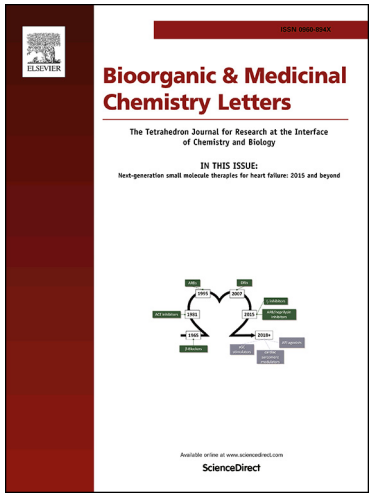

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Design, synthesis, and bioactivity evaluation of novel Bcl-2/HDAC dual-target inhibitors for the treatment of multiple myeloma

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Abstract: Multiple myeloma (MM) is the second most common haematological malignancy. Almost all patients with MM eventually relapse, and most recommended treatment protocols for the patients with relapsed refractory MM comprise a combination of drugs with different mechanisms of action. Therefore novel drugs are in urgent need in clinic. Bcl-2 inhibitors and HDAC inhibitors were proved their anti-MM effect in clinic or under clinical trials, and they were further discovered to have synergistic interactions. In this study, a series of Bcl-2/HDAC dual-target inhibitors were designed and synthesized. Among them, compounds **7e - 7g** showed good inhibitory activities against HDAC6 and high binding affinities to Bcl-2 protein

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simultaneously. They also displayed good growth inhibitory activities against human MM cell line RPMI-8226, which proved their potential value for the treatment of multiple myeloma.

Multiple myeloma (MM) is a malignancy of terminally differentiated plasma cells, and is the second most common haematological malignancy. Along with autologous stem cell transplantation, the use of new drugs such as proteasome inhibitors and immunomodulatory drugs has increased response rates and survival substantially in the past decade.[1, 2] However, almost all patients with MM eventually relapse.[3] As a result, novel drugs are in urgent need in clinic. Histone deacetylase (HDAC) inhibitor panobinostat (Fig. 1) has been approved recently for the treatment of relapsed/refractory MM.[4] Several other novel drugs are being explored under clinical trials and seem to be promising, such as kinesin spindle protein inhibitors,[5] nuclear export protein inhibitors,[6] histone deacetylase 6 inhibitors[7] and Bcl-2 inhibitors.[8]

Anti-apoptotic Bcl-2 proteins have become attractive targets for anti-cancer agents development, because they are associated with progression of a wide variety of human cancers.[9-11] The small-molecular Bcl-2 inhibitor venetoclax (ABT-199) (Fig. 1) has been approved recently for the treatment of patients with chronic lymphocytic leukemia who have a chromosomal abnormality called 17p deletion.[8, 12] At present, ABT-199 is under clinical trials for the treatment of MM.[8] HDACs, which are frequently dysregulated in cancer, can be subdivided into 4 classes: classes I, II and IV (zinc dependent enzymes), and class III (nicotinamide adenine dinucleotide

(NAD)-dependent). Five HDAC inhibitors vorinostat (SAHA), romidpesin, belinostat, chidamide, and panobinostat have been approved as anti-cancer agents.[13, 14] The class IIb HDAC, HDAC6 is distinguished by its ability to deacetylate α-tubulin and HSP90, in addition to the modification of histone.[14, 15] A selective HDAC6 inhibitor ACY-1215 (Fig. 1) is currently under clinical trials for the treatment of MM.[7]

