1.47 $\pm$ 0.76	5.31
		0.3	0.85 $\pm$ 0.06	9.18						

As previously observed for JQ1, OTX015 and JQ35 also revealed the largest SF in combination with entinostat (Tables 5 and S7), leading to a complete reversal of cisplatin resistance in J82 cisR. The application of one epigenetic inhibitor only gave a slight reduction in the IC₅₀ value of cisplatin (Table S7). Overall, the combination of the BETi, OTX015 or JQ35, and entinostat showed comparable sensitizing effects on the IC₅₀ value of cisplatin, as JQ1 did. Compound **20** had an SF of 5.31 in J82 cisR, which is comparable to the effect of the combination of entinostat plus OTX015 or JQ35. In J82, T24, and T24 LTT, the SF of **20** was mostly only <2 (Tables 5 and S8); however, 7.5 μ M of **20** induced a significant decrease in cell viability (approx. 60–70%) in all bladder cancer cell lines. (Figures 10 and S17). OTX015 was chosen for further detailed investigations due to its overall presenting the highest SF in the triple combinations in J82 and J82 cisR (Table 5). We investigated whether OTX015 alone and in combination treatments induced comparable effects as JQ1 regarding apoptosis induction. The results of subG1 induction are shown in Figure 11 and representative cytometry flow images are presented in Figure S18.

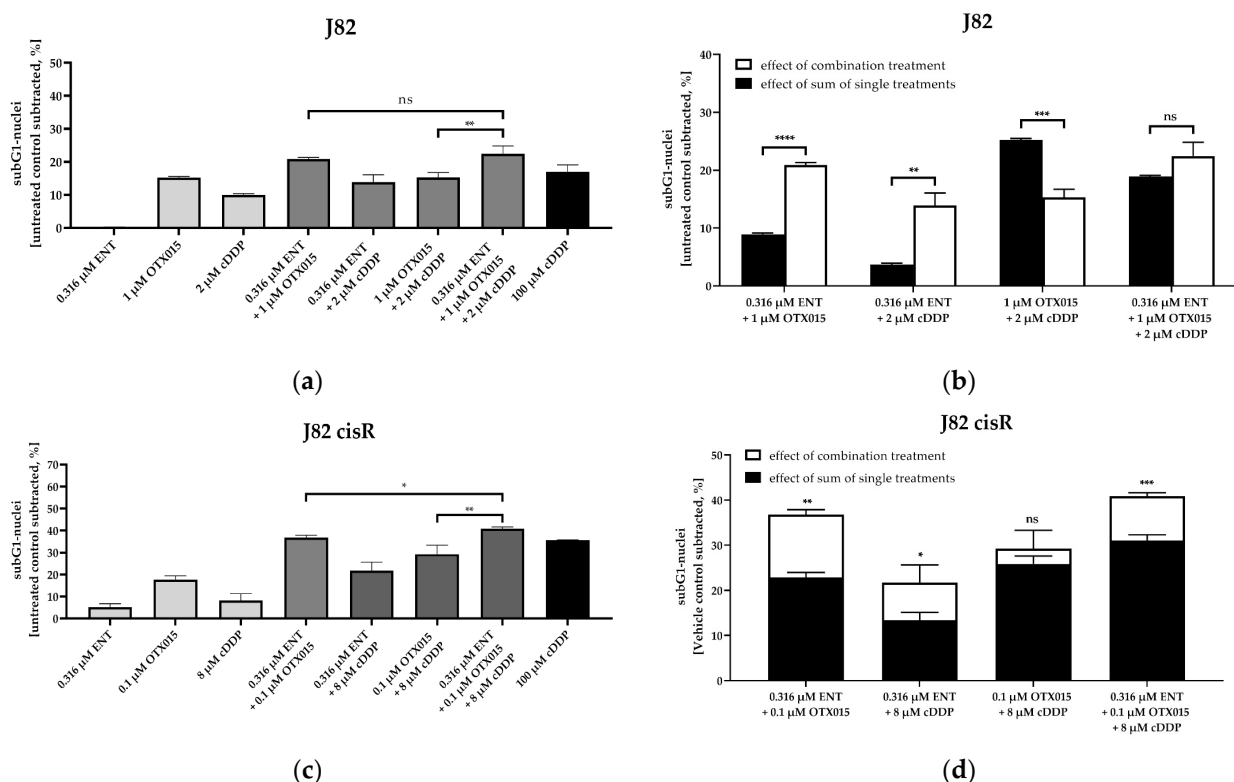


Figure 11. Induction of subG1-nuclei. Cells were preincubated with entinostat and OTX015 for 48 h followed by addition of cisplatin for another 24 h. Apoptotic cells were defined by subG1 fraction

(flow cytometry). Data shown are the mean + SD of one representative experiment out of at least two, each carried out at least in triplicates. Untreated control was subtracted. (a) J82; (c) J82 cisR. (b,d) show the comparison between the sum of the single compound effects (black bars) versus the effect of the combination treatments (white bars) in J82 (b) and J82 cisR (d). Statistical analysis was performed using unpaired *t*-test. Level of significance: ns ($p \geq 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

The triple combination of entinostat, OTX015, and cisplatin exhibited the largest subG1 fraction in J82 and J82 cisR. The triple combination induced significantly more subG1 nuclei than the single or dual combinations in J82 cisR. The same applied to J82, except for the combination of entinostat and OTX015. In J82, the difference between the sum of the single treatments and the triple combination treatment was not significant. Even the dual combination of entinostat and OTX015 was as effective as the triple treatment and showed a significant increase in subG1 nuclei in comparison to the sum of single treatments. In J82 cisR, the number of subG1 nuclei induced by triple treatment was significantly higher than the sum of the single treatments. Overall, OTX015 in combination with entinostat and cisplatin yielded comparable results to JQ1 in the induction of subG1 nuclei (Figures 4 and 11).

Then, we investigated whether the induction of apoptosis was triggered by caspase 3/7 activation as observed for JQ1. The activation of caspase 3/7 was investigated using the using dual and triple combinations consisting of entinostat, OTX015, and cisplatin in J82 and J82 cisR (Figure 12). In addition, we investigated the dual combination of 20 and cisplatin in J82, J82 cisR, T24, and T24 LTT (Figure 13). Representative fluorescence images are presented in Figure S19.

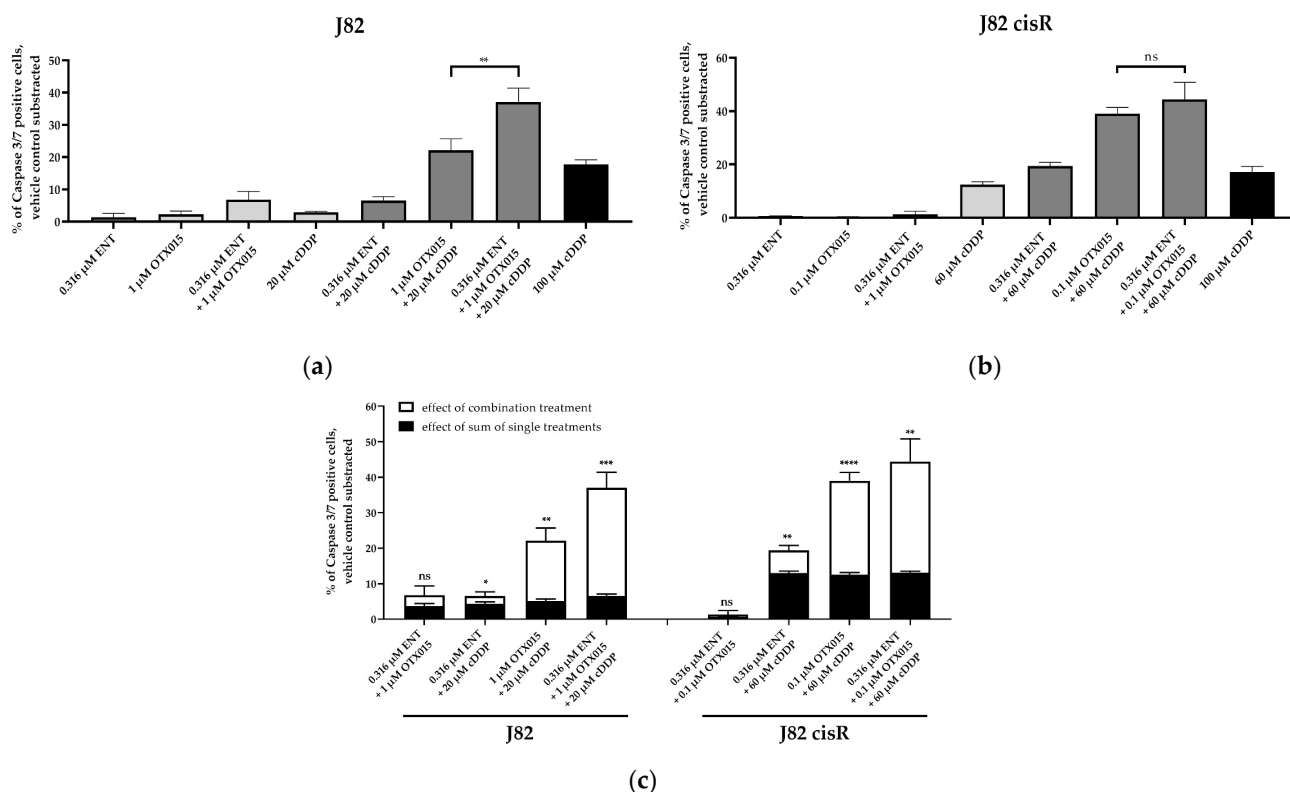


Figure 12. Caspase 3/7 activation in (a) J82 and (b) J82 cisR. Cells were preincubated with entinostat and OTX015 for 48 h followed by addition of cisplatin for another 24 h. (c) shows the comparison between the sum of the single compound effects (black bars) versus the effect of the combination treatments (white bars) in J82 and J82 cisR. Data shown are the mean + SD of one representative experiment out of at least two, each carried out in duplicates. Significance was calculated using unpaired *t*-test. Level of significance: ns ($p \geq 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

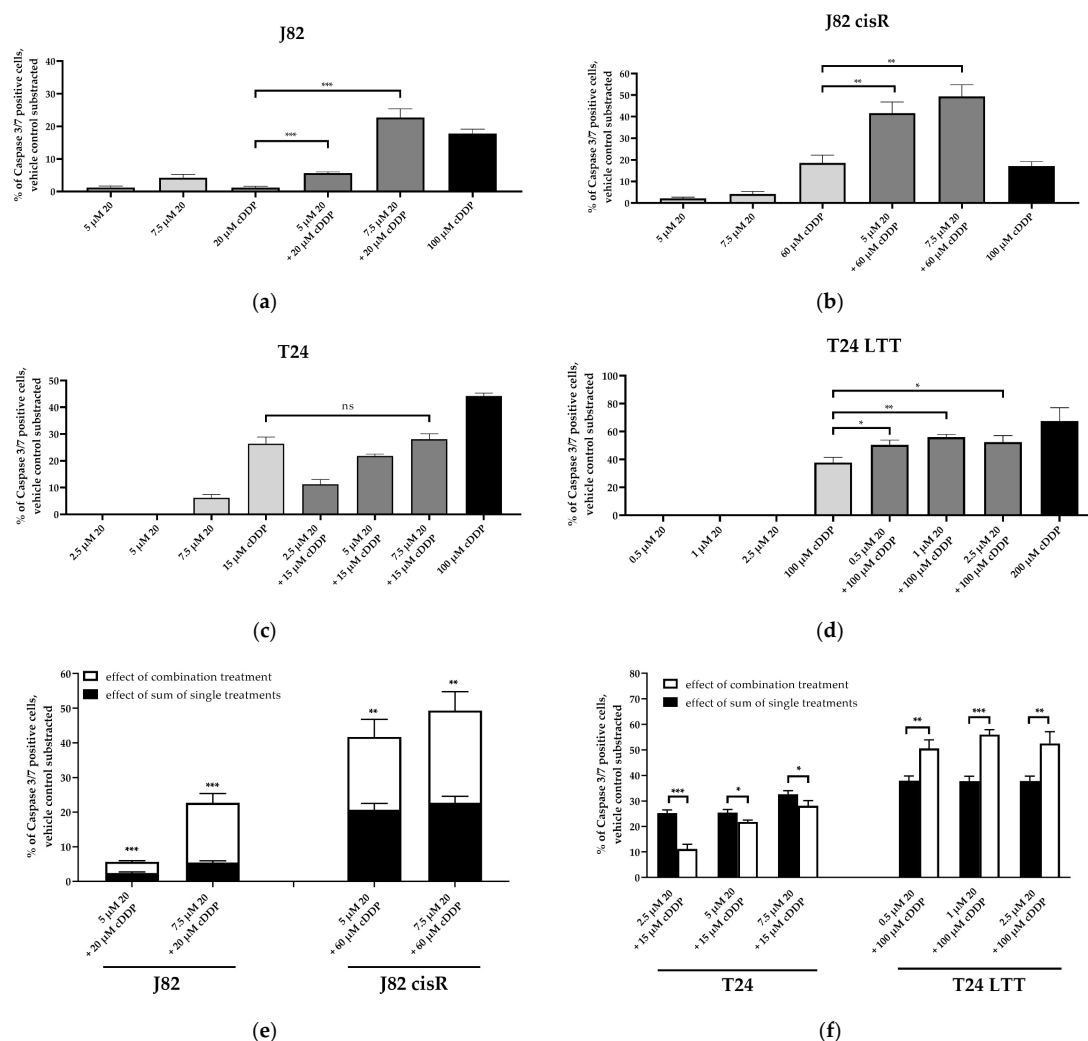


Figure 13. Caspase 3/7 activation in (a) J82, (b) J82 cisR, (c) T24, and (d) T24 LTT. Cells were preincubated with **20** for 48 h followed by addition of cisplatin for another 24 h (T24 LTT 48 h). (e,f) show the comparison between the sum of the single compound effects (black bars) versus the effect of the combination treatments (white bars) in J82 and J82 cisR (e) and T24 and T24 LTT (f). Data shown is the mean + SD of one representative experiment out of at least two, each carried out in duplicates. Significance was calculated using unpaired *t*-test. Level of significance: ns ($p \geq 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$).

The triple combination induced the highest caspase 3/7 activation followed by the combination of OTX015 and cisplatin in J82 and J82 cisR, although the difference was not significant in J82 cisR. The synergistic effect of the triple combination was evident as seen by a huge increase in caspase 3/7 activation in J82 and J82 cisR compared to the sum of the single treatments. These results again demonstrated that OTX015 behaved analogous to JQ1 in the triple combination (Figures 5 and 12). Remarkably, the triple combination with OTX015 exceeded by far the caspase activation induced by 100 μ M cisplatin, again demonstrating the potential of the triple combination for overcoming cisplatin resistance.

Compound **20** only weakly activated caspase 3/7 as a single treatment in all four cell lines, although **20** combines both features as HDACis and BETis (Figure 13). However, the dual combination of **20** and cisplatin revealed a significant increase in caspase 3/7 activation compared to cisplatin alone in J82 and J82 cisR, exceeding by far the effect of 100 μ M cisplatin (Figure 13). The combination treatment also demonstrated a significantly higher caspase 3/7 activation in T24 LTT, although increasing concentrations of **20** did not further increase this effect. Remarkably, already 0.5 μ M **20** in combination with cisplatin

resulted in a higher caspase 3/7 activation in T24 LTT compared to cisplatin alone, although the IC₅₀ of **20** in T24 LTT cells is much higher (4.66 μM, Table 4). In T24 cells, the combination of **20** and cisplatin did not increase caspase 3/7 activation compared to cisplatin alone up to 7.5 μM **20**, which is slightly above its IC₅₀ value in T24 cells (6.95 μM, Table 4). In summary, our results confirm a synergistic effect of **20** and cisplatin for caspase 3/7 activation in J82, J82 cisR, and T24 LTT (Figure 13) and additionally confirm the higher potency of the combination of HDACi and BETi (including **20**) in cisplatin-resistant cell lines (see also Table 4).

Similar to the analysis of protein expression changes upon entinostat and JQ1 treatment (Figure 9), we analyzed altered protein expression upon treatment with OTX015, entinostat, and their combination, as well as with **20** in Western blot (Figure 14).

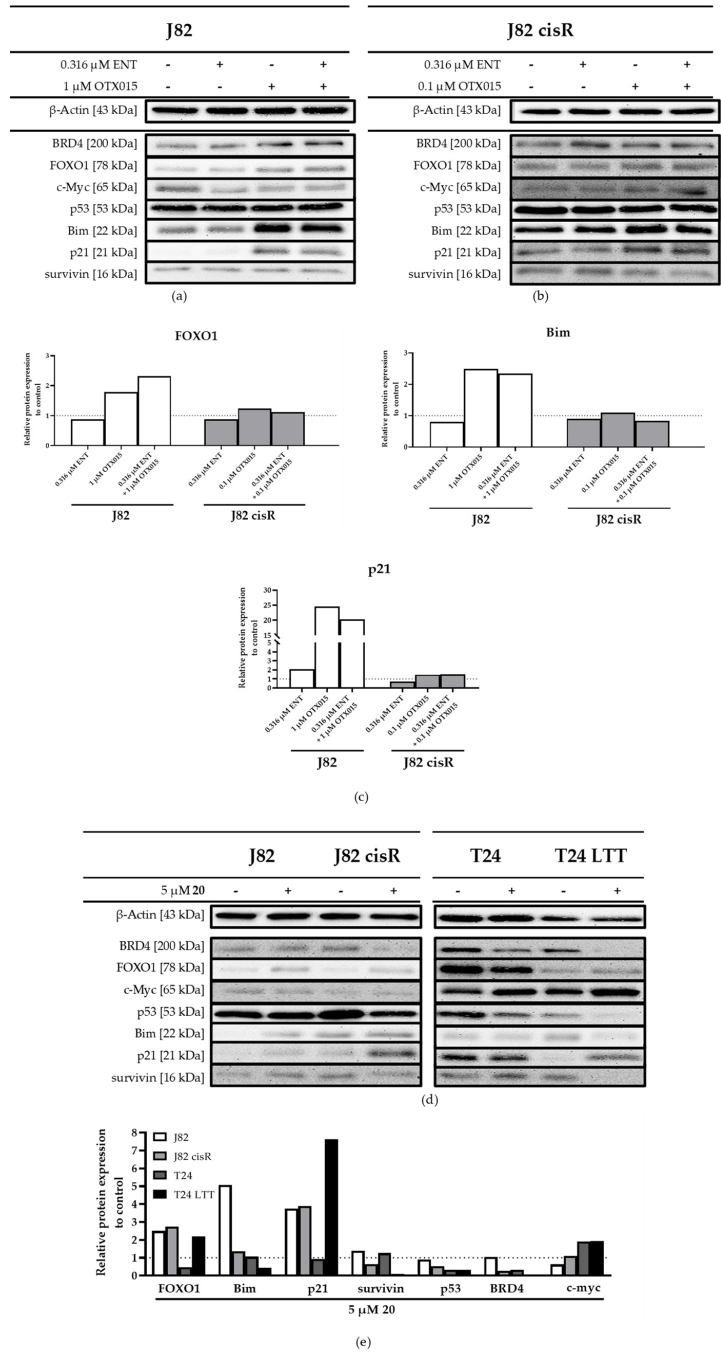


Figure 14. Western blot analysis of (a) J82, (b) J82 cisR and (d) J82, J82 cisR, T24, and T24 LTT. Cells were incubated with entinostat, OTX015, or **20** for 48 h. Data shown are a representative blot out of at

least two different protein samples. Uncropped blots are presented in the Supplementary Materials (Figures S20–S23). Relative protein expression of selected proteins related to β -actin as loading control in (c) J82 and J82 cisR and (e) J82, J82 cisR, T24, and T24 LTT. Ratio of control was set to 1, represented by the dashed line. Western blots were analyzed with ImageJ. The remaining investigated proteins are displayed in Figure S24.

In J82 and J82 cisR cells, expression of FOXO1 and p21 increased upon treatment with OTX015 and the combination of OTX015 plus entinostat but not with entinostat alone (Figure 14). The expression of Bim was clearly increased by OTX015 and the combination treatment in J82, but to a lesser extent in J82 cisR. A decrease in survivin expression was found for OTX015 and OTX015 plus entinostat in J82 cisR, whereas its expression was unchanged in J82. Furthermore, the expression of p53 remained unchanged for all treatments in J82 cisR and J82. Expression of c-Myc slightly increased upon treatment with OTX015 and the combination in agreement to the findings with JQ1 (Figure 9) in J82 cisR. However, in J82 cells, expression of c-Myc decreased upon treatment with OTX015 and the combination, which was different with the BETi JQ1 (Figure 9). BRD4 expression slightly increased upon entinostat treatment but not with OTX015 or the combination in J82 cisR. However, incubation with OTX015 and combination of entinostat and OTX015 led to an increase in BRD4 in J82 cells. In summary, changes in protein expression in J82 and J82 cisR cells were similar for entinostat plus OTX015 as observed for entinostat plus JQ1 (Figures 9 and 14). Major changes in expression were found for FOXO1, p21, and Bim.

Upon treatment with compound **20**, similar expression changes were obtained as with the combination of entinostat and OTX015 (Figure 14). FOXO1 expression increased in J82, J82 cisR, and T24 LTT, but decreased in T24. P21 expression increased upon treatment with **20** in all cell lines except T24. Of interest, increase was most prominent in the cisplatin-resistant cell lines J82 cisR and T24 LTT. Bim expression increased in J82 only and was even decreased in T24 LTT. Survivin expression slightly increased in the cisplatin-sensitive cell lines J82 and T24 but decreased clearly in the cisplatin-resistant cell clones J82 cisR and T24 LTT. P53 was downregulated by **20** in all cell lines. The expression of c-Myc decreased in J82, remained unchanged in J82 cisR, and slightly increased in T24 and T24 LTT cells. Except for in J82, BRD4 was downregulated upon treatment with **20** in all cell lines. In summary, **20** increased the expression of FOXO1 and p21 and decreased expression of survivin, particularly in the cisplatin-resistant clones.

3.5. Selectivity of the Triple Combination Entinostat, BETi, and Cisplatin for Cancer over Non-Cancer Cells

A limiting factor in the clinical administration of anticancer drugs is their toxicity to non-cancer cells. Overall, the triple combination of entinostat, the BETi JQ1 or OTX015, and cisplatin delivered promising anticancer effects in two pairs of cisplatin-sensitive and -resistant bladder cancer cells. Here, we assessed the cytotoxicity of the triple treatments in non-cancer cell lines. The HBLAK cells were chosen as a non-cancerous entity from the bladder and HEK293 cells as a second sample for non-cancer cells. Concentration–effect curves of the compounds were performed in both cell lines. In HBLAK cells, JQ1 was used as the BETi, and in HEK293, JQ1 and OTX015 were used as the BETis (Figure S25, Table 6).

Table 6. IC₅₀ values (MTT, 72 h) of cisplatin, entinostat, JQ1, and OTX015 in non-cancer cell lines HBLAK and HEK293. Data shown are the mean $\pm$ SD of at least three independent experiments, each carried out in triplicates; ND = not determined.

	Cisplatin IC ₅₀ $\pm$ SD [μ M]	Entinostat IC ₅₀ $\pm$ SD [μ M]	JQ1 IC ₅₀ $\pm$ SD [μ M]	OTX015 IC ₅₀ $\pm$ SD [μ M]
HBLAK	1.71 $\pm$ 0.09	1.45 $\pm$ 0.18	2.37 $\pm$ 0.41	ND
HEK293	3.36 $\pm$ 0.09	4.47 $\pm$ 0.77	1.41 $\pm$ 0.26	1.98 $\pm$ 0.42

Both non-cancer cell lines had similar IC_{50} values for cisplatin as the cisplatin-sensitive cancer cell lines J82 or T24 (Table 1). Entinostat had a lower IC_{50} value in HBLAK than in HEK293. The IC_{50} value of entinostat in HBLAK was comparable to the IC_{50} in T24 LTT, and the IC_{50} in HEK293 was similar to the IC_{50} in T24 (Table 1). In both non-cancer cell lines, the IC_{50} values of JQ1 and OTX015 were similar to the IC_{50} values in J82 cisR or T24 cells. Thus, the IC_{50} values were generally in a similar range in cancer and non-cancer cells. Next, the effect of entinostat and JQ1 or OTX015 alone and in combination on the IC_{50} of cisplatin was investigated (Figure 15, Tables 7 and 8). Concentrations used in non-cancer cell lines were as used for the cancer cell lines.

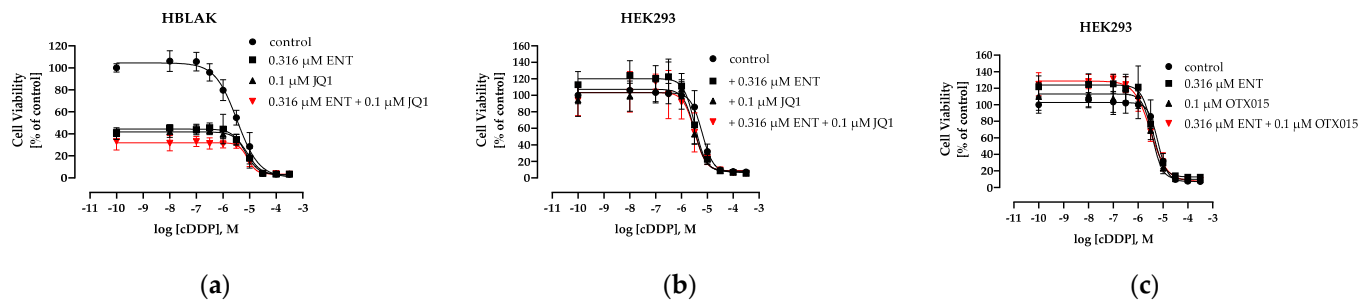


Figure 15. Concentration–effect curves of cisplatin (MTT assay) in (a) HBLAK and (b,c) HEK293. Cells were preincubated with entinostat and/or JQ1 or OTX015 for 48 h followed by addition of cisplatin for another 72 h. Control means concentration–effect curve of cisplatin in absence of epigenetic inhibitors. Data shown are mean $\pm$ SD of at least three independent experiments, each carried out in triplicates.

Table 7. IC_{50} values (MTT) of cisplatin. Cells were preincubated with entinostat and/or JQ1 for 48 h followed by addition of cisplatin for another 72 h. Data shown are mean $\pm$ SD of at least three independent experiments, each carried out in triplicates.

Cell line	Control	+0.316 μ M ENT		+0.1 μ M JQ1		+0.316 μ M ENT +0.1 μ M JQ1	
	Cisplatin $IC_{50} \pm SD$ [μ M]	Cisplatin $IC_{50} \pm SD$ [μ M]	SF	Cisplatin $IC_{50} \pm SD$ [μ M]	SF	Cisplatin $IC_{50} \pm SD$ [μ M]	SF
HBLAK	3.38 $\pm$ 0.18	6.89 $\pm$ 0.81	0.49	8.33 $\pm$ 0.76	0.41	8.45 $\pm$ 0.77	0.40
HEK293	6.26 $\pm$ 0.21	3.29 $\pm$ 0.30	1.90	3.19 $\pm$ 0.32	1.96	3.19 $\pm$ 0.45	1.96

Table 8. IC_{50} values (MTT) of cisplatin. Cells were preincubated with entinostat and/or OTX015 for 48 h followed by addition of cisplatin for another 72 h. Data shown are mean $\pm$ SD of at least three independent experiments, each carried out in triplicates.

Cell Line	Control	+0.316 μ M ENT		+0.1 μ M OTX015		+0.316 μ M ENT +0.1 μ M OTX015	
	Cisplatin $IC_{50} \pm SD$ [μ M]	Cisplatin $IC_{50} \pm SD$ [μ M]	SF	Cisplatin $IC_{50} \pm SD$ [μ M]	SF	Cisplatin $IC_{50} \pm SD$ [μ M]	SF
HEK293	6.26 $\pm$ 0.21	3.84 $\pm$ 0.31	1.63	3.74 $\pm$ 0.31	1.67	3.16 $\pm$ 0.24	1.98

Overall, all combination treatments, including triple combinations, yielded low SFs in both non-cancer cell lines. SFs of around two were calculated for HEK293. Surprisingly, in HBLAK, the IC_{50} values of cisplatin even increased ($SF < 1$) under treatment with the epigenetic inhibitors. However, in HBLAK, treatment with entinostat and/or JQ1 in the absence of cisplatin reduced cell viability by 60–65% (Figure 15a), whereas in HEK293, cell viability was only reduced by up to 20% (Figure 15b,c). All SFs of the triple combination

were <2.1 in non-cancer cells (Tables 7 and 8) and, thus, much lower than the SFs of the cancer cell lines which were up to 15.6 (Table 2).

Finally, the cytotoxic effect of various triple combinations (fixed concentration JQ1, 0.1–0.75 μ M entinostat, 0.2–1.0 μ M cisplatin) were investigated in HEK293 and J82 cisR cells for direct comparison of their toxicities (Figure 16).

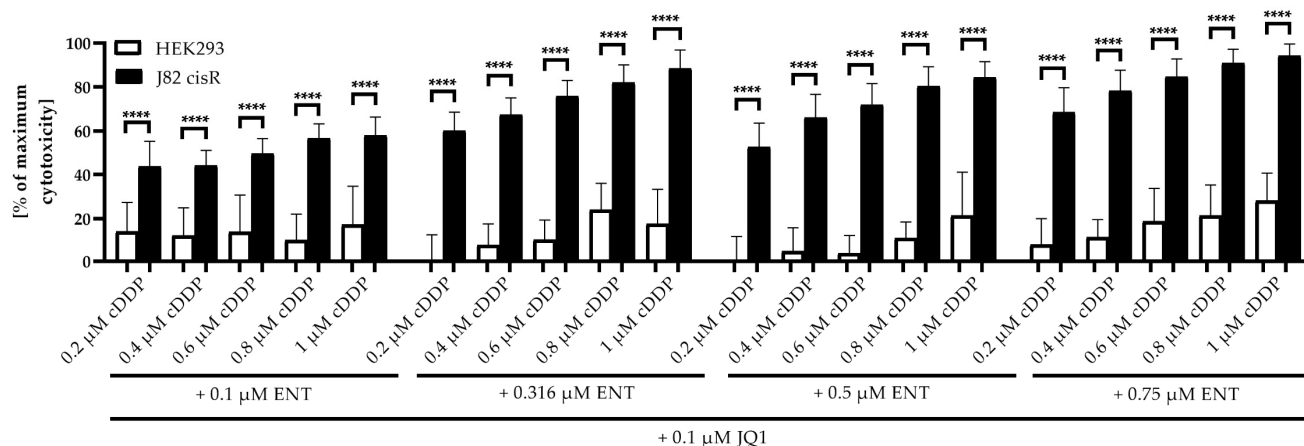


Figure 16. Cytotoxicity (MTT assay) of the triple combination entinostat, JQ1, and cisplatin in HEK293 cells (white bars) and J82 cisR cells (black bars). The cells were preincubated with entinostat and JQ1 for 48 h, followed by the addition of cisplatin for another 72 h. Shown are the mean + SD from at least three independent experiments, each carried out in triplicates. Data were normalized to untreated control, where 100% cell viability was set as 0% cytotoxicity. Significance was calculated using unpaired *t*-test. **** ($p \leq 0.0001$).

Figure 16 shows that all triple combinations had a significantly higher cytotoxicity in the cisplatin-resistant cancer cell line J82 cisR than in the non-cancer cell line HEK293. Since the IC_{50} values of entinostat, JQ1, and cisplatin are approximately the same in cancer and non-cancer cell lines, these results suggest that non-cancer cell lines are much less susceptible to sensitization by epigenetic inhibitors, here HDACis and BETis, against cisplatin than cancer cells (Tables 2, 7 and 8, Figure 16). Even sub-micromolar concentrations of entinostat, JQ1 and cisplatin were sufficient to achieve a high cytotoxic effect in cisplatin-resistant J82 cisR cells due to reversal of cisplatin resistance, whereas cytotoxicity was very low in HEK293 (Figure 16). Thus, our results indicate a strong selectivity of the triple combination for cancer over non-cancer cell line.

4. Discussion

The development of cisplatin resistance is an unresolved problem [71,72]. The use of combination therapies offers the potential to overcome intrinsic or acquired resistance of cisplatin through increased efficacy and selectivity. Especially, non-responders of established cisplatin-based chemotherapy or patients with cisplatin-resistant tumors need new therapeutic approaches, among them bladder cancer patients. The inhibition of epigenetic regulators such as HDACs or BET proteins has often been found to sensitize cancer cells to cisplatin, including bladder cancer cells [14,32,72,73]. In this study, we investigated the triple combination consisting of the class I HDACi entinostat; a BETi, such as JQ1 or OTX015; and cisplatin in two pairs of cisplatin-sensitive and -resistant bladder cancer cells. A previous study has demonstrated a synergistic effect of the class I HDACi romidepsin and JQ1 in bladder carcinoma cell lines [48]. However, this previous study did not investigate the effect of romidepsin and JQ1 on cisplatin. In the present study, we used the class I-selective HDACi entinostat but found only moderate mutual sensitizing effects of entinostat and JQ1 ($SF < 2$, Table S3), resulting in a study design including cisplatin as a further cytotoxic agent with clinical relevance in bladder carcinoma. The triple combination of entinostat, JQ1, and cisplatin in this study resulted in a strongly reduced IC_{50} value

of cisplatin obtaining shift factors of >2 in all four bladder carcinoma cell lines. In accordance with previous results from our groups [74,75], 48 h preincubation of the epigenetic modulators prior to addition of cisplatin for another 72 h was much more effective (SF of cisplatin up to 15.6-fold, Table 2) than co-incubation of all three compounds (SF < 2 , Table S4). The triple combination (applying pre-incubation of the epigenetic modulators) resulted in strong synergistic effects according to Chou-Talalay and Bliss analysis (Table 3, Figures 2 and 3) in all four cell lines. This led to a complete reversal of cisplatin resistance in J82 cisR cells (IC_{50} value reduced from 8.49 to 0.54 μ M, Table 2) and an even lower IC_{50} value of cisplatin than in the native cell line J82 (2.16 μ M), which can be designated as “oversensitization”. Even the highly resistant T24 LTT cell line (IC_{50} value cisplatin 75.0 μ M) was strongly sensitized against cisplatin by a factor of 4.3 applying the epigenetic modulators, although no complete resistance reversal was achieved.

Studies investigating dual combinations consisting of an HDACi plus cisplatin or an HDACi plus a BETi found an enhanced induction of apoptosis through increased caspase 3/7 activation [14,48]. These results are consistent with our findings that the synergistic effect of the triple treatment is based on synergistic increase in caspase 3/7 activation and increased apoptosis (Figures 4 and 5). Among other proteins, JQ1 inhibits BRD4, which is a main regulator for numerous genes of the DNA repair system [76]. Therefore, it appears reasonable that inhibition of BRD4 by JQ1 or OTX015 may contribute to a higher number of double-strand breaks. Indeed, the triple treatment including JQ1 resulted in significantly more double-strand breaks than the dual combination comprising entinostat and cisplatin (Figure 6b), underlining the synergistic effect of the triple combination.

The underlying mechanism for the synergistic effect may at least in part be attributed to increased expression of FOXO1, Bim and p21 in all four cell lines as analyzed by qPCR and Western blot. As the literature confirms, these proteins are increased by class I HDACi entinostat or BETi JQ1 and play a key role for DNA damage-induced cell death. [19,54,70,77]. Further, the literature reports that JQ1-mediated BRD4 inhibition leads to downregulation of mutant p53, resulting in upregulation of p21 in triple-negative breast cancer cells [78]. Both native bladder cell lines used in this study have mutated p53 (J82 missense mutation, T24 non-sense mutation) [79–82]. p53 mutations can cause chemoresistance via different mechanisms, one of which is partially preventing or impeding drug-induced apoptosis. Additionally, mutated p53 can prevent cells from initiating G1 or G2/M arrest, likely due to inhibition of p21 expression [83]. Our Western blot results are consistent with literature reports on expression of mutant p53, as p53 was downregulated in all four cell lines by the dual combination of entinostat and JQ1, whereby p21 was upregulated (Figures 9 and S7). Particularly in J82 cells, p21 expression was strongly enhanced by treatment with JQ1 with or without entinostat. High levels of p21 can then lead to increased cell cycle arrest and apoptosis induction [84]. It was shown in bladder carcinoma cells that overexpression of c-Myc and the resulting increase in MMS19 expression facilitate cell proliferation and the development of cisplatin resistance [85]. Many studies revealed a downregulation of c-Myc by JQ1 [70,86]. Surprisingly, we could observe a downregulation of c-Myc only in T24 and T24 LTT cells, whereas J82 and J82 cisR exhibited the opposite tendency (Figure 9). It can therefore be concluded that the suppression of c-Myc and its downstream signaling in bladder carcinoma cell lines can only partly explain the synergistic effect of entinostat and JQ1 and cisplatin. Further studies are needed to fully understand the mechanisms and network of protein interactions leading to the synergistic effect of the triple combination observed in our study. As common changes in gene expression upon treatment with entinostat and JQ1 in the four investigated bladder cancer cell lines, we have discovered an upregulation of p21, FOXO1, and Bim, all of which will at least in part contribute to the observed synergistic effect with cisplatin.

OTX015, already investigated in clinical trials, demonstrated comparable effects as JQ1. Oral administration and longer half-life are key advantages of OTX015 over JQ1 [26,27,39]. In the triple-combination experiment using entinostat, OTX015, and cisplatin, we observed complete resensitization against cisplatin in the J82 cisR cells as we did with JQ1. The IC_{50}

value of cisplatin was reduced from 7.80 to 0.85 μM , corresponding to an SF of 9.2 (Table 5). Further, the triple treatment of entinostat, OTX015, and cisplatin overall confirmed results obtained with JQ1 concerning caspase 3/7-mediated apoptosis (Figures 11 and 12) and protein expression changes: reduced p53, increased p21, and increased FOXO1 expression in J82 and J82 cisR cells, whereas Bim expression was only increased in J82 (Figure 14a–c). Taken together, JQ1 can be equivalently exchanged by OTX015. Figure 17 summarizes the effects of the triple combination consisting of entinostat, BETi JQ1, or OTX015 or the dual inhibitor **20** and cisplatin on caspase-mediated apoptotic cell death.

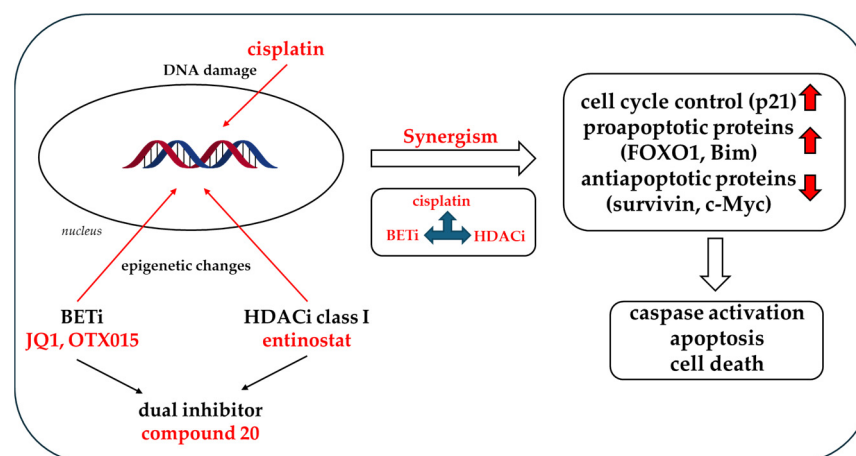


Figure 17. Overview of the effects of the combination of class I HDACi, BETi JQ1, or OTX015 or the dual inhibitor **20** and cisplatin.

In conclusion, the triple combination of entinostat, a BETi, preferably OTX015, and cisplatin yields a highly synergistic caspase-mediated apoptosis and cytotoxicity, completely reversing cisplatin resistance and even leading to oversensitization, meaning to increase cisplatin potency even beyond the potency in the native (non-resistant) cell lines. Thus, this combination is highly promising to be further explored in bladder carcinoma models and eventually in clinical trials, particularly since all components of this combination have already undergone clinical evaluation.

As an additional feature, we have characterized compound **20**, a dual HDACi and BETi previously described [51]. A newer approach in medicinal chemistry follows up on the combination of two or more inhibitory principles in one molecule (multi-target inhibitors) if synergistic effects are likely to simplify the treatment [87,88]. Several dual HDAC and BET inhibitors have already been investigated in various cancer entities, including bladder cancer [51,89–93]. Here, we characterized the dual HDAC and BET inhibitor **20** in combination with cisplatin in the two cisplatin-sensitive and -resistant bladder cancer cell pairs. Compound **20** is a combination of a 4-acyl pyrrole moiety derived from XD14 (BRD4(1) inhibitor) and the zinc-binding group related to panobinostat (HDACi) [51,94]. The combination of **20** and cisplatin led to a strong decrease in the IC_{50} value of cisplatin from 7.80 μM to 1.47 μM (SF 5.31) and thus to a complete cisplatin resensitization of J82 cisR cells (Figure 10). Consequently, the combination of **20** and cisplatin was as effective as the triple combination in J82 cisR cells. In addition, a significant increase in caspase 3/7 activation was observed upon combination of **20** and cisplatin in J82, J82 cisR, and T24 LTT but not T24 cells (Figure 13). This was accompanied by increased expression of FOXO1 and p21 in all cancer cell lines except T24, which may explain the lack of increase in caspase 3/7 activation in T24 by the combination of **20** and cisplatin. Notably, survivin expression was decreased in both cisplatin-resistant cell lines (J82 cisR, T24 LTT, Figure 14d,e). Taken together, the dual inhibitor **20** demonstrated similar changes in protein expression as the combination of entinostat and JQ1 or OTX015. In conclusion, the combination of the dual HDAC and BET inhibitor, **20**, plus cisplatin yielded comparable efficacy as the triple combination of entinostat, JQ1 or OTX015, and cisplatin, thus confirming the efficacy

of the dual targeting approach and simplifying treatment regimes. Further studies are needed to investigate the pharmacokinetics, toxicity, and long-term efficacy of compound 20, including other cancer models.

Ideally, the highly synergistic effect of the triple combination is selective for the cancer cells and does not, or does to a much lesser extent, affect non-cancer cells. The single compounds have similar toxicity in the bladder cancer cell lines and two non-cancer cell lines, the bladder-tissue-derived cell line HBLAK and the kidney cell line HEK293 (Tables 1 and 6). However, in combination with cisplatin, the toxicity is much lower in the non-cancer than in the bladder cancer cells, thus suggesting selectivity for the bladder cancer cells. The reduction in the IC₅₀ of cisplatin upon pretreatment with entinostat and JQ1 or OTX015 was small in HEK293 cells, and the SFs were even <1 in HBLAK cells (Figure 15). A selectivity for cancer over non-cancer cells is crucial for combination treatments aiming to overcome cisplatin resistance in patients. The selectivity of the triple combination for cancer over non-cancer cells is impressively shown for J82 cisR and HEK293 cells in Figure 16 using different concentrations of JQ1, entinostat, and cisplatin relevant for in vivo studies [27,60,95].

5. Conclusions

Our study showed that the triple combination of the HDACi entinostat, a BETi, preferably OTX015, and cisplatin or a dual-acting HDAC/BET inhibitor plus cisplatin is highly synergistic in bladder carcinoma cell lines and completely reverses cisplatin resistance. The synergistic cytotoxicity is due to an enhanced caspase-mediated induction of apoptosis caused by increased expression of p21, FOXO1, and Bim. The triple combination is selective for bladder cancer compared to the non-cancer cell lines HBLAK and HEK293. Thus, this study encourages further preclinical and eventually clinical trials with HDAC and BET inhibitors in combination with cisplatin to overcome cisplatin resistance and foster treatment efficacy in bladder cancer.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers16193374/s1>, Figure S1. Concentration–effect curves determined with a 72 h MTT assay in J82 (a) cisplatin (cDDP), (b) entinostat (ENT), (c) JQ1; J82 cisR (d) cisplatin, (e) entinostat, (f) JQ1; T24 (g) cisplatin, (h) entinostat, (i) JQ1; T24 LTT (j) cisplatin, (k) entinostat, (l) JQ1. Figure S2. Concentration–effect curves of entinostat or JQ1 (MTT assay). Figure S3. Concentration–effect curves of cisplatin (MTT assay). Figure S4. Synergistic cytotoxic effect of combination treatment in J82 (a)–(d), J82 cisR (e)–(h), T24 (i)–(l) and T24 LTT (m)–(p). Figure S5. Representative cytometry flow images are shown for control, entinostat, JQ1, cisplatin and their combination treatments in J82, J82 cisR, T24 and T24 LTT. Figure S6. Representative fluorescent imaging pictures (Thermofisher Arrayscan XTI) are shown for vehicle control, entinostat, JQ1, cisplatin and their combination treatments in J82, J82 cisR, T24 and T24 LTT. Figure S7. The relative protein expression of proteins of interest related to β -actin as loading control is shown in (a) J82, (b) J82 cisR, (c) T24 and (d) T24 LTT; Figure S8. Uncropped western blot, J82: blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c) and the blot below illustrates p53 (d) and survivin (e), the blot on the right-side displays Bim (f), 48 h incubation. Figure S9. Uncropped western blot, J82: blot at right top shows BRD4 (a), c-Myc (b) and β -Actin (c), 48 h incubation. Figure S10. Uncropped western blot, J82 cisR: blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and survivin (e), 48 h incubation. Figure S11. Uncropped western blot, J82 cisR: blot at right top shows at the bottom Bim (a), 48 h incubation. Figure S12. Uncropped western blot, J82 cisR: blot at top shows BRD (a), c-Myc (b) and β -Actin (c), 48 h incubation. Figure S13. Uncropped western blot, T24: blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and survivin (e), the blot on the right-side displays BRD4 (f), c-Myc (g) and Bim (h), 48 h incubation. Figure S14. Uncropped western blot, T24 LTT: blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and survivin (e), the blot on the right-side displays BRD4 (f), c-Myc (g) and Bim (h), 48 h incubation. Figure S15. HDAC enzyme inhibitory activity of 20, vorinostat and Panobinostat; Figure S16. Concentration–effect curves (MTT assay, 72 h) in J82 (a) OTX015, (b) JQ35, (c) 20; in J82 cisR (d) OTX015, (e) JQ35, (f) 20; in T24 (g) 20 and in T24 LTT (h) 20. Figure S17. Concentration–effect curves of cisplatin (MTT assay) of (a) J82; (b) T24; (c) T24 LTT. Figure S18. Representative cytometry flow images are shown for control, entinostat, OTX015, cisplatin and their combination treatments in

J82 and J82 cisR. Figure S19. (a) Representative fluorescent imaging pictures (Thermofisher Arrayscan XTI) are shown for vehicle control, entinostat, OTX015, cisplatin and their combination treatments in J82, J82 cisR. (b) Representative fluorescent imaging pictures (Thermofisher Arrayscan XTI) are shown for vehicle control, 20, cisplatin and their combination treatments in J82, J82 cisR, T24 and T24 LTT. Figure S20. Uncropped western blot, J82, blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and survivin (e), the blot on the right-side displays BRD4 (f), c-Myc (g) and Bim (h), 48 h incubation. Figure S21. Uncropped western blot, J82 cisR, blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and Bim (e), 48 h incubation. Figure S22. Uncropped western blot, J82 cisR, the blot on the right-side displays BRD4 (a), c-Myc (b), β -Actin (c) and survivin (d), 48 h incubation. Figure S23. Uncropped western blot, J82, J82 cisR, T24 and T24 LTT, blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and survivin (e), the blot on the right side displays BRD4 (f), c-Myc (g) and Bim (h), 48 h incubation. Figure S24. The relative protein expression of proteins of interest related to β -actin as loading control is shown in (a) J82, (b) J82 cisR. Figure S25. Concentration–effect curves determined by 72 h MTT assay in (a)–(c) HBLAK and (d)–(g) HEK293. Table S1. Overview of primary antibodies used in Western blot; Table S2. Primer sequences for RT-PCR. Table S3. IC50 values (MTT) of entinostat or JQ1. Table S4. IC50 values (MTT) of cisplatin. Table S5. CI values of the dual combination studies (48 h preincubation with entinostat or JQ1, followed by addition of cisplatin for another 72 h. Table S6. HDAC enzyme inhibitory activity of 20, vorinostat and Panobinostat. Table S7. IC50 values (MTT) of cisplatin; Table S8. IC50 values (MTT) of cisplatin.

