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Design, synthesis and biological evaluation of 1,4-benzodiazepine-2,5-dione-based HDAC inhibitors

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Abstract—New histone deacetylase inhibitors have been synthesized and evaluated for their activity against non-small lung cancer cell line H661. These compounds have been designed with diversely substituted 1,4-benzodiazepine-2,5-dione moieties as cyclic peptide mimic cap structures, and a hydroxamate side chain. Biological evaluations demonstrated that benzodiazepine-based HDACi bearing an aromatic substituent at the *N*1 position exhibited promising antiproliferative and HDAC-inhibitory activities.

Reversible acetylation of histones is one of the most studied epigenetic mechanisms.[1,2] This post-translational modification controls chromatin remodelling by affecting the interactions between histones and DNA with a considerable impact on the accessibility of DNA and regulation of gene expression.[3] The acetylation status of core histones is regulated by opposite activities of two groups of enzymes: HATs (histone acetyltransferases) and HDACs (histone deacetylases). Accumulating evidence indicates that the balance between acetylation and deacetylation of histones, and other proteins like p53 or α-tubulin, plays significant roles in gene transcription and cell proliferation.[4] Alterations in the structure or expression of HDACs have been clearly linked to the pathogenesis of cancer and consequently HDAC inhibitors (HDACi) have emerged as a new class of promising chemotherapeutic agents.[5,6] Indeed, HDACi selectively induce growth arrest, differentiation and apoptosis in tumour cells through the transcriptional activation of a small set of genes that regulate cell proliferation and cell cycle progression like p21.[4,7]

In the last 10 years, the large amount of publications and the wide diversity of natural and synthetic HDACi structures which have been reported testify the great interest in this field.[8,9] Trichostatin A (TSA) **1** was the first natural compound detected for its HDAC inhibition activity.[10] Natural trapoxin B (TPX B) **2**,[11] synthetic cyclic hydroxamic-acid-containing peptide (CHAP1) **3**,[12] suberoylanilide hydroxamic acid (SAHA) **4**[13] and benzamide **5** (MS-275)[14] are representatives of a set of structurally diverse HDACi (Fig. 1). Undergoing research for the design of valuable HDACi relies on crystallographic studies that have pointed out three structural requirements:[15] a terminal group that can bind to the zinc ion at the bottom of the active site, a cap group that makes contacts with the pocket entrance and between both, a linker fitting the tube-like portion of the binding pocket.

To date, at least 18 HDACs have been identified and classified into four classes. HDACi are able to inhibit the activity of class 1, class 2 and class 4 Zn-dependent HDACs, with no marked preference between classes and HDACs. Moreover, little is known about the biological functions of each HDAC enzyme and about the HDACi structure-relationship associated with HDAC class selectivity.[5] To address this issue, it is important to identify new isoform-selective inhibitors of HDAC. Within this framework, specific modulation of the capping structure seems to be appropriate to enable access to selective inhibitors.[16]

In this paper, we report the design, synthesis and biological evaluations of novel HDACi containing 1,4-benzo-