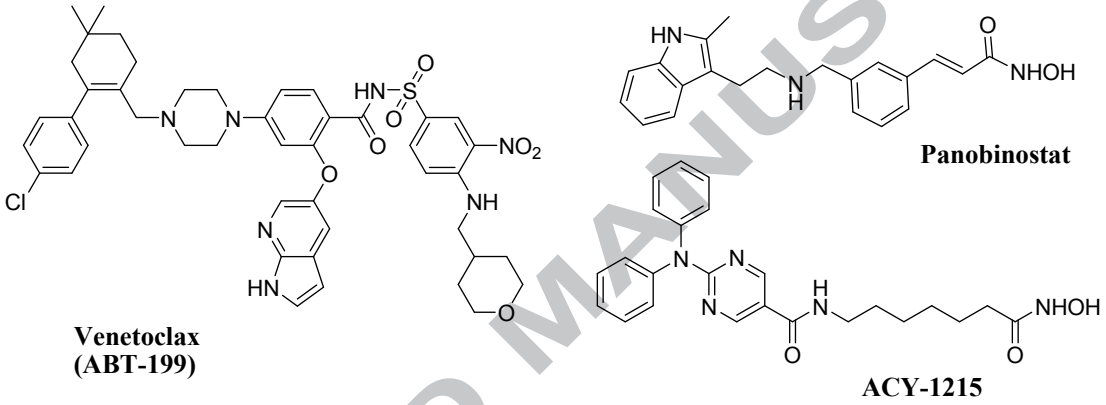


Figure 1. Chemical structures of representative HDAC inhibitors and Bcl-2 inhibitors

Most recommended treatment protocols for the patients with relapsed refractory MM comprise a combination of drugs with different mechanisms of action, such as lenalidomide or bortezomib in combination with dexamethasone, or even triplet regimens.[1, 16] HDAC inhibitors and Bcl-2 inhibitors were in combination with above approved chemotherapeutic agents in clinic or clinical trials for the treatment of MM, [4, 8] and showed synergistic effects with them.[17-20] Additionally, Bcl-2 inhibitors and HDAC inhibitors were discovered to have synergistic interactions in MM cell lines, at least in part, through Bim upregulation.[21, 22] The combination of Bcl-2 inhibitors and HDAC inhibitors were further proved to overcome adaptive bortezomib resistance *in*

vitro.[23] Above research provide the reasonability of the therapeutic strategies combining these two type of agents for the treatment of MM.