Author Contributions: Conceptualization, M.U.K.; methodology, M.U.K., A.H. and L.M.B.; validation, L.M.B. and M.U.K.; formal analysis, L.M.B. and M.U.K.; investigation, L.M.B., F.L., L.B. and L.S.-H.; resources, M.U.K. and F.K.H.; data curation, M.U.K. and L.M.B.; writing—original draft preparation, L.M.B.; writing—review and editing, M.U.K. and A.H.; visualization, L.M.B.; supervision, M.U.K. and A.H.; project administration, M.U.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in this article and the corresponding supplemental information.

Acknowledgments: We gratefully acknowledge receipt of the primary epithelial cell line HBLAK [52], T24 and T24 LLT cell lines from Michèle Hoffmann, Department of Urology, Heinrich-Heine-university, Duesseldorf, Germany.

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424. [CrossRef]
- Dobrush, J.; Oszczudłowski, M. Bladder Cancer: Current Challenges and Future Directions. *Medicina* **2021**, *57*, 749. [CrossRef] [PubMed]
- ECIS. Available online: [https://ecis.jrc.ec.europa.eu/explorer.php?\\$0-4\\$1-All\\$4-1,2\\$3-38\\$6-0,85\\$5-2020,2025\\$7-7\\$21-0\\$2-All\\$CLongtermChart1_1\\$X0_-1-AE27](https://ecis.jrc.ec.europa.eu/explorer.php?$0-4$1-All$4-1,2$3-38$6-0,85$5-2020,2025$7-7$21-0$2-All$CLongtermChart1_1$X0_-1-AE27) (accessed on 31 January 2023).
- Jones, R.J.; Crabb, S.J.; Linch, M.; Birtle, A.J.; McGrane, J.; Enting, D.; Stevenson, R.; Liu, K.; Kularatne, B.; Hussain, S.A. Systemic anticancer therapy for urothelial carcinoma: UK oncologists' perspective. *Br. J. Cancer* **2024**, *130*, 897–907. [CrossRef] [PubMed]
- Aldossary, S.A. Review on Pharmacology of Cisplatin: Clinical Use, Toxicity and Mechanism of Resistance of Cisplatin. *Biomed. Pharmacol. J.* **2019**, *12*, 7–15. [CrossRef]
- Siegmund, M.J.; Marx, C.; Seemann, O.; Schummer, B.; Steidler, A.; Toktomambetova, L.; Köhrmann, K.U.; Rassweiler, J.; Alken, P. Cisplatin-resistant bladder carcinoma cells: Enhanced expression of metallothioneins. *Urol. Res.* **1999**, *27*, 157–163. [CrossRef] [PubMed]
- Bellmunt, J.; von der Maase, H.; Mead, G.M.; Skoneczna, I.; de Santis, M.; Daugaard, G.; Boehle, A.; Chevreau, C.; Paz-Ares, L.; Laufman, L.R.; et al. Randomized phase III study comparing paclitaxel/cisplatin/gemcitabine and gemcitabine/cisplatin in patients with locally advanced or metastatic urothelial cancer without prior systemic therapy: EORTC Intergroup Study 30987. *J. Clin. Oncol.* **2012**, *30*, 1107–1113. [CrossRef]

8. Zhang, S.; Lin, T.; Xiong, X.; Chen, C.; Tan, P.; Wei, Q. Targeting histone modifiers in bladder cancer therapy—Preclinical and clinical evidence. *Nat. Rev. Urol.* **2024**, *21*, 495–511. [[CrossRef](#)] [[PubMed](#)]
9. Natsu, A.; Verma, T.; Khade, B.; Thorat, R.; Gera, P.; Dhara, S.; Gupta, S. Histone acetylation: A key determinant of acquired cisplatin resistance in cancer. *Clin. Epigenetics* **2024**, *16*, 8. [[CrossRef](#)]
10. Morel, D.; Jeffery, D.; Aspeslagh, S.; Almouzni, G.; Postel-Vinay, S. Combining epigenetic drugs with other therapies for solid tumours—Past lessons and future promise. *Nat. Rev. Clin. Oncol.* **2020**, *17*, 91–107. [[CrossRef](#)]
11. Seto, E.; Yoshida, M. Erasers of histone acetylation: The histone deacetylase enzymes. *Cold Spring Harb. Perspect. Biol.* **2014**, *6*, a018713. [[CrossRef](#)]
12. Ponnusamy, L.; Mahalingaiah, P.K.S.; Singh, K.P. Epigenetic reprogramming and potential application of epigenetic-modifying drugs in acquired chemotherapeutic resistance. *Adv. Clin. Chem.* **2020**, *94*, 219–259. [[CrossRef](#)] [[PubMed](#)]
13. Ramaiah, M.J.; Tangutur, A.D.; Manyam, R.R. Epigenetic modulation and understanding of HDAC inhibitors in cancer therapy. *Life Sci.* **2021**, *277*, 119504. [[CrossRef](#)] [[PubMed](#)]
14. Bandolik, J.J.; Hamacher, A.; Schrenk, C.; Weishaupt, R.; Kassack, M.U. Class I-Histone Deacetylase (HDAC) Inhibition is Superior to pan-HDAC Inhibition in Modulating Cisplatin Potency in High Grade Serous Ovarian Cancer Cell Lines. *Int. J. Mol. Sci.* **2019**, *20*, 3052. [[CrossRef](#)]
15. Gueugnon, F.; Cartron, P.-F.; Charrier, C.; Bertrand, P.; Fonteneau, J.-F.; Gregoire, M.; Blanquart, C. New histone deacetylase inhibitors improve cisplatin antitumor properties against thoracic cancer cells. *Oncotarget* **2014**, *5*, 4504–4515. [[CrossRef](#)]
16. Shen, J.; Huang, C.; Jiang, L.; Gao, F.; Wang, Z.; Zhang, Y.; Bai, J.; Zhou, H.; Chen, Q. Enhancement of cisplatin induced apoptosis by suberoylanilide hydroxamic acid in human oral squamous cell carcinoma cell lines. *Biochem. Pharmacol.* **2007**, *73*, 1901–1909. [[CrossRef](#)]
17. Suraweera, A.; O’Byrne, K.J.; Richard, D.J. Combination Therapy with Histone Deacetylase Inhibitors (HDACi) for the Treatment of Cancer: Achieving the Full Therapeutic Potential of HDACi. *Front. Oncol.* **2018**, *8*, 92. [[CrossRef](#)] [[PubMed](#)]
18. Wilson, A.J.; Stubbs, M.; Liu, P.; Ruggeri, B.; Khabele, D. The BET inhibitor INCB054329 reduces homologous recombination efficiency and augments PARP inhibitor activity in ovarian cancer. *Gynecol. Oncol.* **2018**, *149*, 575–584. [[CrossRef](#)]
19. Wang, C.; Hamacher, A.; Petzsch, P.; Köhrer, K.; Niegisch, G.; Hoffmann, M.J.; Schulz, W.A.; Kassack, M.U. Combination of Decitabine and Entinostat Synergistically Inhibits Urothelial Bladder Cancer Cells via Activation of FoxO1. *Cancers* **2020**, *12*, 337. [[CrossRef](#)]
20. Pérez-Salvia, M.; Esteller, M. Bromodomain inhibitors and cancer therapy: From structures to applications. *Epigenetics* **2017**, *12*, 323–339. [[CrossRef](#)]
21. Filippakopoulos, P.; Picaud, S.; Mangos, M.; Keates, T.; Lambert, J.-P.; Barsyte-Lovejoy, D.; Felletar, I.; Volkmer, R.; Müller, S.; Pawson, T.; et al. Histone recognition and large-scale structural analysis of the human bromodomain family. *Cell* **2012**, *149*, 214–231. [[CrossRef](#)]
22. Mochizuki, K.; Nishiyama, A.; Jang, M.K.; Dey, A.; Ghosh, A.; Tamura, T.; Natsume, H.; Yao, H.; Ozato, K. The bromodomain protein Brd4 stimulates G1 gene transcription and promotes progression to S phase. *J. Biol. Chem.* **2008**, *283*, 9040–9048. [[CrossRef](#)] [[PubMed](#)]
23. Sinha, A.; Faller, D.V.; Denis, G.V. Bromodomain analysis of Brd2-dependent transcriptional activation of cyclin A. *Biochem. J.* **2005**, *387*, 257–269. [[CrossRef](#)] [[PubMed](#)]
24. Yang, Z.; He, N.; Zhou, Q. Brd4 recruits P-TEFb to chromosomes at late mitosis to promote G1 gene expression and cell cycle progression. *Mol. Cell. Biol.* **2008**, *28*, 967–976. [[CrossRef](#)] [[PubMed](#)]
25. Bagratuni, T.; Mavrianou, N.; Gavalas, N.G.; Tzannis, K.; Arapinis, C.; Lontos, M.; Christodoulou, M.I.; Thomakos, N.; Haidopoulos, D.; Rodolakis, A.; et al. JQ1 inhibits tumour growth in combination with cisplatin and suppresses JAK/STAT signalling pathway in ovarian cancer. *Eur. J. Cancer* **2020**, *126*, 125–135. [[CrossRef](#)]
26. Guo, J.; Zheng, Q.; Peng, Y. BET proteins: Biological functions and therapeutic interventions. *Pharmacol. Ther.* **2023**, *243*, 108354. [[CrossRef](#)]
27. Shorstova, T.; Foulkes, W.D.; Witcher, M. Achieving clinical success with BET inhibitors as anti-cancer agents. *Br. J. Cancer* **2021**, *124*, 1478–1490. [[CrossRef](#)]
28. Manzotti, G.; Ciarrocchi, A.; Sancisi, V. Inhibition of BET Proteins and Histone Deacetylase (HDACs): Crossing Roads in Cancer Therapy. *Cancers* **2019**, *11*, 304. [[CrossRef](#)]
29. Andrikopoulou, A.; Lontos, M.; Koutsoukos, K.; Dimopoulos, M.-A.; Zagouri, F. Clinical perspectives of BET inhibition in ovarian cancer. *Cell. Oncol.* **2021**, *44*, 237–249. [[CrossRef](#)]
30. Zhu, X.; Holmsen, E.; Park, S.; Willingham, M.C.; Qi, J.; Cheng, S.-Y. Synergistic effects of BET and MEK inhibitors promote regression of anaplastic thyroid tumors. *Oncotarget* **2018**, *9*, 35408–35421. [[CrossRef](#)]
31. Zhang, M.-Y.; Liu, S.-L.; Huang, W.-L.; Tang, D.-B.; Zheng, W.-W.; Zhou, N.; Zhou, H.; Abudurehman, T.; Tang, Z.-H.; Zhou, B.-B.S.; et al. Bromodomains and Extra-Terminal (BET) Inhibitor JQ1 Suppresses Proliferation of Acute Lymphocytic Leukemia by Inhibiting c-Myc-Mediated Glycolysis. *Med. Sci. Monit.* **2020**, *26*, e923411. [[CrossRef](#)]
32. Zanellato, I.; Colangelo, D.; Osella, D. JQ1, a BET Inhibitor, Synergizes with Cisplatin and Induces Apoptosis in Highly Chemoresistant Malignant Pleural Mesothelioma Cells. *Curr. Cancer Drug Targets* **2018**, *18*, 816–828. [[CrossRef](#)] [[PubMed](#)]

33. Li, F.; MacKenzie, K.R.; Jain, P.; Santini, C.; Young, D.W.; Matzuk, M.M. Metabolism of JQ1, an inhibitor of bromodomain and extra terminal bromodomain proteins, in human and mouse liver microsomes. *Biol. Reprod.* **2020**, *103*, 427–436. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Riveiro, M.E.; Astorgues-Xerri, L.; Vazquez, R.; Frapolli, R.; Kwee, I.; Rinaldi, A.; Odore, E.; Rezai, K.; Bekradda, M.; Inghirami, G.; et al. OTX015 (MK-8628), a novel BET inhibitor, exhibits antitumor activity in non-small cell and small cell lung cancer models harboring different oncogenic mutations. *Oncotarget* **2016**, *7*, 84675–84687. [\[CrossRef\]](#)
35. Berenguer-Daizé, C.; Astorgues-Xerri, L.; Odore, E.; Cayol, M.; Cvitkovic, E.; Noel, K.; Bekradda, M.; MacKenzie, S.; Rezai, K.; Lokiec, F.; et al. OTX015 (MK-8628), a novel BET inhibitor, displays in vitro and in vivo antitumor effects alone and in combination with conventional therapies in glioblastoma models. *Int. J. Cancer* **2016**, *139*, 2047–2055. [\[CrossRef\]](#)
36. Lewin, J.; Soria, J.-C.; Stathis, A.; Delord, J.-P.; Peters, S.; Awada, A.; Aftimos, P.G.; Bekradda, M.; Rezai, K.; Zeng, Z.; et al. Phase Ib Trial with Birabresib, a Small-Molecule Inhibitor of Bromodomain and Extraterminal Proteins, in Patients with Selected Advanced Solid Tumors. *J. Clin. Oncol.* **2018**, *36*, 3007–3014. [\[CrossRef\]](#)
37. Berthon, C.; Raffoux, E.; Thomas, X.; Vey, N.; Gomez-Roca, C.; Yee, K.; Taussig, D.C.; Rezai, K.; Roumier, C.; Herait, P.; et al. Bromodomain inhibitor OTX015 in patients with acute leukaemia: A dose-escalation, phase 1 study. *Lancet Haematol.* **2016**, *3*, e186–e195. [\[CrossRef\]](#)
38. Amorim, S.; Stathis, A.; Gleeson, M.; Iyengar, S.; Magarotto, V.; Leleu, X.; Morschhauser, F.; Karlin, L.; Broussais, F.; Rezai, K.; et al. Bromodomain inhibitor OTX015 in patients with lymphoma or multiple myeloma: A dose-escalation, open-label, pharmacokinetic, phase 1 study. *Lancet Haematol.* **2016**, *3*, e196–e204. [\[CrossRef\]](#)
39. Odore, E.; Lokiec, F.; Cvitkovic, E.; Bekradda, M.; Herait, P.; Bourdel, F.; Kahatt, C.; Raffoux, E.; Stathis, A.; Thieblemont, C.; et al. Phase I Population Pharmacokinetic Assessment of the Oral Bromodomain Inhibitor OTX015 in Patients with Haematologic Malignancies. *Clin. Pharmacokinet.* **2016**, *55*, 397–405. [\[CrossRef\]](#) [\[PubMed\]](#)
40. Merck Sharp & Dohme LLC. A Dose Exploration Study with Birabresib (MK-8628) in Participants with Selected Advanced Solid Tumors (MK-8628-006). Available online: <https://www.clinicaltrials.gov/study/NCT02698176?intr=OTX015&rank=4#publications> (accessed on 17 June 2024).
41. Roboz, G.J.; Desai, P.; Lee, S.; Ritchie, E.K.; Winer, E.S.; DeMario, M.; Brennan, B.; Nüesch, E.; Chesne, E.; Brennan, L.; et al. A dose escalation study of RO6870810/TEN-10 in patients with acute myeloid leukemia and myelodysplastic syndrome. *Leuk. Lymphoma* **2021**, *62*, 1740–1748. [\[CrossRef\]](#)
42. Shapiro, G.I.; LoRusso, P.; Dowlati, A.; T Do, K.; Jacobson, C.A.; Vaishampayan, U.; Weise, A.; Caimi, P.F.; Eder, J.P.; French, C.A.; et al. A Phase 1 study of RO6870810, a novel bromodomain and extra-terminal protein inhibitor, in patients with NUT carcinoma, other solid tumours, or diffuse large B-cell lymphoma. *Br. J. Cancer* **2021**, *124*, 744–753. [\[CrossRef\]](#)
43. Fiskus, W.; Sharma, S.; Qi, J.; Valenta, J.A.; Schaub, L.J.; Shah, B.; Peth, K.; Portier, B.P.; Rodriguez, M.; Devaraj, S.G.T.; et al. Highly active combination of BRD4 antagonist and histone deacetylase inhibitor against human acute myelogenous leukemia cells. *Mol. Cancer Ther.* **2014**, *13*, 1142–1154. [\[CrossRef\]](#)
44. Mazur, P.K.; Herner, A.; Mello, S.S.; Wirth, M.; Hausmann, S.; Sánchez-Rivera, F.J.; Lofgren, S.M.; Kuschma, T.; Hahn, S.A.; Vangala, D.; et al. Combined inhibition of BET family proteins and histone deacetylases as a potential epigenetics-based therapy for pancreatic ductal adenocarcinoma. *Nat. Med.* **2015**, *21*, 1163–1171. [\[CrossRef\]](#)
45. Meng, W.; Wang, B.; Mao, W.; Wang, J.; Zhao, Y.; Li, Q.; Zhang, C.; Tang, Y.; Ma, J. Enhanced efficacy of histone deacetylase inhibitor combined with bromodomain inhibitor in glioblastoma. *J. Exp. Clin. Cancer Res.* **2018**, *37*, 241. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Lai, Y.-S.; Chen, J.-Y.; Tsai, H.-J.; Chen, T.-Y.; Hung, W.-C. The SUV39H1 inhibitor chaetocin induces differentiation and shows synergistic cytotoxicity with other epigenetic drugs in acute myeloid leukemia cells. *Blood Cancer J.* **2015**, *5*, e313. [\[CrossRef\]](#)
47. Ramadoss, M.; Mahadevan, V. Targeting the cancer epigenome: Synergistic therapy with bromodomain inhibitors. *Drug Discov. Today* **2018**, *23*, 76–89. [\[CrossRef\]](#)
48. Hölscher, A.S.; Schulz, W.A.; Pinkerneil, M.; Niegisch, G.; Hoffmann, M.J. Combined inhibition of BET proteins and class I HDACs synergistically induces apoptosis in urothelial carcinoma cell lines. *Clin. Epigenetics* **2018**, *10*, 1. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Vasyutin, I.; Zerihun, L.; Ivan, C.; Atala, A. Bladder Organoids and Spheroids: Potential Tools for Normal and Diseased Tissue Modelling. *Anticancer Res.* **2019**, *39*, 1105–1118. [\[CrossRef\]](#)
50. Skowron, M.A.; Petzsch, P.; Hardt, K.; Wagner, N.; Beier, M.; Stepanow, S.; Drechsler, M.; Rieder, H.; Köhrer, K.; Niegisch, G.; et al. Distinctive mutational spectrum and karyotype disruption in long-term cisplatin-treated urothelial carcinoma cell lines. *Sci. Rep.* **2019**, *9*, 14476. [\[CrossRef\]](#) [\[PubMed\]](#)
51. Schäker-Hübner, L.; Warstat, R.; Ahlert, H.; Mishra, P.; Kraft, F.B.; Schliehe-Diecks, J.; Schöler, A.; Borkhardt, A.; Breit, B.; Bhatia, S.; et al. 4-Acyl Pyrrole Capped HDAC Inhibitors: A New Scaffold for Hybrid Inhibitors of BET Proteins and Histone Deacetylases as Antileukemia Drug Leads. *J. Med. Chem.* **2021**, *64*, 14620–14646. [\[CrossRef\]](#)
52. Hoffmann, M.J.; Koutsogiannouli, E.; Skowron, M.A.; Pinkerneil, M.; Niegisch, G.; Brandt, A.; Stepanow, S.; Rieder, H.; Schulz, W.A. The New Immortalized Uroepithelial Cell Line HBLAK Contains Defined Genetic Aberrations Typical of Early Stage Urothelial Tumors. *Bladder Cancer* **2016**, *2*, 449–463. [\[CrossRef\]](#)
53. Engelke, L.H.; Hamacher, A.; Proksch, P.; Kassack, M.U. Ellagic Acid and Resveratrol Prevent the Development of Cisplatin Resistance in the Epithelial Ovarian Cancer Cell Line A2780. *J. Cancer* **2016**, *7*, 353–363. [\[CrossRef\]](#)

54. Vandesompele, J.; de Preter, K.; Pattyn, F.; Poppe, B.; van Roy, N.; de Paepe, A.; Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **2002**, *3*, RESEARCH0034. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Skowron, M.A.; Niegisch, G.; Albrecht, P.; van Koeveringe, G.; Romano, A.; Albers, P.; Schulz, W.A.; Hoffmann, M.J. Various Mechanisms Involve the Nuclear Factor (Erythroid-Derived 2)-Like (NRF2) to Achieve Cytoprotection in Long-Term Cisplatin-Treated Urothelial Carcinoma Cell Lines. *Int. J. Mol. Sci.* **2017**, *18*, 1680. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Bijnsdorp, I.V.; Giovannetti, E.; Peters, G.J. Analysis of drug interactions. *Methods Mol. Biol.* **2011**, *731*, 421–434. [\[CrossRef\]](#) [\[PubMed\]](#)
57. Chou, T.C.; Talalay, P. Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **1984**, *22*, 27–55. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Zhao, W.; Sachsenmeier, K.; Zhang, L.; Sult, E.; Hollingsworth, R.E.; Yang, H. A New Bliss Independence Model to Analyze Drug Combination Data. *J. Biomol. Screen.* **2014**, *19*, 817–821. [\[CrossRef\]](#)
59. Di Veroli, G.Y.; Fornari, C.; Wang, D.; Mollard, S.; Bramhall, J.L.; Richards, F.M.; Jodrell, D.I. CombeneFit: An interactive platform for the analysis and visualization of drug combinations. *Bioinformatics* **2016**, *32*, 2866–2868. [\[CrossRef\]](#)
60. Kurmasheva, R.T.; Bandyopadhyay, A.; Favours, E.; Del Pozo, V.; Ghilu, S.; Phelps, D.A.; Erickson, S.W.; Peer, C.J.; Figg, W.D.; Smith, M.A.; et al. Evaluation of entinostat alone and in combination with standard-of-care cytotoxic agents against rhabdomyosarcoma xenograft models. *Pediatr. Blood Cancer* **2019**, *66*, e27820. [\[CrossRef\]](#)
61. Pflieger, M.; Hamacher, A.; Öz, T.; Horstick-Muche, N.; Boesen, B.; Schrenk, C.; Kassack, M.U.; Kurz, T. Novel α,β -unsaturated hydroxamic acid derivatives overcome cisplatin resistance. *Bioorg. Med. Chem.* **2019**, *27*, 115036. [\[CrossRef\]](#)
62. Rogakou, E.P.; Pilch, D.R.; Orr, A.H.; Ivanova, V.S.; Bonner, W.M. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* **1998**, *273*, 5858–5868. [\[CrossRef\]](#)
63. Kuo, L.J.; Yang, L.-X. Gamma-H2AX—A novel biomarker for DNA double-strand breaks. *In Vivo* **2008**, *22*, 305–309. [\[PubMed\]](#)
64. Rothkamm, K.; Barnard, S.; Moquet, J.; Ellender, M.; Rana, Z.; Burdak-Rothkamm, S. DNA damage foci: Meaning and significance. *Environ. Mol. Mutagen.* **2015**, *56*, 491–504. [\[CrossRef\]](#)
65. Turinetti, V.; Giachino, C. Multiple facets of histone variant H2AX: A DNA double-strand-break marker with several biological functions. *Nucleic Acids Res.* **2015**, *43*, 2489–2498. [\[CrossRef\]](#) [\[PubMed\]](#)
66. Panier, S.; Boulton, S.J. Double-strand break repair: 53BP1 comes into focus. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 7–18. [\[CrossRef\]](#) [\[PubMed\]](#)
67. Chronis, F.; Rogakou, E.P. Interplay between γ H2AX and 53BP1 Pathways in DNA Double-Strand Break Repair Response. In *Apoptosis, Senescence, and Cancer*; Gewirtz, D.A., Holt, S.E., Grant, S., Eds.; Humana Press: Totowa, NJ, USA, 2007. [\[CrossRef\]](#)
68. Schultz, L.B.; Chehab, N.H.; Malikzay, A.; Halazonetis, T.D. p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. *J. Cell Biol.* **2000**, *151*, 1381–1390. [\[CrossRef\]](#)
69. Ward, I.M.; Minn, K.; Jorda, K.G.; Chen, J. Accumulation of checkpoint protein 53BP1 at DNA breaks involves its binding to phosphorylated histone H2AX. *J. Biol. Chem.* **2003**, *278*, 19579–19582. [\[CrossRef\]](#)
70. Pang, Y.; Bai, G.; Zhao, J.; Wei, X.; Li, R.; Li, J.; Hu, S.; Peng, L.; Liu, P.; Mao, H. The BRD4 inhibitor JQ1 suppresses tumor growth by reducing c-Myc expression in endometrial cancer. *J. Transl. Med.* **2022**, *20*, 336. [\[CrossRef\]](#)
71. Ghosh, S. Cisplatin: The first metal based anticancer drug. *Bioorg. Chem.* **2019**, *88*, 102925. [\[CrossRef\]](#)
72. Li, F.; Zheng, Z.; Chen, W.; Li, D.; Zhang, H.; Zhu, Y.; Mo, Q.; Zhao, X.; Fan, Q.; Deng, F.; et al. Regulation of cisplatin resistance in bladder cancer by epigenetic mechanisms. *Drug Resist. Updat.* **2023**, *68*, 100938. [\[CrossRef\]](#)
73. Thy, S.; Hommel, A.; Meneceur, S.; Bartkowiak, A.L.; Schulz, W.A.; Niegisch, G.; Hoffmann, M.J. Epigenetic Treatment of Urothelial Carcinoma Cells Sensitizes to Cisplatin Chemotherapy and PARP Inhibitor Treatment. *Cancers* **2021**, *13*, 1376. [\[CrossRef\]](#)
74. Alves Avelar, L.A.; Schrenk, C.; Sönnichsen, M.; Hamacher, A.; Hansen, F.K.; Schliehe-Diecks, J.; Borkhardt, A.; Bhatia, S.; Kassack, M.U.; Kurz, T. Synergistic induction of apoptosis in resistant head and neck carcinoma and leukemia by alkoxyamide-based histone deacetylase inhibitors. *Eur. J. Med. Chem.* **2021**, *211*, 113095. [\[CrossRef\]](#) [\[PubMed\]](#)
75. Krieger, V.; Hamacher, A.; Cao, F.; Stenzel, K.; Gertzen, C.G.W.; Schäker-Hübner, L.; Kurz, T.; Gohlke, H.; Dekker, F.J.; Kassack, M.U.; et al. Synthesis of Peptoid-Based Class I-Selective Histone Deacetylase Inhibitors with Chemosensitizing Properties. *J. Med. Chem.* **2019**, *62*, 11260–11279. [\[CrossRef\]](#) [\[PubMed\]](#)
76. Donati, B.; Lorenzini, E.; Ciarrocchi, A. BRD4 and Cancer: Going beyond transcriptional regulation. *Mol. Cancer* **2018**, *17*, 164. [\[CrossRef\]](#)
77. Tan, Y.; Wang, L.; Du, Y.; Liu, X.; Chen, Z.; Weng, X.; Guo, J.; Chen, H.; Wang, M.; Wang, X. Inhibition of BRD4 suppresses tumor growth in prostate cancer via the enhancement of FOXO1 expression. *Int. J. Oncol.* **2018**, *53*, 2503–2517. [\[CrossRef\]](#) [\[PubMed\]](#)
78. Zhou, J.X.; Agborbesong, E.; Li, L.X.; Li, X. Bromodomain Protein BRD4-Mediated Mutant p53 Transcription Promotes TNBC Progression. *Int. J. Mol. Sci.* **2022**, *23*, 5163. [\[CrossRef\]](#)
79. Pandey, S.; Bourn, J.; Cekanova, M. Mutations of p53 decrease sensitivity to the anthracycline treatments in bladder cancer cells. *Oncotarget* **2018**, *9*, 28514–28531. [\[CrossRef\]](#)
80. Wang, H.-T.; Lee, H.-W.; Weng, M.-W.; Liu, Y.; Huang, W.C.; Lepor, H.; Wu, X.-R.; Tang, M.-S. The role of TAp63 γ and P53 point mutations in regulating DNA repair, mutational susceptibility and invasion of bladder cancer cells. *eLife* **2021**, *10*, e71184. [\[CrossRef\]](#)

81. Rieger, K.M.; Little, A.F.; Swart, J.M.; Kastrinakis, W.V.; Fitzgerald, J.M.; Hess, D.T.; Libertino, J.A.; Summerhayes, I.C. Human bladder carcinoma cell lines as indicators of oncogenic change relevant to urothelial neoplastic progression. *Br. J. Cancer* **1995**, *72*, 683–690. [\[CrossRef\]](#)
82. Di Micco, P.; Antolin, A.A.; Mitsopoulos, C.; Villasclaras-Fernandez, E.; Sanfelice, D.; Dolciemi, D.; Ramagiri, P.; Mica, I.L.; Tym, J.E.; Gingrich, P.W.; et al. canSAR: Update to the cancer translational research and drug discovery knowledgebase. *Nucleic Acids Res.* **2023**, *51*, D1212–D1219. [\[CrossRef\]](#)
83. Hu, J.; Cao, J.; Topatana, W.; Juengpanich, S.; Li, S.; Zhang, B.; Shen, J.; Cai, L.; Cai, X.; Chen, M. Targeting mutant p53 for cancer therapy: Direct and indirect strategies. *J. Hematol. Oncol.* **2021**, *14*, 157. [\[CrossRef\]](#)
84. Bollmann, L.M.; Skerhut, A.J.; Asfaha, Y.; Horstick, N.; Hanenberg, H.; Hamacher, A.; Kurz, T.; Kassack, M.U. The Novel Class IIa Selective Histone Deacetylase Inhibitor YAK540 Is Synergistic with Bortezomib in Leukemia Cell Lines. *Int. J. Mol. Sci.* **2022**, *23*, 3398. [\[CrossRef\]](#) [\[PubMed\]](#)
85. Ren, D.; Li, L.; Wang, S.; Zuo, Y. The c-MYC transcription factor conduces to resistance to cisplatin by regulating MMS19 in bladder cancer cells. *Tissue Cell* **2023**, *82*, 102096. [\[CrossRef\]](#) [\[PubMed\]](#)
86. Shi, X.; Liu, C.; Liu, B.; Chen, J.; Wu, X.; Gong, W. JQ1: A novel potential therapeutic target. *Pharmazie* **2018**, *73*, 491–493. [\[CrossRef\]](#) [\[PubMed\]](#)
87. Roy, R.; Ria, T.; RoyMaha Patra, D.; Sk, U.H. Single Inhibitors versus Dual Inhibitors: Role of HDAC in Cancer. *ACS Omega* **2023**, *8*, 16532–16544. [\[CrossRef\]](#)
88. Yang, Y.; Mou, Y.; Wan, L.-X.; Zhu, S.; Wang, G.; Gao, H.; Liu, B. Rethinking therapeutic strategies of dual-target drugs: An update on pharmacological small-molecule compounds in cancer. *Med. Res. Rev.* **2024**, *online ahead of print*. [\[CrossRef\]](#)
89. Atkinson, S.J.; Soden, P.E.; Angell, D.C.; Bantscheff, M.; Chung, C.; Giblin, K.A.; Smithers, N.; Furze, R.C.; Gordon, L.; Drewes, G.; et al. The structure based design of dual HDAC/BET inhibitors as novel epigenetic probes. *Med. Chem. Commun.* **2014**, *5*, 342–351. [\[CrossRef\]](#)
90. Kling, M.J.; Keshewani, V.; Mishra, N.K.; Alexander, G.; McIntyre, E.M.; Ray, S.; Challagundla, K.B.; Joshi, S.S.; Coulter, D.W.; Chaturvedi, N.K. A novel dual epigenetic approach targeting BET proteins and HDACs in Group 3 (MYC-driven) Medulloblastoma. *J. Exp. Clin. Cancer Res.* **2022**, *41*, 321. [\[CrossRef\]](#)
91. Zhang, X.; Zegar, T.; Weiser, T.; Hamdan, F.H.; Berger, B.-T.; Lucas, R.; Balourdas, D.-I.; Ladigan, S.; Cheung, P.F.; Liffers, S.-T.; et al. Characterization of a dual BET/HDAC inhibitor for treatment of pancreatic ductal adenocarcinoma. *Int. J. Cancer* **2020**, *147*, 2847–2861. [\[CrossRef\]](#)
92. Amemiya, S.; Yamaguchi, T.; Hashimoto, Y.; Noguchi-Yachide, T. Synthesis and evaluation of novel dual BRD4/HDAC inhibitors. *Bioorg. Med. Chem.* **2017**, *25*, 3677–3684. [\[CrossRef\]](#)
93. Burmeister, A.; Stephan, A.; Alves Avelar, L.A.; Müller, M.R.; Seiwert, A.; Höfmann, S.; Fischer, F.; Torres-Gomez, H.; Hoffmann, M.J.; Niegisch, G.; et al. Establishment and Evaluation of Dual HDAC/BET Inhibitors as Therapeutic Options for Germ Cell Tumors and Other Urological Malignancies. *Mol. Cancer Ther.* **2022**, *21*, 1674–1688. [\[CrossRef\]](#)
94. Lucas, X.; Wohlwend, D.; Hügler, M.; Schmidtkunz, K.; Gerhardt, S.; Schüle, R.; Jung, M.; Einsle, O.; Günther, S. 4-Acyl pyrroles: Mimicking acetylated lysines in histone code reading. *Angew. Chem. Int. Ed. Engl.* **2013**, *52*, 14055–14059. [\[CrossRef\]](#) [\[PubMed\]](#)
95. Panteix, G.; Beaujard, A.; Garbit, F.; Chaduiron-Faye, C.; Guillaumont, M.; Gilly, F.; Baltassat, P.; Bressolle, F. Population pharmacokinetics of cisplatin in patients with advanced ovarian cancer during intraperitoneal hyperthermia chemotherapy. *Anticancer Res.* **2002**, *22*, 1329–1336. [\[PubMed\]](#)

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Triple Combination of Entinostat, a Bromodomain Inhibitor, and Cisplatin Is a Promising Treatment Option for Bladder Cancer

Supplemental Information

Table S1. Overview of primary antibodies used in western blot.

Protein of interest	expected size (kDa)	Primary antibody (company, Catalog nr.)
BRD4	200	Cell Signaling Technologie; E2A7X
FOXO1	78	Cell Signaling Technologie; C29H4
c-Myc	65	Cell Signaling Technologie; D84C12
p53	53	Santa Cruz; sc-126
β -Actin	43	Santa Cruz; sc47778
Bim	22	bio-technie; NBP1-76963
p21	21	Cell Signaling Technology; #2947
survivin	16	bio-technie; AF886

Table S2. Primer sequences for RT-PCR.

Gene	Primer Forward	Primer Reverse
<i>survivin</i>	TGAGAACGAGCCAGACTTGG	TGTTCTCTATGGGGTCGTCA
<i>FOXO1</i>	ATGTGTTGCCCAACCAAAGC	AGTGTAACCTGCTCACTAACCC
<i>FOXO3a</i>	AAGGATCACTGAGGAAGGG	GTGTCAAGTTTGAGGGTCTGC
<i>GUSB</i>	ACCTCCAAGTATCCCAAGGGT	GTCTTGCTCCACGCTGGT
<i>HPRT1</i>	CCTGGCGTCGTGATTAGTGA	CGAGCAAGACGTTTCAGTCCT
<i>TBP</i>	GTGACCCAGCATCACTGTTTC	GAGCATCTCCAGCACACTCT
<i>p53</i>	AGTCAGATCCTAGCGTCG	TCAGGAAGTAGTTTCCATAGG
<i>Bim</i>	AGACAGAGCCACAAGCTTCC	CAATACGCCGCAACTCTTGG
<i>p21</i>	TGCCGAAGTCAGTTCCTTGT	GTTCTGACATGGCGCCTCC

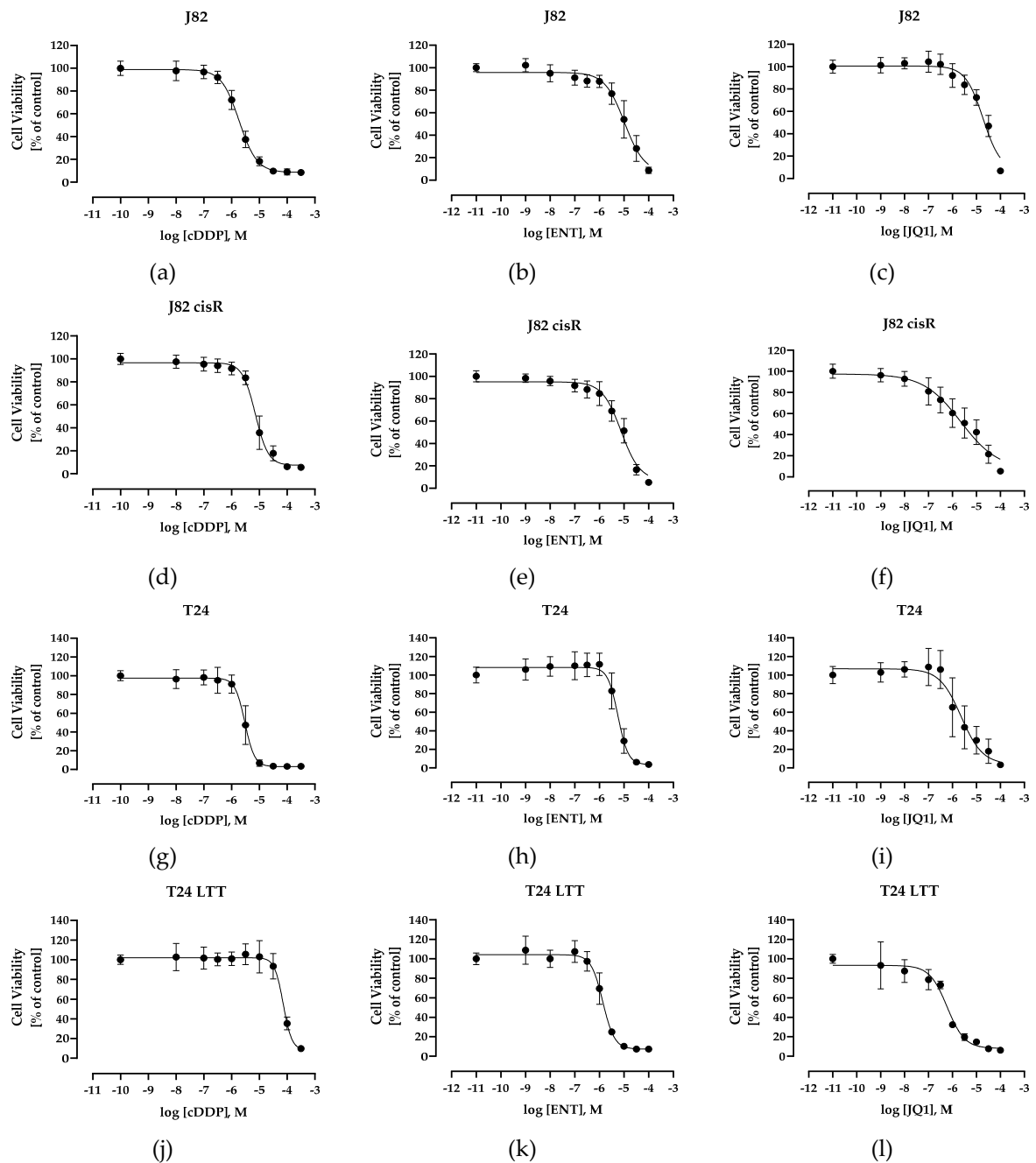
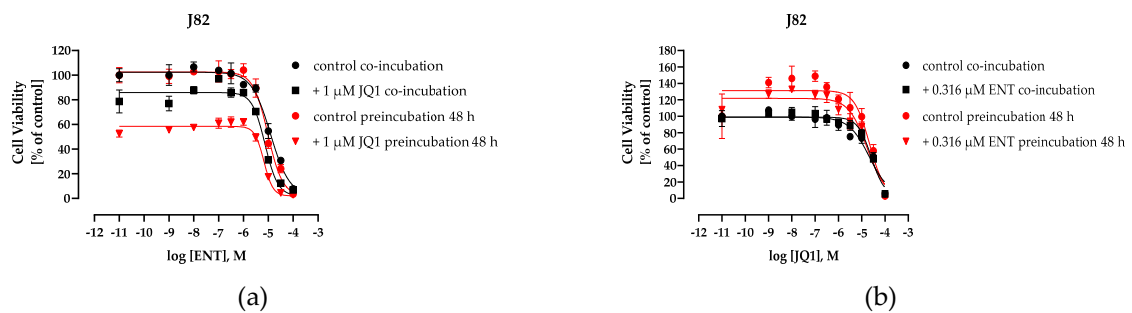


Figure S1. Concentration-effect-curves determined with a 72 h MTT-Assay in J82 (a) cisplatin (cDDP), (b) entinostat (ENT), (c) JQ1; J82 cisR (d) cisplatin, (e) entinostat, (f) JQ1; T24 (g) cisplatin, (h) entinostat, (i) JQ1; T24 LTT (j) cisplatin, (k) entinostat, (l) JQ1. Data shown are the mean $\pm$ SD of at least three independent experiments, each carried out in triplicates.



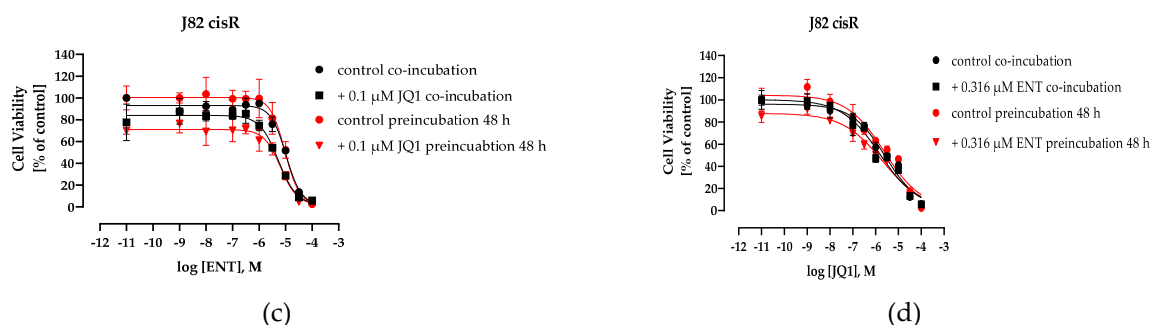


Figure S2. Concentration-effect-curves of entinostat or JQ1 (MTT-Assay). Cells were either preincubated with entinostat or JQ1 for 48 h followed by the addition of the second epigenetic inhibitor (JQ1 or entinostat) for another 72 h or co-incubated with both epigenetic inhibitors for 72 h. (a)-(b) J82; (c)-(d) J82 cisR. Control means concentration-effect-curve of entinostat or JQ1 in the absence of the second epigenetic inhibitor. Data shown are the mean $\pm$ SD of at least one representative experiment, carried out in triplicates.

Table S3. IC₅₀ values (MTT) of entinostat or JQ1. Cells were co-incubated with one fixed concentration of entinostat or JQ1 for 72 h or preincubated with one fixed concentration of entinostat or JQ1 for 48 h followed by addition of the other epigenetic inhibitor for another 72 h. Data shown are the mean $\pm$ SD of at least one representative experiment carried out in triplicates. Shift factors (SF) were calculated by dividing the IC₅₀ value of the control by the IC₅₀ value of the additional treatment.

cell line	control	co-incubation JQ1			control	co-incubation ENT		
	ENT IC ₅₀ $\pm$ SD [μ M]	JQ1 [μ M]	ENT IC ₅₀ $\pm$ SD [μ M]	SF	JQ1 IC ₅₀ $\pm$ SD [μ M]	ENT [μ M]	JQ1 IC ₅₀ $\pm$ SD [μ M]	SF
J82	12.3 $\pm$ 1.08	1	7.34 $\pm$ 0.77	1.68	21.5 $\pm$ 4.05	0.316	25.7 $\pm$ 2.71	0.84
J82 cisR	10.4 $\pm$ 0.94	0.1	5.27 $\pm$ 0.64	1.97	2.64 $\pm$ 0.52	0.316	1.54 $\pm$ 0.36	1.71

cell line	control	preincubation JQ1 48 h			control	preincubation ENT 48 h		
	ENT IC ₅₀ $\pm$ SD [μ M]	JQ1 [μ M]	ENT IC ₅₀ $\pm$ SD [μ M]	SF	JQ1 IC ₅₀ $\pm$ SD [μ M]	ENT [μ M]	JQ1 IC ₅₀ $\pm$ SD [μ M]	SF
J82	9.59 $\pm$ 0.81	1	6.65 $\pm$ 0.49	1.44	21.6 $\pm$ 4.99	0.316	16.9 $\pm$ 2.79	1.28
J82 cisR	9.38 $\pm$ 0.89	0.1	6.46 $\pm$ 0.73	1.45	2.41 $\pm$ 0.51	0.316	1.99 $\pm$ 0.37	1.21

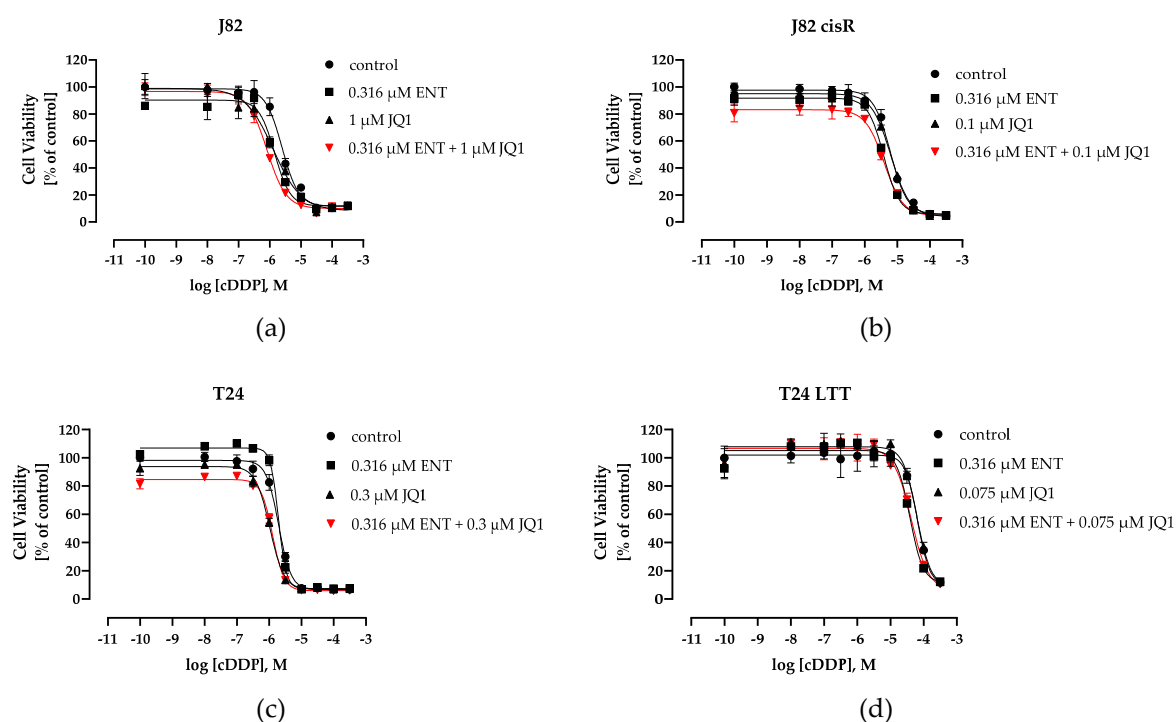


Figure S3. Concentration-effect-curves of cisplatin (MTT-Assay). Cells were co-incubated with entinostat, JQ1 and cisplatin for 72 h. (a) J82 (b) J82 cisR (c) T24 (d) T24 LTT. The control illustrates cell viability after treatment with ascending concentrations of cisplatin. Data shown are the mean $\pm$ SD of one representative experiment carried out in triplicates.

Table S4. IC₅₀ values (MTT) of cisplatin. Cells were co-incubated with entinostat, JQ1 and cisplatin for 72 h. Data shown are the mean $\pm$ SD of at least one representative experiment carried out in triplicates.

cell line	control	co-incubation								
	cisplatin IC ₅₀ $\pm$ SD [μ M]	ENT [μ M]	cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	JQ1 [μ M]	cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	ENT+ JQ1 [μ M]	cisplatin IC ₅₀ $\pm$ SD [μ M]	SF
J82	2.49 $\pm$ 0.12	0.316	1.43 $\pm$ 0.15	1.74	1	1.31 $\pm$ 0.18	1.90	0.316 + 1	0.816 $\pm$ 0.06	3.05
J82 cisR	6.17 $\pm$ 0.23	0.316	3.86 $\pm$ 0.18	1.60	0.1	6.30 $\pm$ 0.30	0.98	0.316 + 0.1	3.95 $\pm$ 0.29	1.56
T24	1.97 $\pm$ 0.06	0.316	1.94 $\pm$ 0.07	1.02	0.3	1.05 $\pm$ 0.04	1.88	0.316 + 0.3	1.28 $\pm$ 0.04	1.54
T24 LTT	65.3 $\pm$ 4.17	0.316	38.2 $\pm$ 4.06	1.71	0.075	62.8 $\pm$ 7.45	1.04	0.316 + 0.075	42.2 $\pm$ 4.77	1.55

Table S5. CI values of the dual combination studies (48 h preincubation with entinostat or JQ1, followed by addition of cisplatin for another 72 h. CI values were calculated using compuSyn software from the mean of at least three independent experiments, each carried out in triplicates. CI < 1.0 indicate a synergistic effect (green), CI = 1 indicate an additive effect, CI > 1 indicate an antagonistic effect (red); * means fraction affected was less than 0.20.

J82									
JQ1					ENT				
[μM]					[μM]				
					0.1	0.316	0.5	0.75	
cisplatin [μM]	0.05	*	*	*	0.804	*	*	*	*
	0.1	*	*	1.48	0.800	*	*	*	*
	0.15	*	*	1.04	0.733	*	*	*	*
	0.2	*	1.80	0.887	0.624	*	*	*	*
	0.3	*	0.926	0.509	0.391	*	*	*	3.52

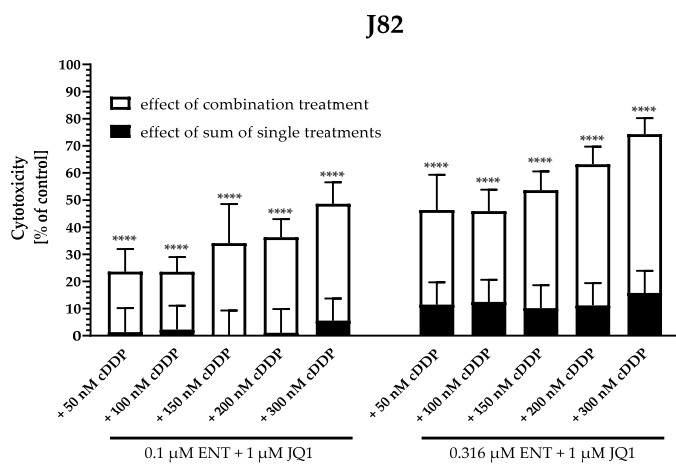
J82 cisR									
JQ1					ENT				
[μM]					[μM]				
					0.1	0.316	0.5	0.75	
cisplatin [μM]	0.2	*	2.21	1.72	0.683	*	0.934	1.53	1.23
	0.4	*	3.54	1.13	0.584	*	*	*	1.38
	0.6	*	3.12	1.16	0.383	*	*	*	1.30
	0.8	*	2.51	0.828	0.275	*	1.30	1.47	0.989
	1	*	1.61	0.533	0.209	*	0.823	1.31	0.701

T24									
JQ1					ENT				
[μM]					[μM]				
					0.316	0.5	0.75		
cisplatin [μM]	0.2	*	1.15	0.446	0.392	*	*	*	
	0.4	*	0.730	0.322	0.325	*	*	*	

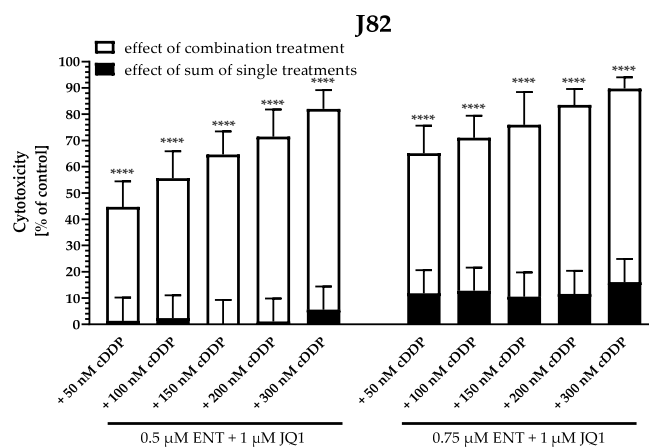
0.6	*	0.522	0.302	0.291	*	*	*
0.8	*	0.419	0.271	0.308	*	*	*
1	*	0.358	0.260	0.277	*	*	*

T24 LTT

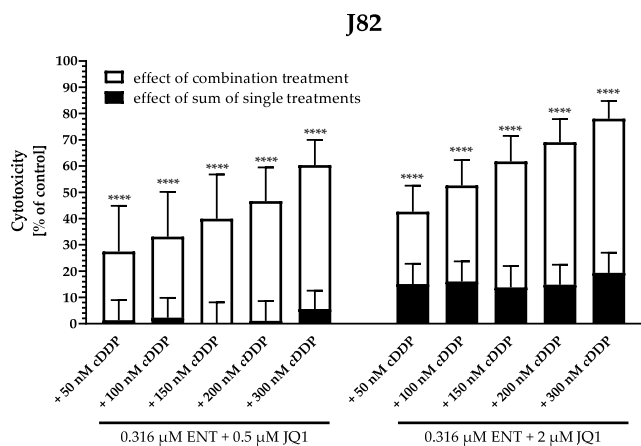
JQ1					ENT			
[μM]					[μM]			
0.050.0750.150.3					0.3160.50.75			
cisplatin [μM]	5	*	*	*	0.955	*	*	1.53
	10	*	*	*	0.803	*	*	1.40
	15	*	*	*	0.759	*	*	0.869
	20	*	*	*	0.565	*	0.910	0.558
	25	*	*	1.85	0.504	0.808	0.632	0.422



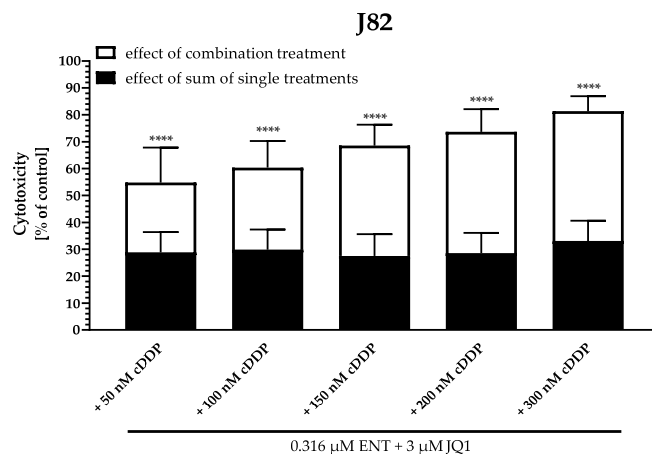
(a)



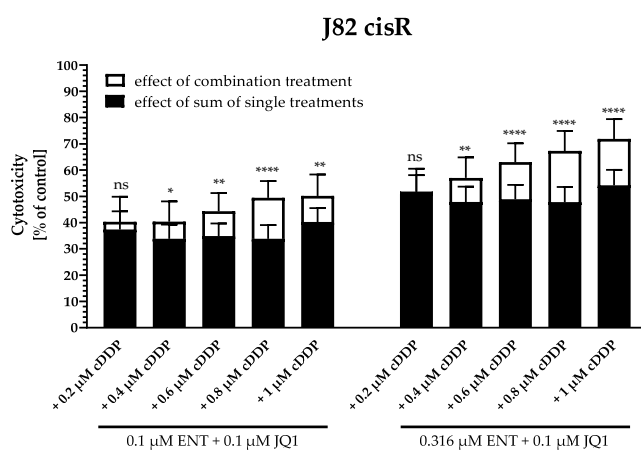
(b)



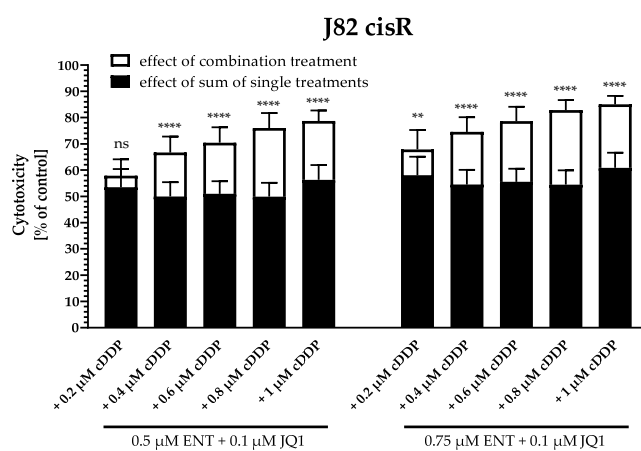
(c)



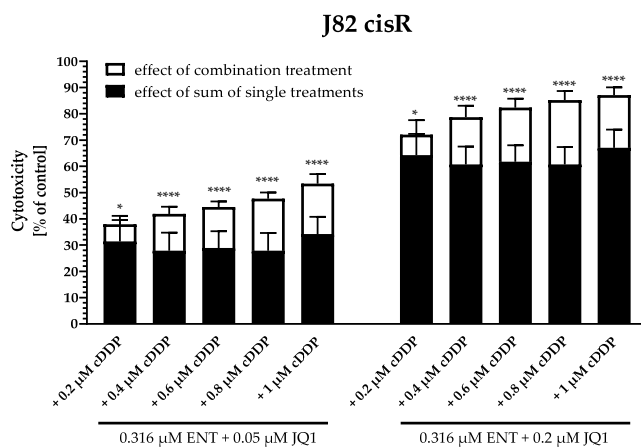
(d)



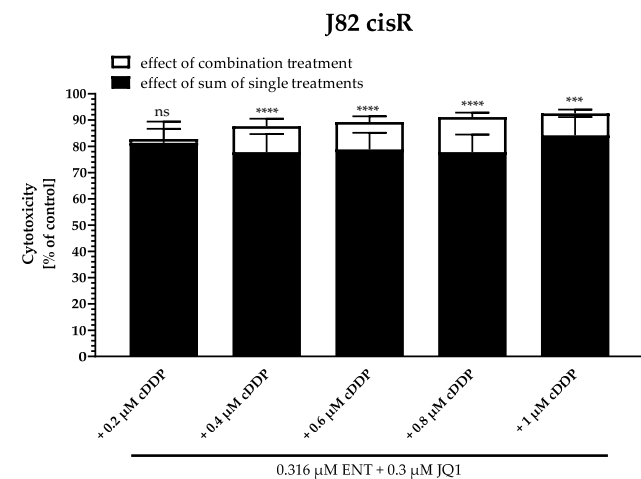
(e)



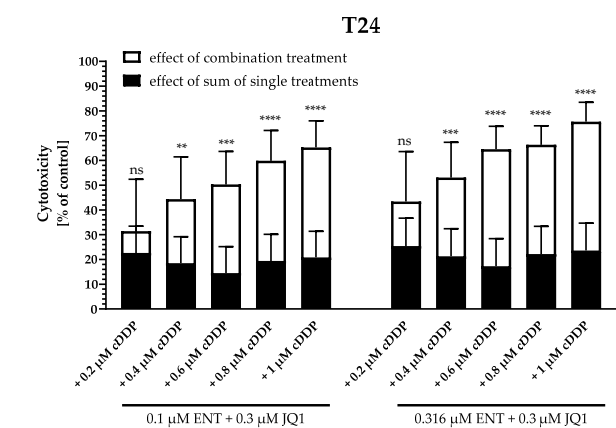
(f)



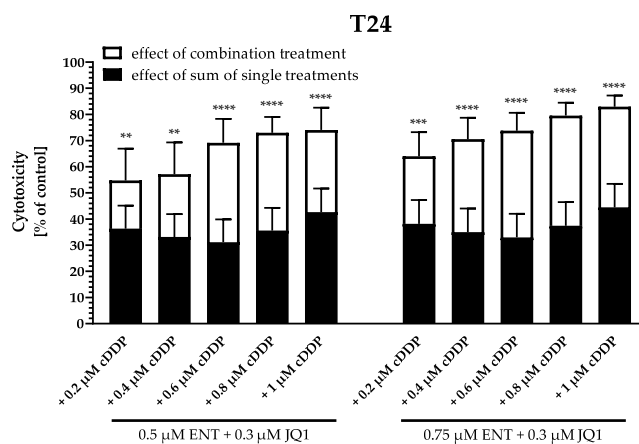
(g)



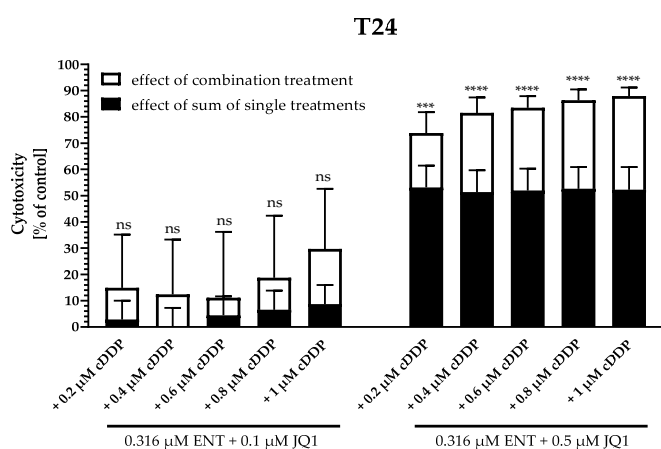
(h)



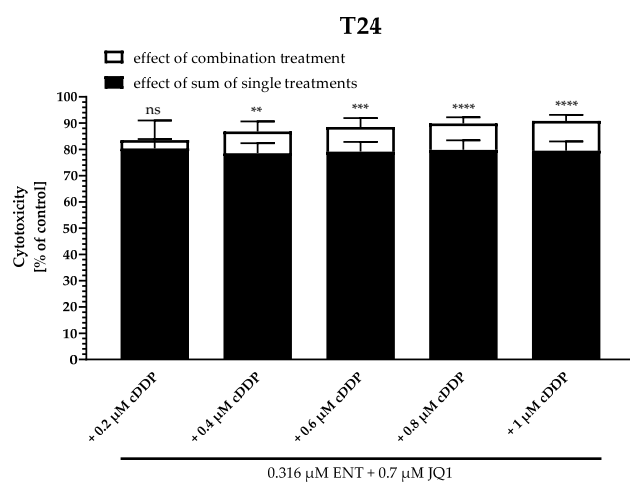
(i)



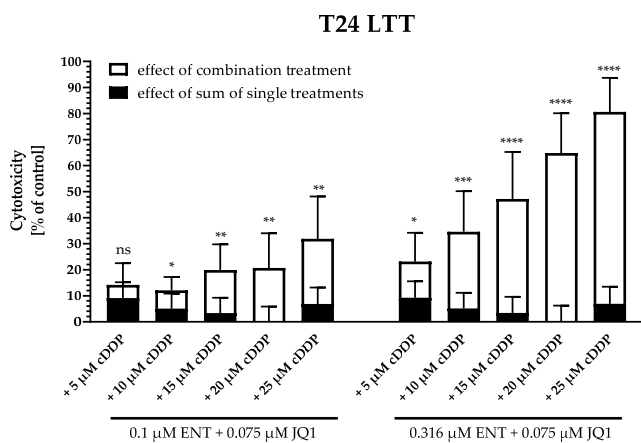
(j)



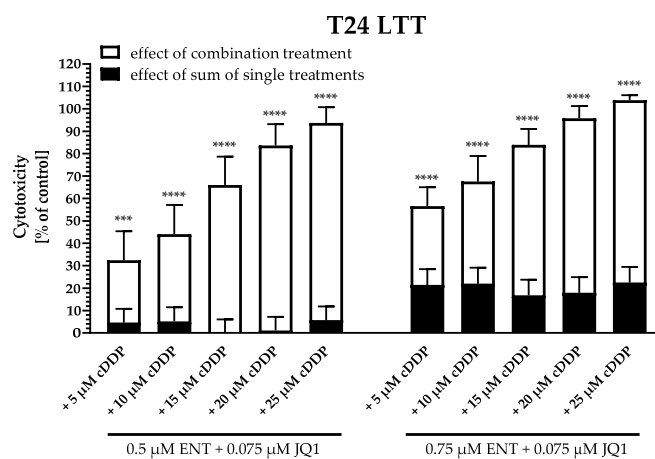
(k)



(l)



(m)



(n)

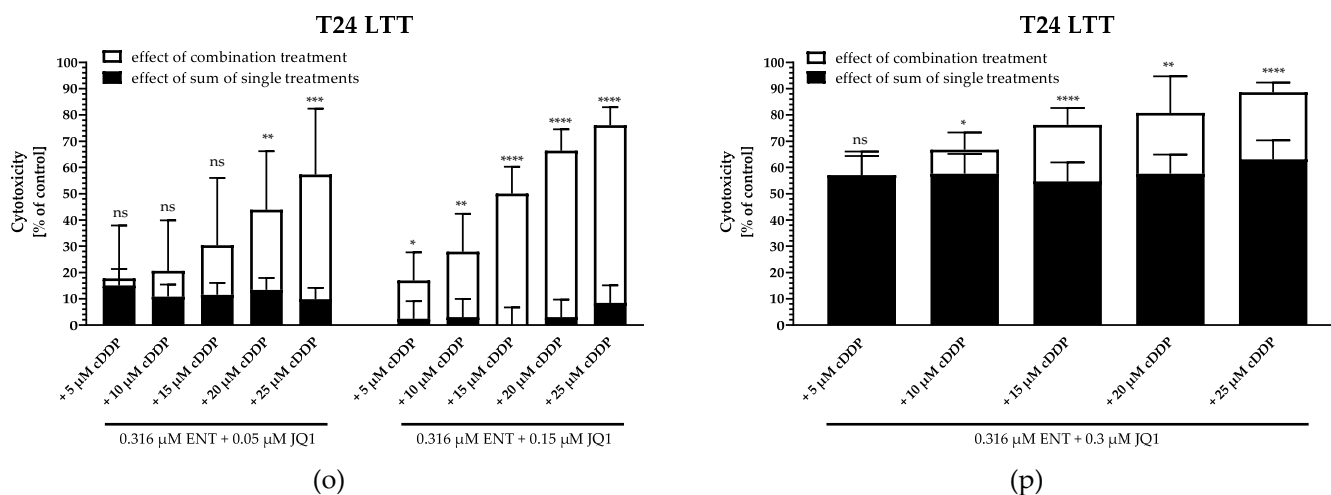
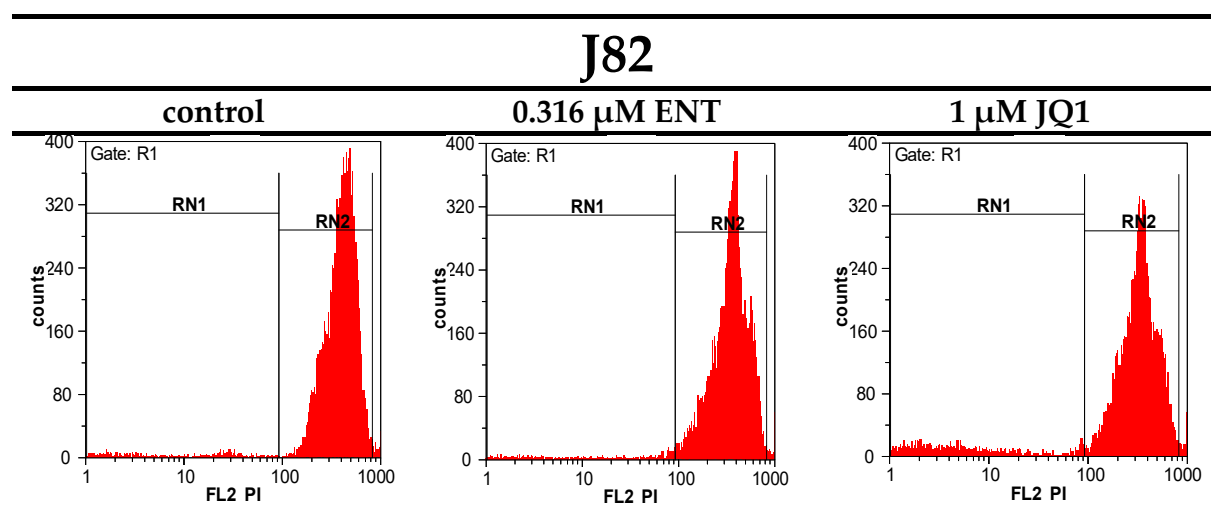
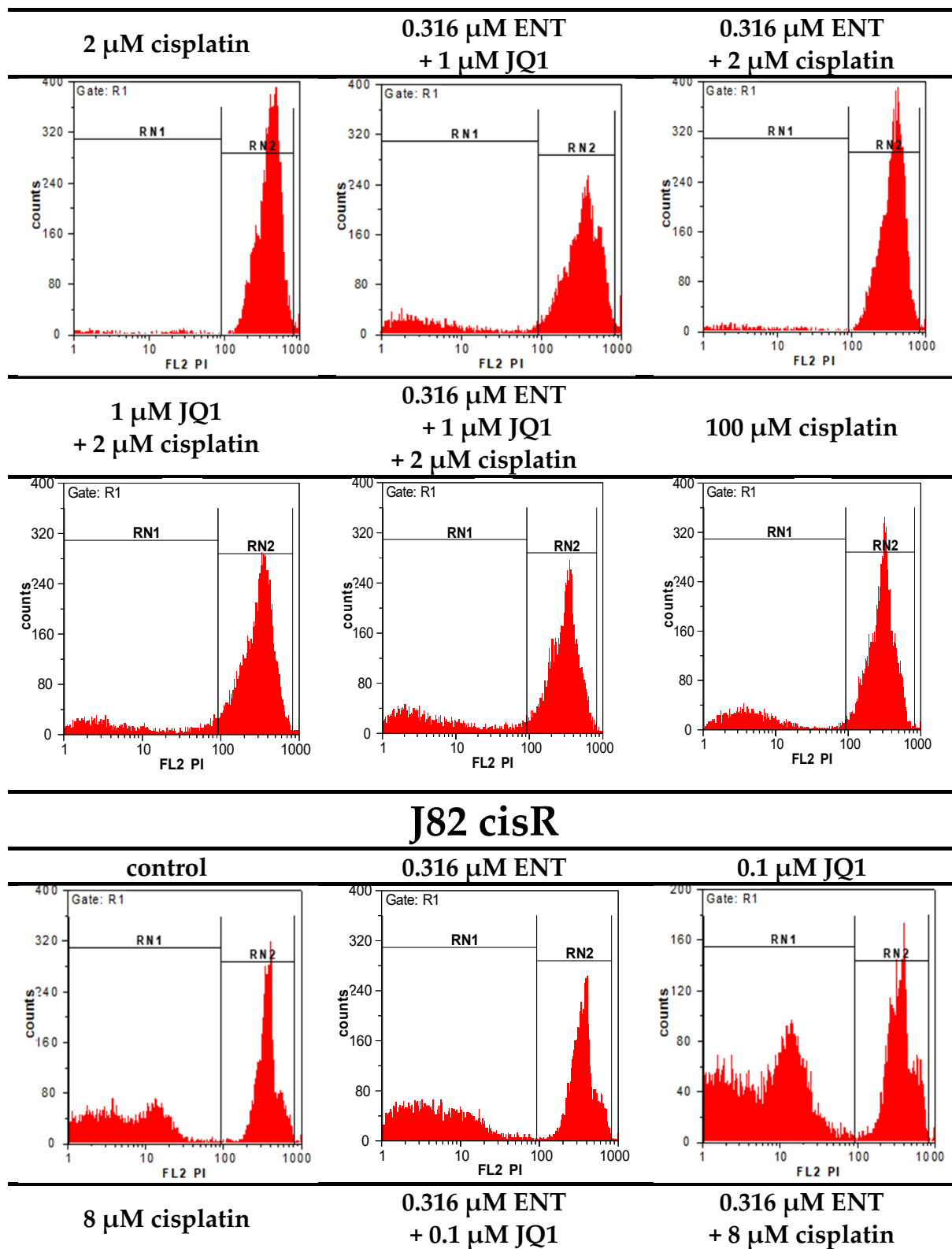
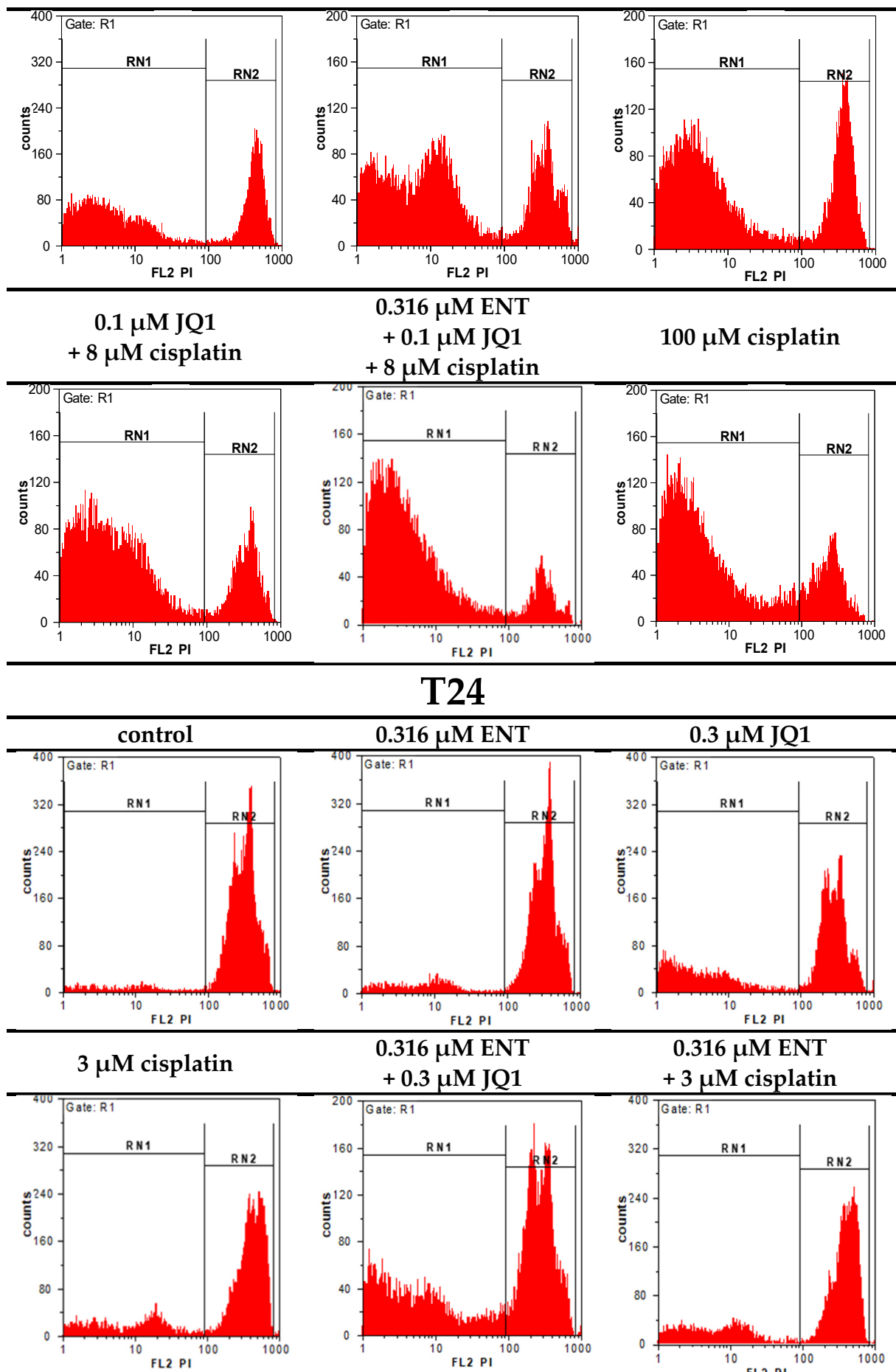
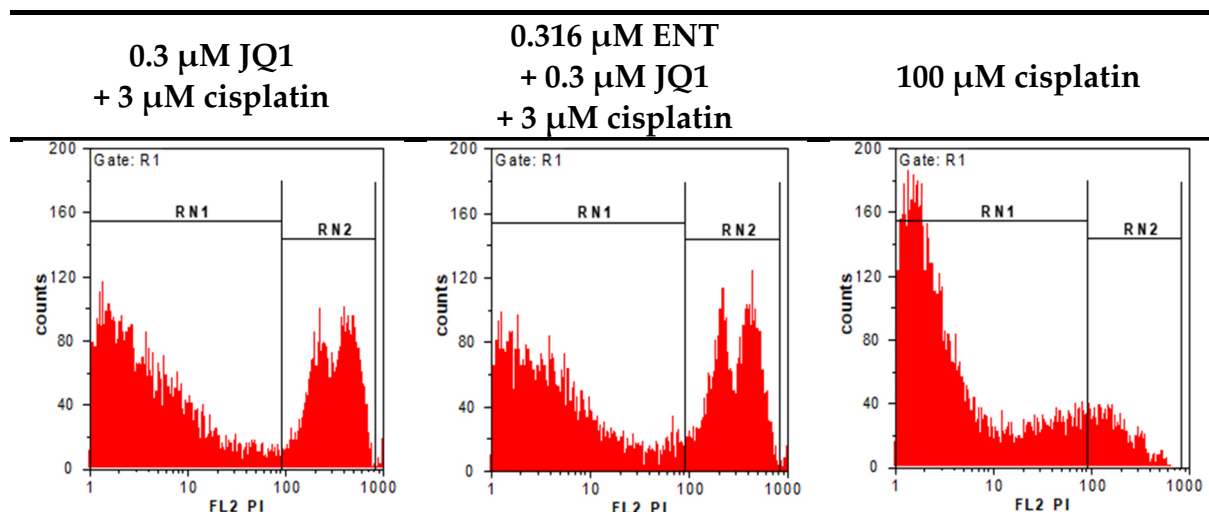


Figure S4. Synergistic cytotoxic effect of combination treatment in J82 (a)-(d), J82 cisR (e)-(h), T24 (i)-(l) and T24 LTT (m)-(p). Cells were preincubated with entinostat and JQ1 for 48 h followed by an addition of cisplatin for another 72 h. Shown are the mean + SD of at least three independent experiments, each carried out in triplicates. Data were normalized to untreated control. Black bars illustrate the additive effects (sum) of the individual treatments, white bars are the data of the combination treatment. Significance was calculated using unpaired t-test. ns ($p \geq 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

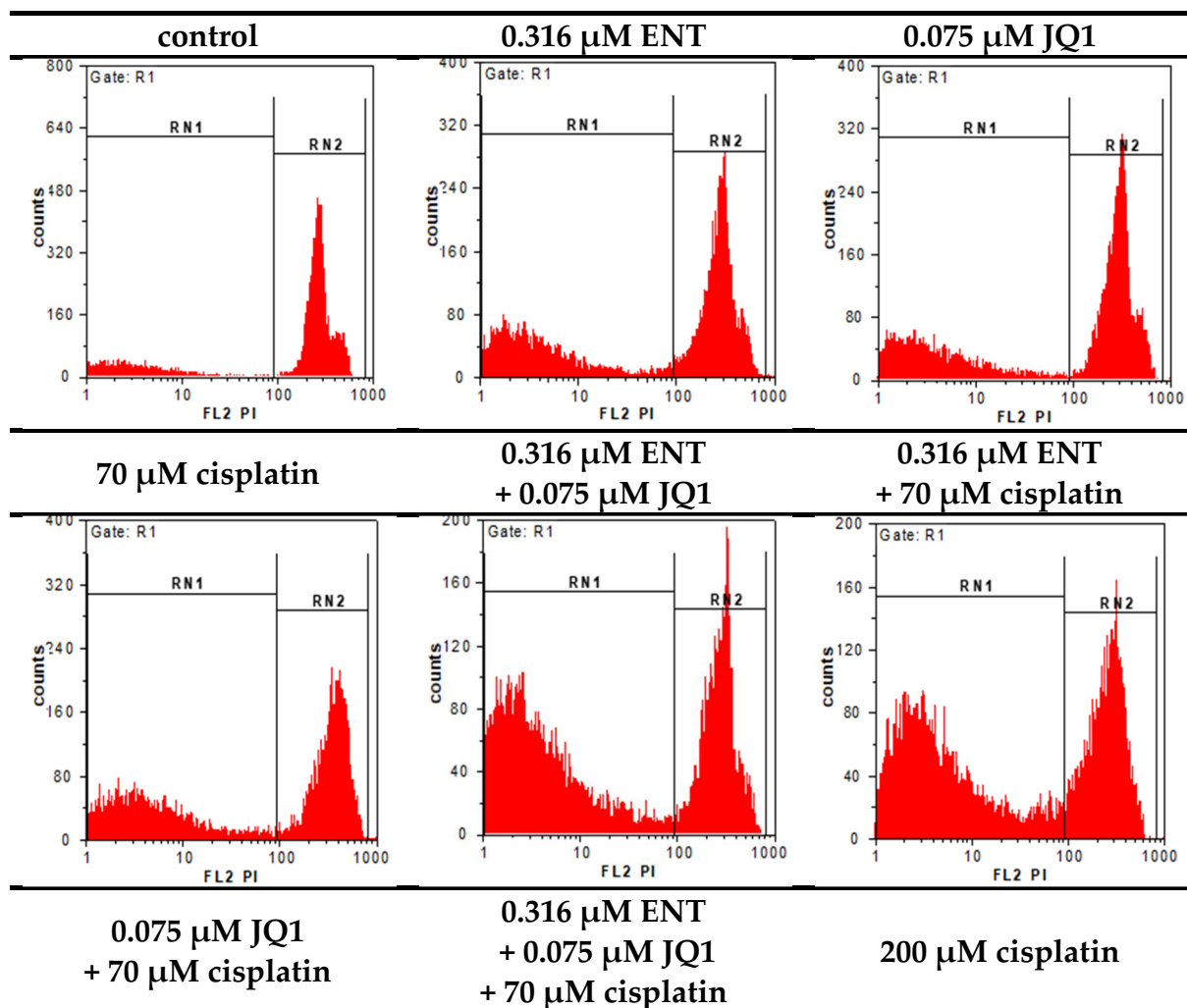








T24 LTT



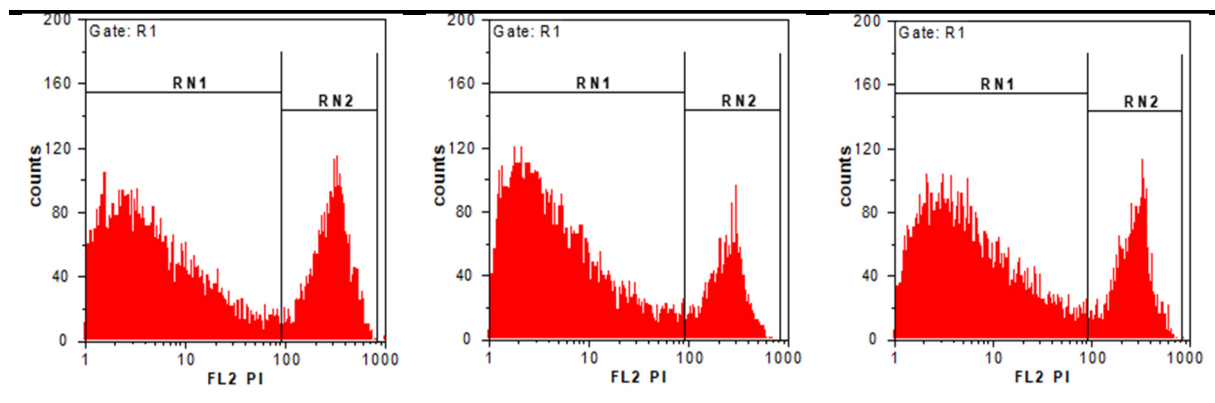
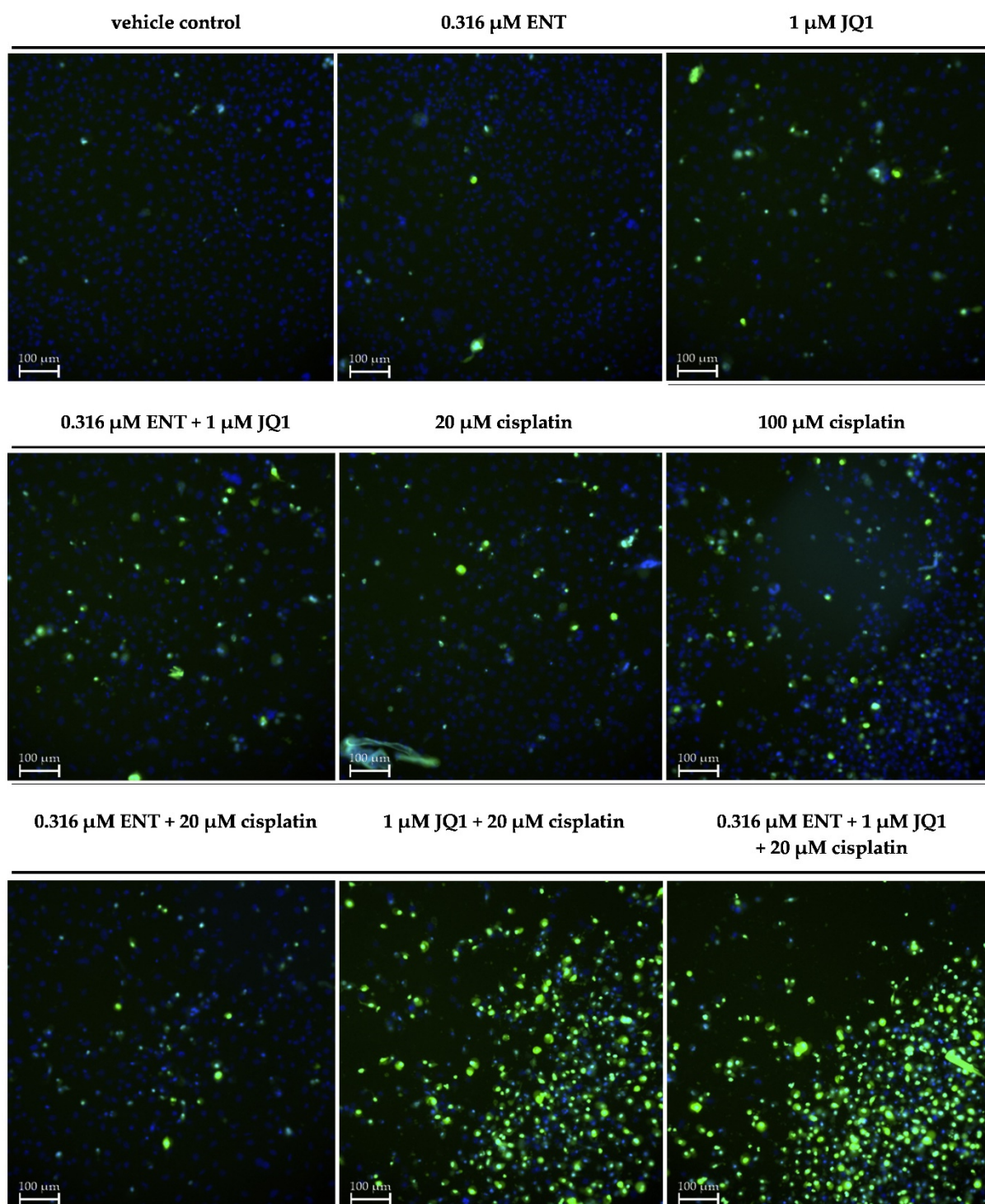
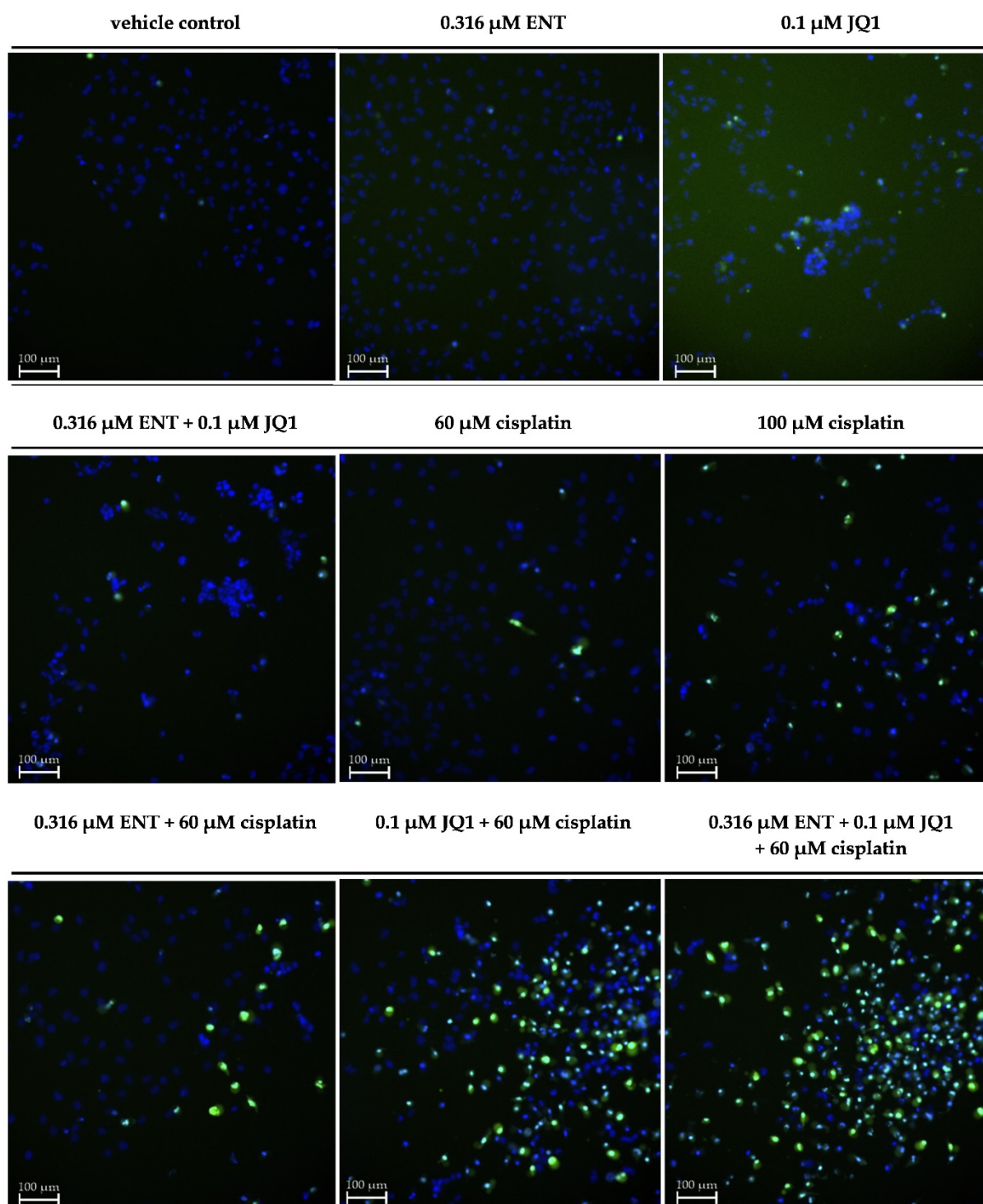


Figure S5. Representative cytometry flow images are shown for control, entinostat, JQ1, cisplatin and their combination treatments in J82, J82 cisR, T24 and T24 LTT. Cells were preincubated with entinostat and JQ1 for 48 h followed by addition of cisplatin for another 24 h. RN1 was defined as the subG1 fraction.

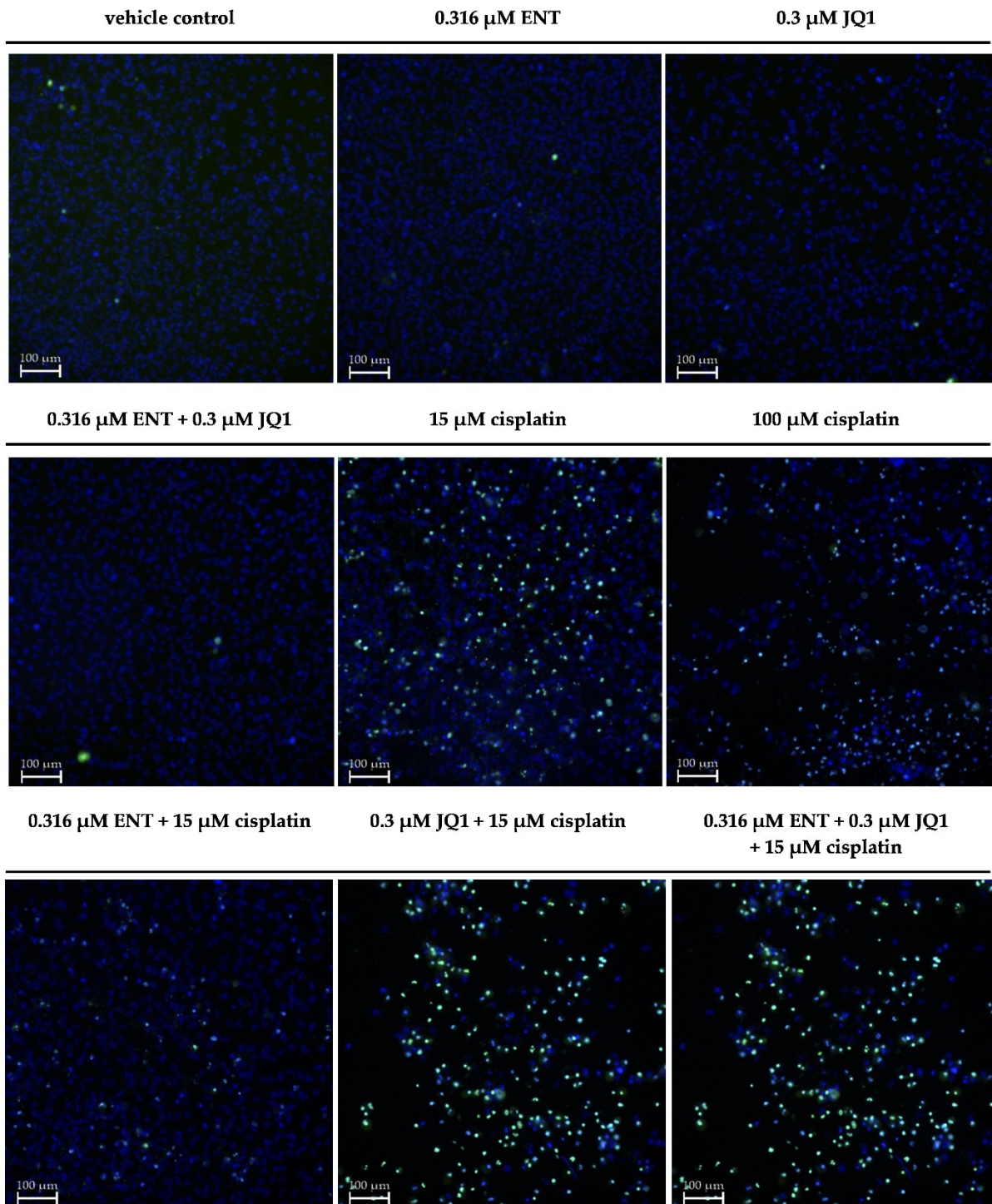
J82



J82 cisR



T24



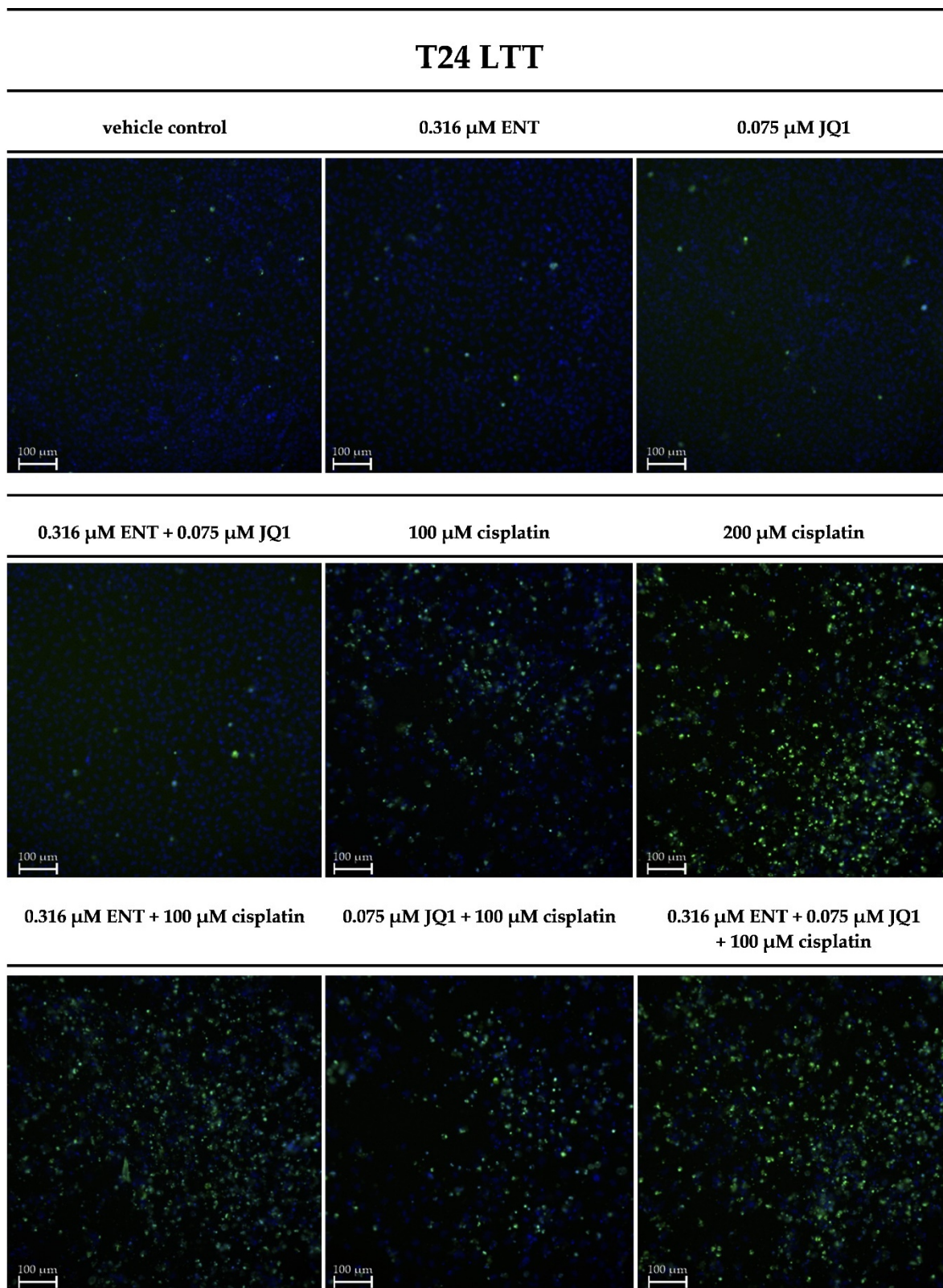


Figure S6. Representative fluorescent imaging pictures (Thermofisher Arrayscan XTI) are shown for vehicle control, entinostat, JQ1, cisplatin and their combination treatments in J82, J82 cisR, T24 and T24 LTT. Nuclei were stained using Hoechst 33342 (blue). Caspase 3/7 activation is shown in green. Cells were preincubated with entinostat and JQ1 for 48 h followed by addition of cisplatin for another 24 h (T24 LTT 48 h).