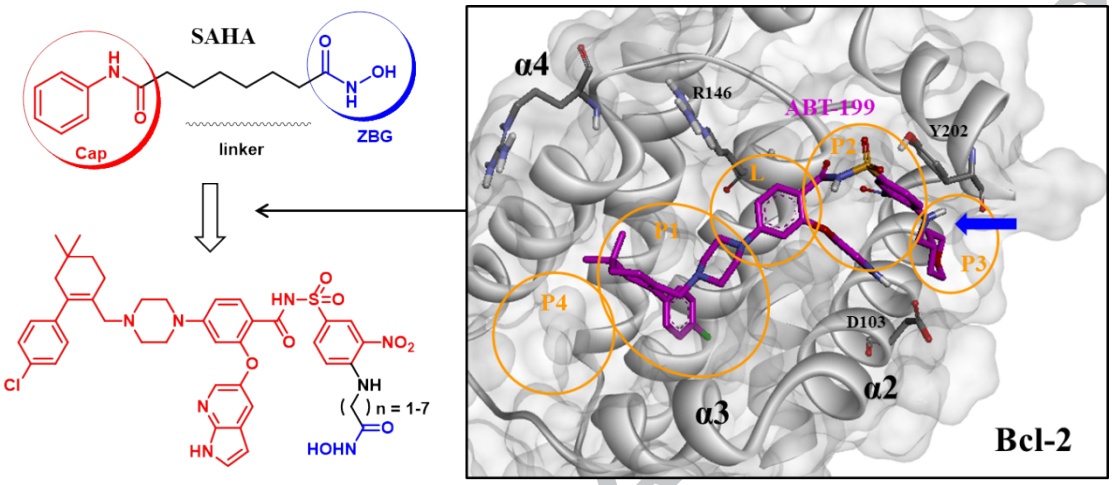


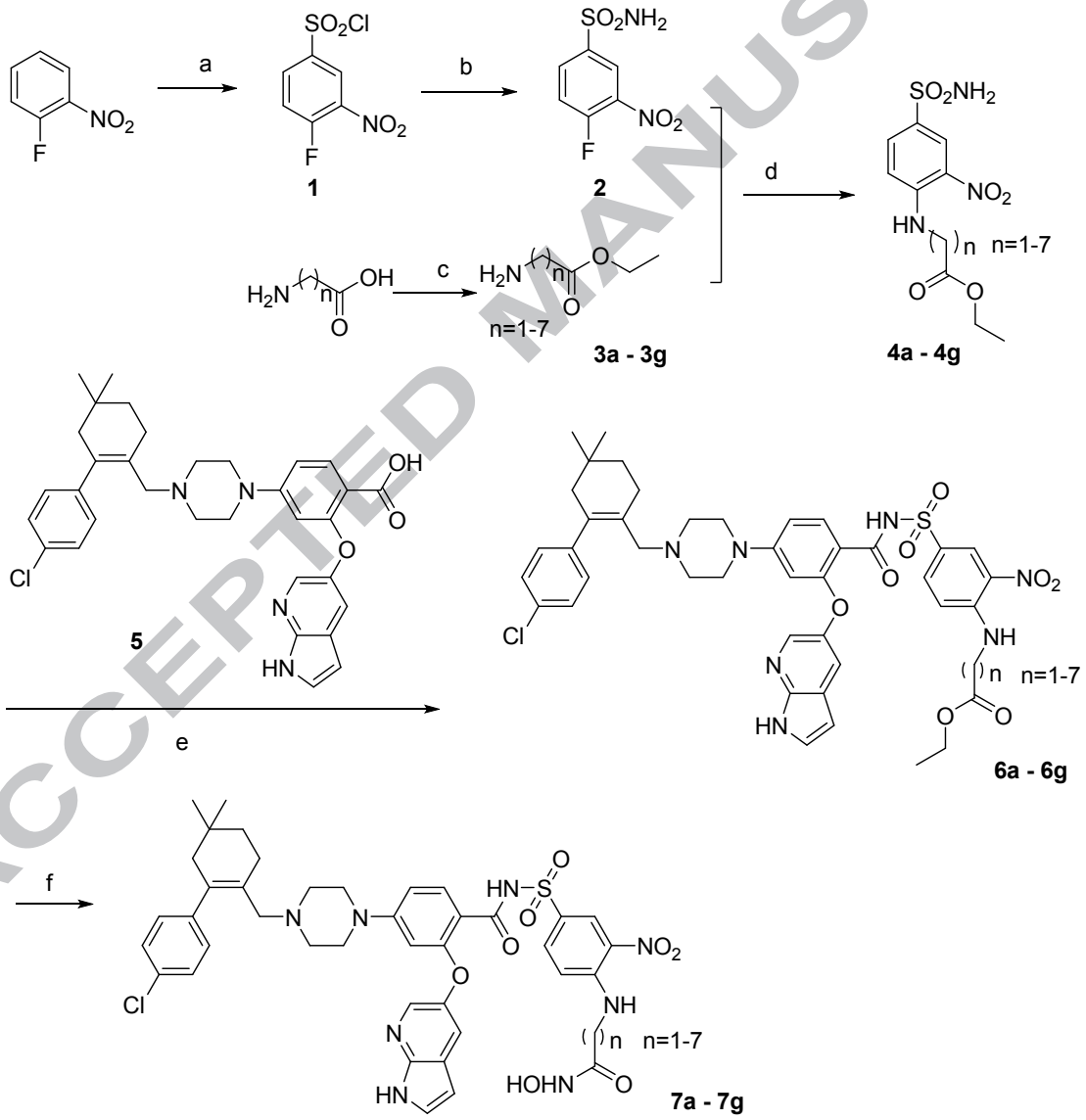
Figure 2. Design of title compounds. Three structural features of HDAC inhibitors a cap region, a zinc-binding group (ZBG), and a linker region were labeled on the structure of SAHA. The binding modes of ABT-199 (purple sticks) in complex with Bcl-2 (using crystal structure PDB ID: 4MAN as templet) was displayed in the black box. Five pockets of the binding groove of Bcl-2 were showed as orange circles labeled as P1, L, P2, P3 and P4. The place where the hydroxamic acid group and linkers were attached (blue arrow) was exposed to the solvent.