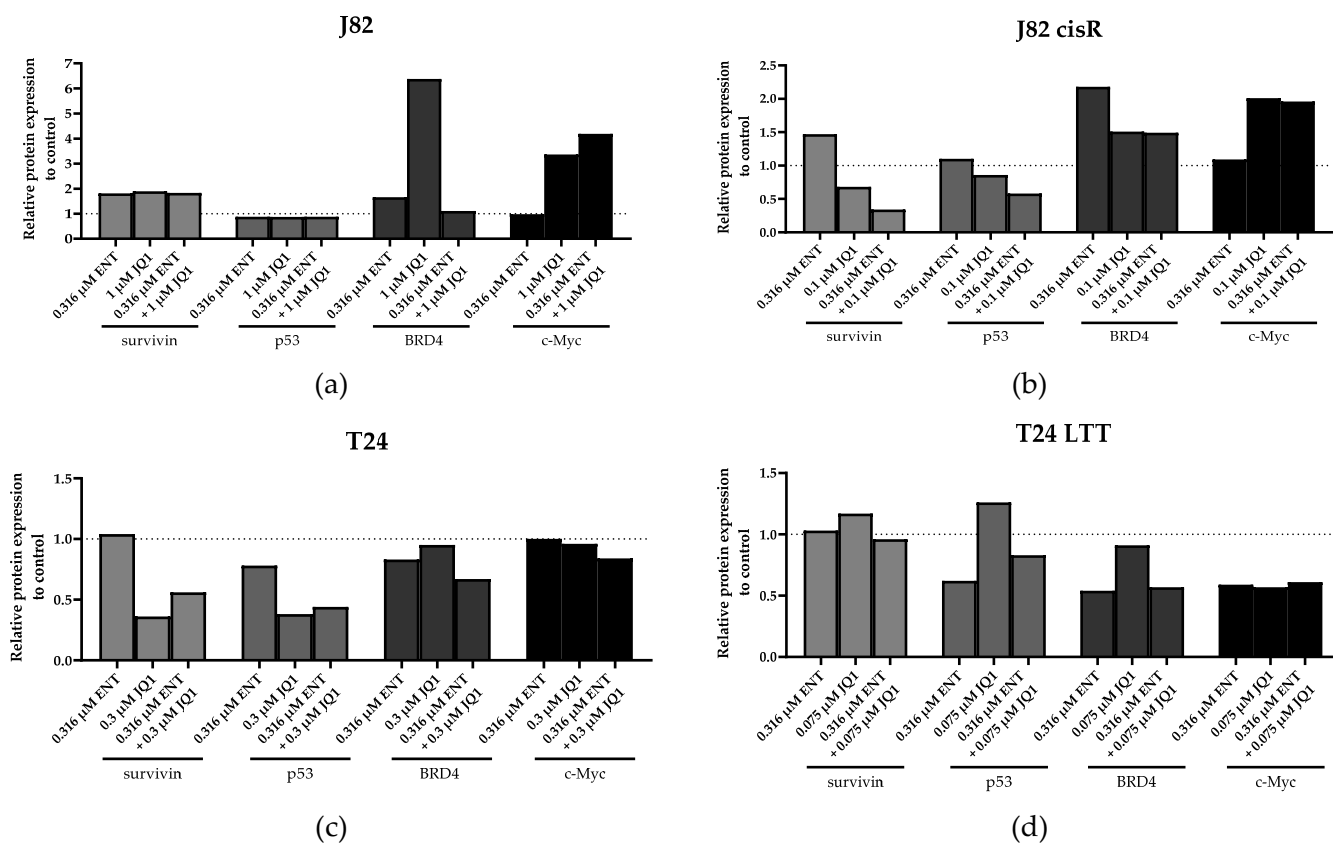


Figure S7. The relative protein expression of proteins of interest related to β -actin as loading control is shown in (a) J82, (b) J82 cisR, (c) T24 and (d) T24 LTT. Ratio of control was set to 1, represented by the dashed line. The western blots were analyzed with ImageJ software (ImageJ 1.54g (<http://imagej.org>)). Data shown is one representative blot out of at least two different protein samples. Cells were incubated with entinostat and JQ1 for 48 h.

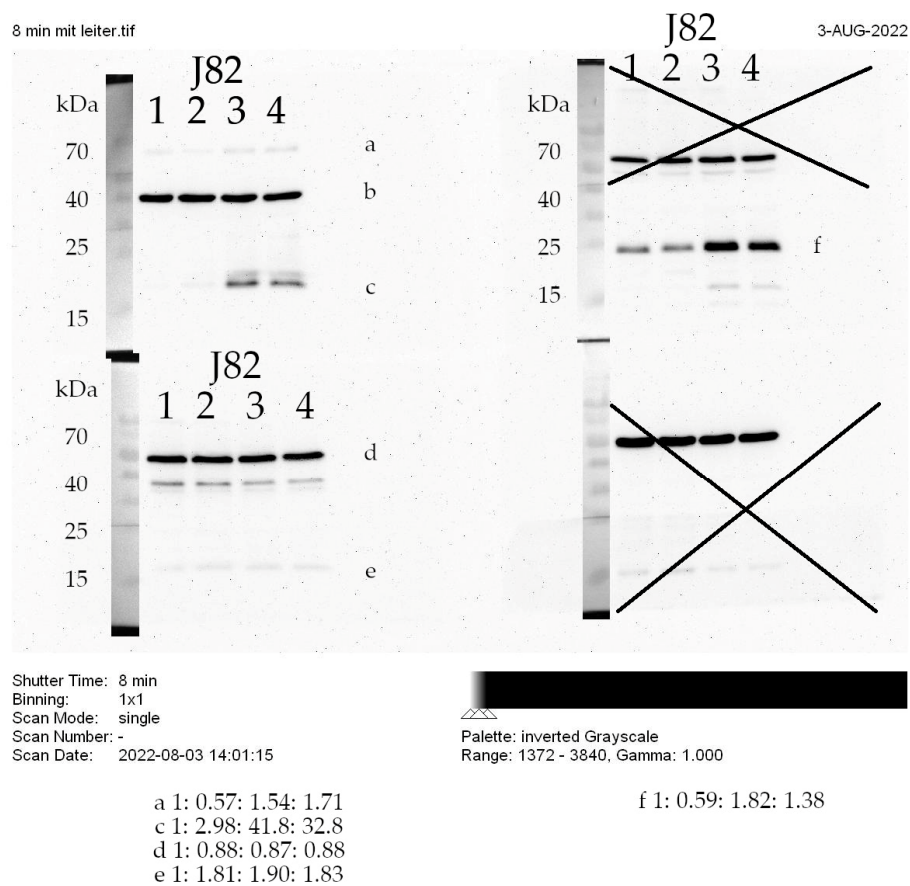


Figure S8. Uncropped western blot, J82: blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c) and the blot below illustrates p53 (d) and survivin (e), the blot on the right-side displays Bim (f), 48 h incubation. Order of samples left to right: control (1), entinostat (2), JQ1 (3), entinostat plus JQ1 (4). Densitometric analysis with ImageJ software of relative protein expression in relation to β -Actin is presented (a; c-f). Ratio of control was set to 1.

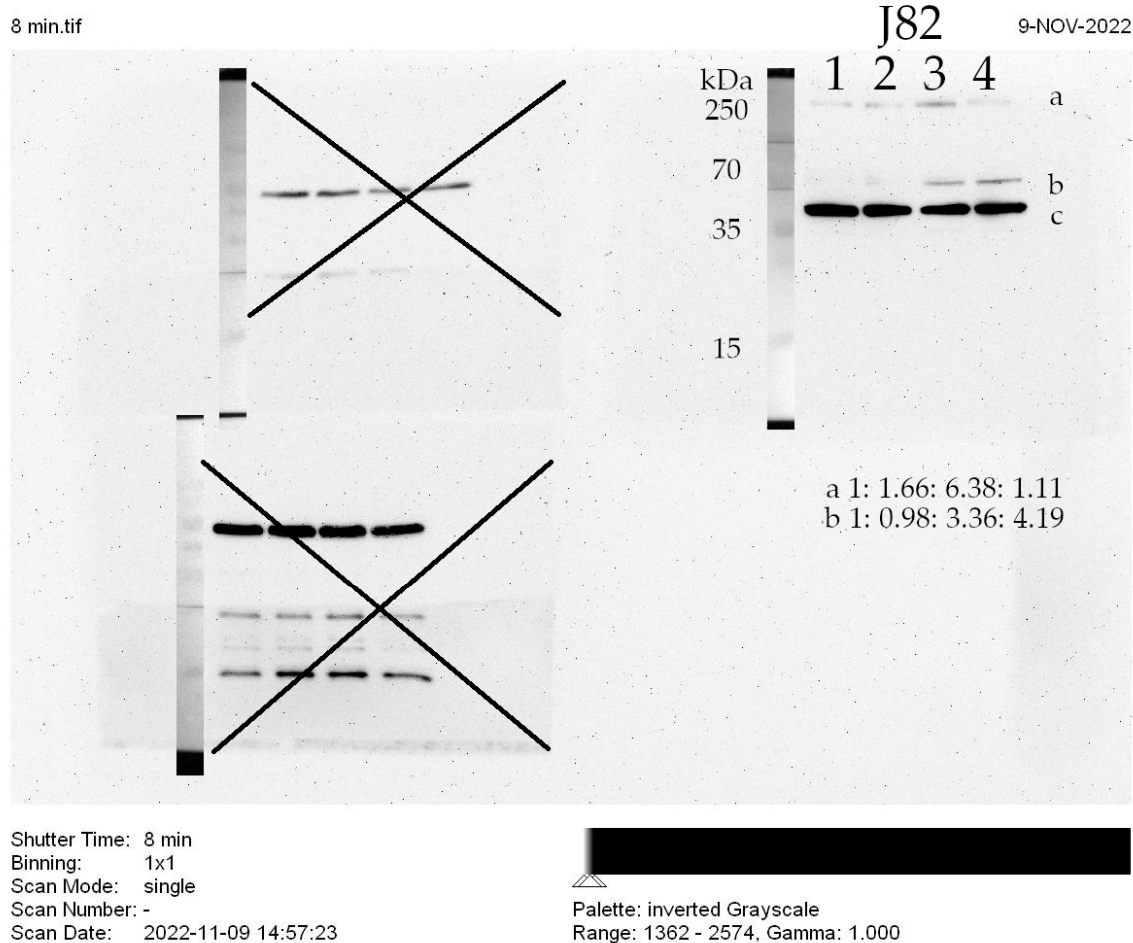
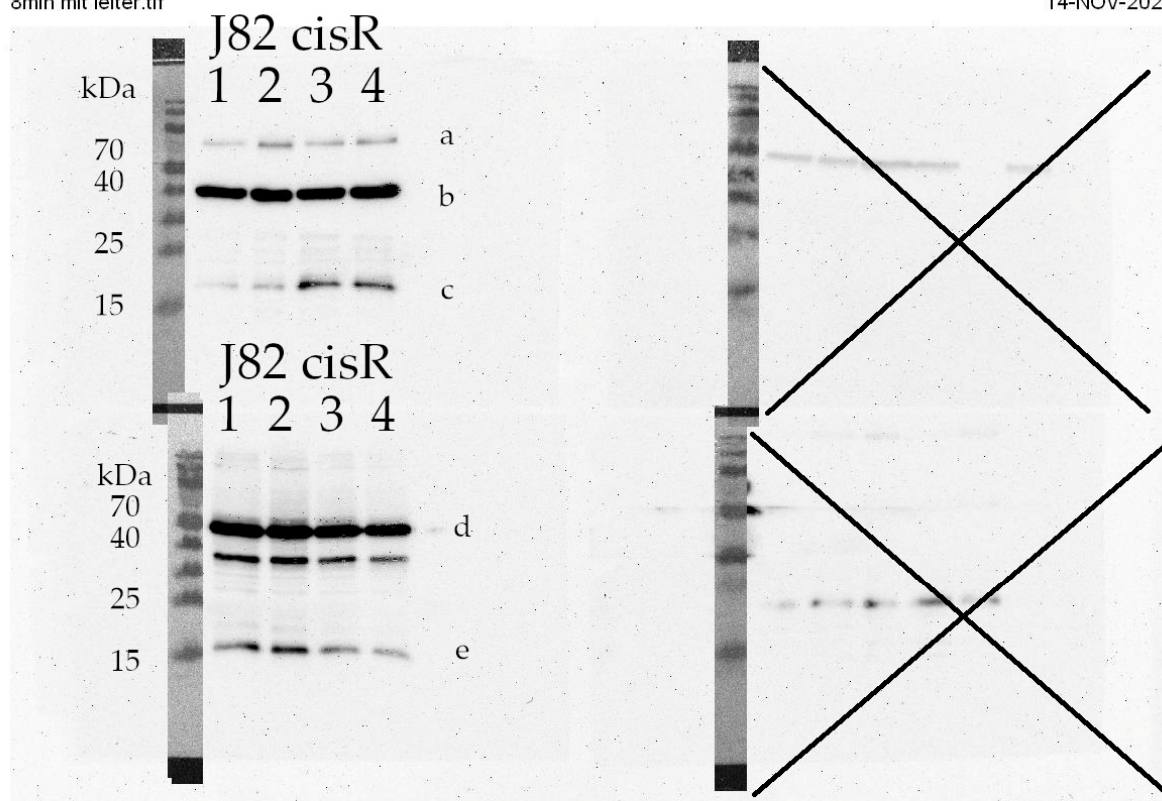


Figure S9. Uncropped western blot, J82: blot at right top shows BRD4 (a), c-Myc (b) and β -Actin (c), 48 h incubation. Order of samples left to right: control (1), entinostat (2), JQ1 (3), entinostat plus JQ1 (4). Densitometric analysis with ImageJ software of relative protein expression in relation to β -Actin is presented (a-b). Ratio of control was set to 1.



Shutter Time: 8 min
 Binning: 1x1
 Scan Mode: single
 Scan Number: -
 Scan Date: 2023-11-14 15:48:00



Palette: inverted Grayscale
 Range: 1349 - 2822, Gamma: 1.000

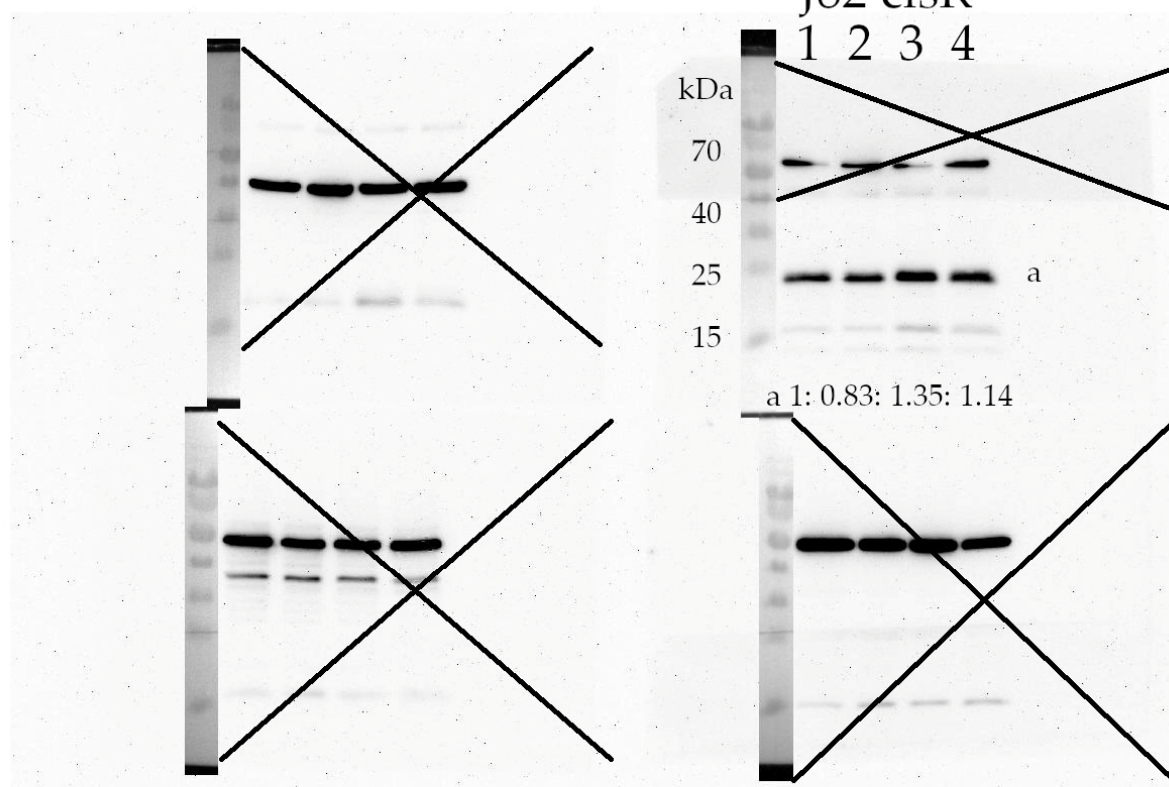
a 1: 3.13: 1.79: 2.02
 c 1: 2.20: 9.75: 6.83
 d 1: 1.10: 0.86: 0.58
 e 1: 1.47: 0.68: 0.34

Figure S10. Uncropped western blot, J82 cisR: blot at left top shows FOXO1 (a), β-Actin (b) and p21 (c), the blot below illustrates p53 (d) and survivin (e), 48 h incubation. Order of samples left to right: control (1), entinostat (2), JQ1 (3), entinostat plus JQ1 (4). Densitometric analysis with ImageJ software of relative protein expression in relation to β-Actin is presented (a; c-e). Ratio of control was set to 1.

8 min mit leiter.tif

J82 cisR

9-AUG-2022



Shutter Time: 8 min
Binning: 1x1
Scan Mode: single
Scan Number: -
Scan Date: 2022-08-09 17:00:01

Palette: inverted Grayscale
Range: 1390 - 4076, Gamma: 1.000

Figure S11. Uncropped western blot, J82 cisR: blot at right top shows at the bottom Bim (a), 48 h incubation. Order of samples left to right: control (1), entinostat (2), JQ1 (3), entinostat plus JQ1 (4). Densitometric analysis with ImageJ software of relative protein expression in relation to β -Actin is presented (a). Ratio of control was set to 1.

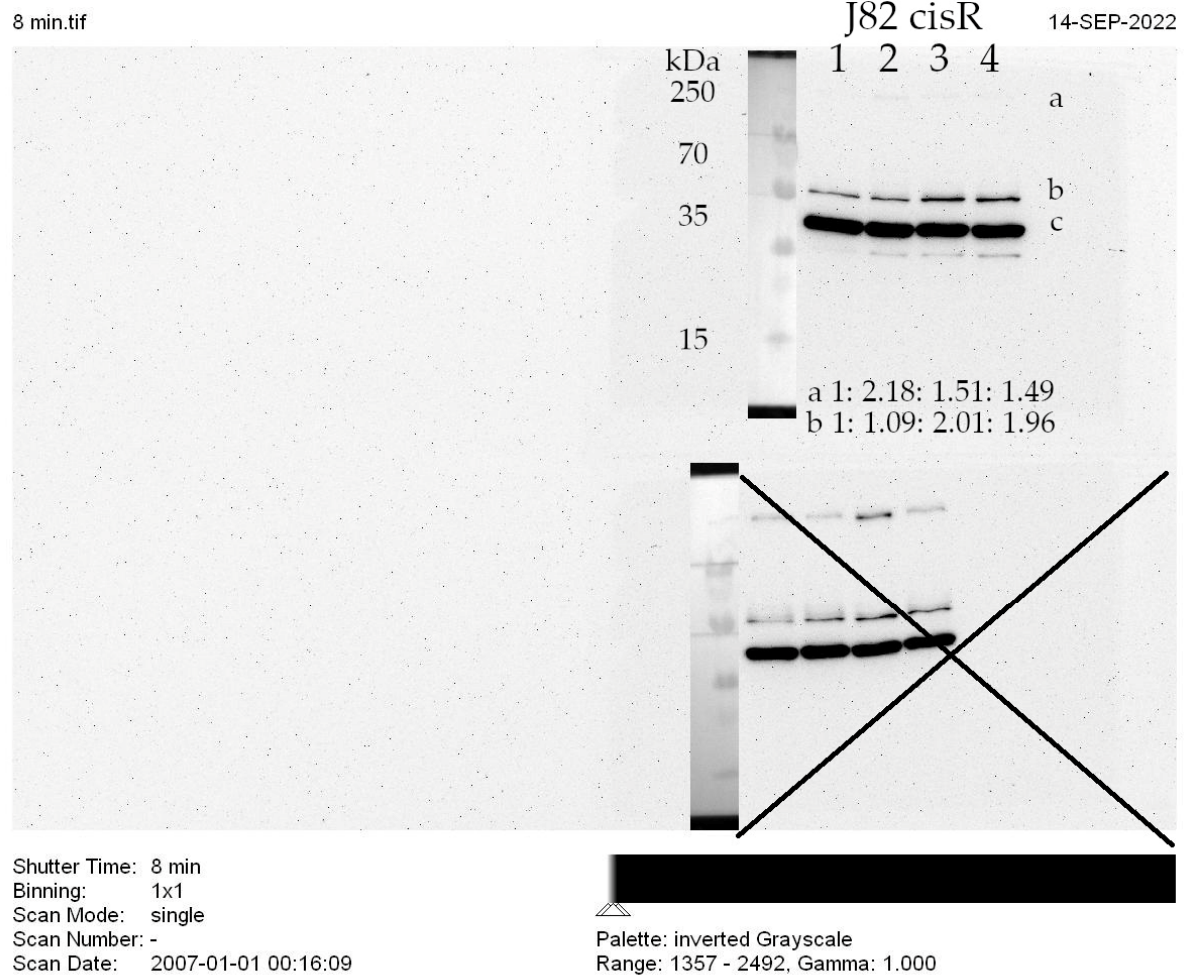


Figure S12. Uncropped western blot, J82 cisR: blot at top shows BRD (a), c-Myc (b) and β -Actin (c), 48 h incubation. Order of samples left to right: control (1), entinostat (2), JQ1 (3), entinostat plus JQ1 (4). Densitometric analysis with ImageJ software of relative protein expression in relation to β -Actin is presented (a-b). Ratio of control was set to 1.

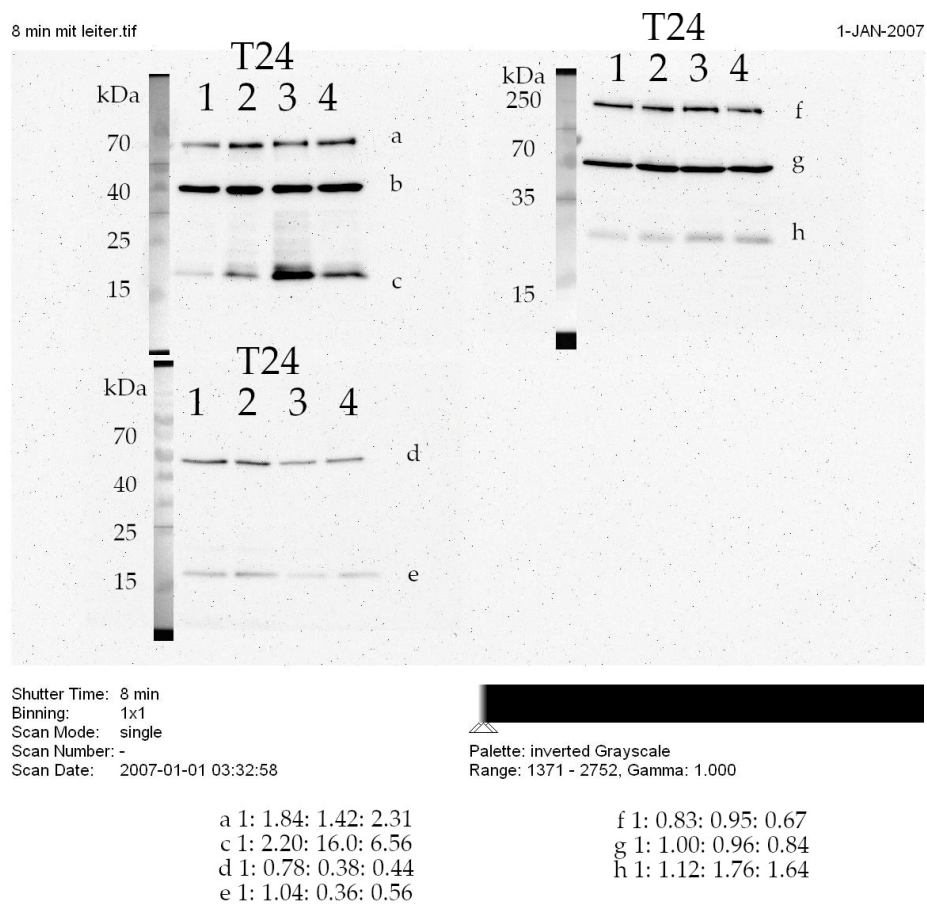


Figure S13. Uncropped western blot, T24: blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and survivin (e), the blot on the right-side displays BRD4 (f), c-Myc (g) and Bim (h), 48 h incubation. Order of samples left to right: control (1), entinostat (2), JQ1 (3), entinostat plus JQ1 (4). Densitometric analysis with ImageJ software of relative protein expression in relation to β -Actin is presented (a; c-h). Ratio of control was set to 1.

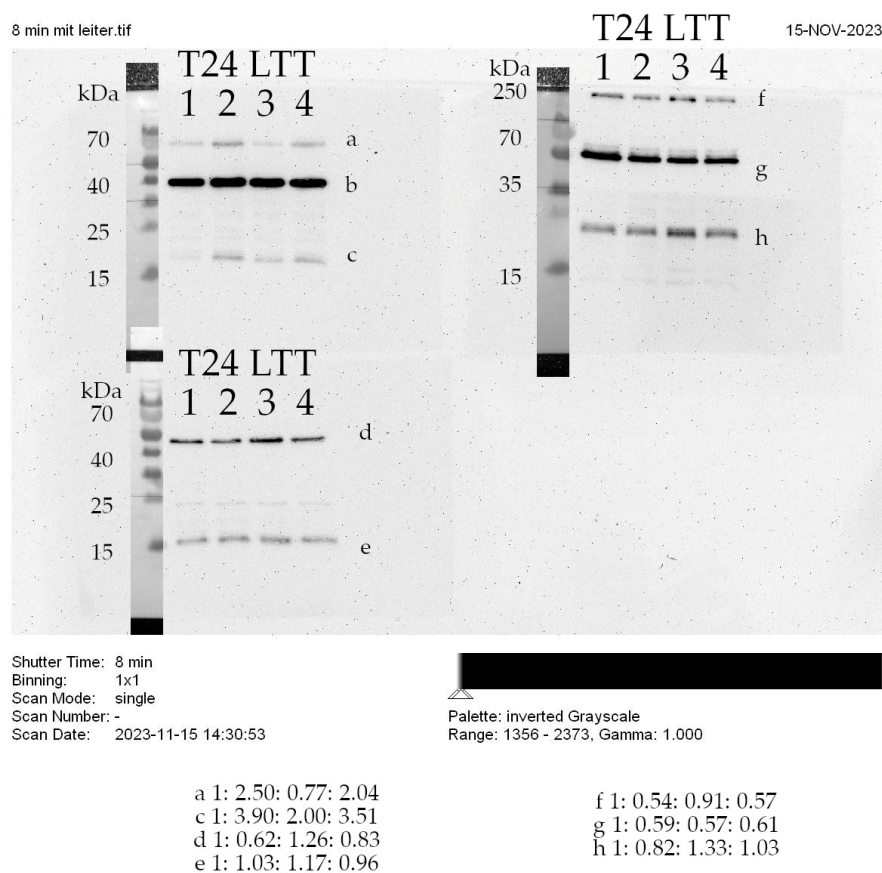


Figure S14. Uncropped western blot, T24 LTT: blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and survivin (e), the blot on the right-side displays BRD4 (f), c-Myc (g) and Bim (h), 48 h incubation. Order of samples left to right: control (1), entinostat (2), JQ1 (3), entinostat plus JQ1 (4). Densitometric analysis with ImageJ software of relative protein expression in relation to β -Actin is presented (a; c-h). Ratio of control was set to 1.

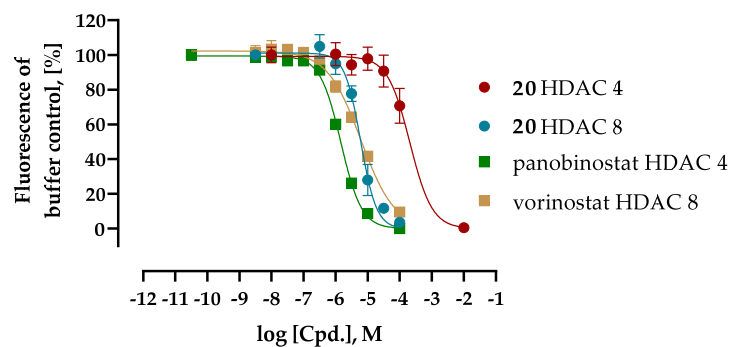


Figure S15. HDAC enzyme inhibitory activity of 20, vorinostat and panobinostat.

Table S6. HDAC enzyme inhibitory activity of **20**, vorinostat and panobinostat. Data shown are IC₅₀ values ± SD of at least two independent experiments, each carried out in duplicates. ND is not determined.

	HDAC4	HDAC8
Cpd.	IC ₅₀ ± SD	IC ₅₀ ± SD
	[μM]	[μM]
20	207 ± 50.0	6.03 ± 0.30
vorinostat	ND	6.13 ± 0.36
panobinostat	1.46 ± 0.04	ND

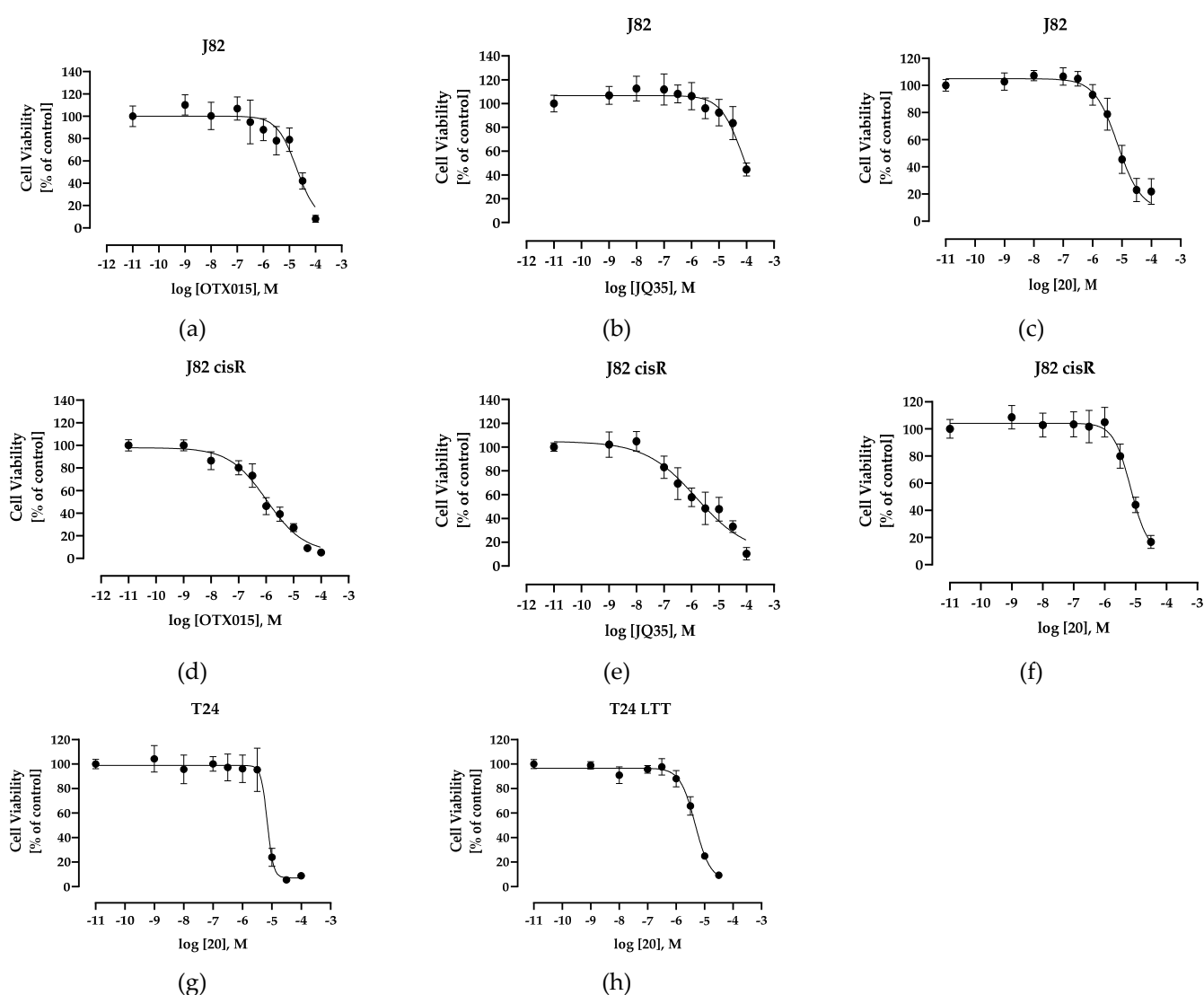


Figure S16. Concentration-effect-curves (MTT-Assay, 72 h) in J82 (a) OTX015, (b) JQ35, (c) **20**; in J82 cisR (d) OTX015, (e) JQ35, (f) **20**; in T24 (g) **20** and in T24 LTT (h) **20**. Data shown are the mean ± SD of at least three independent experiments, each carried out in triplicates.

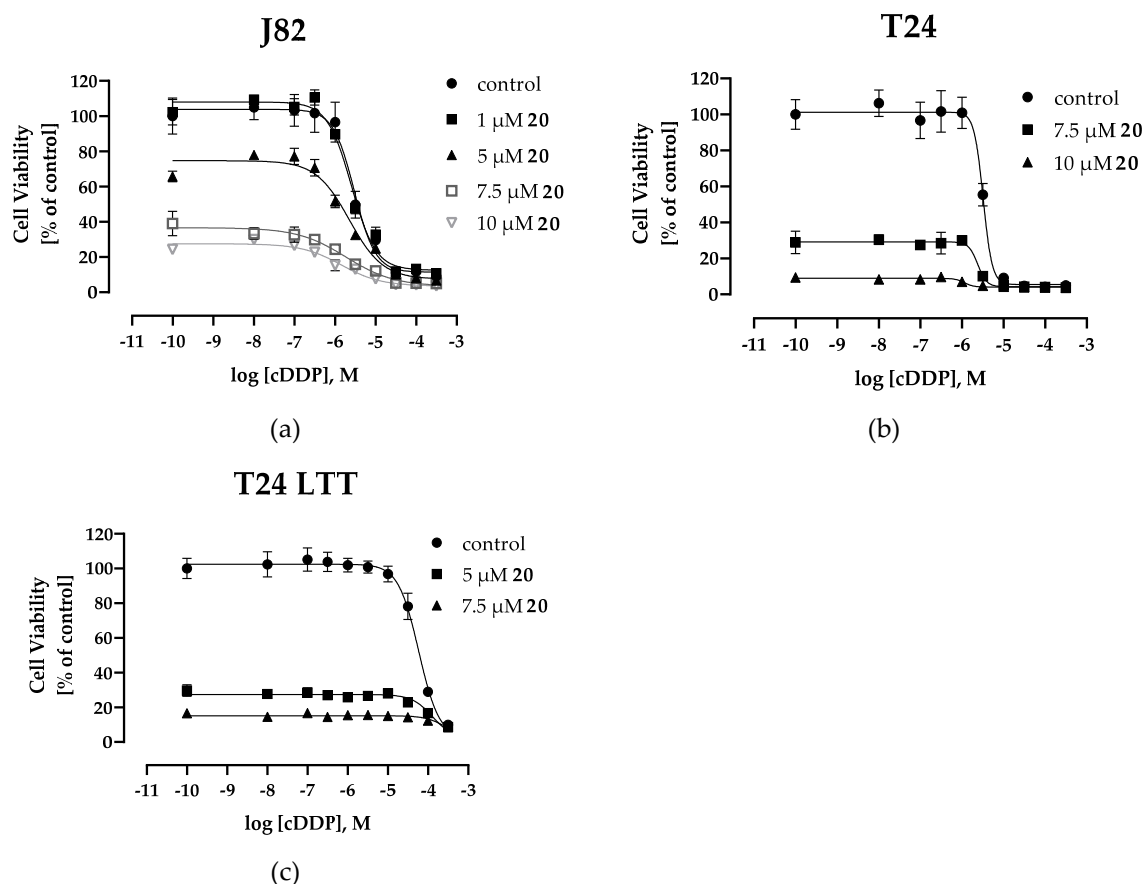


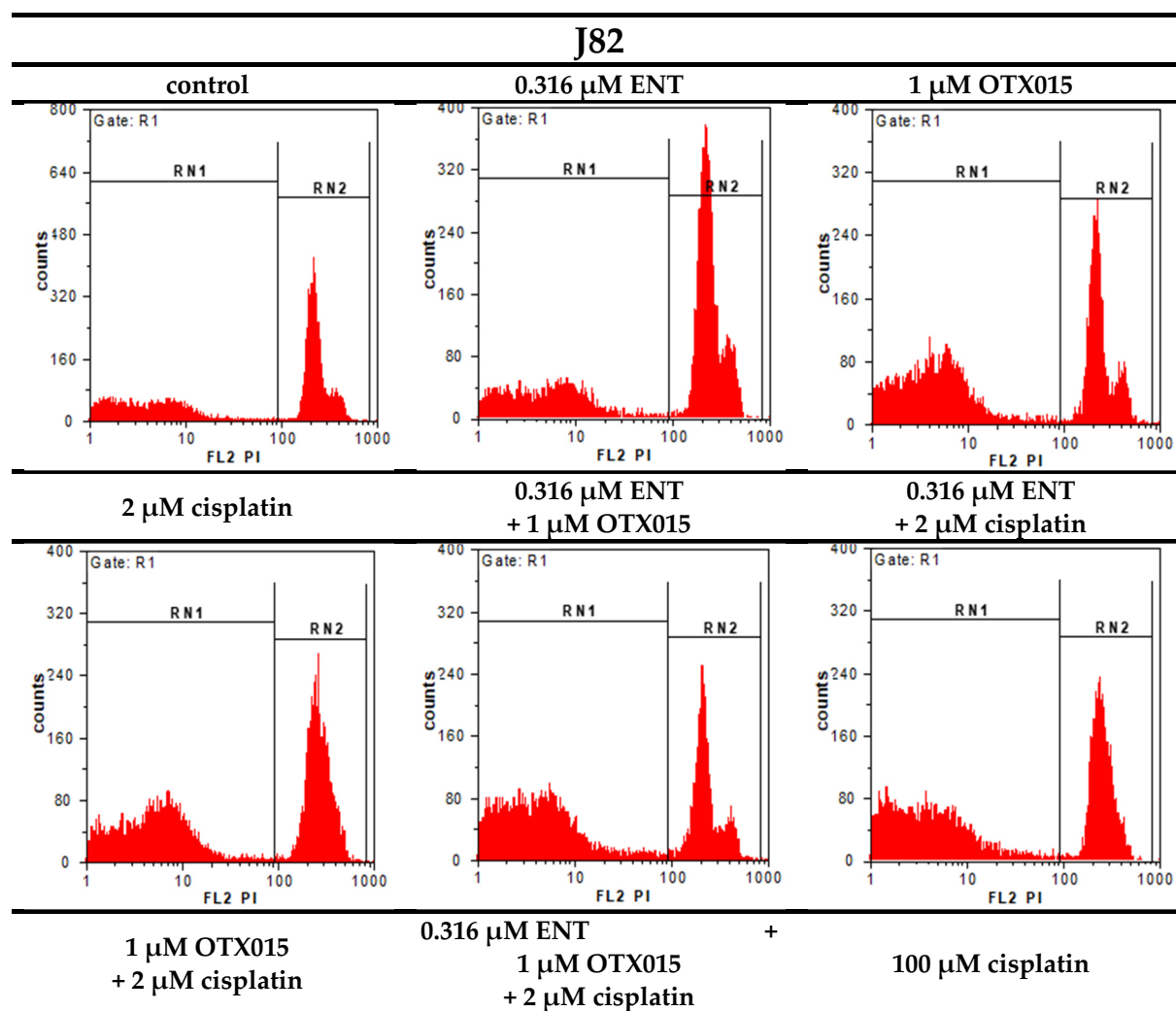
Figure S17. Concentration-effect-curves of cisplatin (MTT-Assay) of (a) J82; (b) T24; (c) T24 LTT. Cells were preincubated with 20 for 48 h followed by addition of cisplatin for another 72 h. Data shown are the mean of one representative experiment carried out in triplicates.

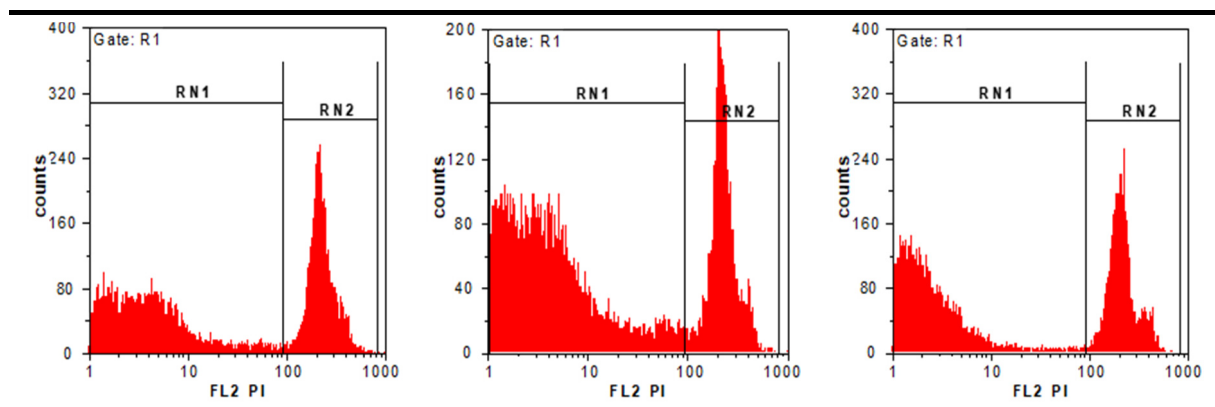
Table S7. IC₅₀ values (MTT) of cisplatin. Cells were preincubated with entinostat and/or OTX015; JQ35 for 48 h followed by addition of cisplatin for another 72 h. Control was untreated for 48 h followed by addition of cisplatin for another 72 h. Data shown are the mean $\pm$ SD of at least three independent experiments, each carried out in triplicates.

cell line	control									
	cisplatin IC ₅₀ $\pm$ SD [μ M]	ENT	cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	OTX015	cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	JQ35	cisplatin IC ₅₀ $\pm$ SD [μ M]	SF
J82	3.02 $\pm$ 0.10	0.316 μ M	1.93 $\pm$ 0.25	1.56	1 μ M	1.36 $\pm$ 0.23	2.22	3 μ M	2.18 $\pm$ 0.17	1.39
J82 cisR	7.80 $\pm$ 0.23	0.316 μ M	4.29 $\pm$ 0.18	1.82	0.1 μ M	4.17 $\pm$ 0.32	1.87	0.1 μ M	4.88 $\pm$ 0.59	1.60
					0.3 μ M	2.32 $\pm$ 0.15	3.36			

Table S8. IC₅₀ values (MTT) of cisplatin. Cells were preincubated with **20** for 48 h followed by addition of cisplatin for another 72 h. Control was untreated for 48 h followed by addition of cisplatin for another 72 h. Data shown are the mean of one representative experiment carried out in triplicates. ND = not determined.

cell line	control	+ 1 μ M 20		+ 5 μ M 20		+ 7.5 μ M 20		+ 10 μ M 20	
	cisplatin IC ₅₀ $\pm$ SD [μ M]	cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	cisplatin IC ₅₀ $\pm$ SD [μ M]	SF	cisplatin IC ₅₀ $\pm$ SD [μ M]	SF
J82	2.98 $\pm$ 0.21	2.55 $\pm$ 0.26	1.17	2.26 $\pm$ 0.38	1.32	1.86 $\pm$ 0.56	1.60	1.43 $\pm$ 0.36	2.08
J82 cisR	7.64 $\pm$ 0.38	ND	ND	3.27 $\pm$ 0.63	2.33	1.47 $\pm$ 0.76	5.19	ND	ND
T24	3.23 $\pm$ 0.10	ND	ND	ND	ND	2.40 $\pm$ 0.22	1.34	1.15 $\pm$ 0.39	2.82
T24 LTT	58.7 $\pm$ 2.60	ND	ND	128 $\pm$ 18.4	0.46	285 $\pm$ 31.8	0.21	ND	ND





J82 cisR

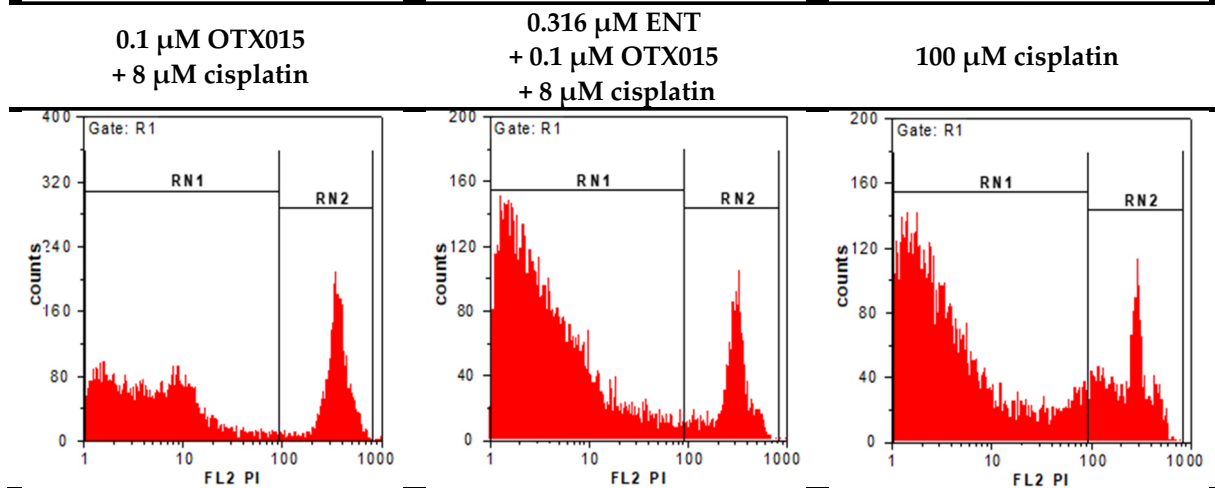
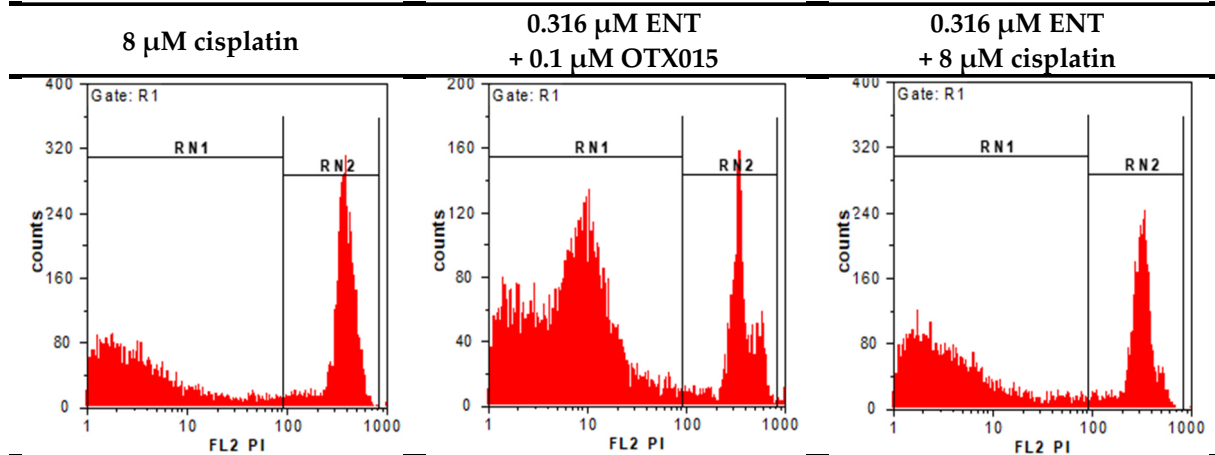
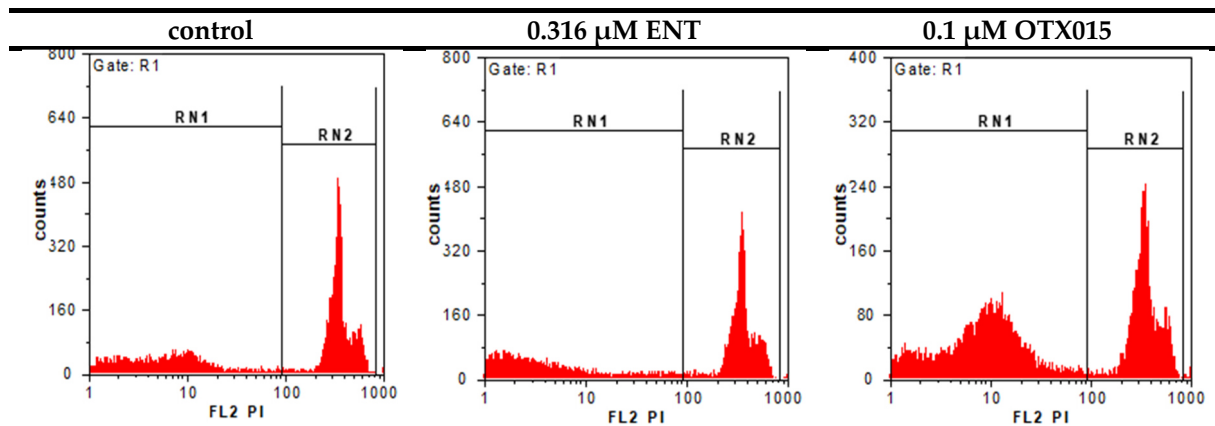
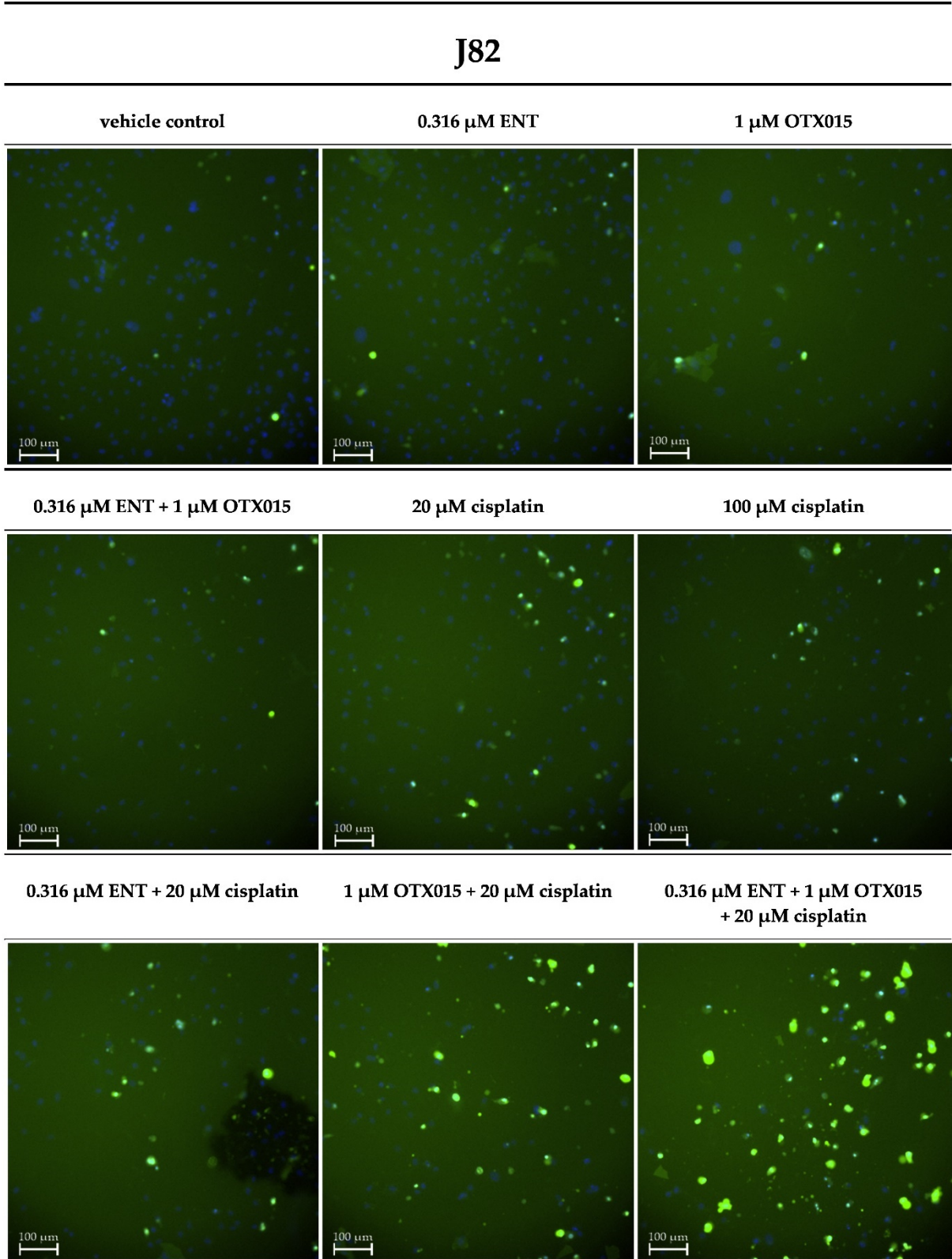
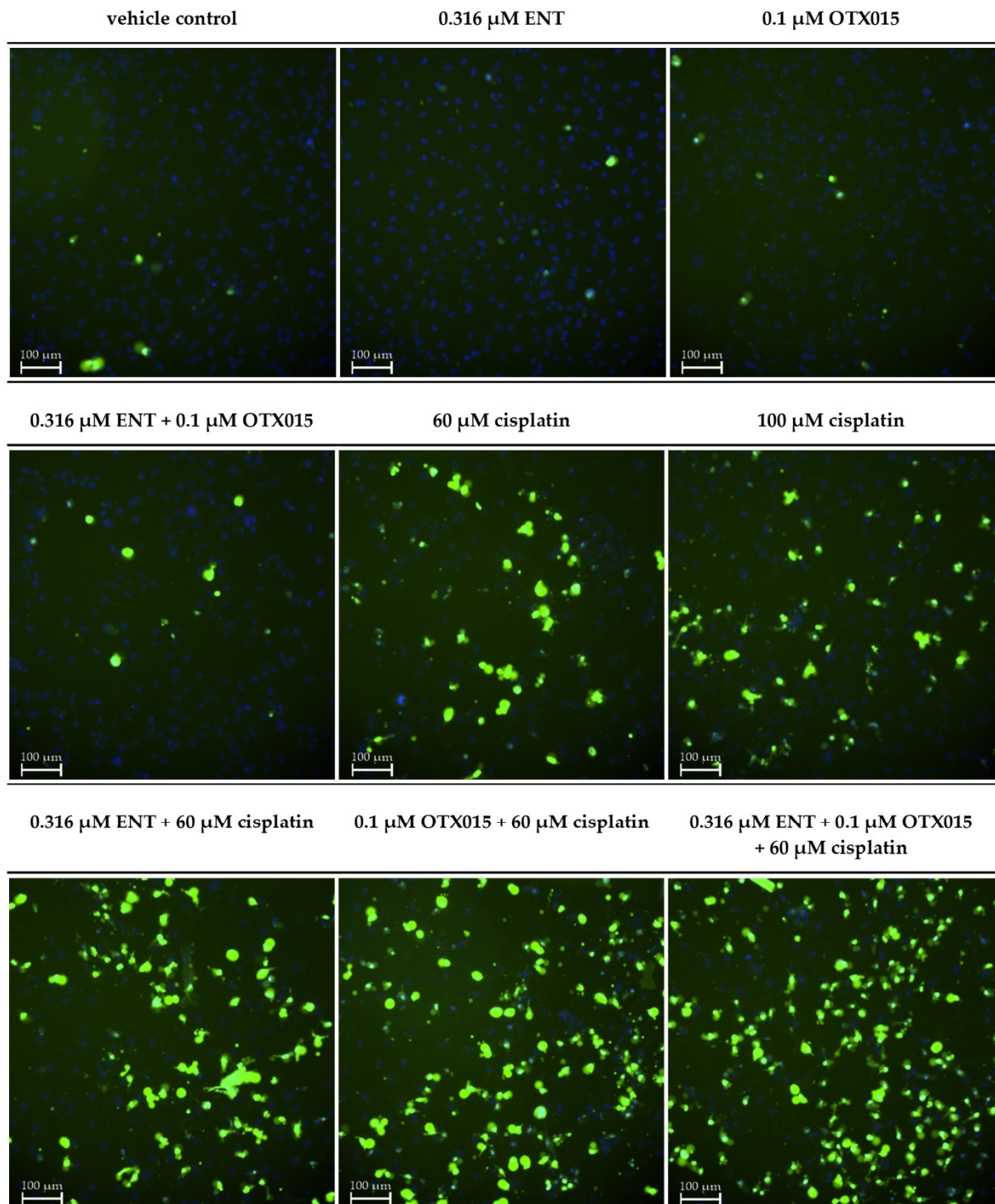


Figure S18. Representative cytometry flow images are shown for control, entinostat, OTX015, cisplatin and their combination treatments in J82 and J82 cisR. Cells were preincubated with entinostat and JQ1 for 48 h followed by addition of cisplatin for another 24 h. RN1 was defined as the subG1 fraction.

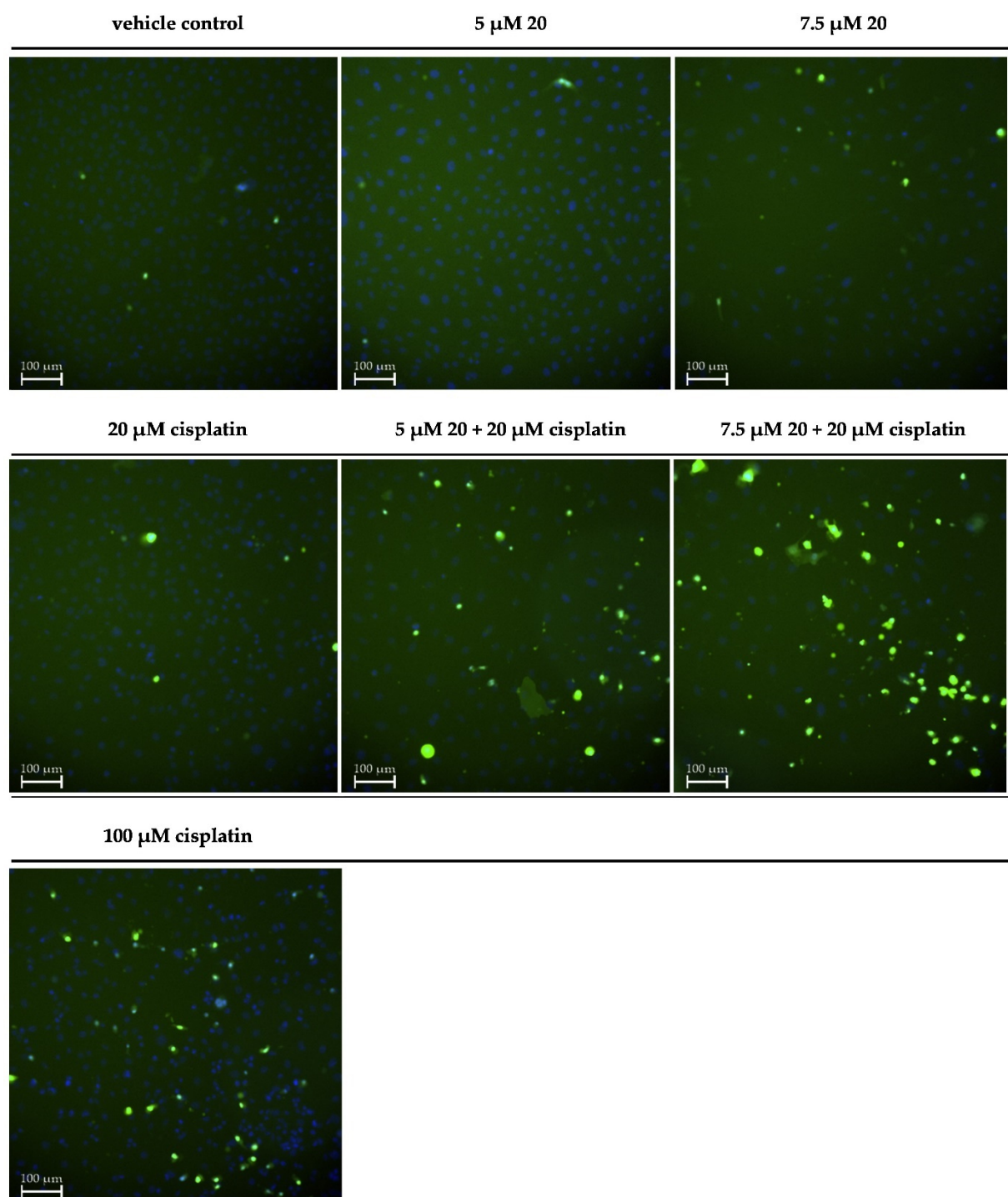


J82 cisR

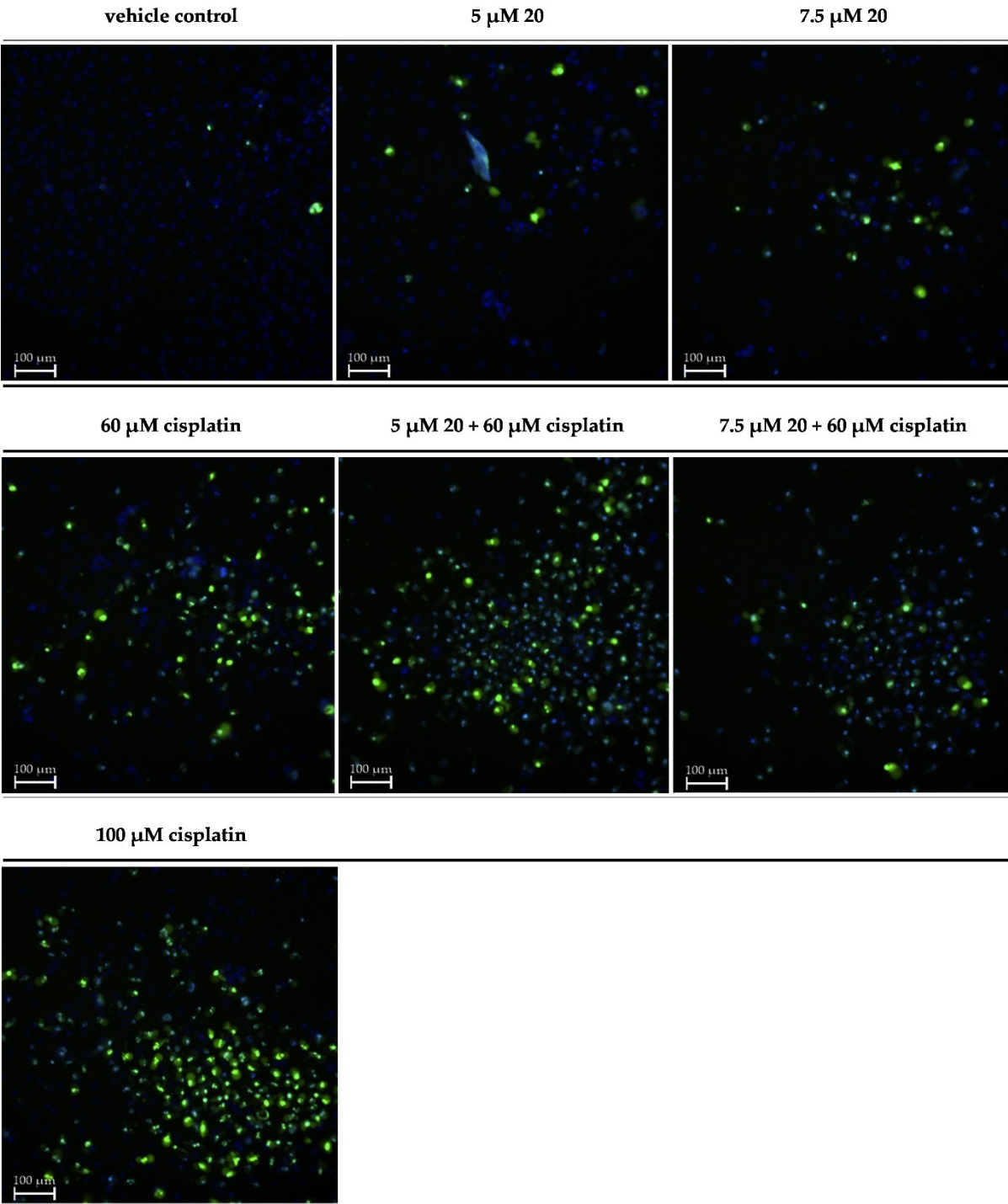


(a)

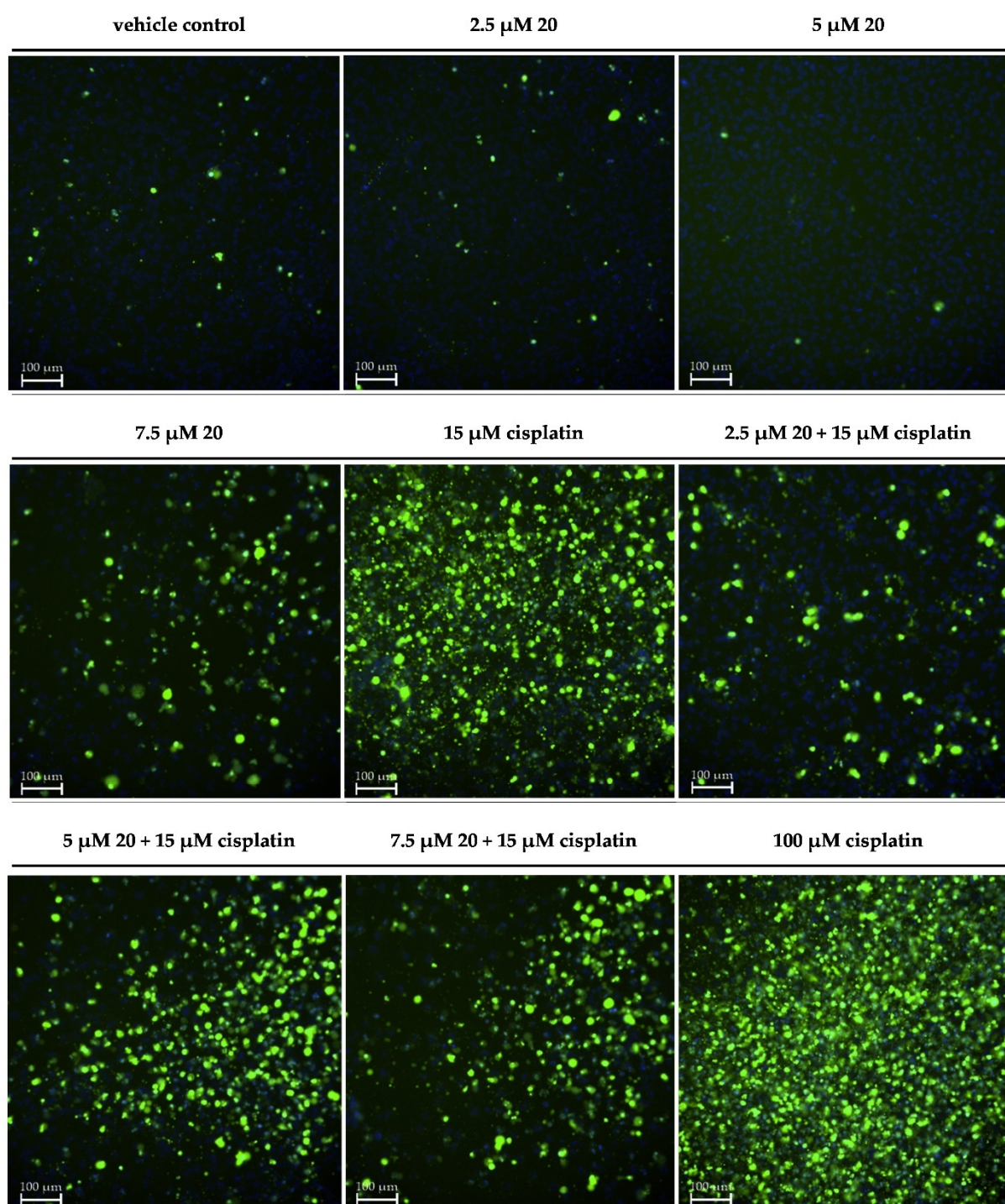
J82



J82 cisR



T24



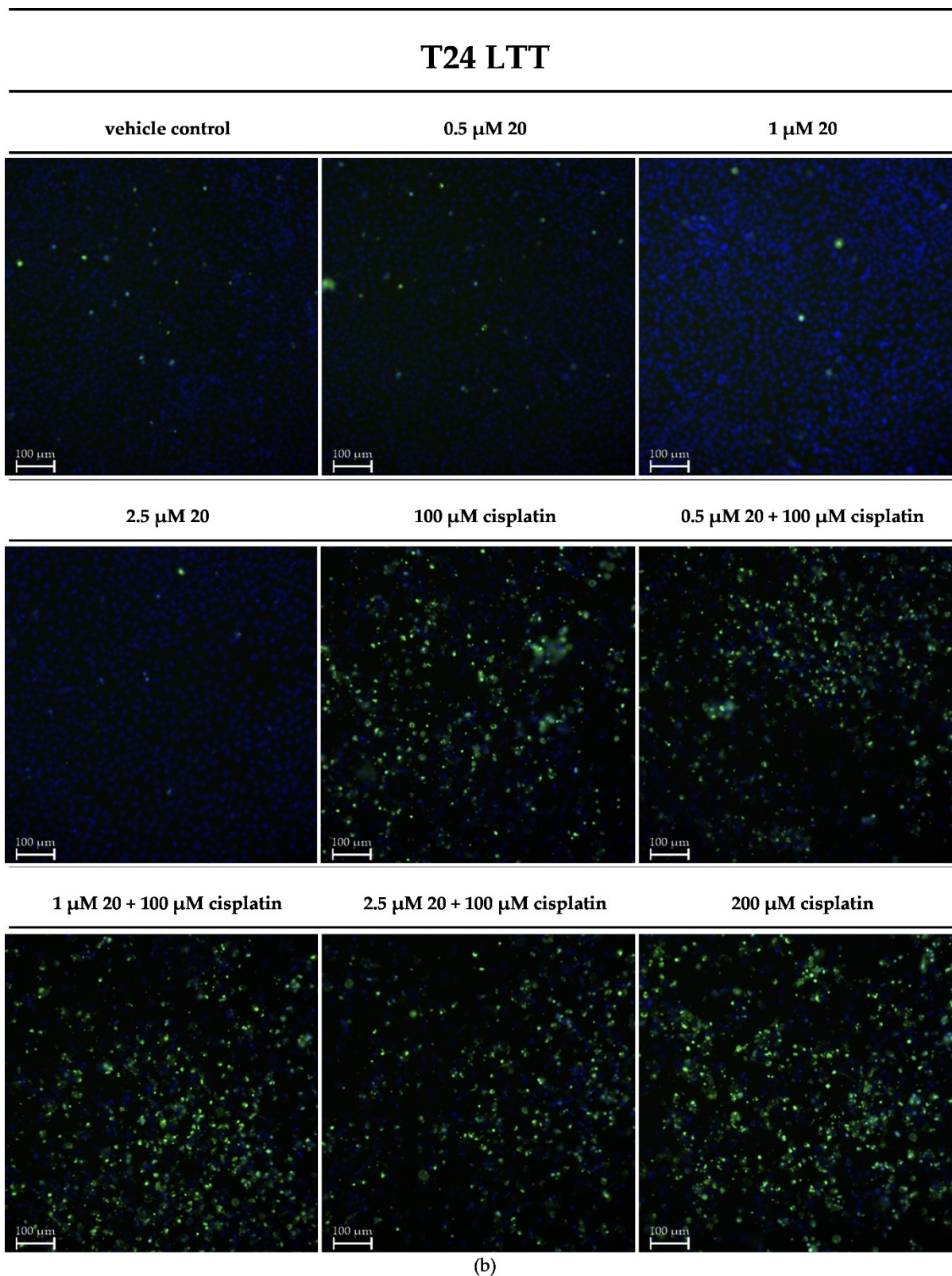


Figure S19. (a) Representative fluorescent imaging pictures (Thermofisher Arrayscan XTI) are shown for vehicle control, entinostat, OTX015, cisplatin and their combination treatments in J82, J82 cisR. Cells were preincubated with entinostat and OTX015 for 48 h followed by addition of cisplatin for another 24 h. **(b)** Representative fluorescent imaging pictures (Thermofisher Arrayscan XTI) are shown for vehicle control, 20, cisplatin and their combination treatments in J82, J82 cisR, T24 and T24 LTT. Cells were preincubated with 20 for 48 h followed by addition of cisplatin for another 24 h (T24 LTT 48 h). Nuclei were stained using Hoechst 33342 (blue). Caspase 3/7 activation is presented in green.

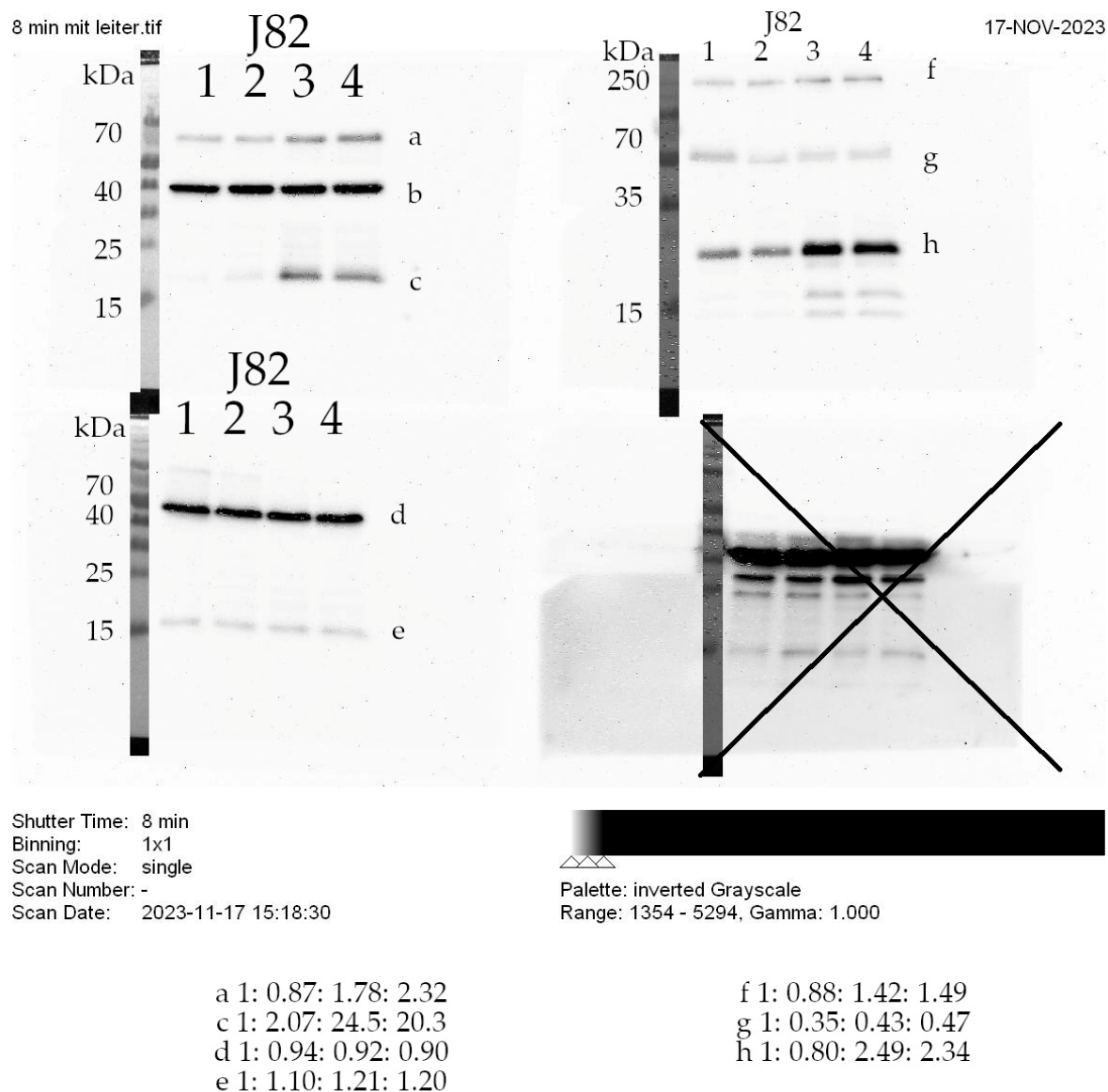


Figure S20. Uncropped western blot, J82, blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and survivin (e), the blot on the right-side displays BRD4 (f), c-Myc (g) and Bim (h), 48 h incubation. Order of samples left to right: control (1), entinostat (2), OTX015 (3), entinostat plus OTX015 (4). Densitometric analysis with ImageJ software of relative protein expression in relation to β -Actin is presented (a; c-h). Ratio of control was set to 1.

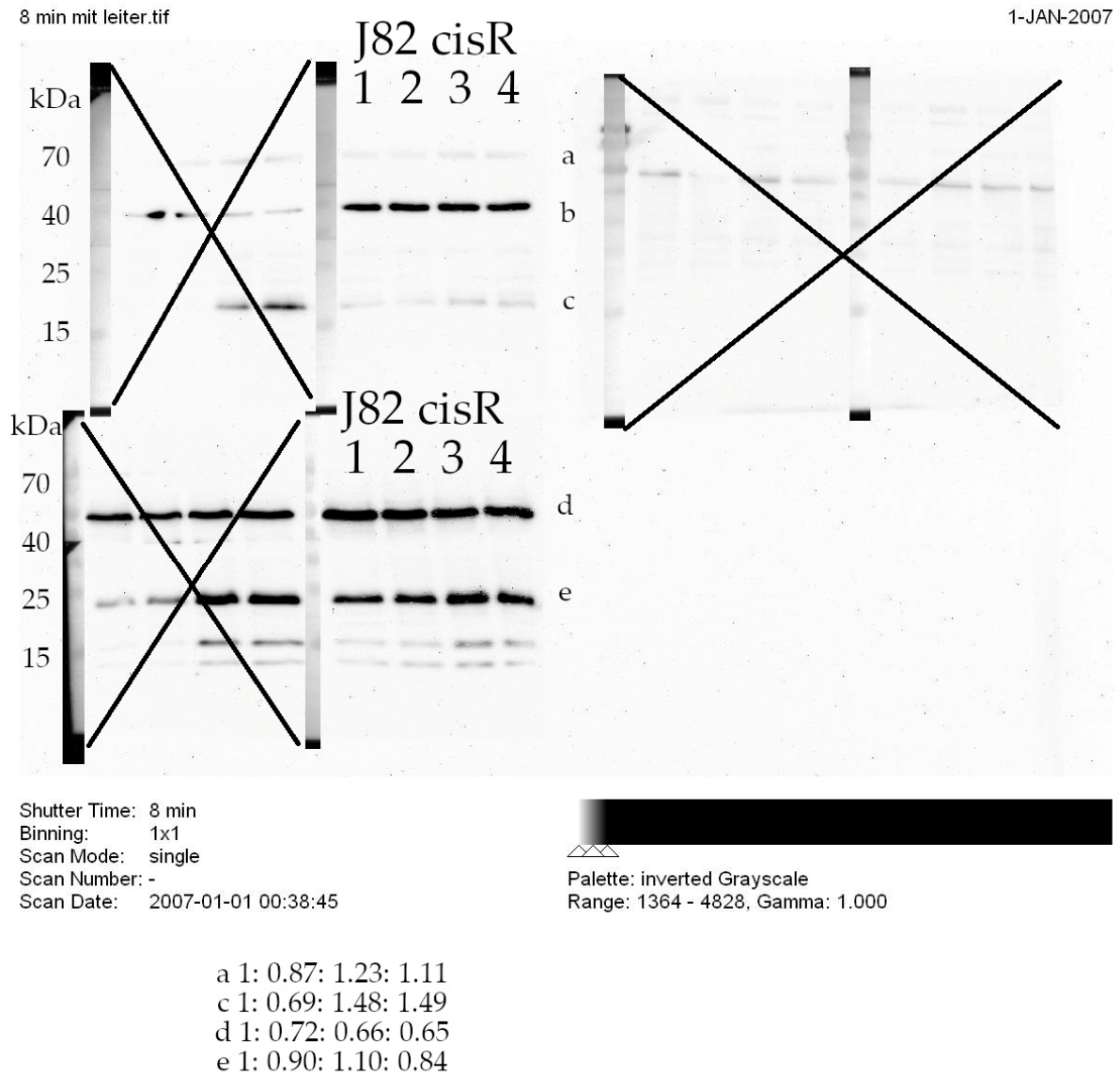


Figure S21. Uncropped western blot, J82 cisR, blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and Bim (e), 48 h incubation. Order of samples left to right: control (1), entinostat (2), OTX015 (3), entinostat plus OTX015 (4). Densitometric analysis with ImageJ software of relative protein expression in relation to β -Actin is presented (a; c-e). Ratio of control was set to 1.

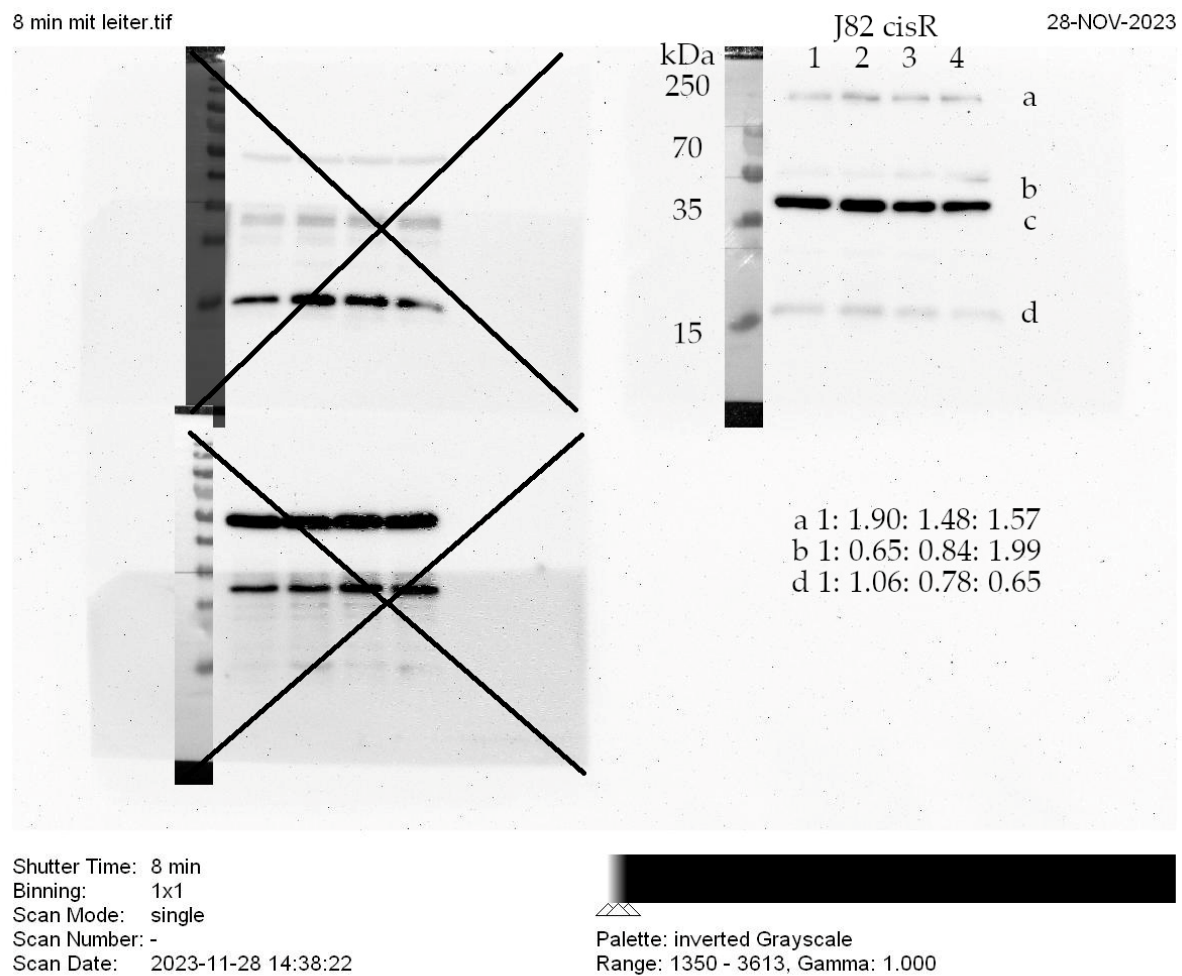


Figure S22. Uncropped western blot, J82 cisR, the blot on the right-side displays BRD4 (a), c-Myc (b), β -Actin (c) and survivin (d), 48 h incubation. Order of samples left to right: control (1), entinostat (2), OTX015 (3), entinostat plus OTX015 (4). Densitometric analysis with ImageJ software of relative protein expression in relation to β -Actin is presented (a-b; d). Ratio of control was set to 1.

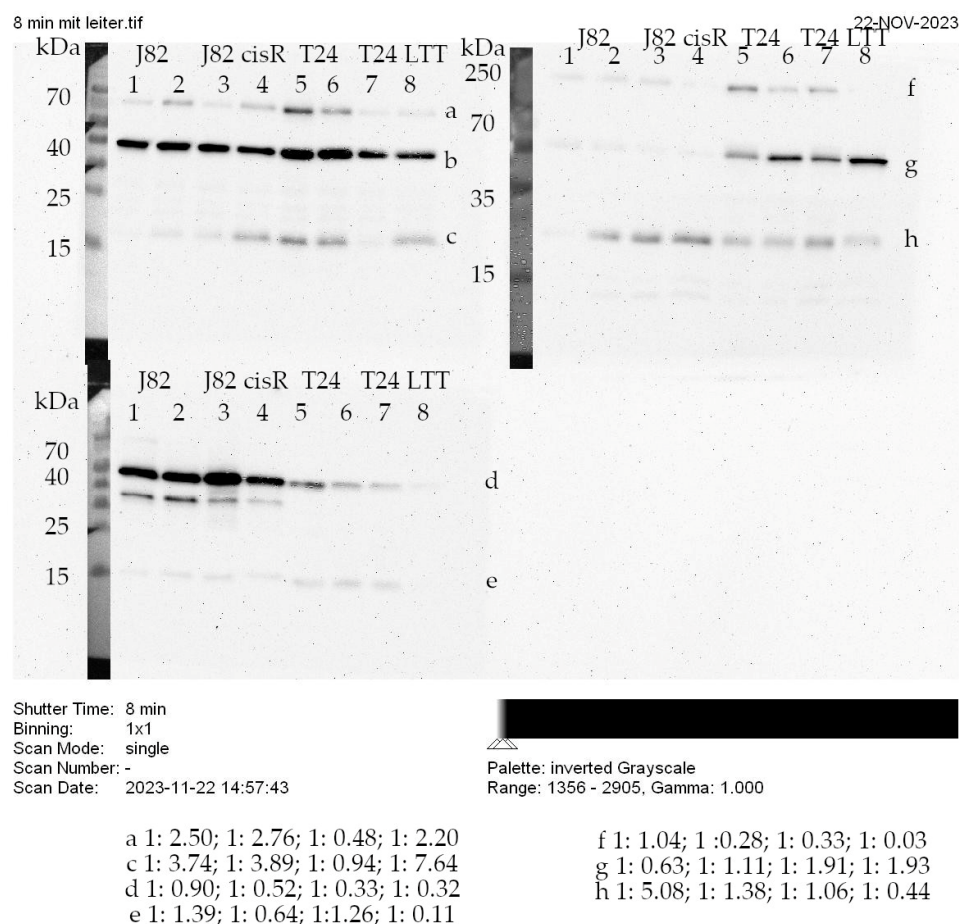


Figure S23. Uncropped western blot, J82, J82 cisR, T24 and T24 LTT, blot at left top shows FOXO1 (a), β -Actin (b) and p21 (c), the blot below illustrates p53 (d) and survivin (e), the blot on the right side displays BRD4 (f), c-Myc (g) and Bim (h), 48 h incubation. Order of samples left to right: control (1), 5 μ M **20** (2), control (3), 5 μ M **20** (4), control (5), 5 μ M **20** (6), control (7), 5 μ M **20** (8). Densitometric analysis with ImageJ software of relative protein expression in relation to β -Actin is presented (a; c-h). Ratio of control was set to 1.

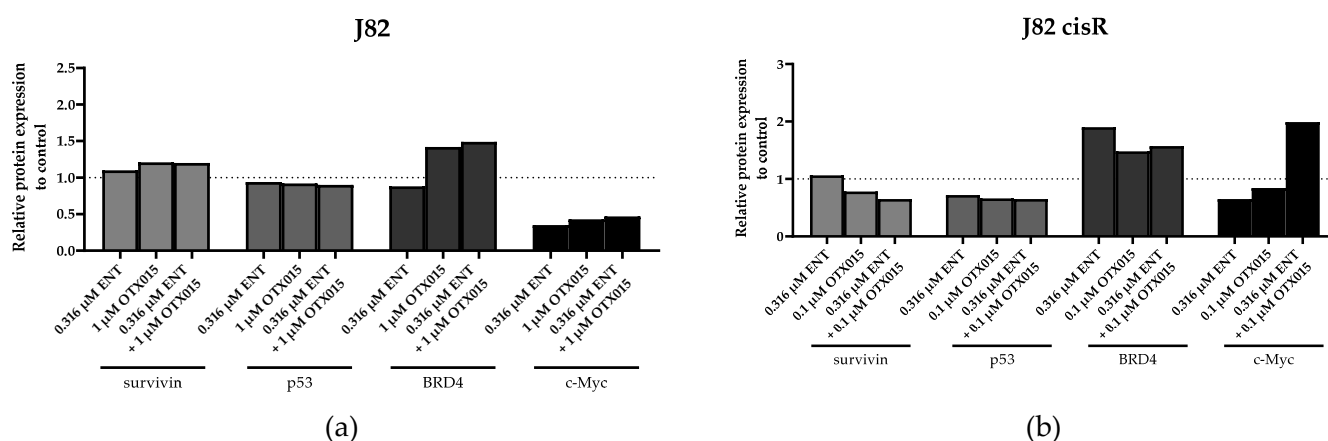


Figure S24. The relative protein expression of proteins of interest related to β -actin as loading control is shown in (a) J82, (b) J82 cisR. Ratio of control was set to 1, represented by the dashed line. Cells were incubated with entinostat and OTX015 for 48 h. The western blots were analyzed with ImageJ software. The data presented a representative blot out of at least two different protein samples.