Considered the more predictable pharmacokinetic profile, less drug-drug interactions, the improved convenience, the potential superior efficacy and lower toxicity, drugs with optimal multi-target activities may represent a valuable complement or even alternative to therapeutic regimens based on drug combinations.[24, 25] In this study, a series of Bcl-2/HDAC dual-target inhibitors were designed. It is well known that the

HDAC inhibitors have a general structure consisting of a cap region, a zinc-binding group (ZBG), and a linker region which connects the two (Fig. 2). Hydroxamic acid group is the most common used ZBG. It chelates the zinc ion in the active site and is critical for the inhibitory activity. However, the cap region, the so-called surface recognition domain, occludes the entrance of the active site pocket. It is predominantly responsible for selectivity and tolerates a large change.[13, 26] In addition, the docking binding mode of ABT-199 and Bcl-2 protein (Fig. 2) was obtained using the complex crystal structure of its analog as template (PDB ID: 4MAN).[27] Based on the three-dimensional pharmacophore we constructed before,[28-30] the groups binding in the P1, L and P2 pockets of binding groove played major roles in the interaction with Bcl-2 protein. While the tetrahydropyranyl methyl group binding in the P3 pocket exposed to the solvent and had less effect on the binding. As a result, Bcl-2/HDAC dual-target inhibitors were designed by the replacement of the tetrahydropyranyl methyl group of ABT-199 by hydroxamic acid group with different linkers (Fig. 2).

The synthetic routes of title compounds were shown in Scheme 1. Intermediate **1** was obtained by electrophilic substitution reaction of 1-fluoro-2-nitrobenzene under the condition of chlorosulfonic acid, and then intermediate **2** was obtained under the condition of aqueous ammonia in ice bath. Using different amino acid as start material, esterification reaction was carried out under the condition of thionyl chloride and ethanol to produce the intermediates **3a - 3g**. Intermediates **4a -4g** was yielded from intermediates **2** and intermediates **3a - 3g** by nucleophilic substitution reaction

under the condition of DIEA in DMSO. Compound **5** was synthesized by the method reported.[27] Then intermediates **4a -4g** and **5** were treated with condensation agent EDCI to obtain intermediate **6a – 6g**. It was ammoniated in methanol solution of hydroxylamine hydrochloride in which KOH had been dissociated, title compounds **7a – 7g** was obtained finally.



Reagents and conditions: a) HSO_3Cl， reflux; b) NH_4OH, 0℃; c) $SOCl_2$, EtOH, rt; d) DIEA, DMSO, 80℃; e) EDCI, DMAP, DCM; f) NH_2OH, MeOH, KOH, rt.

Scheme 1. Synthesis of title compounds.

The inhibitory activities against HDAC1 and HDAC6 of title compounds were evaluated in a fluorescent assay using SAHA and ACY-1215 as positive control. The results were displayed in Fig. 3A. Compounds **7e - 7g** with linker containing 5-7 carbons showed good inhibitory activities against HDAC6. They also displayed obvious selectivity to HDAC6 over HDAC1 (selectivity index > 10), which was comparable with that of ACY-1215, the selective HDAC6 inhibitor under clinical trials. Additionally, these results were consistent with the biological effect in cells based on the western blot analysis (Table 1). After incubation in human MM cell line RPMI-8226 at 10 μM for 24 h, compound **7e - 7g** increased the level of Ac-tubulin significantly, and only compound **7g** increased the level of Ac-H3 obviously.

A

No.	HDAC6 (IC_{50}, nM)	HDAC1 (IC_{50}, nM)
7a	$>10^3$	$>10^3$
7b	$>10^3$	$>10^3$
7c	$>10^3$	$>10^3$
7d	$>10^3$	$>10^3$
7e	28 ± 4	847 ± 88
7f	28 ± 6	665 ± 81
7g	19 ± 1	281 ± 70
SAHA	13 ± 1	35 ± 1
ACY-1215	5 ± 1	74 ± 18

B

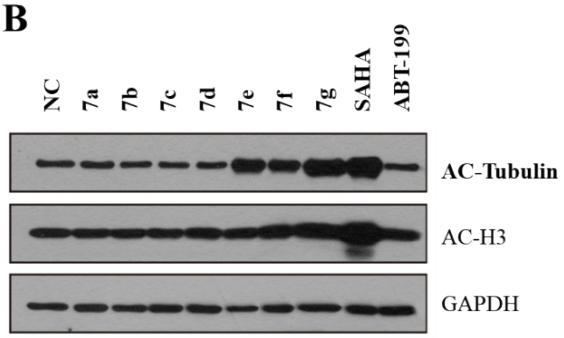


Figure 3. A. Inhibitory activities against HDAC1 and HDAC6 of title compounds. **B.** Western blot analysis of the level of Ac-H3 and Ac-tubulin in RPMI-8226 cell line after incubating with title compounds at 10 μM for 24 h. Protein expression was detected by immunoblot analysis with the specific antibody.