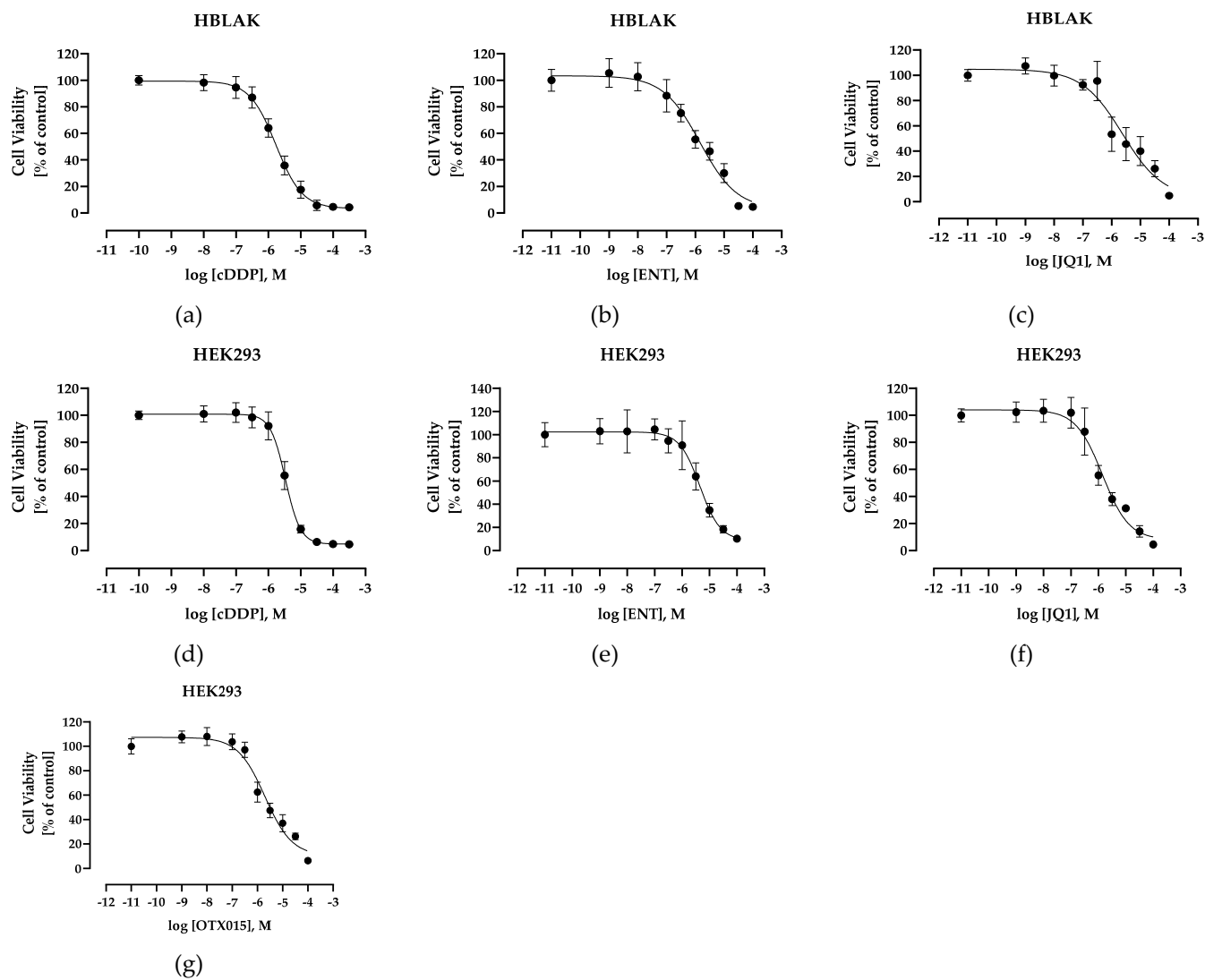


Figure S25. Concentration-effect-curves determined by 72 h MTT-Assay in (a)-(c) HBLAK and (d)-(g) HEK293. Data shown are the mean $\pm$ SD of at least three independent experiments, each carried out in triplicates.

4. Discussion and Conclusion

Two different cancer cell types, AML and muscle-invasive bladder carcinoma (MIBCa), were selected for combination therapies comprising HDACi. Both AML, with a low 5-year survival rate of 30%, and MIBCa, the tenth most common cancer in the world, require new innovative therapeutic approaches to improve OS or overcome drug resistance [18, 19]. The findings for AML are presented first, followed by the results for MIBCa.

4.1 Combination of Class IIa HDACi with the PI BTZ

BTZ is a highly cytotoxic agent whose application may be limited by its cytotoxicity. Therefore, it seems reasonable to combine BTZ with agents that selectively increase the cytotoxicity of BTZ in cancer cells to allow a dose reduction of BTZ to minimize adverse effects. Class IIa HDACi may be suitable combination partners as they may be superior to pan- or class I HDACi due to lower toxicity. The combination of the class IIa HDACi TMP269 and the PI carfilzomib has already been tested in a study in multiple myeloma. It was shown to induce increased apoptosis [104, 164]. This approach of combining class IIa HDACi and PI was applied to the treatment of three AML cell lines (THP-1, HL-60, MONO-MAC-6) and one chronic myeloid leukemia cell line (K562). The study *The Novel Class IIa Selective Histone Deacetylase Inhibitor YAK540 Is Synergistic with Bortezomib in Leukemia Cell Lines*, characterized the new class IIa selective HDACi YAK540 for the first time [210]. YAK540 was designed based on the structure of TMP269 with a trifluoromethyloxadiazole (TFMO) group as the ZBG to improve the selectivity for class IIa HDACs and the physicochemical properties such as improved solubility compared to TMP269. YAK540 did not cause solubility problems at the concentrations used as TMP269 did. BTZ was selected as combination partner as the first approved PI for multiple myeloma. Several clinical trials with BTZ in AML have already been completed, (NCT05982756; NCT04173585 [213, 214]) while others are still ongoing, such as the combination of BTZ with sorafenib and decitabine and CPX-351. (NCT01861314; NCT01371981; NCT07008638 [215-217]). TMP269 was included as the first representative of the class IIa HDACi with a TFMO ZBG.

Compared to TMP269, YAK540 demonstrated a lower IC_{50} value for HDAC4 inhibition (0.114 μ M vs. 0.157 μ M). The selectivity index of YAK540 for HDAC4 over HDAC2 (265-fold), HDAC6 (100-fold), and HDAC8 (82-fold) was at least a factor of 82. For TMP269, the selectivity index for HDAC4 over HDAC2 (> 637-fold), HDAC6 (52-fold), and HDAC8 (27-fold) was overall lower compared to YAK540, with the exception of HDAC2. These results indicate an increased selectivity of YAK540 compared to TMP269. YAK540 demonstrated overall only a slight cytotoxic effect on the leukemia cell lines (mostly > 100 μ M). TMP269 on the other hand was more cytotoxic (IC_{50} values

4. Discussion and Conclusion

around 20 μM). In contrast to both of the aforementioned compounds, the IC_{50} values of BTZ were in the double-digit nanomolar range (Table 1).

Table 1. Cytotoxic activity (MTT, 72 h) of BTZ, YAK540, TMP269. Data shown are the mean of pooled data from $n \geq 4$. Table taken from [210].

Cell line	Bortezomib		YAK540		TMP269	
	IC_{50} [nM]	$\text{pIC}_{50} \pm \text{SEM}$	IC_{50} [μM]	$\text{pIC}_{50} \pm \text{SEM}$	IC_{50} [μM]	$\text{pIC}_{50} \pm \text{SEM}$
HL-60	10.7	7.97 ± 0.006	> 100		16.7	4.78 ± 0.016
K562	20.9	7.68 ± 0.014	> 100		23.0	4.64 ± 0.024
MONO-MAC-6	12.4	7.91 ± 0.012	91.4	4.04 ± 0.022	15.9	4.80 ± 0.023
THP-1	6.28	8.20 ± 0.020	> 100		13.0	4.89 ± 0.035

In the following experiments, the combination of YAK540 or TMP269 with BTZ induced significantly more caspase 3/7-mediated apoptosis than the single treatments in the AML cancer cell line THP-1 (Figure 22).

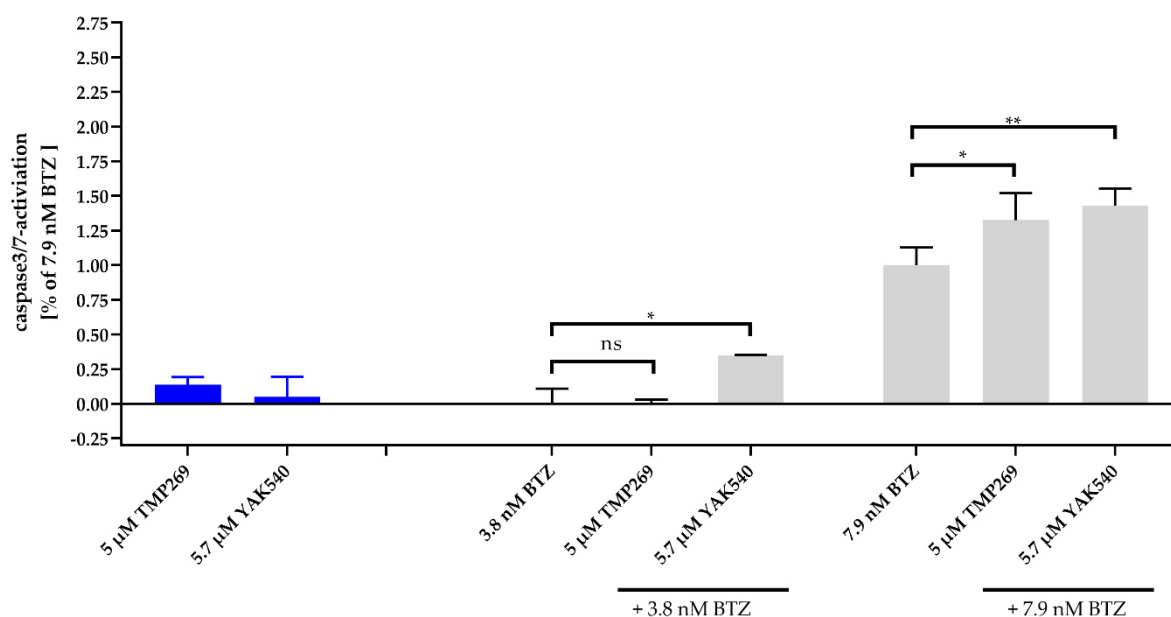


Figure 22. Caspase 3/7-activation in THP-1 cells. Data are presented as a ratio of caspase activation to the effect of 7.9 nM BTZ. Cells were incubated for 48h. Untreated control was subtracted, and data normalized to 7.9 nM BTZ. Data shown is the mean $\pm$ SD, $n \geq 2$. Statistical analysis was performed by t-test with significance level: ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$). Figure taken from [210].

Therefore, the literature-known results of increased apoptosis induction in multiple myeloma could be confirmed in AML cells for the combination of class IIa HDACi and a PI [164]. Subsequent synergism analyses according to Chou-Talalay showed synergistic effects for the combination treatment with YAK540 or TMP269 and BTZ in all four leukemia cell lines. Figure 23 illustrates the calculated synergy levels with the Bliss model in combenefit for YAK540 in all four leukemia cell lines [218, 219].

4. Discussion and Conclusion

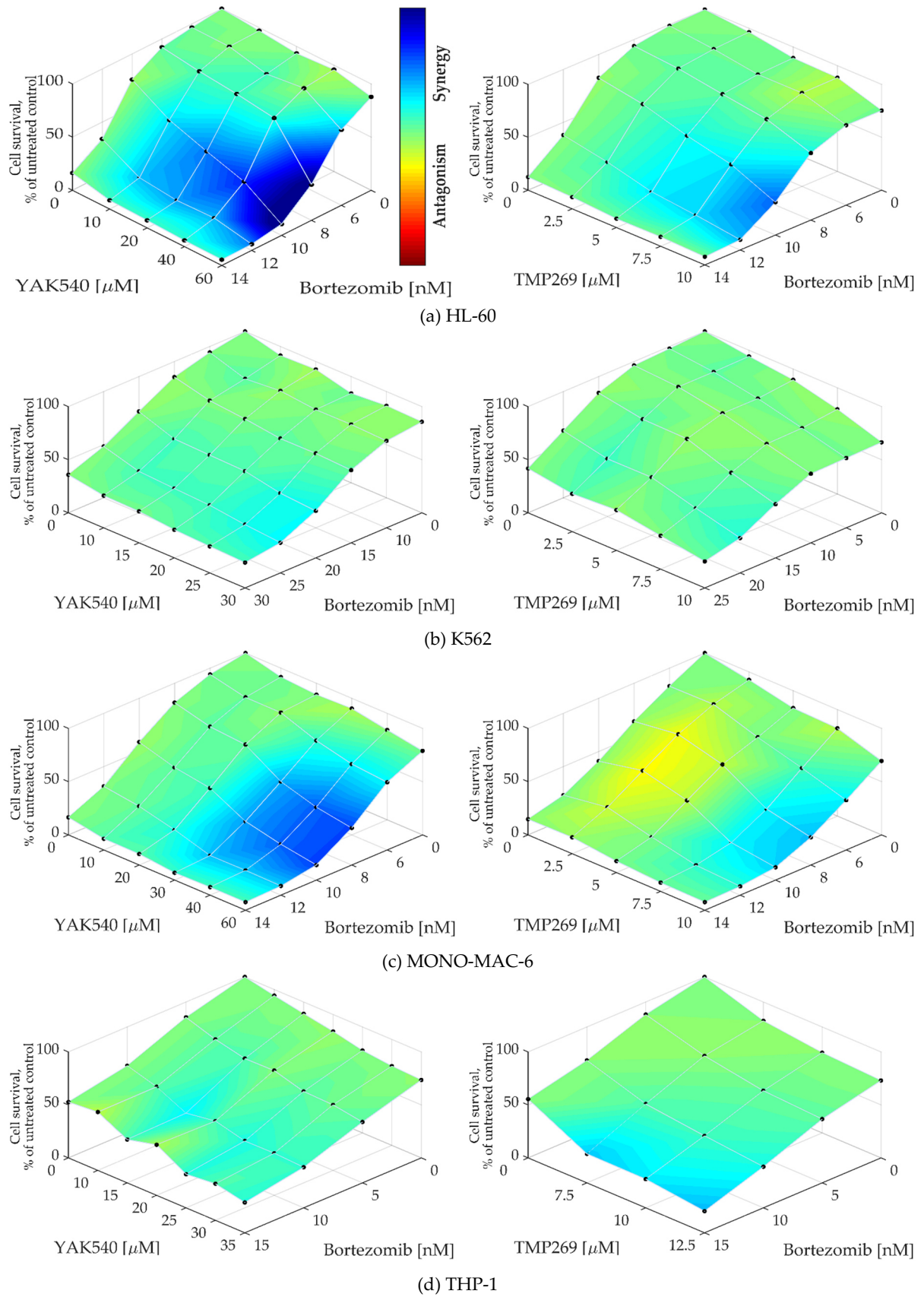


Figure 23. Synergy levels were calculated with the Bliss model [219] in Combeneft [218], depending on experimental concentration-response surface of combinations of BTZ and YAK540 or TMP269 (72 h incubation) in **(a)** HL-60, **(b)** K562, **(c)** MONO-MAC-6 and **(d)** THP-1 cell line. Data shown are from at least two independent experiments, each carried out at least in duplicates. Figure taken and modified from [210].

4. Discussion and Conclusion

The calculated synergy levels were higher overall in all chosen leukemia cell lines (with the exception of THP-1) when YAK540 is combined with BTZ as opposed to TMP269, as evidenced by the more intense blue coloration when comparing the illustrations for the respective cell line. Therefore, the combination of BTZ plus YAK540 revealed a more potent synergistic effect than the combination of BTZ plus TMP269.

Further, the combination of YAK540 and BTZ increased the protein expression of p21 in the leukemia cell lines HL-60, MONO-MAC-6 and K562. In contrast, enhanced p21 expression by the combination of TMP269 and BTZ was only observed in the MONO-MAC-6 cell line (Figure 24).

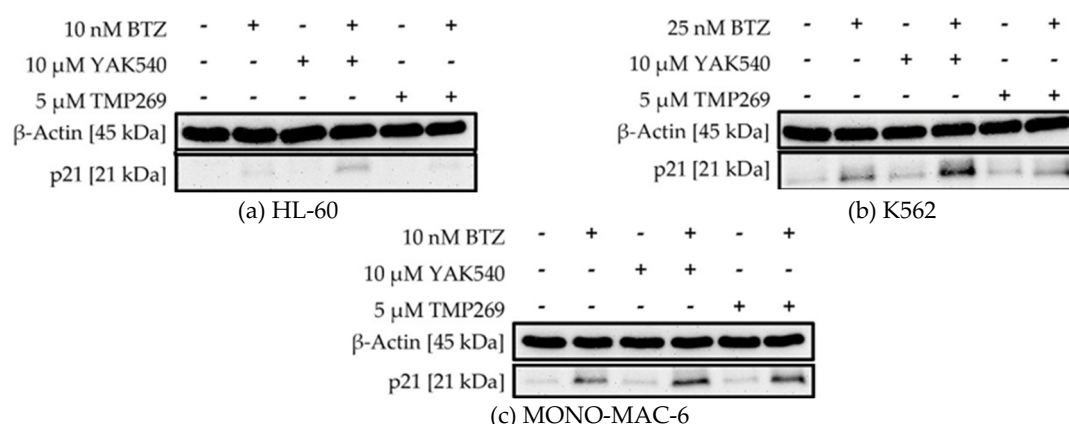


Figure 24. Western Blot analysis of (a) HL-60, (b) K562 and (c) MONO-MAC-6 after treatment with BTZ and YAK540 or TMP269 for 24 h. The data presents one representative blot out of two. Figure taken and modified from [210].

p21 leads to cell cycle arrest and can result in reduced cell viability in tumor cells [220]. Increased levels of p21 by pan- or class I-HDACi have been observed before [221-225]. This study demonstrated that the treatment of cells with the combination of class IIa HDACi YAK540 or TMP269 and BTZ resulted in elevated p21 levels. Prior studies have already demonstrated that the silencing or downregulation of HDAC4 resulted in increased p21 levels [226, 227]. Consequently, our findings confirms that augmented p21 expression can be accomplished by employing class IIa HDACi. This indicates that enhanced p21 expression can be attained through the use of pan- or class I HDACi, as well as class IIa HDACi. In contrast to pan- or class I-HDACi, one special feature of class IIa HDACi is their lower toxicity (e.g. YAK540 $IC_{50} \geq 91.4 \mu$ M), which could be advantageous in a clinical context.

Finally, the combination of the class IIa HDACi YAK540 or TMP269 and BTZ was shown to be significantly more cytotoxic in cancer cells than in the non-cancer cell line HEK293 (Figure 25). This suggests a potential selective toxicity of BTZ in combination with class IIa HDACi such as YAK540 in cancer cells compared to non-cancerous cells.

4. Discussion and Conclusion

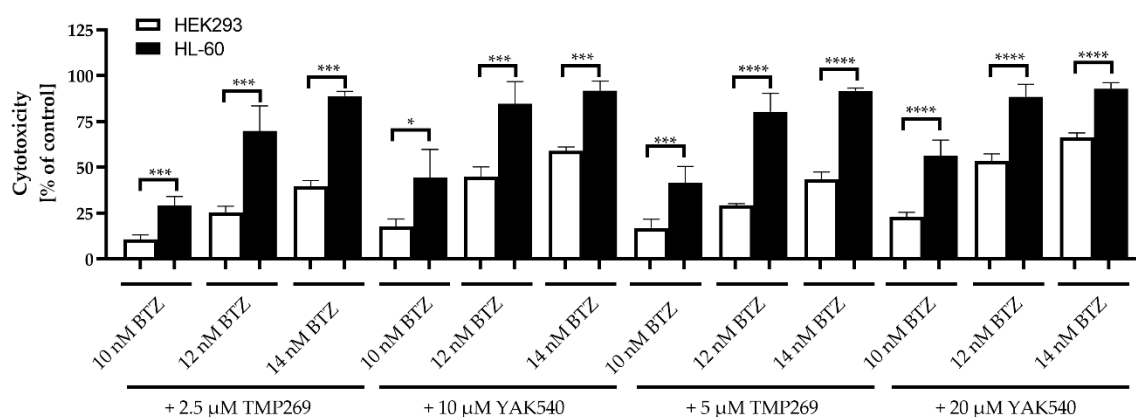


Figure 25. Comparison of cytotoxicity (MTT assay) of the combination BTZ and class IIa HDACi YAK540 or TMP269 in HL-60 (black bars) and HEK293 (white bars) cell line after 72 h incubation. Data shown is the mean $\pm$ SD, $n \geq 3$. Statistical analysis was conducted by t-test with significance levels: * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$).

In summary, the novel class IIa HDACi YAK540 was found to be more class IIa HDAC selective than TMP269. Further, YAK540 is clearly less cytotoxic ($IC_{50} > 91 \mu M$) and induces higher p21 expression than TMP269. YAK540 in combination with the PI BTZ also shows superior synergistic effects in several AML and one CML cell line. Thus, the combination of a low-toxic class IIa HDACi such as YAK540 with a proteasome inhibitor such as BTZ may represent a new therapeutic option in the future reducing BTZ toxicity by synergistic co-treatment.

Based on these results, further para-substituted TFMO-based HDACi were synthesized using the structure of YAK540 and TMP269 in the study *5-(trifluoromethyl)-1,2,4-oxadiazole (TFMO)-based highly selective class IIa HDAC inhibitors exhibit synergistic anticancer activity in combination with bortezomib* [211]. The aim was to identify more potent and selective class IIa HDACi than TMP269 or YAK540. To this end, a series of synthesized compounds were characterized.

FFK24 had the lowest IC_{50} value at HDAC4 of all TFMO compounds in the series with 12 nM. Other groups had also worked on the optimization of TFMO compounds, resulting in compound 12 with an IC_{50} of 10 nM for HDAC4 and NT160 with an IC_{50} of 0.08 nM for HDAC4 [162, 163]. Accordingly, the potency of FFK24 at HDAC4 is lower than that of NT160 but in the same range as that of compound 12. Nevertheless, FFK24 demonstrated good selectivity for HDAC4 over HDAC2, HDAC6 and HDAC8 (at least a factor of 364). Further studies of the synthesized compound series in cancer cells revealed the highest cellular HDAC-inhibition for FFK24 (IC_{50} value of 0.077 μM) and a 142-fold selectivity for class IIa HDACs. The most promising compounds of the series (FFK24, 1b, 2a and 2c) were characterized with an extended HDAC enzyme profile underlining the class IIa HDAC selectivity of these compounds (Table 2).

4. Discussion and Conclusion

Table 2. HDAC enzyme inhibitory activity profile of frontrunner compounds. Data presented are the mean of at least two experiments performed in duplicates. SD is < 15 % of the mean. # Some data of YAK540 and TMP269 is taken from [210, 228]; vorinostat data is taken from [229], ND is not determined. Table taken from [211].

Cpd.	IC ₅₀ [μM]								
	Class I				Class IIa			Class IIb	Class IV
	HDACs				HDACs			HDAC	HDAC
	1	2	3	8	4	5	7	6	11
FFK24	4.02	6.13	3.82	4.26	0.012	0.019	0.046	5.79	66.0
1b	14.6	8.13	6.86	>100	0.025	0.154	0.096	2.03	>100
2a	>100	>100	>100	>100	0.118	1.09	1.97	>100	>100
#YAK540	20.7	30.2	15.0	9.39	0.114	0.268	0.284	11.4	>100
2c	2.21	9.77	2.36	7.13	0.141	0.151	0.263	18.1	>100
TMP269	>100	>100#	>100	4.2#	0.157#	0.097	0.043	8.2#	>100
#vorinostat	0.111	0.146	0.01	11.9	42.2	ND	ND	0.102	ND

The next step was to investigate the cytotoxicity of the selected compounds. FFK24 showed an IC₅₀ of 9.24 μM in THP-1 cells (MTT assay), the lowest IC₅₀ of all TFMO derivatives analyzed, even compared to the reference compounds TMP269 and YAK540. Further, FFK24 exhibited the most promising results overall. Since the combination treatment of class IIa HDACi with BTZ has already been proven to be synergistic in AML cells, FFK24 was combined with BTZ in further cellular experiments to confirm the findings [164, 210]. For comparison, YAK540 and TMP269 were used as additional class IIa HDACi. The strongest synergistic effect was observed with the combination of FFK24 and BTZ (Figure 26a).

4. Discussion and Conclusion

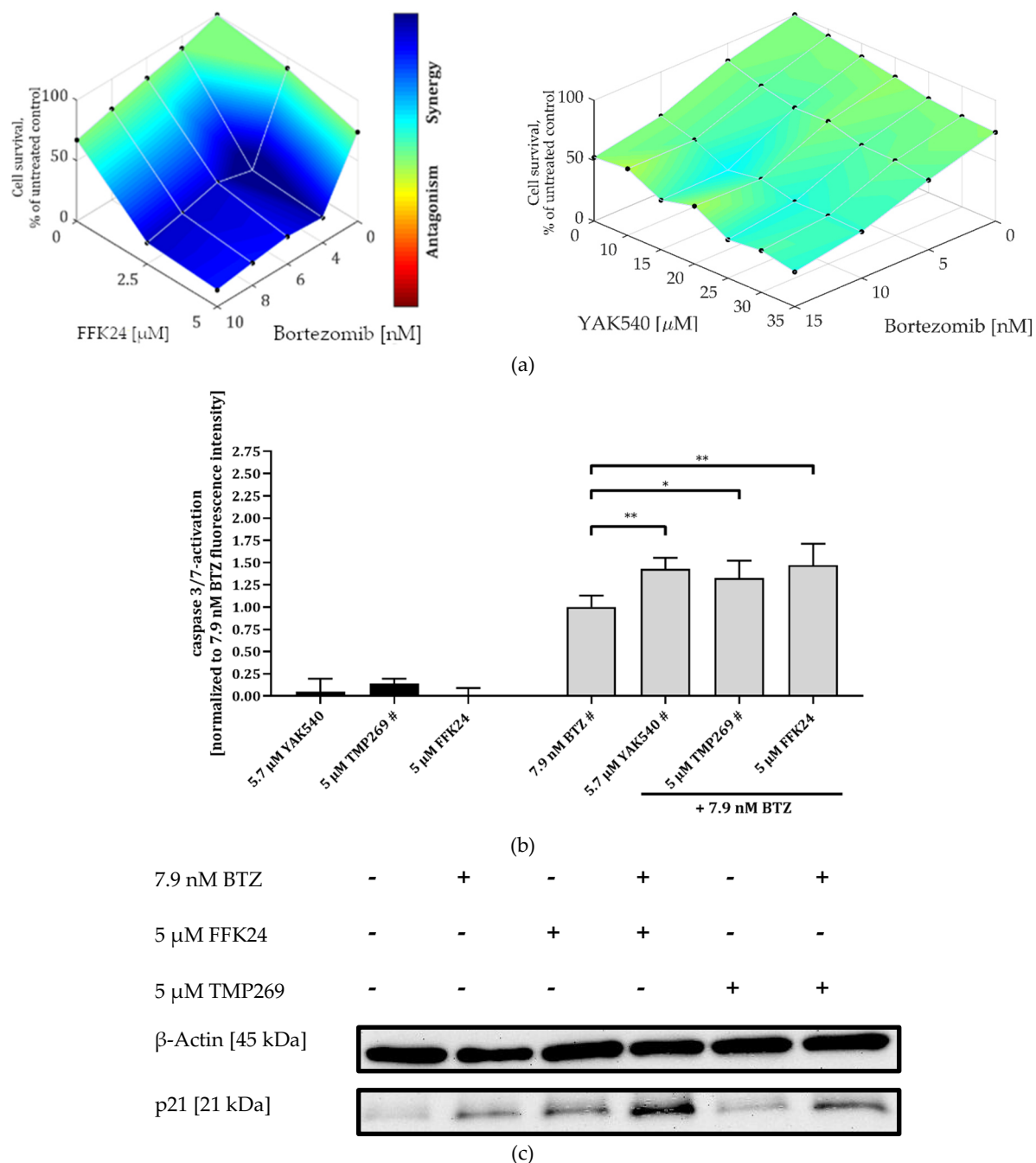


Figure 26. (a) Synergy levels calculated with Bliss model [219] in Combeneft [218], depending on experimental concentration-response surface of combinations of BTZ and FFK24 or YAK540 in THP-1 cell line for 72 h. Data shown are from at least two independent experiments each carried out at least in duplicates. (b) Caspase3/7-activation by single and combination treatment of FFK24, YAK540 or TMP269 and BTZ. THP-1 cells were co-incubated for 48 h. The presented data are normalized to the effect of 7.9 nM BTZ and are the mean $\pm$ SD. $n \geq 2$ each with at least 3 replicates. Statistical analysis was performed using t-test. Level of significance: * ($p \leq 0,05$); ** ($p \leq 0,01$); # Data taken from [210]. (c) Western Blot analysis of p21 in THP-1 cells after treatment with 5 μ M FFK24 or 5 μ M TMP269 alone or in combination with 7.9 nM BTZ for 24 h. Data presented is a representative blot out of two. Figures taken and modified from [211].

As previously demonstrated, the synergistic effect can be explained by an increased induction of apoptosis through enhanced caspase 3/7 activation (Figure 26b) [210]. Further mechanistic investigations revealed higher expression of p21 at both gene and protein levels when BTZ and FFK24 were combined. FFK24 led to a greater increase in

4. Discussion and Conclusion

expression of p21 than TMP269 (Figure 26c). Additionally, FFK24, the most promising TFMO HDACi of the series, was evaluated in solid tumor cells with high HDAC4 expression. An HDAC4 overexpression clone of the head and neck cancer cell line Cal27 (Cal27_HDAC4) was used next to the native vector control cell line.

A previous study from our group with the high HDAC4 expression cell line Cal27_HDAC4 has shown that the combination of the class IIa HDACi CHDI0039 and BTZ enhanced caspase 3/7 activation particularly in cells with increased HDAC4 expression [230]. Therefore, we wanted to confirm these results with the more selective and more potent class IIa HDACi FFK24. FFK24 and BTZ indeed exhibited a significantly higher cytotoxicity (MTT assay) in Cal27_HDAC4 than in the native Cal27 cell line (IC_{50} FFK24: 8.63 μ M (HDAC4 overexpression) vs. 22.5 μ M (native HDAC4), BTZ: 4.73 nM vs. 7.60 nM). In contrast, the compound YAK540 was not significantly different toxic in native or HDAC4 overexpressing Cal27 cells and overall less toxic (77.7 μ M and 69.8 μ M, respectively). Subsequent synergism studies according to Chou Talalay revealed that the combination of BTZ and the class IIa HDACi FFK24 was only synergistic if HDAC4 was overexpressed. However, when CHDI0039 was used in the same experimental setup, a synergistic effect independent of HDAC4 expression could be observed [230]. Further, there were differences between FFK24 and CHDI0039 in terms of cell growth inhibition. FFK24 inhibited the HDAC4 clones 2.6-fold more than the control cells, whereas CHDI0039 had a comparable effect in both cell types.

Moreover, FFK24 was investigated in the native Cal27 and the HDAC4 overexpressing Cal27 cell lines in the *in vivo* chorio-allantoic membrane assay (Figure 27).

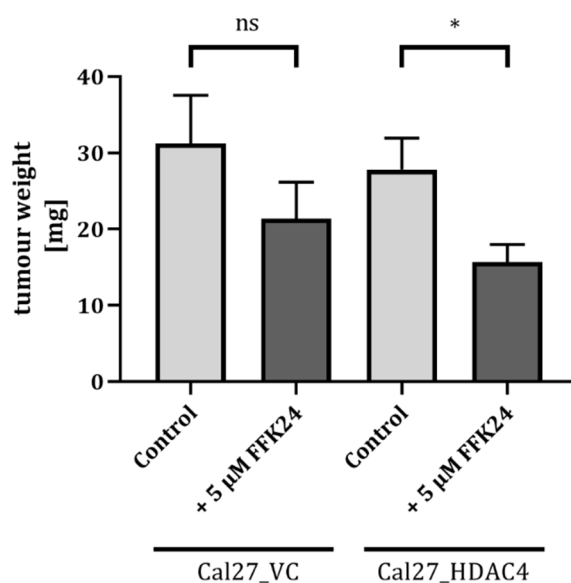


Figure 27. Chorio-allantoic membrane assay of FFK24 in Cal27_VC and Cal27_HDAC4 cells. Data shown are mean tumour weight in mg with $n \geq 5$. Statistical analysis was performed using t-test. Level of significance: ns ($p > 0,05$); * ($p \leq 0,05$). Figure taken from [211].

4. Discussion and Conclusion

The treatment with FFK24 resulted in significantly reduced tumor growth in Cal27_HDAC4, but not in native Cal27. Interestingly, these data are consistent with data from our previous study in which CHDI0039 was evaluated as a class IIa HDACi in the chorio-allantoic membrane assay on the same cell lines [230]. Overall, our data suggest that class IIa HDACi may demonstrate enhanced efficacy in cancer cells with HDAC4 overexpression.

The widely accepted pharmacophore model for class IIa selective HDACi incorporates a ZBG, a linker, a lower pocket group, and a cap [104, 210]. Although CHDI0039 and FFK24 are potent class IIa HDACi and designed according to the pharmacophore model, they may exert different phenotypic effects. The main difference between them is the ZBG (Figure 28). CHDI0039 binds the zinc atom in class IIa HDACs via the hydroxamic acid group, whereas the TFMO moiety of FFK24 and YAK540 acts as a non-chelating ZBG [161, 210, 211, 231].

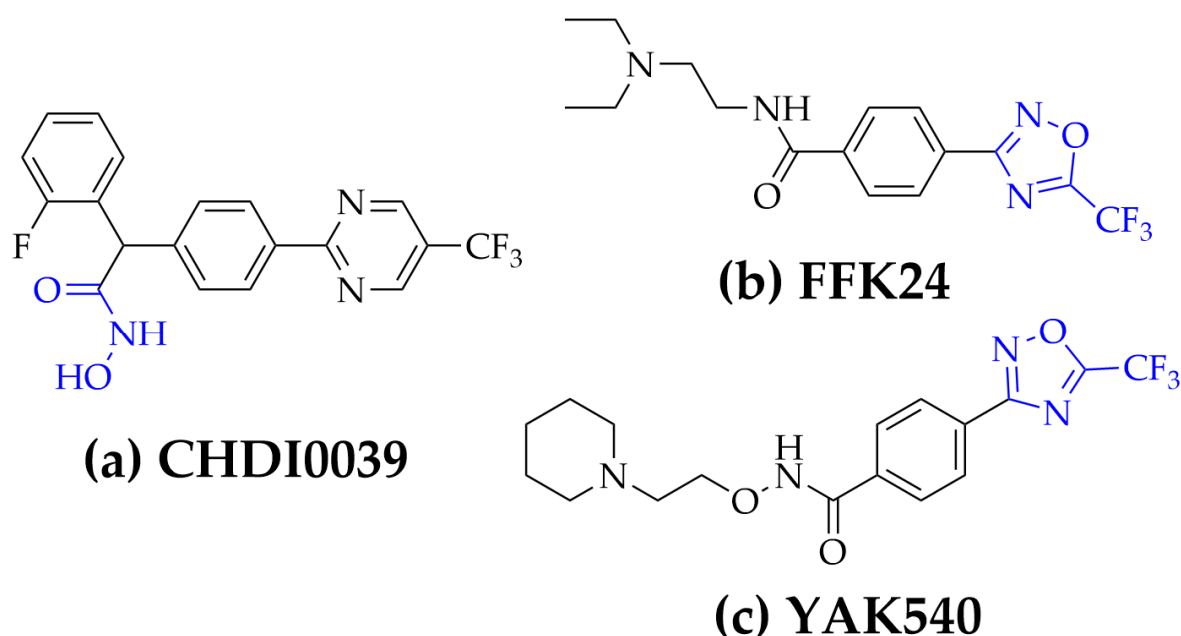


Figure 28. Structures of class IIa HDACi. (a) CHDI0039, (b) FFK24, (c) YAK540. The zinc-binding group of CHDI0039 is a hydroxamic acid. However, for FFK24 and YAK540, the zinc-binding group is the 5-(trifluoromethyl)-1,2,4-oxadiazole (TFMO)-group [211, 231].

A different ZBG may result in different off-target effects and different cellular distribution. Since HDACs primarily exist as multiprotein complexes, a differing ZBG may also engender different effects on class IIa HDAC protein complexes. One advantage of the TFMO ZBG in FFK24 and YAK540 may be that it causes fewer off-target effects due to its weaker electrostatic interaction with the metal ion [104, 161, 228]. Overall, further characterization of the class IIa HDACi is necessary for optimal use in a clinical context, for the identification of off-target effects, and for understanding the full mechanism of action of class IIa HDACi [104, 228].

4. Discussion and Conclusion

In both aforementioned studies, BTZ was selected as the combination partner for the class IIa selective HDACi. BTZ is the first approved proteasome inhibitor for the treatment of newly diagnosed multiple myeloma and relapsed/refractory multiple myeloma as well as mantle cell lymphoma [197]. Meanwhile, combination therapies involving BTZ have been approved, including combination regimes with melphalan and prednisone or rituximab, cyclophosphamide, doxorubicin, and prednisone [232]. Presently, additional clinical trials are underway to investigate new combination therapies (NCT07018050,[233]). Moreover, BTZ has undergone investigation in solid tumors, including those derived from breast, prostate, ovarian, and pancreatic cancers (NCT00183937, NCT00059618) [233-237]. The use of BTZ is limited due to adverse effects, particularly in solid tumors and other hematological malignancies. The most critical adverse effects associated with BTZ are peripheral neuropathy, renal toxicity, thrombocytopenia and neutropenia [234, 238, 239]. The potential for BTZ to induce toxic adverse effects may be mitigated by the administration of a reduced dosage. Then, the most effective approach to ensure the consistent effectiveness of the therapy would be to combine BTZ with other drugs. Low-toxicity class IIa HDACis, in particular, could be suitable combination partners for BTZ or other PIs due to the synergistic effect of their combination [104, 210, 211]. Both our studies suggest that class IIa HDACis are promising combination partners for BTZ in AML or even in solid cancers. The addition of class IIa HDACi significantly increased the toxicity of BTZ in cancer cells in a synergistic manner. Interestingly, toxicity in non-cancer cells increased substantially less, demonstrating greater selectivity of the combination for cancer cells than non-cancer cells [210]. Especially when HDAC4 (or generally class IIa HDACs) is overexpressed in cancer cells, the use of selective class IIa HDACi may have beneficial effects as they are more cytotoxic in cancer cells with high HDAC4 expression. At the same time they are less toxic in cells without HDAC4 overexpression [230]. In combination with PI such as BTZ, the efficacy of BTZ could be increased compared to single BTZ therapy and/or therapy-limiting adverse effects of BTZ could be reduced by lowering the dose of BTZ while still maintaining the same efficacy of therapy through synergistic class IIa HDACi co-treatment. Further preclinical and clinical studies are warranted to evaluate the full potential of class IIa selective and potent HDACi as combination anticancer agents.

4.2 Combination of the Class I HDACi Entinostat, a BETi, and Cisplatin

The second part of this thesis addressed the problem of cisplatin chemoresistance in patients with muscle-invasive bladder carcinoma (MIBC). Although enfortumab vedotin (EV) plus pembrolizumab is the new standard therapy for patients eligible for combination therapies with metastatic urothelial carcinoma according to the latest European Association of Urology (EAU) guideline on muscle-invasive and metastatic bladder cancer (march 2025), platinum-based combination therapies (MVAC and GC) maintain their significance as an alternative. Especially for patients who cannot receive EV treatment because of uncontrolled diabetes, peripheral neuropathy grade ≥ 2 , or pre-existing significant skin disorders, platinum-based combination therapy is the preferred treatment option [53]. However, the development of cisplatin resistance can limit its use in existing treatment regimens [20, 240]. New combination therapies may be a way to develop more and superior treatment options for patients in the future. In particular, epigenetic modulators may prove to be efficacious combination partners to address cisplatin resistance. Moreover, the epigenetic combination partners may also enhance sensitivity to treatments other than cisplatin. Existing research has already shown promising synergistic results in vitro in different cancer entities with dual combinations of entinostat and cisplatin, BETis and cisplatin, as well as entinostat and BETis [127, 208, 241-252]. Based on these approaches, the study: *Triple Combination of Entinostat, a Bromodomain Inhibitor, and Cisplatin Is a Promising Treatment Option for Bladder Cancer* investigated the triple combination of entinostat, a BETi and cisplatin in native and cisplatin-resistant bladder cancer cells [212]. The aim of these cell studies was to investigate whether the triple combination could provide an improved approach to overcome cisplatin resistance, following the existing research data on dual combinations.

First, the IC_{50} values for entinostat, JQ1 and cisplatin were determined in both bladder cancer cell pairs (J82, J82 cisR and T24, T24 LTT). Surprisingly, both cisplatin-resistant cell lines (J82 cisR and T24 LTT) were at least 4-fold more sensitive to the BETi JQ1 than the native cell lines. Further experiments confirmed these results for the BETis OTX015 and JQ35. Concentrations of the inhibitors in the combination studies were chosen according to a maximum of an IC_{25} to ensure that the toxicity of the individual treatments was low. Further, to generate potential clinical relevance of this combination, the concentrations were chosen in the range of in vivo studies [186, 253]. Therefore, in addition to the BETi JQ1 as an initial proof of principle, OTX015 and JQ35 were investigated in combination with entinostat and cisplatin. OTX015 and JQ35 were already in clinical trials due to their superior physicochemical and biopharmaceutical properties. A particular advantage of OTX015 is its oral administration [186, 254-256]. Entinostat as a representative of the class I HDACi is also utilized in clinical testing in various combination therapies for indications such as breast cancer, cholangiocarcinoma, pancreatic adenocarcinoma and others [257, 258].

4. Discussion and Conclusion

Using different incubation schemes, a 48-hour preincubation of the cells with both epigenetic inhibitors (entinostat, JQ1) followed by another 72-hour incubation with cisplatin was found to be most effective in reducing the IC₅₀ value of cisplatin. In three out of four cell lines (except T24), the triple combination decreased the IC₅₀ value most, up to a factor of 15.6 (J82 cisR). Consequently, the triple combination completely resensitized J82 cisR cells to cisplatin to the level of the native cell line (J82 cisplatin IC₅₀: 2.16 μ M) and even beyond. Moreover, in the highly resistant T24 LTT cells, the triple combination reduced the IC₅₀ of cisplatin from 75 μ M to 17.5 μ M (T24 cisplatin IC₅₀: 4.15 μ M).

Subsequent experiments confirmed that the use of both inhibitors made the cancer cells significantly more sensitive to cisplatin. Synergism studies according to Chou Talalay have demonstrated a strong synergistic effect of the triple combination in all four cancer cell lines. In addition, the calculation of the synergy level was performed using the Bliss model. An illustrative representation of this analysis is provided in Figure 29a-d.

4. Discussion and Conclusion

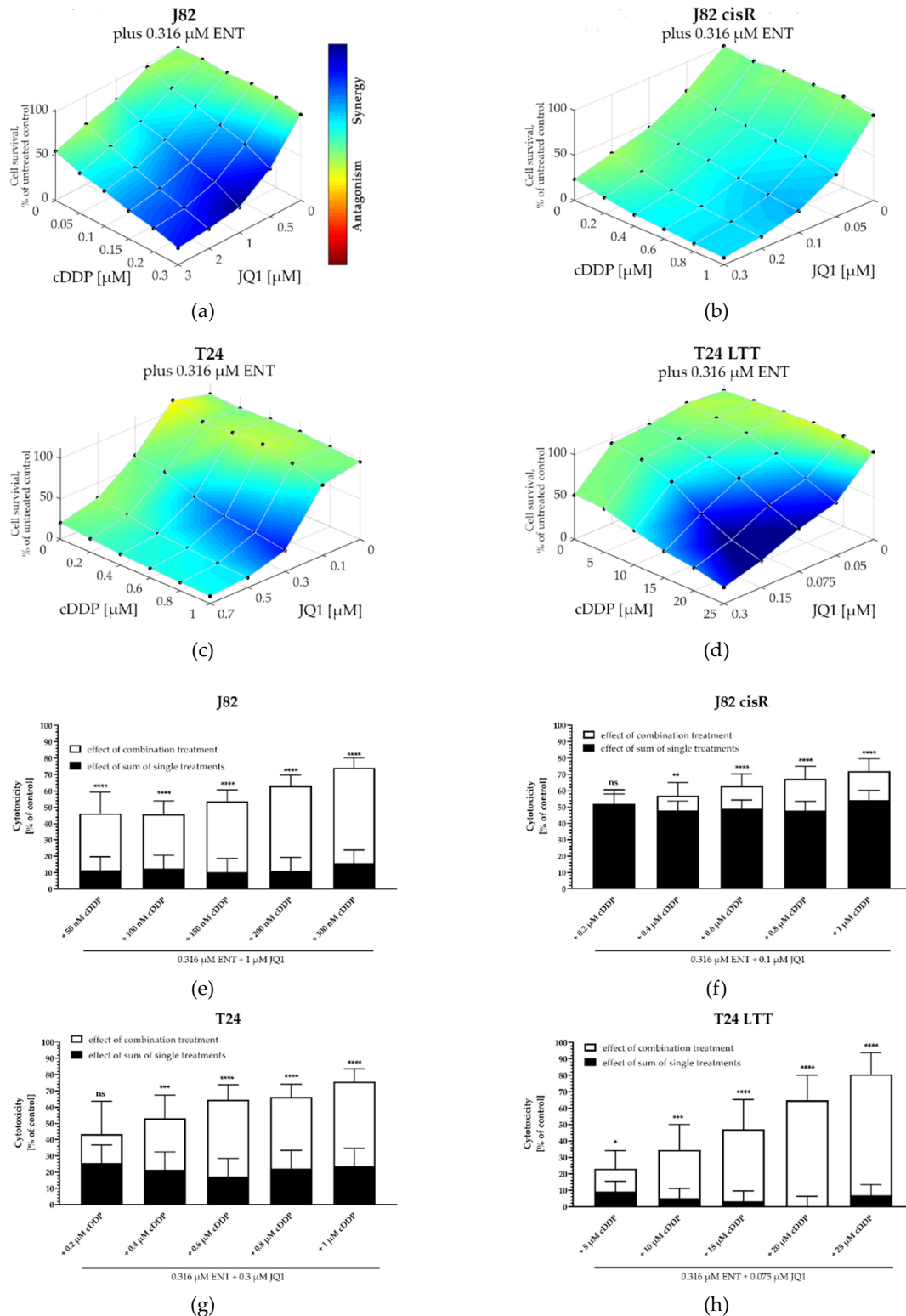


Figure 29. Synergy levels of the triple combination studies were calculated and visualized using the Bliss model in combenefit [218, 219]. Cells were incubated for 48 h with entinostat and JQ1, followed by the addition of cisplatin for another 72 h. (a) J82, (b) J82 cisR, (c) T24 and (d) T24 LTT cell line. Additionally, the synergistic cytotoxic effect of combination treatment is shown in a representative illustration for the (e) J82, (f) J82 cisR, (g) T24 and (h) T24 LTT cell line. Cells were incubated with entinostat and JQ1 for 48 h followed by an addition of cisplatin for another 72 h. Shown are the mean $\pm$ SD of at least three independent experiments, each carried out in triplicates. Data were normalized to untreated control. Black bars illustrate the additive effects (sum) of the individual treatments, white bars are the data of the combination treatment. Significance was calculated using unpaired t-test. ns ($p \geq 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$). Figure was derived from illustrations of [212].

In contrast, the dual combination of one epigenetic inhibitor and cisplatin had no or only a moderate synergistic effect in the cancer cells except in T24. In the T24 cell line, the synergistic effect of the dual combination (JQ1 plus cisplatin) was comparable to the triple treatment. Existing research has already noted a synergistic combination of JQ1 and cisplatin [241, 259]. We were able to prove that the triple treatment was even more effective in reducing the IC₅₀ value of cisplatin than the dual combination of JQ1 and cisplatin in various bladder cancer cell lines. The significant increase in toxicity of the triple combination could also be graphically visualized by comparing the cytotoxicity of the sum of the single treatments with the cytotoxicity of the combination treatment (Figure 29e-h).

Based on these results, it was shown in this study that the synergistic effect was caused by an enhanced cell cycle arrest with increased caspase 3/7 activity. Figure 30a-b illustrates the caspase 3/7 activity of cisplatin and the triple treatment in J82 and J82 cisR, including a representative fluorescence image. Further, microscopic analysis of the cell nuclei revealed the highest number of double-strand breaks in J82 and J82 cisR cells occurred upon triple combination treatment (detected as corresponding foci of γ -H2AX and 53BP1), as visualized in Figure 30c-d.

4. Discussion and Conclusion

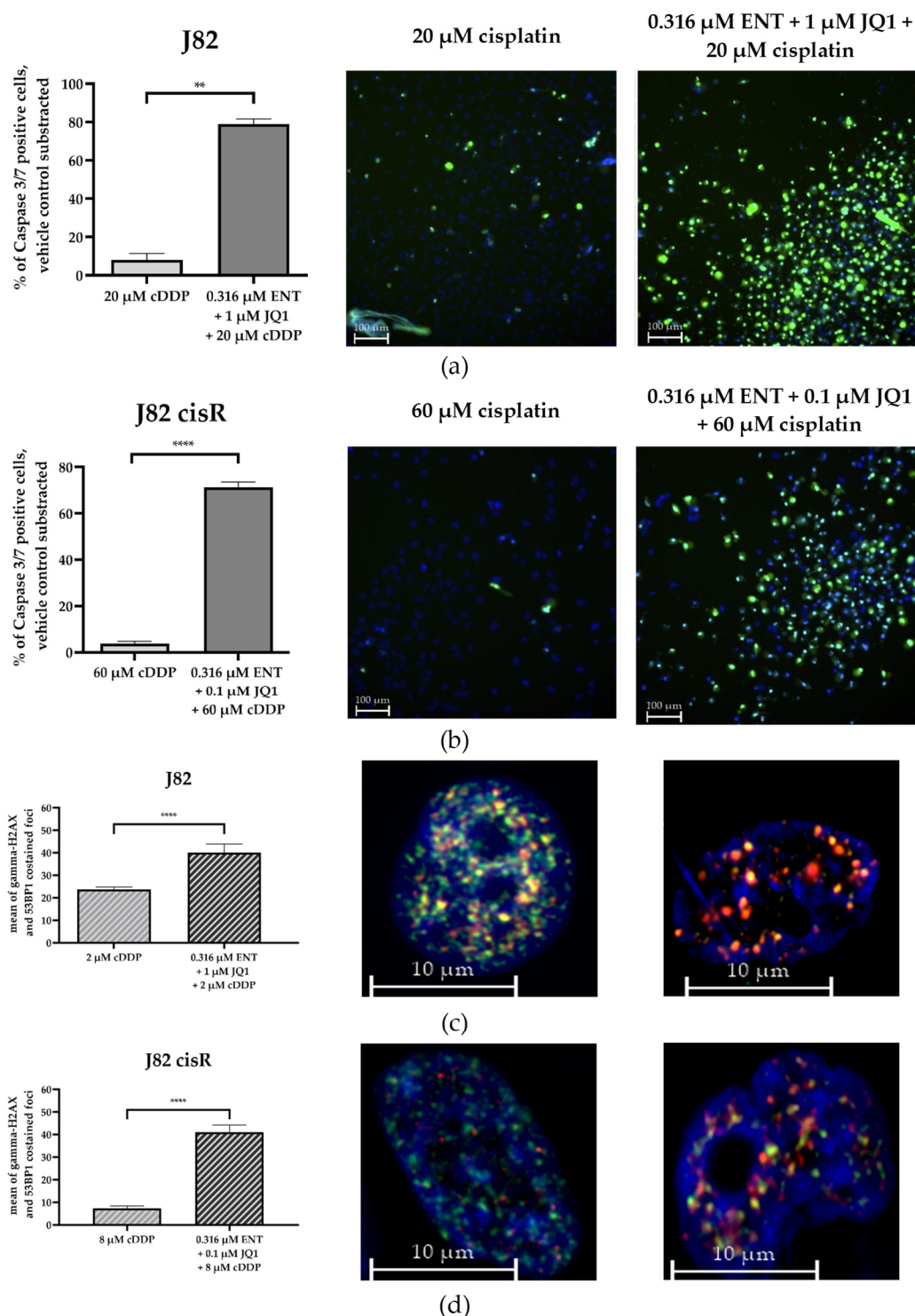


Figure 30. The excerpts from graphs taken from [212] illustrate the activation of caspase 3/7 and provide representative fluorescent imaging pictures (ThermoFisher Arrayscan XTI) of (a) J82 and (b) J82 cisR. Cells were incubated with entinostat and JQ1 for 48 h followed by the addition of cisplatin for another 24 h. Data shown is the mean $\pm$ SD of one representative experiment out of at least two, each carried out in duplicates. The following excerpt taken from [212] displays the number of co-stained foci for γ -H2AX (green) and 53BP1 (red) as well as representative fluorescence images of (c) J82 and (d) J82 cisR. Cells were incubated with entinostat and JQ1 for 48 h followed by addition of cisplatin for another 24 h. Double-stained foci (γ -H2AX and 53BP1) in at least 19 representative random cells were counted. Data shown are the mean $\pm$ SEM of counted foci. Nuclei were stained using Hoechst 33342 (blue). Significance was calculated using unpaired t-test in GraphPad Prism. Level of significance: ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

4. Discussion and Conclusion

A previous study examined the combination of class I selective HDACi romidepsin and BETi JQ1 in eight urothelial carcinoma cell lines, revealing a synergistic effect and enhanced caspase-mediated apoptosis [208]. In addition, the combination of class I HDACi entinostat and cisplatin induced caspase 3/7-mediated apoptosis in five ovarian cancer cell lines as well [127]. In consideration of the findings from these preceding studies, our study demonstrated that the triple combination consisting of a class I HDACi, BETi, and cisplatin resulted in a more potent induction of apoptosis via caspase 3/7 as well as a higher number of double-strand breaks compared to the dual combination approaches.

Next, qPCR was performed to determine the effects of the epigenetic inhibitors after 48-hour incubation on genes involved in apoptosis induction in the cancer cells. FOXO1, Bim and p21 were upregulated by the triple combination (in J82 and J82 cisR). This was subsequently confirmed by Western Blot at the protein level in all four bladder cancer cell lines using the same incubation scheme except for Bim where the triple combination did not increase Bim expression in the resistant cell lines J82 cisR and T24 LTT (Figure 31).

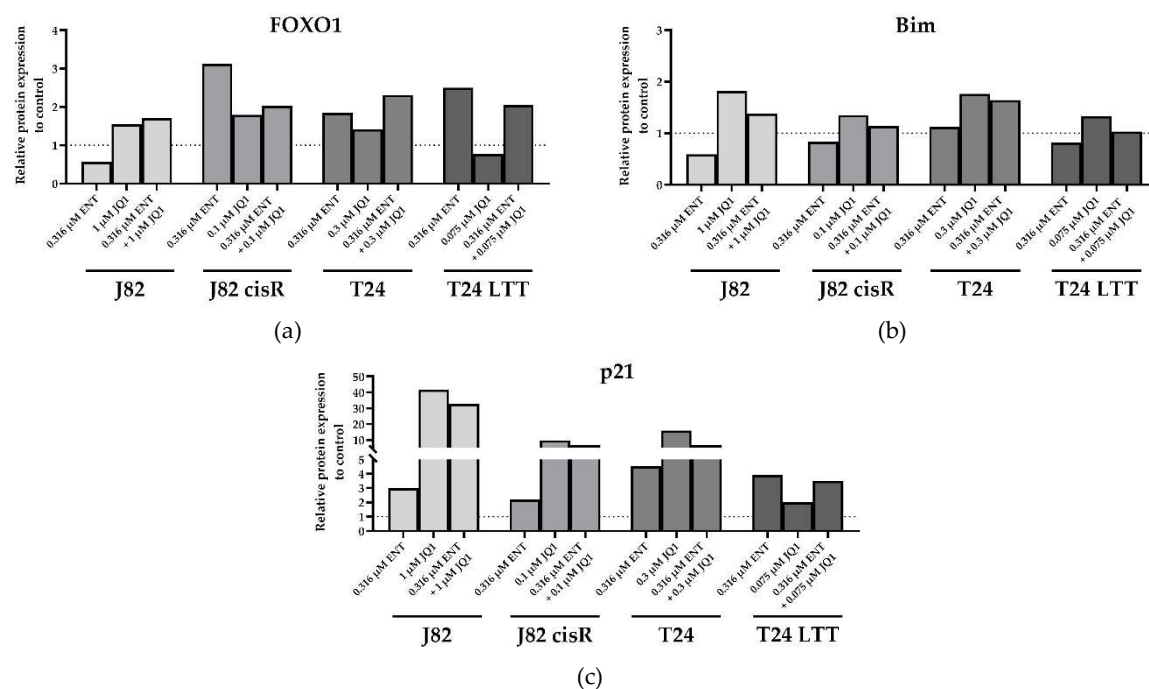


Figure 31. Relative protein expression of (a) FOXO1, (b) Bim and (c) p21 related to β -actin as loading control analyzed with ImageJ in J82, J82 cisR, T24 and T24 LTT. Ratio of control was set to 1, represented by the dashed line. Figure taken from [212].

In the literature, reduced c-Myc expression has been mechanistically linked to JQ1 [260, 261]. However, in our study, the protein expression of c-Myc exhibited a mixed pattern: the expression of c-Myc was increased with the triple combination in J82 and J82 cisR, whereas the protein expression of c-Myc was lower in T24 and T24 LTT. This suggests that the synergistic effect of the triple combination cannot be attributed solely

4. Discussion and Conclusion

to one protein such as c-Myc but is the result of a complex change in expression of several pro- and antiapoptotic proteins.

BETi OTX015 and JQ35 were also evaluated in triple combinations with entinostat and cisplatin. Both compounds showed a good reduction of the IC₅₀ value of cisplatin (up to 9.18-fold) using similar concentrations and identical incubation schemes as performed with JQ1 (Figure 32).

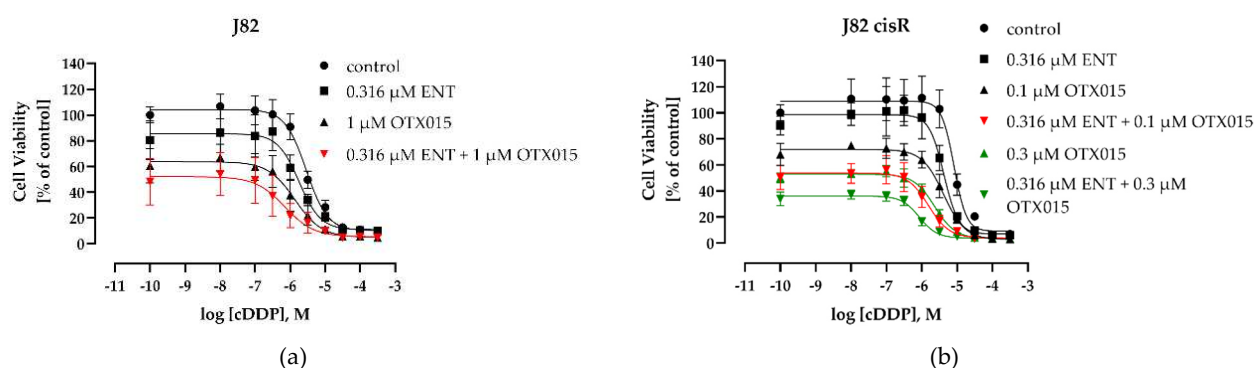


Figure 32. Concentration-effect-curves of cisplatin (MTT-Assay) of (a) J82 and (b) J82 cisR. Cells were preincubated with entinostat and/or OTX015 for 48 h followed by the addition of cisplatin for another 72 h. Control means concentration-effect-curve of cisplatin in absence of epigenetic inhibitors. Data shown are the mean $\pm$ SD of at least one experiment, carried out in triplicates. Figure taken from [212].

In a second step, OTX015 was selected for further evaluation based on the highest cisplatin IC₅₀ reduction with the triple combination in J82 and J82 cisR. Overall, the triple combination of entinostat, OTX015 and cisplatin displayed similar caspase-mediated apoptosis induction compared to the use of JQ1 in triple combination (Figure 33a). The combination of entinostat and OTX015 also resulted in a significant increase in protein expression of p21, FOXO1 and Bim in J82 but not in J82 cisR after 48 hours of incubation, as previously seen with entinostat and JQ1 (Figure 33b-d).

4. Discussion and Conclusion

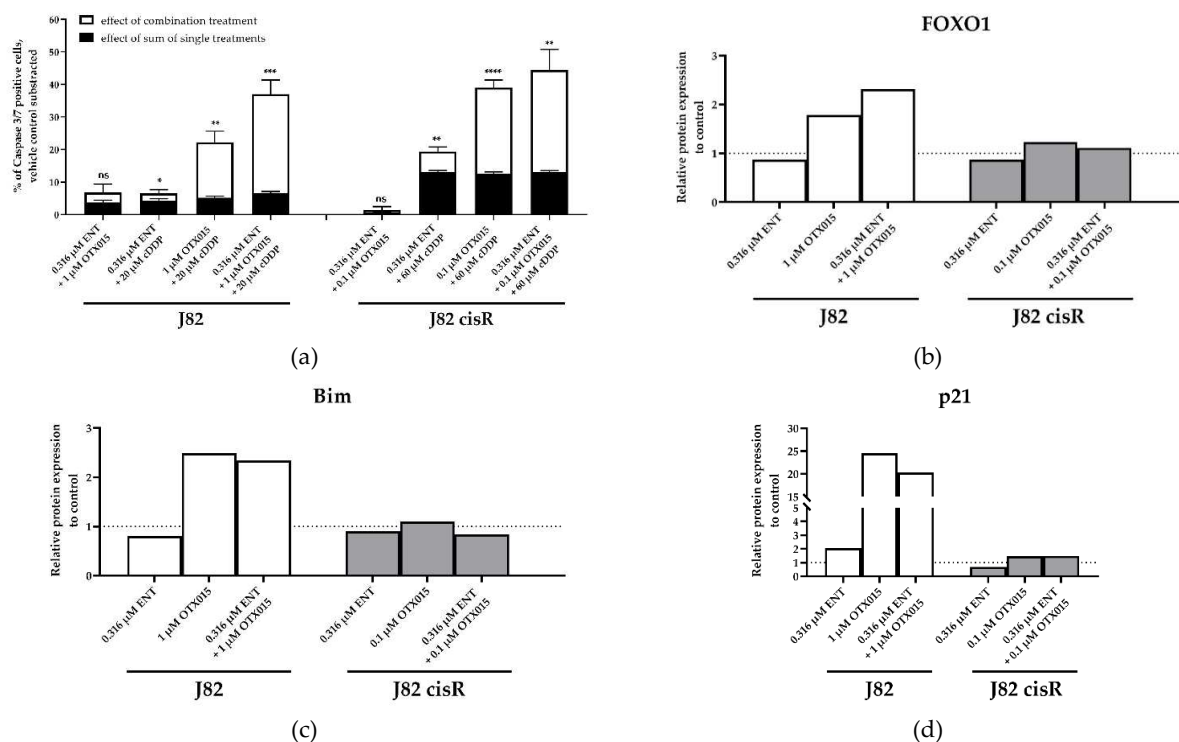


Figure 33. (a) Comparison of caspase 3/7 activity between the sum of the single compound effects (black bars) versus the effect of the combination treatments (white bars) in J82 and J82 cisR. Data shown is the mean $\pm$ SD of one representative experiment out of at least two, each carried out in duplicates. Significance was calculated using unpaired t-test. Level of significance: ns ($p \geq 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$). Relative protein expression of (b) FOXO1, (c) Bim and (d) p21 related to β -actin as loading control analyzed with ImageJ in J82 and J82 cisR. Ratio of control was set to 1, represented by the dashed line. Figures taken from [212].

Thus, the proof of principle established with JQ1 as a model BETi could be transferred to OTX015, a BETi investigated in clinical studies with advantages over JQ1 due to its favorable biopharmaceutical properties.

Lastly, the cytotoxic effect of the triple combination was investigated in non-cancer cell lines. The incubation of the non-cancerous urothelial cell line HBLAK with the epigenetic inhibitors entinostat and JQ1 resulted in an increase in the IC_{50} of cisplatin. Moreover, a slight decrease in the IC_{50} value of cisplatin was observed in the non-cancer cell line HEK293. Then, the cytotoxicity of the triple combination was compared to the same concentrations in J82 cisR and the HEK293 cell line. The triple combination showed a significantly higher cytotoxic effect in J82 cisR than in HEK293, tested with 0.1 μ M JQ1, four different concentrations of entinostat, and five different concentrations of cisplatin (Figure 34)

4. Discussion and Conclusion

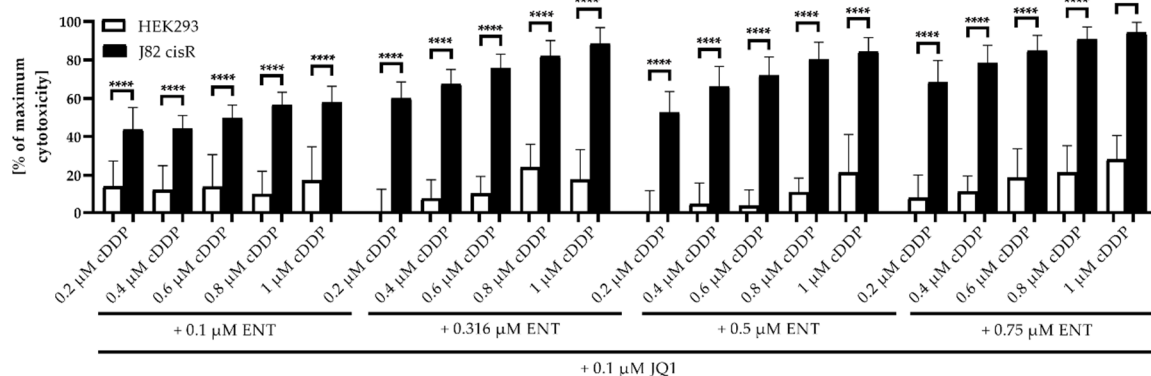


Figure 34. Comparison of cytotoxicity (MTT assay) of the triple combination entinostat, JQ1, and cisplatin in HEK293 cells (white bars) and J82 cisR cells (black bars). Cells were incubated with entinostat and JQ1 for 48 h followed by the addition of cisplatin for another 72 h. Shown are the mean $\pm$ SD from at least three independent experiments, each carried out in triplicates. Data were normalized to untreated control where 100% cell viability was set as 0% cytotoxicity. Significance was calculated using unpaired t-test. Level of significance: **** ($p \leq 0.0001$). Figure taken from [212].

This finding suggests that the triple combination is highly selective for cancerous cells, as compared to non-cancerous cells. Moreover, the high level of cytotoxicity observed in J82 cisR could already be induced by sub-micromolar concentrations of the compounds.

In a next project, the dual inhibitor **compound 20** containing structural features of a BETi and HDACi was evaluated in combination with cisplatin as an alternative approach to the triple therapy (Figure 35).

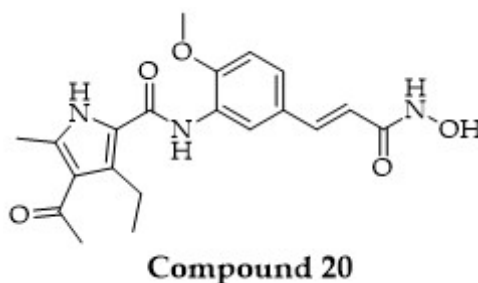


Figure 35. Structure of **compound 20**. **Compound 20** is a multi-target inhibitor with a stable linker composed of pan-bromodomain and extra-terminal motif inhibitor (BETi) XD14 and HDACi panobinostat [171].

Dual inhibitors have the advantage of targeting multiple targets simultaneously, simplifying treatment regimens and improving patient compliance. Recent years have seen an increase of research into various dual inhibitors, including dual HDACi and BETi [171-180, 262].

The cytotoxicity of the combination of **compound 20** with cisplatin is comparable to the triple combination in J82 cisR. In contrast, the IC_{50} of cisplatin was less reduced in the other three cell lines compared to the triple combination with JQ1. All cell lines except T24 showed a significant increase in caspase 3/7 activity by **compound 20** and cisplatin (Figure 36a-d).

4. Discussion and Conclusion

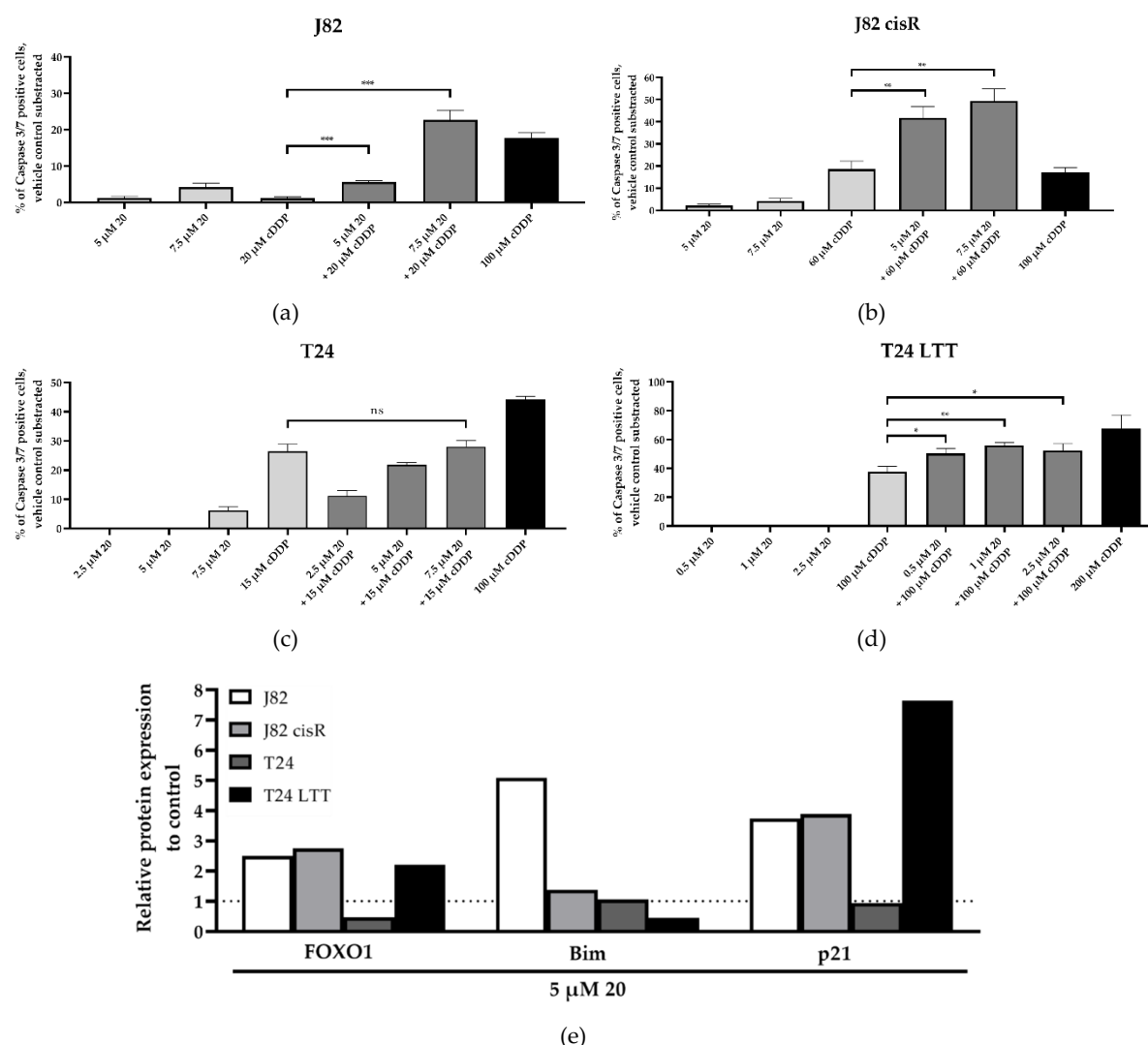


Figure 36. Caspase 3/7 activation in (a) J82, (b) J82 cisR, (c) T24 and (d) T24 LTT cell line. Cells were incubated with **compound 20** for 48 h followed by the addition of cisplatin for another 24 h (T24 LTT 48 h). Data shown is the mean $\pm$ SD of one representative experiment out of at least two, each carried out in duplicates. Significance was calculated using unpaired t-test. Level of significance: ns ($p \geq 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$). (e) Relative protein expression of FOXO1, Bim and p21 related to β -actin as loading control in J82, J82 cisR, T24 and T24 LTT. Ratio of control was set to 1, represented by the dashed line. Western blots were analyzed with ImageJ. The figure was derived from illustrations of [212].

The proteins FOXO1 and p21 were more strongly expressed in all cancer cell lines except T24 after 48 hours of incubation (Figure 36e). Interestingly, survivin was less expressed upon **compound 20** treatment in both cisplatin-resistant cell lines and more expressed in the native cell lines. In summary, the changes in protein expression upon treatment with **compound 20** demonstrated similarities in J82, J82 cisR and T24 LTT to the combination treatment with entinostat and JQ1 or OTX015, especially for FOXO1, p21, and Bim. The T24 cell line represents an exception in this regard, as no enhanced protein expression of FOXO1, p21, and Bim was observed. Consequently, the findings indicate that the dual BETi and HDACi **compound 20** can induce effects comparable to those observed with the combination of entinostat and JQ1 or OTX015, particularly

4. Discussion and Conclusion

in the resistant cell lines, despite the fact that **compound 20** did not induce these effects in the T24 cell line.

This work confirms that epigenetic changes through the combination of class I HDACi entinostat with a BETi (JQ1, OTX015) or the dual HDACi and BETi **compound 20** sensitize bladder cancer cells to cisplatin. The synergistic effect of the triple treatment leads – to various degrees in the different cell lines – to higher expression of proapoptotic proteins such as FOXO1 and Bim as well as lower expression of antiapoptotic proteins such as survivin and c-Myc. Moreover, enhanced p21 expression eventually results in an increased cell cycle control. These changes in protein expression result in increased caspase 3/7 activation, apoptosis and cell death (Figure 37).

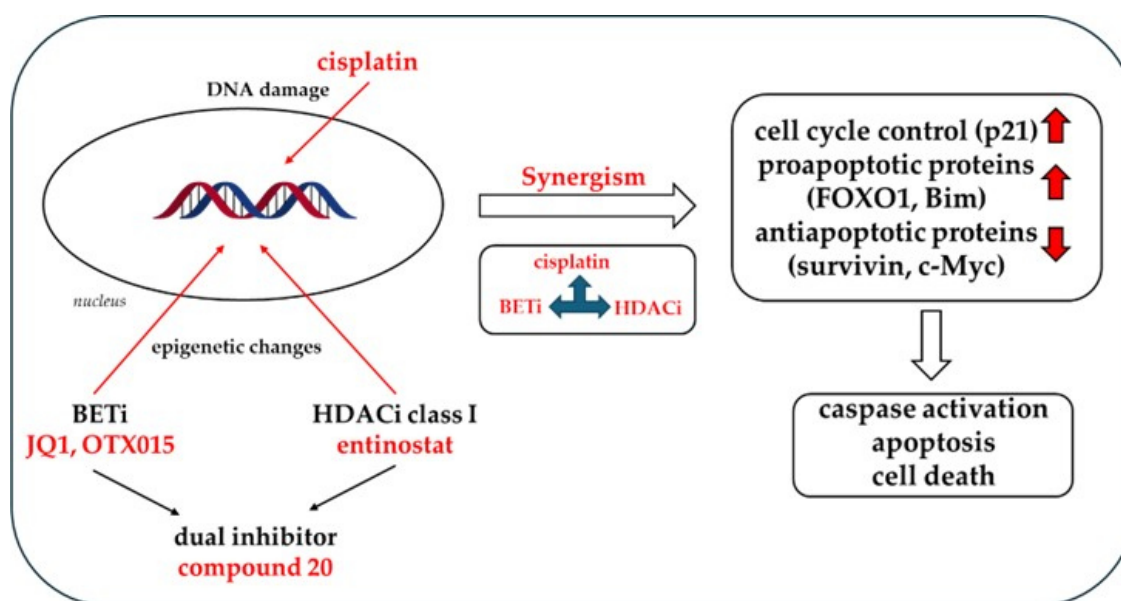


Figure 37. Overview of the results of triple combination of entinostat, a BETi and cisplatin in bladder cancer cell lines. Figure taken from [212].

Both, the triple combination (entinostat plus JQ1 or OTX015 plus cisplatin) and the combination of a dual HDAC and BET inhibitor (**compound 20**) with cisplatin, are promising approaches for developing novel treatment options for chemoresistant cancer cells. Specifically for OTX015, a triple treatment approach could lead to the resumption of clinical trials that were previously discontinued due to a lack of efficacy [133, 256, 263]. Thus far, only single-treatment and dual-combination therapies involving OTX015 have been tested in clinical trials [133, 264]. Therefore, further preclinical and clinical studies are encouraged by the results of this thesis to determine whether the suggested triple combination as new approach, especially including OTX015, will lead to improved clinical efficacy in bladder and other cancers.

4.3 Conclusion

The following conclusions can be drawn from both parts of this thesis:

- HDACi are promising combination partners for various compounds such as PI, BETi and cisplatin to maximize the cytotoxic effect of cytotoxic agents in several cancer entities in a synergistic manner
- Epigenetic modulators may help to overcome chemoresistance in cancer
- The combination of class IIa HDACi and PI may be a new approach for the treatment of AML
- The triple combination of HDACi, BETi and cisplatin or dual HDACi and BETi with cisplatin may be a potential combination in muscle invasive bladder cancer, especially in the case of intrinsic or acquired cisplatin resistance.
- Combining HDACi with OTX015 and cisplatin could enable the resumption of OTX015 clinical trials.

5. References

1. Roy PS, Saikia BJ. Cancer and cure: A critical analysis. *Indian journal of cancer*. 2016;53(3):441–2.
2. Graham TA, Sottoriva A. Measuring cancer evolution from the genome. *The Journal of pathology*. 2017;241(2):183–91.
3. O'Connor MJ. Targeting the DNA Damage Response in Cancer. *Molecular cell*. 2015;60(4):547–60.
4. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer discovery*. 2022;12(1):31–46.
5. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646–74.
6. Senga SS, Grose RP. Hallmarks of cancer-the new testament. *Open biology*. 2021;11(1):200358.
7. Greaves M, Maley CC. Clonal evolution in cancer. *Nature*. 2012;481(7381):306–13.
8. Brown JS, Amend SR, Austin RH, Gatenby RA, Hammarlund EU, Pienta KJ. Updating the Definition of Cancer. *Molecular cancer research : MCR*. 2023;21(11):1142–7.
9. Welch DR, Hurst DR. Defining the Hallmarks of Metastasis. *Cancer research*. 2019;79(12):3011–27.
10. Turajlic S, Swanton C. Metastasis as an evolutionary process. *Science (New York, NY)*. 2016;352(6282):169–75.
11. Boutry J, Tissot S, Ujvari B, Capp J-P, Giraudeau M, Nedelcu AM, et al. The evolution and ecology of benign tumors. *Biochimica et biophysica acta Reviews on cancer*. 2022;1877(1):188643.
12. Wang J-J, Lei K-F, Han F. Tumor microenvironment: recent advances in various cancer treatments. *European review for medical and pharmacological sciences*. 2018;22(12):3855–64.
13. Krebsregisterdaten Zf. Unterschiedliche Zellarten bilden Ursprung von Krebs [updated 07.12.2023; cited 2023 07.12.]. Available from: https://www.krebsdaten.de/Krebs/DE/Content/Krebsarten/Krebs_gesamt/krebs_gesamt_node.html.
14. Whiteley AE, Price TT, Cantelli G, Sipkins DA. Leukaemia: a model metastatic disease. *Nature reviews Cancer*. 2021;21(7):461–75.
15. Kinnersley B, Houlston RS, Bondy ML. Genome-Wide Association Studies in Glioma. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 2018;27(4):418–28.
16. Gottesman MM. Mechanisms of cancer drug resistance. *Annual review of medicine*. 2002;53:615–27.
17. Li J, Gao N, Gao Z, Liu W, Pang B, Dong X, et al. The Emerging Role of Exosomes in Cancer Chemoresistance. *Frontiers in cell and developmental biology*. 2021;9:737962.
18. Shimony S, Stahl M, Stone RM. Acute myeloid leukemia: 2023 update on diagnosis, risk-stratification, and management. *American journal of hematology*. 2023;98(3):502–26.
19. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians*. 2018;68(6):394–424.
20. Aldossary SA. Review on Pharmacology of Cisplatin: Clinical Use, Toxicity and Mechanism of Resistance of Cisplatin. *Biomedical and Pharmacology Journal*. 2019;12(1):07–15.
21. Pelcovits A, Niroula R. Acute Myeloid Leukemia: A Review. *Rhode Island medical journal* (2013). 2020;103(3):38–40.
22. Rubnitz JE, Gibson B, Smith FO. Acute myeloid leukemia. *Hematology/oncology clinics of North America*. 2010;24(1):35–63.
23. Nong T, Mehra S, Taylor J. Common Driver Mutations in AML: Biological Impact, Clinical Considerations, and Treatment Strategies. *Cells*. 2024;13(16).
24. Hindley A, Catherwood MA, McMullin MF, Mills KI. Significance of NPM1 Gene Mutations in AML. *Int J Mol Sci*. 2021;22(18).

5. References

25. Arber DA, Orazi A, Hasserjian RP, Borowitz MJ, Calvo KR, Kvasnicka H-M, et al. International Consensus Classification of Myeloid Neoplasms and Acute Leukemias: integrating morphologic, clinical, and genomic data. *Blood*. 2022;140(11):1200–28.
26. Khoury JD, Solary E, Abla O, Akkari Y, Alaggio R, Apperley JF, et al. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms. *Leukemia*. 2022;36(7):1703–19.
27. Döhner H, Wei AH, Appelbaum FR, Craddock C, DiNardo CD, Dombret H, et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. *Blood*. 2022;140(12):1345–77.
28. Sargas C, Ayala R, Larráyoiz MJ, Chillón MC, Rodríguez-Arboli E, Bilbao C, et al. Comparison of the 2022 and 2017 European LeukemiaNet risk classifications in a real-life cohort of the PETHEMA group. *Blood cancer journal*. 2023;13(1):77.
29. Christoph Röllig FAA, Jan Braess, Michael Heuser, Markus G. Manz, Jakob Passweg, Dirk Reinhardt, Richard F. Schlenk, Armin Zebisch. Akute Myeloische Leukämie (AML) August 2023 [cited 2025 01.08.]. Available from: <https://www.onkopedia.com/de/onkopedia/guidelines/akute-myeloische-leukaemie-aml/@@guideline/html/index.html>.
30. Strickland SA, Vey N. Diagnosis and treatment of therapy-related acute myeloid leukemia. *Crit Rev Oncol Hematol*. 2022;171:103607.
31. Kantarjian H, Kadia T, DiNardo C, Daver N, Borthakur G, Jabbour E, et al. Acute myeloid leukemia: current progress and future directions. *Blood cancer journal*. 2021;11(2):41.
32. Fleischmann M, Schnetzke U, Hochhaus A, Scholl S. Management of Acute Myeloid Leukemia: Current Treatment Options and Future Perspectives. *Cancers*. 2021;13(22).
33. Li J, Fu S, Ye C, Li J. Combination therapy involving azacitidine for acute myeloid leukemia patients ineligible for intensive chemotherapy. *Leuk Res*. 2025;148:107638.
34. Perl AE, Hosono N, Montesinos P, Podoltsev N, Martinelli G, Panoskaltsis N, et al. Clinical outcomes in patients with relapsed/refractory FLT3-mutated acute myeloid leukemia treated with gilteritinib who received prior midostaurin or sorafenib. *Blood cancer journal*. 2022;12(5):84.
35. DiNardo CD, Ravandi F, Agresta S, Konopleva M, Takahashi K, Kadia T, et al. Characteristics, clinical outcome, and prognostic significance of IDH mutations in AML. *American journal of hematology*. 2015;90(8):732–6.
36. Liu H. Emerging agents and regimens for AML. *Journal of hematology & oncology*. 2021;14(1):49.
37. Kayser S, Levis MJ. The clinical impact of the molecular landscape of acute myeloid leukemia. *Haematologica*. 2023;108(2):308–20.
38. McMurry H, Fletcher L, Traer E. IDH Inhibitors in AML-Promise and Pitfalls. *Curr Hematol Malig Rep*. 2021;16(2):207-17.
39. Lo-Coco F, Avvisati G, Vignetti M, Thiede C, Orlando SM, Iacobelli S, et al. Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. *The New England journal of medicine*. 2013;369(2):111–21.
40. Avvisati G, Lo-Coco F, Paoloni FP, Petti MC, Diverio D, Vignetti M, et al. AIDA 0493 protocol for newly diagnosed acute promyelocytic leukemia: very long-term results and role of maintenance. *Blood*. 2011;117(18):4716–25.
41. Lo-Coco F, Avvisati G, Vignetti M, Breccia M, Gallo E, Rambaldi A, et al. Front-line treatment of acute promyelocytic leukemia with AIDA induction followed by risk-adapted consolidation for adults younger than 61 years: results of the AIDA-2000 trial of the GIMEMA Group. *Blood*. 2010;116(17):3171–9.
42. Aubrey BJ, Blonquist T, McMasters M, Hobbs G, McAfee S, Rosenblatt J, et al. A phase I clinical trial of lenalidomide combined with bortezomib for acute myeloid leukemia or myelodysplastic syndrome relapsing after allogeneic stem cell transplantation. *Leuk Res*. 2025;153:107693.

5. References

43. Csizmar CM, Kim DH, Sachs Z. The role of the proteasome in AML. *Blood Cancer J*. 2016;6(12):e503.
44. Marrero RJ, Shastri VM, Nicolet D, Mrozek K, Walker CJ, Blum WG, et al. Cytarabine Pharmacogenomics and Outcomes Among Children and Young Adults With Acute Myeloid Leukemia. *JAMA Netw Open*. 2025;8(6):e2516296.
45. Roboz GJ, Mandrekar SJ, Desai P, Laumann K, Walker AR, Wang ES, et al. Randomized trial of 10 days of decitabine +/- bortezomib in untreated older patients with AML: CALGB 11002 (Alliance). *Blood Adv*. 2018;2(24):3608-17.
46. Dyrskjot L, Hansel DE, Efstathiou JA, Knowles MA, Galsky MD, Teoh J, et al. Bladder cancer. *Nature reviews Disease primers*. 2023;9(1):58.
47. Dobruch J, Oszczudłowski M. Bladder Cancer: Current Challenges and Future Directions. *Medicina (Kaunas, Lithuania)*. 2021;57(8).
48. Kirkali Z, Chan T, Manoharan M, Algaba F, Busch C, Cheng L, et al. Bladder cancer: epidemiology, staging and grading, and diagnosis. *Urology*. 2005;66(6 Suppl 1):4-34.
49. UK CR. Stages of bladder cancer [cited 2024 25.03.]. Available from: <https://www.cancerresearchuk.org/about-cancer/bladder-cancer/types-stages-grades/stages>.
50. Woldu SL, Bagrodia A, Lotan Y. Guideline of guidelines: non-muscle-invasive bladder cancer. *BJU international*. 2017;119(3):371-80.
51. Han RF, Pan JG. Can intravesical bacillus Calmette-Guérin reduce recurrence in patients with superficial bladder cancer? A meta-analysis of randomized trials. *Urology*. 2006;67(6):1216-23.
52. Sylvester RJ, van der MEIJDEN APM, Lamm DL. Intravesical bacillus Calmette-Guerin reduces the risk of progression in patients with superficial bladder cancer: a meta-analysis of the published results of randomized clinical trials. *The Journal of urology*. 2002;168(5):1964-70.
53. EAU Guidelines. Edn. presented at the EAU Annual Congress Madrid 2025. EAU guideline on muscle-invasive and metastatic bladder cancer 2025 [cited 2025 05.09.2025]. Available from: <https://d56bochluxqnz.cloudfront.net/documents/full-guideline/EAU-Guidelines-on-Muscle-Invasive-Bladder-Cancer-2025.pdf>.
54. Neoadjuvant chemotherapy in invasive bladder cancer: update of a systematic review and meta-analysis of individual patient data advanced bladder cancer (ABC) meta-analysis collaboration. *European urology*. 2005;48(2):202-5; discussion 5-6.
55. Patel VG, Oh WK, Galsky MD. Treatment of muscle-invasive and advanced bladder cancer in 2020. *CA: a cancer journal for clinicians*. 2020;70(5):404-23.
56. Yin M, Joshi M, Meijer RP, Glantz M, Holder S, Harvey HA, et al. Neoadjuvant Chemotherapy for Muscle-Invasive Bladder Cancer: A Systematic Review and Two-Step Meta-Analysis. *The oncologist*. 2016;21(6):708-15.
57. Stenhejm DD, Tran D, Nkrumah MA, Gupta S. PD1/PDL1 inhibitors for the treatment of advanced urothelial bladder cancer. *OncoTargets and therapy*. 2018;11:5973-89.
58. Zander H, Muller-Egert S, Zwiewka M, Gross S, van Zandbergen G, Engelbergs J. [Checkpoint inhibitors for cancer therapy]. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz*. 2020;63(11):1322-30.
59. Bellmunt J, Wit Rd, Vaughn DJ, Fradet Y, Lee J-L, Fong L, et al. Pembrolizumab as Second-Line Therapy for Advanced Urothelial Carcinoma. *The New England journal of medicine*. 2017;376(11):1015-26.
60. Sharma P, Retz M, Siefker-Radtke A, Baron A, Necchi A, Bedke J, et al. Nivolumab in metastatic urothelial carcinoma after platinum therapy (CheckMate 275): a multicentre, single-arm, phase 2 trial. *The Lancet Oncology*. 2017;18(3):312-22.
61. Brower V. Anti-PD-L1 inhibitor durvalumab in bladder cancer. *The Lancet Oncology*. 2016;17(7):e275.
62. Bellmunt J, Powles T, Vogelzang NJ. A review on the evolution of PD-1/PD-L1 immunotherapy for bladder cancer: The future is now. *Cancer treatment reviews*. 2017;54:58-67.
63. Alt M, Stecca C, Tobin S, Di Jiang M, Sridhar SS. Enfortumab Vedotin in urothelial cancer. *Therapeutic advances in urology*. 2020;12:1756287220980192.

5. References

64. Powles T, Rosenberg JE, Sonpavde GP, Loriot Y, Durán I, Lee J-L, et al. Enfortumab Vedotin in Previously Treated Advanced Urothelial Carcinoma. *The New England journal of medicine*. 2021;384(12):1125–35.
65. Challita-Eid PM, Satpayev D, Yang P, An Z, Morrison K, Shostak Y, et al. Enfortumab Vedotin Antibody-Drug Conjugate Targeting Nectin-4 Is a Highly Potent Therapeutic Agent in Multiple Preclinical Cancer Models. *Cancer research*. 2016;76(10):3003–13.
66. Ghosh S. Cisplatin: The first metal based anticancer drug. *Bioorganic chemistry*. 2019;88:102925.
67. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. *European journal of pharmacology*. 2014;740:364–78.
68. Lumpf T, Stößer S, Fischer F, Hartwig A, Köberle B. Role of Epigenetics for the Efficacy of Cisplatin. *International journal of molecular sciences*. 2024;25(2).
69. Hamaya S, Oura K, Morishita A, Masaki T. Cisplatin in Liver Cancer Therapy. *International journal of molecular sciences*. 2023;24(13).
70. Fuertes MA, Alonso C, Perez JM. Biochemical modulation of Cisplatin mechanisms of action: enhancement of antitumor activity and circumvention of drug resistance. *Chem Rev*. 2003;103(3):645–62.
71. Mandke P, Vasquez KM. Interactions of high mobility group box protein 1 (HMGB1) with nucleic acids: Implications in DNA repair and immune responses. *DNA Repair (Amst)*. 2019;83:102701.
72. Fedele M, Fusco A. HMGA and cancer. *Biochim Biophys Acta*. 2010;1799(1-2):48–54.
73. Yang JY, Lei XY, He KY, Guo JR, Liu MJ, Li JQ, et al. HMGA1 drives chemoresistance in esophageal squamous cell carcinoma by suppressing ferroptosis. *Cell Death Dis*. 2024;15(2):158.
74. Hsu WH, Zhao X, Zhu J, Kim IK, Rao G, McCutcheon J, et al. Checkpoint Kinase 1 Inhibition Enhances Cisplatin Cytotoxicity and Overcomes Cisplatin Resistance in SCLC by Promoting Mitotic Cell Death. *J Thorac Oncol*. 2019;14(6):1032–45.
75. Engeland K. Cell cycle regulation: p53-p21-RB signaling. *Cell Death Differ*. 2022;29(5):946–60.
76. Marteijn JA, Lans H, Vermeulen W, Hoeijmakers JH. Understanding nucleotide excision repair and its roles in cancer and ageing. *Nat Rev Mol Cell Biol*. 2014;15(7):465–81.
77. Marija Petrovic DT. Biochemical and molecular mechanisms of action of cisplatin in cancer cells. *Medicine and Biology*. 2016;18(No 1):12–8.
78. Karran P. Mechanisms of tolerance to DNA damaging therapeutic drugs. *Carcinogenesis*. 2001;22(12):1931–7.
79. Sawant A, Kothandapani A, Zhitkovich A, Sobol RW, Patrick SM. Role of mismatch repair proteins in the processing of cisplatin interstrand cross-links. *DNA Repair (Amst)*. 2015;35:126–36.
80. Li Z, Pearlman AH, Hsieh P. DNA mismatch repair and the DNA damage response. *DNA Repair (Amst)*. 2016;38:94–101.
81. Li LS, Morales JC, Veigl M, Sedwick D, Greer S, Meyers M, et al. DNA mismatch repair (MMR)-dependent 5-fluorouracil cytotoxicity and the potential for new therapeutic targets. *Br J Pharmacol*. 2009;158(3):679–92.
82. Wang JY, Edelmann W. Mismatch repair proteins as sensors of alkylation DNA damage. *Cancer Cell*. 2006;9(6):417–8.
83. Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, et al. Molecular mechanisms of cisplatin resistance. *Oncogene*. 2012;31(15):1869–83.
84. Fu R, Zhao B, Chen M, Fu X, Zhang Q, Cui Y, et al. Moving beyond cisplatin resistance: mechanisms, challenges, and prospects for overcoming recurrence in clinical cancer therapy. *Medical oncology (Northwood, London, England)*. 2023;41(1):9.
85. Zhu H, Luo H, Zhang W, Shen Z, Hu X, Zhu X. Molecular mechanisms of cisplatin resistance in cervical cancer. *Drug design, development and therapy*. 2016;10:1885–95.
86. Amable L. Cisplatin resistance and opportunities for precision medicine. *Pharmacological research*. 2016;106:27–36.