Then, binding affinities to Bcl-2 protein of compounds **7e - 7g** were further evaluated

by a fluorescence polarization (FP)-based method using ABT-199 as positive control (Table 1). All of the compounds showed high binding affinities, which was comparable with that of ABT-199. It indicated that the replacement of the tetrahydropyranyl methyl group of ABT-199 by hydroxamic acid group with different aliphatic chains had little effect on the binding to Bcl-2 protein. Based on the above results, compounds **7e - 7g** were potent Bcl-2/HDAC dual-target inhibitors. They were evaluated the growth inhibitory activities against human MM cell lines RPMI-8226 and U266 (Table 1), and ABT-199, ACY-1215 and SAHA were used as positive controls. RPMI-8226 cell line seems to be sensitive to these compounds than U266 cell line. On RPMI-8226 cell line, these compounds displayed obviously better growth inhibitory activities than positive controls. It indicated that the Bcl-2/HDAC dual-target inhibitors show potential value for the treatment of MM.

Table 1

Binding affinities to Bcl-2 protein and growth inhibitory activities against human MM cell lines RPMI-8226 and U266 of **7e - 7g**

	Bcl-2 (IC_{50}, μM)	**RPMI-8226 (IC_{50}, μM)**	**U266 (IC_{50}, μM)**
7e	0.23 ± 0.01	0.3 ± 0.0	27.1± 4.2
7f	0.24 ± 0.02	0.3 ± 0.1	9.8± 2.2
7g	0.25 ± 0.01	0.2 ± 0.0	8.7± 1.3
ABT-199	0.23 ± 0.06	2.7± 0.3	94.0 ± 10.5
ACY-1215	/	11.0 ± 1.7	21.2 ± 2.4
SAHA	/	10.6 ± 2.1	42.6 ± 3.8

In summary, encouraged by the synergistic interactions between Bcl-2 inhibitors and HDAC inhibitors, a series of Bcl-2/HDAC dual-target inhibitors were designed and synthesized in this study. Among them, compounds **7e - 7g** showed good inhibitory activities against HDAC6 and high binding affinities to Bcl-2 protein simultaneously. They displayed good growth inhibitory activities against human MM cell line RPMI-8226, obviously better than Bcl-2 inhibitor ABT-199 and HDAC inhibitors ACY-1215 and SAHA. The studies presented here prove the potential value of Bcl-2/HDAC dual-target inhibitors for the treatment of multiple myeloma, and provide a new structural type for the development of novel antitumor agents.

Acknowledgments

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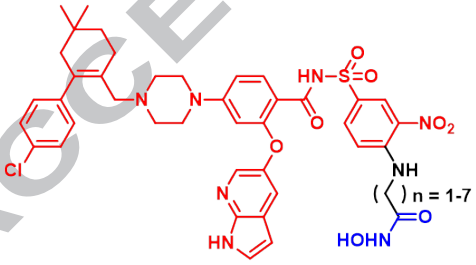
Graphical Abstracts



No.	HDAC6 (IC_{50}, nM)	Bcl-2 (IC_{50}, μM)	RPMI-8226 (IC_{50}, μM)
7g (n = 7)	19 ± 1	0.25 ± 0.01	0.2 ± 0.0
ABT-199	/	0.23 ± 0.06	2.7 ± 0.3
SAHA	13 ± 1	/	10.6 ± 2.1

Highlights

A series of Bcl-2/HDAC dual-target inhibitors were designed and synthesized.

Compounds **7e - 7g** showed good inhibitory activities against HDAC6 and Bcl-2 protein.

Compounds **7e - 7g** displayed good inhibitory activities against RPMI-8226 cell line.