5. References

87. Li F, Zheng Z, Chen W, Li D, Zhang H, Zhu Y, et al. Regulation of cisplatin resistance in bladder cancer by epigenetic mechanisms. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy*. 2023;68:100938.
88. Faridounnia M, Folkers GE, Boelens R. Function and Interactions of ERCC1-XPF in DNA Damage Response. *Molecules*. 2018;23(12).
89. Nafchi HM, Solatzadeh H, Hajimaghsoudi E, Babakhanzadeh E. Personalizing cancer therapy: the role of pharmacogenetics in overcoming drug resistance and toxicity. *Mol Biol Rep*. 2025;52(1):785.
90. Malottki K, Popat S, Deeks JJ, Riley RD, Nicholson AG, Billingham L. Problems of variable biomarker evaluation in stratified medicine research--A case study of ERCC1 in non-small-cell lung cancer. *Lung Cancer*. 2016;92:1-7.
91. Deans C, Maggert KA. What do you mean, "epigenetic"? *Genetics*. 2015;199(4):887–96.
92. Miranda Furtado CL, Dos Santos Luciano MC, Da Silva Santos R, Furtado GP, Moraes MO, Pessoa C. Epidrugs: targeting epigenetic marks in cancer treatment. *Epigenetics*. 2019;14(12):1164–76.
93. Morel D, Jeffery D, Aspeslagh S, Almouzni G, Postel-Vinay S. Combining epigenetic drugs with other therapies for solid tumours - past lessons and future promise. *Nature reviews Clinical oncology*. 2020;17(2):91–107.
94. Seto E, Yoshida M. Erasers of histone acetylation: the histone deacetylase enzymes. *Cold Spring Harbor perspectives in biology*. 2014;6(4):a018713.
95. Thibauudeau TA, Smith DM. A Practical Review of Proteasome Pharmacology. *Pharmacological reviews*. 2019;71(2):170–97.
96. Maemoto Y, Shimizu Y, Katoh R, Ito A. Naturally occurring small molecule compounds that target histone deacetylases and their potential applications in cancer therapy. *The Journal of antibiotics*. 2021;74(10):667–76.
97. Prives C, Manley JL. Why is p53 acetylated? *Cell*. 2001;107(7):815–8.
98. Nagasaka M, Miyajima C, Aoki H, Aoyama M, Morishita D, Inoue Y, et al. Insights into Regulators of p53 Acetylation. *Cells*. 2022;11(23).
99. Luo J, Su F, Chen D, Shiloh A, Gu W. Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature*. 2000;408(6810):377–81.
100. Marmorstein R, Zhou M-M. Writers and readers of histone acetylation: structure, mechanism, and inhibition. *Cold Spring Harbor perspectives in biology*. 2014;6(7):a018762.
101. Ramaiah MJ, Tangutur AD, Manyam RR. Epigenetic modulation and understanding of HDAC inhibitors in cancer therapy. *Life sciences*. 2021;277:119504.
102. Sykes SM, Mellert HS, Holbert MA, Li K, Marmorstein R, Lane WS, et al. Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol Cell*. 2006;24(6):841-51.
103. Barneda-Zahonero B, Parra M. Histone deacetylases and cancer. *Molecular oncology*. 2012;6(6):579–89.
104. Asfaha Y, Schrenk C, Alves Avelar LA, Hamacher A, Pflieger M, Kassack MU, et al. Recent advances in class IIa histone deacetylases research. *Bioorganic & medicinal chemistry*. 2019;27(22):115087.
105. Kim H-J, Bae S-C. Histone deacetylase inhibitors: molecular mechanisms of action and clinical trials as anti-cancer drugs. *American Journal of Translational Research*. 2010;3(2):166–79.
106. Zheng HC, Jiang HM. Shuttling of cellular proteins between the plasma membrane and nucleus (Review). *Mol Med Rep*. 2022;25(1).
107. de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J*. 2003;370(Pt 3):737-49.
108. Liu Y, Tong X, Hu W, Chen D. HDAC11: A novel target for improved cancer therapy. *Biomed Pharmacother*. 2023;166:115418.
109. Choi JH, Kwon HJ, Yoon BI, Kim JH, Han SU, Joo HJ, et al. Expression profile of histone deacetylase 1 in gastric cancer tissues. *Japanese journal of cancer research : Gann*. 2001;92(12):1300–4.

5. References

110. Choi JH, Song YS, Yoon JS, Song KW, Lee YY. Enhancer of zeste homolog 2 expression is associated with tumor cell proliferation and metastasis in gastric cancer. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica*. 2010;118(3):196–202.
111. Minamiya Y, Ono T, Saito H, Takahashi N, Ito M, Mitsui M, et al. Expression of histone deacetylase 1 correlates with a poor prognosis in patients with adenocarcinoma of the lung. *Lung cancer (Amsterdam, Netherlands)*. 2011;74(2):300–4.
112. Zhang Z, Yamashita H, Toyama T, Sugiura H, Ando Y, Mita K, et al. Quantitation of HDAC1 mRNA expression in invasive carcinoma of the breast*. *Breast cancer research and treatment*. 2005;94(1):11–6.
113. Weichert W, Röske A, Niesporek S, Noske A, Buckendahl A-C, Dietel M, et al. Class I histone deacetylase expression has independent prognostic impact in human colorectal cancer: specific role of class I histone deacetylases in vitro and in vivo. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2008;14(6):1669–77.
114. Fritzsche FR, Weichert W, Röske A, Gekeler V, Beckers T, Stephan C, et al. Class I histone deacetylases 1, 2 and 3 are highly expressed in renal cell cancer. *BMC cancer*. 2008;8:381.
115. Adams H, Fritzsche FR, Dirnhofer S, Kristiansen G, Tzankov A. Class I histone deacetylases 1, 2 and 3 are highly expressed in classical Hodgkin's lymphoma. *Expert opinion on therapeutic targets*. 2010;14(6):577–84.
116. Giannopoulou AF, Velentzas AD, Konstantakou EG, Avgeris M, Katarachia SA, Papandreou NC, et al. Revisiting Histone Deacetylases in Human Tumorigenesis: The Paradigm of Urothelial Bladder Cancer. *Int J Mol Sci*. 2019;20(6).
117. Sun JY, Xu L, Tseng H, Ciccarelli B, Fulciniti M, Hunter ZR, et al. Histone deacetylase inhibitors demonstrate significant preclinical activity as single agents, and in combination with bortezomib in Waldenström's macroglobulinemia. *Clinical lymphoma, myeloma & leukemia*. 2011;11(1):152–6.
118. Milde T, Oehme I, Korshunov A, Kopp-Schneider A, Remke M, Northcott P, et al. HDAC5 and HDAC9 in medulloblastoma: novel markers for risk stratification and role in tumor cell growth. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2010;16(12):3240–52.
119. Ouaiissi M, Sielezneff I, Silvestre R, Sastre B, Bernard J-P, Lafontaine JS, et al. High histone deacetylase 7 (HDAC7) expression is significantly associated with adenocarcinomas of the pancreas. *Annals of surgical oncology*. 2008;15(8):2318–28.
120. Buckwalter JM, Chan W, Shuman L, Wildermuth T, Ellis-Mohl J, Walter V, et al. Characterization of Histone Deacetylase Expression Within In Vitro and In Vivo Bladder Cancer Model Systems. *Int J Mol Sci*. 2019;20(10).
121. Quan P, Schatz C, Haybaeck J. Histone deacetylase in human sarcomas. *Int J Biol Markers*. 2025;3936155251385254.
122. Weichert W. HDAC expression and clinical prognosis in human malignancies. *Cancer Lett*. 2009;280(2):168-76.
123. Bernt KM. HDAC expression patterns in pediatric ALL. *Leuk Res*. 2013;37(10):1191-2.
124. Wilson AJ, Byun D-S, Nasser S, Murray LB, Ayyanar K, Arango D, et al. HDAC4 promotes growth of colon cancer cells via repression of p21. *Molecular biology of the cell*. 2008;19(10):4062–75.
125. Mottet D, Pirotte S, Lamour V, Hagedorn M, Javerzat S, Bikfalvi A, et al. HDAC4 represses p21(WAF1/Cip1) expression in human cancer cells through a Sp1-dependent, p53-independent mechanism. *Oncogene*. 2009;28(2):243–56.
126. Stronach EA, Alfraidi A, Rama N, Datler C, Studd JB, Agarwal R, et al. HDAC4-regulated STAT1 activation mediates platinum resistance in ovarian cancer. *Cancer research*. 2011;71(13):4412–22.
127. Bandolik JJ, Hamacher A, Schrenk C, Weishaupt R, Kassack MU. Class I-Histone Deacetylase (HDAC) Inhibition is Superior to pan-HDAC Inhibition in Modulating Cisplatin Potency in

5. References

- High Grade Serous Ovarian Cancer Cell Lines. *International journal of molecular sciences*. 2019;20(12).
128. Alves Avelar LA, Schrenk C, Sönnichsen M, Hamacher A, Hansen FK, Schliehe-Diecks J, et al. Synergistic induction of apoptosis in resistant head and neck carcinoma and leukemia by alkoxyamide-based histone deacetylase inhibitors. *European journal of medicinal chemistry*. 2021;211:113095.
129. Boyson SP, Gao C, Quinn K, Boyd J, Paculova H, Fietze S, et al. Functional Roles of Bromodomain Proteins in Cancer. *Cancers*. 2021;13(14).
130. Kulikowski E, Rakai BD, Wong NCW. Inhibitors of bromodomain and extra-terminal proteins for treating multiple human diseases. *Medicinal research reviews*. 2021;41(1):223–45.
131. Fujisawa T, Filippakopoulos P. Functions of bromodomain-containing proteins and their roles in homeostasis and cancer. *Nature reviews Molecular cell biology*. 2017;18(4):246–62.
132. Filippakopoulos P, Picaud S, Mangos M, Keates T, Lambert J-P, Barsyte-Lovejoy D, et al. Histone Recognition and Large-Scale Structural Analysis of the Human Bromodomain Family. *Cell*. 2012;149(1):214–31.
133. Guo J, Zheng Q, Peng Y. BET proteins: Biological functions and therapeutic interventions. *Pharmacology & therapeutics*. 2023;243:108354.
134. Cheung KL, Kim C, Zhou MM. The Functions of BET Proteins in Gene Transcription of Biology and Diseases. *Front Mol Biosci*. 2021;8:728777.
135. Chiang CM. Phospho-BRD4: transcription plasticity and drug targeting. *Drug Discov Today Technol*. 2016;19:17–22.
136. Sarnik J, Popławski T, Tokarz P. BET Proteins as Attractive Targets for Cancer Therapeutics. *International journal of molecular sciences*. 2021;22(20).
137. Sahai V, Redig AJ, Collier KA, Eckerdt FD, Munshi HG. Targeting BET bromodomain proteins in solid tumors. *Oncotarget*. 2016;7(33):53997–4009.
138. Kulig P, Łuczowska K, Bakinowska E, Baumert B, Machaliński B. Epigenetic Alterations as Vital Aspects of Bortezomib Molecular Action. *Cancers*. 2023;16(1).
139. Zhao L, Zhao J, Zhong K, Tong A, Jia D. Targeted protein degradation: mechanisms, strategies and application. *Signal transduction and targeted therapy*. 2022;7(1):113.
140. Bard JAM, Goodall EA, Greene ER, Jonsson E, Dong KC, Martin A. Structure and Function of the 26S Proteasome. *Annual review of biochemistry*. 2018;87:697–724.
141. Lee H, Kim S, Lee D. The versatility of the proteasome in gene expression and silencing: Unraveling proteolytic and non-proteolytic functions. *Biochimica et biophysica acta Gene regulatory mechanisms*. 2023;1866(4):194978.
142. Wang J, Xiang Y, Fan M, Fang S, Hua Q. The Ubiquitin-Proteasome System in Tumor Metabolism. *Cancers*. 2023;15(8).
143. Nandi D, Tahiliani P, Kumar A, Chandu D. The ubiquitin-proteasome system. *Journal of biosciences*. 2006;31(1):137–55.
144. Park J, Cho J, Song EJ. Ubiquitin-proteasome system (UPS) as a target for anticancer treatment. *Archives of pharmacal research*. 2020;43(11):1144–61.
145. Sanaei M, Kavooosi F. Histone Deacetylases and Histone Deacetylase Inhibitors: Molecular Mechanisms of Action in Various Cancers. *Advanced biomedical research*. 2019;8:63.
146. Barrett RRG, Nash C, Diennet M, Cotnoir-White D, Doyle C, Mader S, et al. Dual-function antiandrogen/HDACi hybrids based on enzalutamide and entinostat. *Bioorg Med Chem Lett*. 2022;55:128441.
147. Zhang L, Zhang J, Jiang Q, Zhang L, Song W. Zinc binding groups for histone deacetylase inhibitors. *J Enzyme Inhib Med Chem*. 2018;33(1):714–21.
148. Yudaev P, Aleksandrova Y, Neganova M. Recent Insights into the Creation of Histone Deacetylase Inhibitors for the Treatment of Human Diseases. *Int J Mol Sci*. 2025;26(17).
149. Li Y, Seto E. HDACs and HDAC Inhibitors in Cancer Development and Therapy. *Cold Spring Harbor perspectives in medicine*. 2016;6(10).

5. References

150. Ho TCS, Chan AHY, Ganesan A. Thirty Years of HDAC Inhibitors: 2020 Insight and Hindsight. *J Med Chem.* 2020;63(21):12460-84.
151. Sun Y, Hong JH, Ning Z, Pan D, Fu X, Lu X, et al. Therapeutic potential of tucidinostat, a subtype-selective HDAC inhibitor, in cancer treatment. *Front Pharmacol.* 2022;13:932914.
152. Lamb YN. Givinostat: First Approval. *Drugs.* 2024;84(7):849-56.
153. Duvyzat (givinostat): An overview of Duvyzat and why it is authorised in the EU: European Medicines Agency; 2025 [cited 2025 09.11.]. Available from: https://www.ema.europa.eu/en/documents/overview/duvyzat-epar-medicine-overview_en.pdf.
154. Wang C, Hamacher A, Petzsch P, Köhrer K, Niegisch G, Hoffmann MJ, et al. Combination of Decitabine and Entinostat Synergistically Inhibits Urothelial Bladder Cancer Cells via Activation of FoxO1. *Cancers.* 2020;12(2).
155. Petrich A, Nabhan C. Use of class I histone deacetylase inhibitor romidepsin in combination regimens. *Leukemia & lymphoma.* 2016;57(8):1755–65.
156. Kiany S, Harrison D, Gordon N. The Histone Deacetylase Inhibitor Entinostat/Syndax 275 in Osteosarcoma. *Advances in experimental medicine and biology.* 2020;1257:75–83.
157. Frys S, Simons Z, Hu Q, Barth MJ, Gu JJ, Mavis C, et al. Entinostat, a novel histone deacetylase inhibitor is active in B-cell lymphoma and enhances the anti-tumour activity of rituximab and chemotherapy agents. *British journal of haematology.* 2015;169(4):506–19.
158. Center MSKC. Combination Therapy With Entinostat and Pembrolizumab in Relapsed and Refractory Lymphomas: NCT03179930: clinicaltrials.gov; [cited 2024 21.03.]. Available from: <https://www.clinicaltrials.gov/study/NCT03179930?term=NCT03179930&rank=1>.
159. Network HCR. Study of Entinostat With Nivolumab Plus Ipilimumab in Previously Treated Renal Cell Carcinoma: NCT03552380: clinicaltrials.gov; [cited 2024 21.03.]. Available from: <https://www.clinicaltrials.gov/study/NCT03552380?term=NCT03552380&rank=1>.
160. Connolly RM, Zhao F, Miller KD, Lee M-J, Piekarz RL, Smith KL, et al. E2112: Randomized Phase III Trial of Endocrine Therapy Plus Entinostat or Placebo in Hormone Receptor-Positive Advanced Breast Cancer. A Trial of the ECOG-ACRIN Cancer Research Group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2021;39(28):3171–81.
161. Lobera M, Madauss KP, Pohlhaus DT, Wright QG, Trocha M, Schmidt DR, et al. Selective class IIa histone deacetylase inhibition via a nonchelating zinc-binding group. *Nature chemical biology.* 2013;9(5):319–25.
162. Stott AJ, Maillard MC, Beaumont V, Allcock D, Aziz O, Borchers AH, et al. Evaluation of 5-(Trifluoromethyl)-1,2,4-oxadiazole-Based Class IIa HDAC Inhibitors for Huntington's Disease. *ACS medicinal chemistry letters.* 2021;12(3):380–8.
163. Turkman N, Liu D, Pirola I. Design, synthesis, biochemical evaluation, radiolabeling and in vivo imaging with high affinity class-IIa histone deacetylase inhibitor for molecular imaging and targeted therapy. *European journal of medicinal chemistry.* 2022;228:114011.
164. Kikuchi S, Suzuki R, Ohguchi H, Yoshida Y, Lu D, Cottini F, et al. Class IIa HDAC inhibition enhances ER stress-mediated cell death in multiple myeloma. *Leukemia.* 2015;29(9):1918–27.
165. Li X, Li X, Liu F, Li S, Shi D. Rational Multitargeted Drug Design Strategy from the Perspective of a Medicinal Chemist. *Journal of medicinal chemistry.* 2021;64(15):10581–605.
166. Fu RG, Sun Y, Sheng WB, Liao DF. Designing multi-targeted agents: An emerging anticancer drug discovery paradigm. *Eur J Med Chem.* 2017;136:195-211.
167. Ai T, Cui H, Chen L. Multi-targeted histone deacetylase inhibitors in cancer therapy. *Current medicinal chemistry.* 2012;19(4):475–87.
168. Patel VK, Shirbhate E, Tiwari P, Kore R, Veerasamy R, Mishra A, et al. Multi-targeted HDAC Inhibitors as Anticancer Agents: Current Status and Future Prospective. *Current medicinal chemistry.* 2023;30(24):2762–95.
169. Gediya LK, Khandelwal A, Patel J, Belosay A, Sabnis G, Mehta J, et al. Design, synthesis, and evaluation of novel mutual prodrugs (hybrid drugs) of all-trans-retinoic acid and histone

5. References

- deacetylase inhibitors with enhanced anticancer activities in breast and prostate cancer cells in vitro. *Journal of medicinal chemistry*. 2008;51(13):3895–904.
170. Tomita A, Kiyoi H, Naoe T. Mechanisms of action and resistance to all-trans retinoic acid (ATRA) and arsenic trioxide (As₂O₃) in acute promyelocytic leukemia. *Int J Hematol*. 2013;97(6):717–25.
171. Schäker-Hübner L, Warstat R, Ahlert H, Mishra P, Kraft FB, Schliehe-Diecks J, et al. 4-Acyl Pyrrole Capped HDAC Inhibitors: A New Scaffold for Hybrid Inhibitors of BET Proteins and Histone Deacetylases as Antileukemia Drug Leads. *Journal of medicinal chemistry*. 2021;64(19):14620–46.
172. Duan W, Hou J, Chu X, Li X, Zhang J, Li J, et al. Synthesis and biological evaluation of novel histone deacetylases inhibitors with nitric oxide releasing activity. *Bioorganic & medicinal chemistry*. 2015;23(15):4481–8.
173. Gryder BE, Rood MK, Johnson KA, Patil V, Raftery ED, Yao L-PD, et al. Histone deacetylase inhibitors equipped with estrogen receptor modulation activity. *Journal of medicinal chemistry*. 2013;56(14):5782–96.
174. Guerrant W, Patil V, Canzonieri JC, Yao L-P, Hood R, Oyelere AK. Dual-acting histone deacetylase-topoisomerase I inhibitors. *Bioorganic & medicinal chemistry letters*. 2013;23(11):3283–7.
175. Lamblin M, Dabbas B, Spingarn R, Mendoza-Sanchez R, Wang T-T, An B-S, et al. Vitamin D receptor agonist/histone deacetylase inhibitor molecular hybrids. *Bioorganic & medicinal chemistry*. 2010;18(11):4119–37.
176. Shukla S, Khan S, Kumar S, Sinha S, Farhan M, Bora HK, et al. Cucurbitacin B Alters the Expression of Tumor-Related Genes by Epigenetic Modifications in NSCLC and Inhibits NNK-Induced Lung Tumorigenesis. *Cancer prevention research (Philadelphia, Pa)*. 2015;8(6):552–62.
177. Tang C, Li C, Zhang S, Hu Z, Wu J, Dong C, et al. Novel Bioactive Hybrid Compound Dual Targeting Estrogen Receptor and Histone Deacetylase for the Treatment of Breast Cancer. *Journal of medicinal chemistry*. 2015;58(11):4550–72.
178. Wang L, Chen G, Chen K, Ren Y, Li H, Jiang X, et al. Dual targeting of retinoid X receptor and histone deacetylase with DW22 as a novel antitumor approach. *Oncotarget*. 2015;6(12):9740–55.
179. Zhang X, Kong Y, Zhang J, Su M, Zhou Y, Zang Y, et al. Design, synthesis and biological evaluation of colchicine derivatives as novel tubulin and histone deacetylase dual inhibitors. *European journal of medicinal chemistry*. 2015;95:127–35.
180. Zhang X, Su M, Chen Y, Li J, Lu W. The design and synthesis of a new class of RTK/HDAC dual-targeted inhibitors. *Molecules (Basel, Switzerland)*. 2013;18(6):6491–503.
181. Shimizu T, LoRusso PM, Papadopoulos KP, Patnaik A, Beeram M, Smith LS, et al. Phase I first-in-human study of CUDC-101, a multitargeted inhibitor of HDACs, EGFR, and HER2 in patients with advanced solid tumors. *Clin Cancer Res*. 2014;20(19):5032–40.
182. Galloway TJ, Wirth LJ, Colevas AD, Gilbert J, Bauman JE, Saba NF, et al. A Phase I Study of CUDC-101, a Multitarget Inhibitor of HDACs, EGFR, and HER2, in Combination with Chemoradiation in Patients with Head and Neck Squamous Cell Carcinoma. *Clin Cancer Res*. 2015;21(7):1566–73.
183. Wang Z, Li L, Chu C, Wei X, Liu Q, Wang R, et al. CUDC-101 is a potential target inhibitor for the EGFR-overexpression bladder cancer cells. *Int J Oncol*. 2023;63(6).
184. Cao W, Yao S, Li A, Chen H, Zhang E, Cao L, et al. CUDC-101 as a dual-target inhibitor of EGFR and HDAC enhances the anti-myeloma effects of bortezomib by regulating G2/M cell cycle arrest. *J Zhejiang Univ Sci B*. 2023;24(5):442–54.
185. Li F, MacKenzie KR, Jain P, Santini C, Young DW, Matzuk MM. Metabolism of JQ1, an inhibitor of bromodomain and extra terminal bromodomain proteins, in human and mouse liver microsomes†. *Biology of reproduction*. 2020;103(2):427–36.
186. Shorstova T, Foulkes WD, Witcher M. Achieving clinical success with BET inhibitors as anti-cancer agents. *British journal of cancer*. 2021;124(9):1478–90.

5. References

187. Wang C, Zhang Y, Chen W, Wu Y, Xing D. New-generation advanced PROTACs as potential therapeutic agents in cancer therapy. *Mol Cancer*. 2024;23(1):110.
188. Nalawansha DA, Crews CM. PROTACs: An Emerging Therapeutic Modality in Precision Medicine. *Cell Chem Biol*. 2020;27(8):998-1014.
189. Raina K, Lu J, Qian Y, Altieri M, Gordon D, Rossi AMK, et al. PROTAC-induced BET protein degradation as a therapy for castration-resistant prostate cancer. *Proceedings of the National Academy of Sciences*. 2016;113(26):7124–9.
190. Saenz DT, Fiskus W, Qian Y, Manshouri T, Rajapakshe K, Raina K, et al. Novel BET protein proteolysis-targeting chimera exerts superior lethal activity than bromodomain inhibitor (BETi) against post-myeloproliferative neoplasm secondary (s) AML cells. *Leukemia*. 2017;31(9):1951–61.
191. Sun B, Fiskus W, Qian Y, Rajapakshe K, Raina K, Coleman KG, et al. BET protein proteolysis targeting chimera (PROTAC) exerts potent lethal activity against mantle cell lymphoma cells. *Leukemia*. 2018;32(2):343–52.
192. Zhou XL, Zhao F, Xu YT, Guan YY, Yu T, Zhang YZ, et al. A comprehensive review of BET-targeting PROTACs for cancer therapy. *Bioorg Med Chem*. 2022;73:117033.
193. Hamilton EP, Wang JS, Oza AM, Patel MR, Ulahannan SV, Bauer T, et al. First-in-human Study of AZD5153, A Small-molecule Inhibitor of Bromodomain Protein 4, in Patients with Relapsed/Refractory Malignant Solid Tumors and Lymphoma. *Mol Cancer Ther*. 2023;22(10):1154-65.
194. Bamborough P, Chung CW, Goodwin NC, Mitchell DJ, Neipp CE, Phillipou A, et al. Design and Characterization of 1,3-Dihydro-2H-benzo[d]azepin-2-ones as Rule-of-5 Compliant Bivalent BET Inhibitors. *ACS Med Chem Lett*. 2023;14(9):1231-6.
195. To KKW, Xing E, Larue RC, Li PK. BET Bromodomain Inhibitors: Novel Design Strategies and Therapeutic Applications. *Molecules*. 2023;28(7).
196. Li T, Zhang W, Wang Y, Xu G, Miao F, Chen P, et al. Lysine-Targeting, Covalent Inhibitors of Bromodomain BD1 of BET Proteins in Live Cells and Animals. *Angew Chem Int Ed Engl*. 2025;64(28):e202424832.
197. Chen D, Frezza M, Schmitt S, Kanwar J, Dou QP. Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. *Curr Cancer Drug Targets*. 2011;11(3):239-53.
198. Csizmar CM, Kim D-H, Sachs Z. The role of the proteasome in AML. *Blood cancer journal*. 2016;6(12):e503.
199. Guzman ML, Neering SJ, Upchurch D, Grimes B, Howard DS, Rizzieri DA, et al. Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood*. 2001;98(8):2301–7.
200. Kagoya Y, Yoshimi A, Kataoka K, Nakagawa M, Kumano K, Arai S, et al. Positive feedback between NF- κ B and TNF- α promotes leukemia-initiating cell capacity. *The Journal of clinical investigation*. 2014;124(2):528–42.
201. Richardson PG, Sonneveld P, Schuster MW, Irwin D, Stadtmauer EA, Facon T, et al. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. *The New England journal of medicine*. 2005;352(24):2487–98.
202. Manasanch EE, Orlowski RZ. Proteasome inhibitors in cancer therapy. *Nature reviews Clinical oncology*. 2017;14(7):417–33.
203. Robak P, Robak T. Bortezomib for the Treatment of Hematologic Malignancies: 15 Years Later. *Drugs in R&D*. 2019;19(2):73–92.
204. Meregalli C. An Overview of Bortezomib-Induced Neurotoxicity. *Toxics*. 2015;3(3):294–303.
205. Moreau P, Masszi T, Grzasko N, Bahlis NJ, Hansson M, Pour L, et al. Ixazomib, an Investigational Oral Proteasome Inhibitor (PI), in Combination with Lenalidomide and Dexamethasone (IRd), Significantly Extends Progression-Free Survival (PFS) for Patients (Pts) with Relapsed and/or Refractory Multiple Myeloma (RRMM): The Phase 3 Tourmaline-MM1 Study (NCT01564537). *Blood*. 2015;126(23):727.

5. References

206. Siegel DS, Martin T, Wang M, Vij R, Jakubowiak AJ, Lonial S, et al. A phase 2 study of single-agent carfilzomib (PX-171-003-A1) in patients with relapsed and refractory multiple myeloma. *Blood*. 2012;120(14):2817–25.
207. Kraft FB, Biermann L, Schaker-Hubner L, Hanl M, Hamacher A, Kassack MU, et al. Hydrazide-Based Class I Selective HDAC Inhibitors Completely Reverse Chemoresistance Synergistically in Platinum-Resistant Solid Cancer Cells. *J Med Chem*. 2024;67(19):17796-819.
208. Hölscher AS, Schulz WA, Pinkerneil M, Niegisch G, Hoffmann MJ. Combined inhibition of BET proteins and class I HDACs synergistically induces apoptosis in urothelial carcinoma cell lines. *Clinical epigenetics*. 2018;10:1.
209. Bhadury J, Nilsson LM, Muralidharan SV, Green LC, Li Z, Gesner EM, et al. BET and HDAC inhibitors induce similar genes and biological effects and synergize to kill in Myc-induced murine lymphoma. *Proceedings of the National Academy of Sciences*. 2014;111(26):E2721-30.
210. Bollmann LM, Skerhut AJ, Asfaha Y, Horstick N, Hanenberg H, Hamacher A, et al. The Novel Class Ila Selective Histone Deacetylase Inhibitor YAK540 Is Synergistic with Bortezomib in Leukemia Cell Lines. *International journal of molecular sciences*. 2022;23(21).
211. Asfaha Y, Bollmann LM, Skerhut AJ, Fischer F, Horstick N, Roth D, et al. 5-(Trifluoromethyl)-1,2,4-oxadiazole (TFMO)-based highly selective class Ila HDAC inhibitors exhibit synergistic anticancer activity in combination with bortezomib. *European journal of medicinal chemistry*. 2024;263:115907.
212. Bollmann LM, Lange F, Hamacher A, Biermann L, Schäker-Hübner L, Hansen FK, et al. Triple Combination of Entinostat, a Bromodomain Inhibitor, and Cisplatin Is a Promising Treatment Option for Bladder Cancer. *Cancers*. 2024;16(19):3374.
213. Hospital Z. Bortezomib Combined With DAG Regimen in the Treatment of Refractory/Relapsed AML: NCT05982756: Zhongnan Hospital; [cited 25.09. 2024]. Available from: <https://www.clinicaltrials.gov/study/NCT05982756?cond=AML&intr=Bortezomib&rank=4>.
214. Schlenk PDRF. TEAM-Trial: Targeting Epigenetic Therapy Resistance in AML With Bortezomib (TEAM): Prof. Dr. Richard F Schlenk, University Hospital Heidelberg; [cited 2025 20.08.]. Available from: <https://clinicaltrials.gov/study/NCT04173585>.
215. Institute NC. Bortezomib, Sorafenib Tosylate, and Decitabine in Treating Patients With Acute Myeloid Leukemia: National Cancer Institute; [cited 2025 20.08.]. Available from: <https://clinicaltrials.gov/study/NCT01861314>.
216. Institute NC. Bortezomib and Sorafenib Tosylate in Treating Patients With Newly Diagnosed Acute Myeloid Leukemia: National Cancer Institute; [cited 2025 20.08.]. Available from: <https://clinicaltrials.gov/study/NCT01371981>
217. Masonic Cancer Center UoM. Phase I/II Clinical Trial of Proteasome Inhibitor in Combination With CPX-351 for the Treatment of Newly-Diagnosed TP53-mutated Acute Myeloid Leukemia (AML): Masonic Cancer Center, University of Minnesota; [cited 2025 20.08.]. Available from: <https://clinicaltrials.gov/study/NCT07008638?term=NCT07008638&rank=1>.
218. Di Veroli GY, Fornari C, Wang D, Mollard S, Bramhall JL, Richards FM, et al. Combeneft: an interactive platform for the analysis and visualization of drug combinations. *Bioinformatics*. 2016;32(18):2866-8.
219. Zhao W, Sachsenmeier K, Zhang L, Sult E, Hollingsworth RE, Yang H. A New Bliss Independence Model to Analyze Drug Combination Data. *J Biomol Screen*. 2014;19(5):817-21.
220. Shamloo B, Usluer S. p21 in Cancer Research. *Cancers*. 2019;11(8).
221. Gui C-Y, Ngo L, Xu WS, Richon VM, Marks PA. Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. *Proceedings of the National Academy of Sciences*. 2004;101(5):1241–6.
222. Bellucci L, Dalvai M, Kocanova S, Moutahir F, Bystricky K. Activation of p21 by HDAC inhibitors requires acetylation of H2A.Z. *PloS one*. 2013;8(1):e54102.
223. Bose P, Dai Y, Grant S. Histone deacetylase inhibitor (HDACI) mechanisms of action: emerging insights. *Pharmacology & therapeutics*. 2014;143(3):323–36.

5. References

224. Hrgovic I, Doll M, Kleemann J, Wang XF, Zoeller N, Pinter A, et al. The histone deacetylase inhibitor trichostatin a decreases lymphangiogenesis by inducing apoptosis and cell cycle arrest via p21-dependent pathways. *BMC Cancer*. 2016;16(1):763.
225. Huang YH, Huang YM, Huang WJ, Yu MC, Chuang CH, Hsu YF, et al. The hydroxamate based HDAC inhibitor WMJ-J-09 induces colorectal cancer cell death by targeting tubulin and downregulating survivin. *Sci Rep*. 2025;15(1):19590.
226. Spaety ME, Gries A, Badie A, Venkatasamy A, Romain B, Orvain C, et al. HDAC4 Levels Control Sensibility toward Cisplatin in Gastric Cancer via the p53-p73/BIK Pathway. *Cancers (Basel)*. 2019;11(11).
227. Kang ZH, Wang CY, Zhang WL, Zhang JT, Yuan CH, Zhao PW, et al. Histone deacetylase HDAC4 promotes gastric cancer SGC-7901 cells progression via p21 repression. *PLoS One*. 2014;9(6):e98894.
228. Di Giorgio E, Gagliostro E, Brancolini C. Selective class IIa HDAC inhibitors: myth or reality. *Cell Mol Life Sci*. 2015;72(1):73-86.
229. Krieger V, Hamacher A, Cao F, Stenzel K, Gertzen CGW, Schaker-Hubner L, et al. Synthesis of Peptoid-Based Class I-Selective Histone Deacetylase Inhibitors with Chemosensitizing Properties. *J Med Chem*. 2019;62(24):11260-79.
230. Schrenk C, Bollmann LM, Haist C, Bister A, Wiek C, Wecker M, et al. Synergistic Interaction of the Class IIa HDAC Inhibitor CHDI0039 with Bortezomib in Head and Neck Cancer Cells. *International journal of molecular sciences*. 2023;24(6).
231. Luckhurst CA, Aziz O, Beaumont V, Burli RW, Breccia P, Maillard MC, et al. Development and characterization of a CNS-penetrant benzhydryl hydroxamic acid class IIa histone deacetylase inhibitor. *Bioorg Med Chem Lett*. 2019;29(1):83-8.
232. Velcade : EPAR - Product Information [cited 2025 07.09.]. Available from: https://www.ema.europa.eu/en/documents/product-information/velcade-epar-product-information_en.pdf.
233. Qilu Pharmaceutical Co. L. Phase II Study of QLS32015 Combination Therapy in the Treatment of Multiple Myeloma: Qilu Pharmaceutical Co., Ltd.; [cited 2025 22.08.]. Available from: <https://www.clinicaltrials.gov/study/NCT07018050?intr=Bortezomib&sort=StudyFirstPostDate&rank=7>.
234. Sogbein O, Paul P, Umar M, Chaari A, Batuman V, Upadhyay R. Bortezomib in cancer therapy: Mechanisms, side effects, and future proteasome inhibitors. *Life Sci*. 2024;358:123125.
235. Curran MP, McKeage K. Bortezomib: a review of its use in patients with multiple myeloma. *Drugs*. 2009;69(7):859-88.
236. Center MDAC. PS-341 Plus Carboplatin in Platinum and Taxane Resistant Recurrent Ovarian Cancer, Primary Peritoneal Cancer, and Fallopian Tube Cancer: M.D. Anderson Cancer Center; [cited 2025 22.08.]. Available from: <https://clinicaltrials.gov/study/NCT00059618>.
237. California UoS. Study of Bortezomib and Docetaxel for Patients With Hormone Refractory Prostate Cancer: University of Southern California; [cited 2025 22.08.]. Available from: <https://clinicaltrials.gov/study/NCT00183937>.
238. Tan CRC, Abdul-Majeed S, Cael B, Barta SK. Clinical Pharmacokinetics and Pharmacodynamics of Bortezomib. *Clin Pharmacokinet*. 2019;58(2):157-68.
239. Meregalli C. An Overview of Bortezomib-Induced Neurotoxicity. *Toxics*. 2015;3(3):294-303.
240. Siegsmond MJ, Marx C, Seemann O, Schummer B, Steidler A, Toktomambetova L, et al. Cisplatin-resistant bladder carcinoma cells: enhanced expression of metallothioneins. *Urological research*. 1999;27(3):157-63.
241. Bagratuni T, Mavrianou N, Gavalas NG, Tzannis K, Arapinis C, Liontos M, et al. JQ1 inhibits tumour growth in combination with cisplatin and suppresses JAK/STAT signalling pathway in ovarian cancer. *European Journal of Cancer*. 2020;126:125-35.

5. References

242. Fiskus W, Sharma S, Qi J, Valenta JA, Schaub LJ, Shah B, et al. Highly active combination of BRD4 antagonist and histone deacetylase inhibitor against human acute myelogenous leukemia cells. *Molecular cancer therapeutics*. 2014;13(5):1142–54.
243. Mazur PK, Herner A, Mello SS, Wirth M, Hausmann S, Sánchez-Rivera FJ, et al. Combined inhibition of BET family proteins and histone deacetylases as a potential epigenetics-based therapy for pancreatic ductal adenocarcinoma. *Nature Medicine*. 2015;21(10):1163–71.
244. Meng W, Wang B, Mao W, Wang J, Zhao Y, Li Q, et al. Enhanced efficacy of histone deacetylase inhibitor combined with bromodomain inhibitor in glioblastoma. *Journal of experimental & clinical cancer research : CR*. 2018;37(1):241.
245. Lai Y-S, Chen J-Y, Tsai H-J, Chen T-Y, Hung W-C. The SUV39H1 inhibitor chaetocin induces differentiation and shows synergistic cytotoxicity with other epigenetic drugs in acute myeloid leukemia cells. *Blood cancer journal*. 2015;5(5):e313.
246. Ramadoss M, Mahadevan V. Targeting the cancer epigenome: synergistic therapy with bromodomain inhibitors. *Drug discovery today*. 2018;23(1):76–89.
247. Gueugnon F, Cartron P-F, Charrier C, Bertrand P, Fonteneau J-F, Gregoire M, et al. New histone deacetylase inhibitors improve cisplatin antitumor properties against thoracic cancer cells. *Oncotarget*. 2014;5(12):4504–15.
248. Shen J, Huang C, Jiang L, Gao F, Wang Z, Zhang Y, et al. Enhancement of cisplatin induced apoptosis by suberoylanilide hydroxamic acid in human oral squamous cell carcinoma cell lines. *Biochemical pharmacology*. 2007;73(12):1901–9.
249. Suraweera A, O'Byrne KJ, Richard DJ. Combination Therapy With Histone Deacetylase Inhibitors (HDACi) for the Treatment of Cancer: Achieving the Full Therapeutic Potential of HDACi. *Frontiers in oncology*. 2018;8:92.
250. Manzotti G, Ciarrocchi A, Sancisi V. Inhibition of BET Proteins and Histone Deacetylase (HDACs): Crossing Roads in Cancer Therapy. *Cancers*. 2019;11(3).
251. Wilson AJ, Stubbs M, Liu P, Ruggeri B, Khabele D. The BET inhibitor INCB054329 reduces homologous recombination efficiency and augments PARP inhibitor activity in ovarian cancer. *Gynecologic oncology*. 2018;149(3):575–84.
252. Andrikopoulou A, Lontos M, Koutsoukos K, Dimopoulos M-A, Zagouri F. Clinical perspectives of BET inhibition in ovarian cancer. *Cellular oncology (Dordrecht, Netherlands)*. 2021;44(2):237–49.
253. Kurmasheva RT, Bandyopadhyay A, Favours E, Del Pozo V, Ghilu S, Phelps DA, et al. Evaluation of entinostat alone and in combination with standard-of-care cytotoxic agents against rhabdomyosarcoma xenograft models. *Pediatric blood & cancer*. 2019;66(8):e27820.
254. Roboz GJ, Desai P, Lee S, Ritchie EK, Winer ES, DeMario M, et al. A dose escalation study of RO6870810/TEN-10 in patients with acute myeloid leukemia and myelodysplastic syndrome. *Leukemia & lymphoma*. 2021;62(7):1740–8.
255. Shapiro GI, LoRusso P, Dowlati A, T Do K, Jacobson CA, Vaishampayan U, et al. A Phase 1 study of RO6870810, a novel bromodomain and extra-terminal protein inhibitor, in patients with NUT carcinoma, other solid tumours, or diffuse large B-cell lymphoma. *British journal of cancer*. 2021;124(4):744–53.
256. Sharp M, LLC D. A Dose Exploration Study With Birabresib (MK-8628) in Participants With Selected Advanced Solid Tumors (MK-8628-006) [cited 2024 04.04.]. Available from: <https://www.clinicaltrials.gov/study/NCT02698176?intr=OTX015&rank=4#publications>.
257. Yeruva SLH, Zhao F, Miller KD, Tevaarwerk AJ, Wagner LI, Gray RJ, et al. E2112: randomized phase iii trial of endocrine therapy plus entinostat/placebo in patients with hormone receptor-positive advanced breast cancer. *NPJ breast cancer*. 2018;4:1.
258. Hopkins SKCCaJ. A Clinical Trial of Entinostat in Combination With Nivolumab for Patients With Previously Treated Unresectable or Metastatic Cholangiocarcinoma and Pancreatic Adenocarcinoma: NCT03250273: Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins; [cited 2024 28.10.]. Available from: <https://clinicaltrials.gov/study/NCT03250273>.

5. References

- 259. Zanelato I, Colangelo D, Osella D. JQ1, a BET Inhibitor, Synergizes with Cisplatin and Induces Apoptosis in Highly Chemoresistant Malignant Pleural Mesothelioma Cells. *Current cancer drug targets*. 2018;18(8):816–28.
- 260. Shi X, Liu C, Liu B, Chen J, Wu X, Gong W. JQ1: a novel potential therapeutic target. *Die Pharmazie*. 2018;73(9):491–3.
- 261. Pang Y, Bai G, Zhao J, Wei X, Li R, Li J, et al. The BRD4 inhibitor JQ1 suppresses tumor growth by reducing c-Myc expression in endometrial cancer. *Journal of translational medicine*. 2022;20(1):336.
- 262. Galloway TJ, Wirth LJ, Colevas AD, Gilbert J, Bauman JE, Saba NF, et al. A Phase I Study of CUDC-101, a Multitarget Inhibitor of HDACs, EGFR, and HER2, in Combination with Chemoradiation in Patients with Head and Neck Squamous Cell Carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2015;21(7):1566–73.
- 263. Ma C, Cheng J, Gu J, Wang Q. Epigenetic drugs in cancer therapy: mechanisms, immune modulation, and therapeutic applications. *Mol Biomed*. 2025;6(1):132.
- 264. Wahi A, Manchanda N, Jain P, Jadhav HR. Targeting the epigenetic reader "BET" as a therapeutic strategy for cancer. *Bioorg Chem*. 2023;140:106833.

List of Publications

Bollmann, L.M.; Skerhut, A.J.; Asfaha, Y.; Horstick, N.; Hanenberg, H.; Hamacher, A.; Kurz, T.; Kassack, M.U. The Novel Class IIa Selective Histone Deacetylase Inhibitor YAK540 Is Synergistic with Bortezomib in Leukemia Cell Lines. *Int. J. Mol. Sci.* 2022, 23, doi:10.3390/ijms232113398.

Shared first authorship: **Bollmann, L.M.**; Skerhut, A.J.; Asfaha, Y.

Shared last authorship: Kurz, T.; Kassack, M.U.

Asfaha, Y.; **Bollmann, L.M.**; Skerhut, A.J.; Fischer, F.; Horstick, N.; Roth, D.; Wecker, M.; Mammen, C.; Smits, S.H.J.; Fluegen, G.; et al. 5-(Trifluoromethyl)-1,2,4-oxadiazole (TFMO)-based highly selective class IIa HDAC inhibitors exhibit synergistic anticancer activity in combination with bortezomib. *Eur. J. Med. Chem.* 2024, 263, 115907, doi:10.1016/j.ejmech.2023.115907.

Shared first authorship: Asfaha, Y.; **Bollmann, L.M.**; Skerhut, A.J.

Shared last authorship: Kassack, M.U.; Kurz, T.

Bollmann, L.M.; Lange, F.; Hamacher, A.; Biermann, L.; Schäker-Hübner, L.; Hansen, F.K.; Kassack, M.U. Triple Combination of Entinostat, a Bromodomain Inhibitor, and Cisplatin Is a Promising Treatment Option for Bladder Cancer. *Cancers (Basel)* 2024, 16, 3374, doi:10.3390/cancers16193374.

Lead authorship: **Bollmann, L.M.**

Senior authorship: Kassack, M.U.

Schrenk, C.; **Bollmann, L.M.**; Haist, C.; Bister, A.; Wiek, C.; Wecker, M.; Roth, D.; Petzsch, P.; Köhrer, K.; Hamacher, A.; et al. Synergistic Interaction of the Class IIa HDAC Inhibitor CHDI0039 with Bortezomib in Head and Neck Cancer Cells. *Int. J. Mol. Sci.* 2023, 24, doi:10.3390/ijms24065553.

Lead authorship: Schrenk, C.

Senior authorship: Kassack, M.U.

Conference Poster

Lukas M. Bollmann, Alexandra Hamacher, Yodita Asfaha, Fabian Fischer, Christian Schrenk, Georg Flügen, Helmut Hanenberg, Alexander J. Skerhut, Thomas Kurz, Matthias U. Kassack, Treatment of leukemia and solid cancer by class IIa histone deacetylase and proteasome inhibitors is a promising synergistic combination, *Clinical Epigenetics International Conference*, 24-26 May **2023** in Szczecin, Poland

Acknowledgement

Die Promotionszeit gab mir den Raum mich selbst mit unterschiedlichen spannenden Themen zu befassen und mich im beruflich sowie privat weiterzuentwickeln. Viele Menschen haben mich auf diesem Weg begleitet und auf unterschiedlicher Weise unterstützt. Dafür möchte ich an dieser Stelle meine Dankbarkeit ausdrücken.

Zuallererst möchte ich mich bei meinem Doktorvater Prof. Dr. Matthias Kassack für die Gelegenheit Teil seiner Arbeitsgruppe werden zu können bedanken. Seit meiner Jugendzeit hatte ich den Wunsch eines Tages zu forschen. Diesen Wunsch konnte ich mir dank seiner Unterstützung erfüllen. Ebenso bin ich ihm dankbar für die spannende Auswahl an Themen und das Vertrauen in mich, diese Themen selbstständig zu bearbeiten und zu gestalten. Ich konnte auf sein fachliches Feedback vertrauen, dass mich immer wieder motiviert und inspiriert hat auch neue Wege auszuprobieren.

Als nächstes gilt mein Dank auch Prof. Dr. Holger Gohlke für seine Übernahme meiner Mentorenschaft. Seine Mentorenschaft erschaffte eine Verbindung zwischen den jeweiligen Arbeitskreisen, die neben anregendem fachlichen Austausch auch Gelegenheit zum privaten Austausch bot.

Besonders bedanken möchte ich mich bei Frau Dr. Hamacher. Ich habe Frau Hamacher während meiner Promotionszeit sehr zu schätzen gelernt. Zum einen als Ansprechpartnerin bei Problemen jeglicher Belange, denn sie hatte immer, trotz eigener beruflicher Auslastung, ein offenes Ohr für die Schwierigkeiten und Herausforderungen. Zum anderen werde ich mich an viele lustige und spannende Unterhaltungen erinnern, die auch so manchen langwierigen Praktikumstag sehr kurzweilig verfliegen ließ. Ihre enthusiastische Art der Lehre hat mich während der Promotionszeit noch mehr beeindruckt, als schon zuvor als Student. Ich hoffe, dass ich mir in der Zeit an der Uni eine kleine Scheibe davon abgeschnitten habe.

Mein Dank gilt auch der übrigen Arbeitsgruppe, insbesondere Lukas Biermann, Jingyu Li, Laura Pradel, Alexander Skerhut, Fritz Lange, Christian Schrenk, Jan Bandolik, Kathrin Christoph und Nadine Horstick-Muche. Ich werde mich noch lange an die guten wie schlechten gemeinsam durchgestandenen Tage im Büro erinnern.

Weiterhin möchte ich mich bei allen Kooperationspartnern für die erfolgreiche Zusammenarbeit bedanken: dem Forschungslabor Urologie AG Harnblasenkarzinom unter der Leitung von Dr. Michèle Hoffmann; Dennis Roth und Maria Wecker (Klinik für Chirurgie (A), Medizinische Fakultät, Universitätsklinikum der Heinrich-Heine-Universität) und Prof. Dr. Finn Hansen (Pharmazeutisches Institut Abteilung Pharmazeutische und Zellbiologische Chemie, Universität Bonn).

Ein herzlicher Dank geht an alle meine Freunde und Bekannte, die mich auf diesem Weg bis hierhin begleitet, unterstützt, gefördert und mich zu dem Menschen gemacht

Acknowledgement

haben, der ich heute bin. Insbesondere möchte ich mich bei meinen ehemaligen Kommilitonen Antonella Blotta, Annika Müller, Roxana Schuhmann, Susanne Pfeiffer und Rebecca Schiffer bedanken. Nicht nur während des Studiums, auch während der Promotionszeit wart ihr eine wichtige Stütze für mich.

Zuletzt möchte ich meiner Familie danken. Zuerst meinen Eltern Heinz und Maria Bollmann, die mir diesen Weg überhaupt erst ermöglicht haben. Durch ihre Unterstützung sowohl während als auch anschließend an mein Studium konnte ich meinen Wunsch des Forschens verfolgen. Dank ihrer Unterstützung konnte ich meinen Weg bis hierhin gehen, wofür ich sehr dankbar bin.

Auch möchte ich mich bei meinen Brüdern Sebastian Bollmann und Thomas Bollmann herzlich bedanken für ihre Unterstützung in dieser Zeit. Beide standen mir jederzeit als Ansprechpartner zur Seite und sorgten regelmäßig für Ablenkung, wenn mir der Uni-Alltag über den Kopf wuchs.

Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Diese Dissertation wurde in keinem anderen Prüfungsverfahren vorgelegt. Ich habe bislang noch keinen erfolglosen Promotionsversuch unternommen.

(Ort, Datum)

(Unterschrift)