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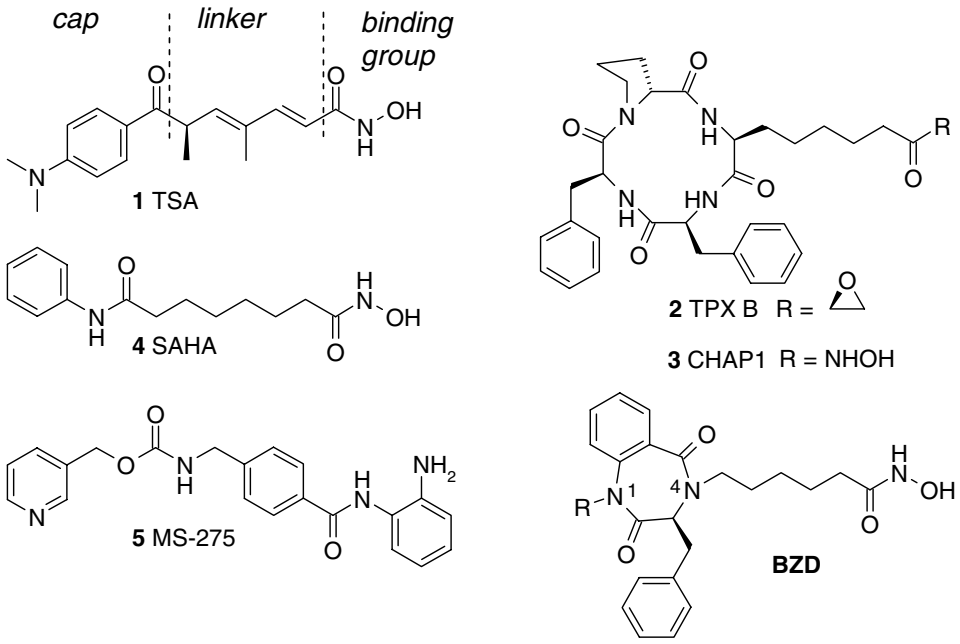
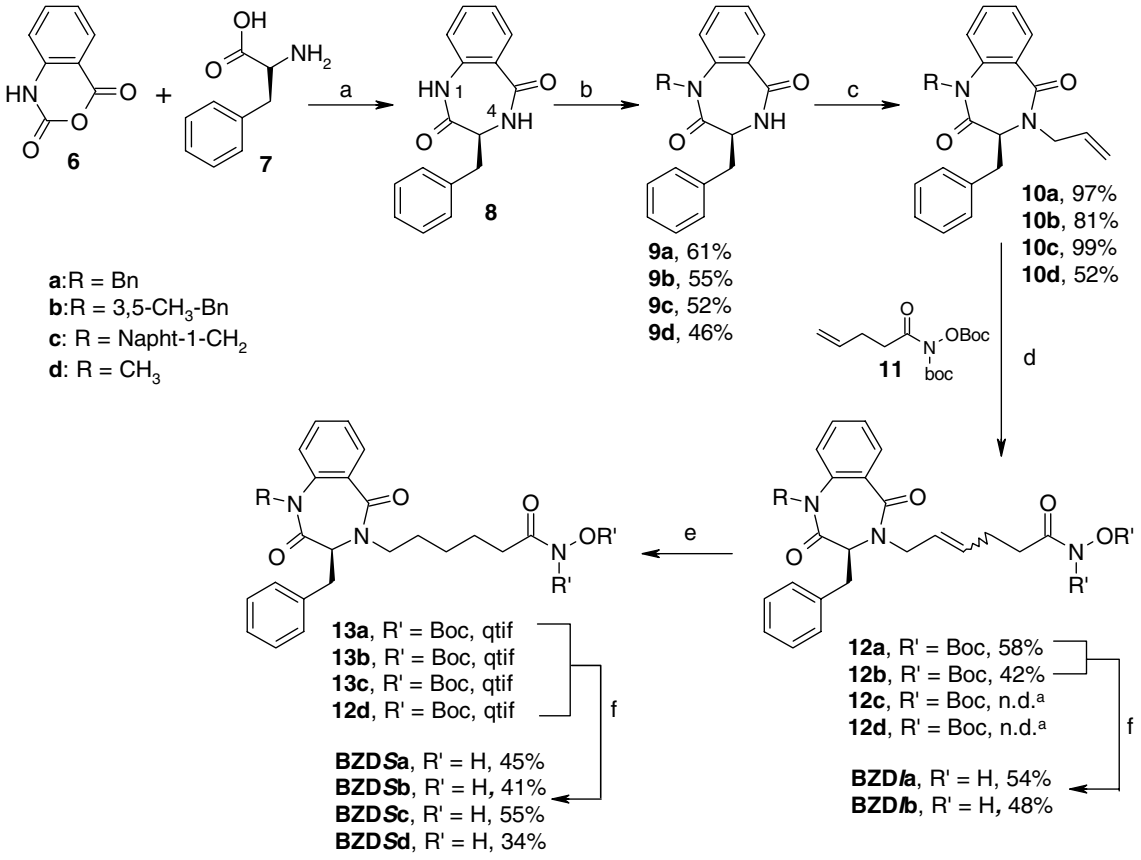


Figure 1. Examples of HDACi.

diazepine-2,5-dione scaffold. Our choice for such heterocycles was motivated by the fact that 1,4-benzodiazepine-2,5-diones are used as constrained peptidomimetics and provide a synthetically versatile platform for structural modification.[17] Thus, by analogy with the CHAP structures, we focused our design on cyclic hydroxamic-acid-containing peptidomimetic, such as **BZD** analogues (Fig. 1). Modulation of the **BZD** R group will provide different cap structures in order to evaluate the influence of the *N*1-benzodiazepine substitution on the resulting activity. It is worth mentioning that a similar approach has been recently reported by Etzkorn et al., who have used with success other types of cyclic peptide mimics.[18]

The 1,4-benzodiazepine-2,5-dione **8** was prepared according to the Sun procedure by condensation of isatoic anhydride **6** with L-phenylalanine **7** (Scheme 1).[19] The synthetic route for our **BZD** analogues relies on Blass's versatile method for the selective *N*-alkylation of a central 1,4-benzodiazepine-2,5-dione core using potassium fluoride on alumina.[20] To finalize the cap structure, *N*1-alkylation of **8** was accomplished with 1.0 equiv of various alkyl bromides (R–Br) in the presence of KF/Al_2O_3 in DMF at 25 °C over 48 h. In order to explore the effect of *N*1-substituent on antiproliferative and HDAC-inhibitory activities, we chose different aromatic structures (R = benzyl, 3,5-dimethyl-benzyl, naphthalen-1-yl methyl) and one alkyl structure (R = methyl). Thus, four different monoalkylated benzodiazepine-2,5-diones **9a–d** were obtained. From compounds **9**, *N*4-alkylation to introduce the hydroxamic acid moiety can be realized using previous KF/Al_2O_3 methodology with refluxing DME.[20] First attempts by means of a hydroxamate derivative of bromo-6-hexanoic acid were unsuccessful. This result prompted us to opt for a cross-metathesis strategy involving allylated benzodiazepines **10** and an appropriate hydroxamate precursor **11**. Furthermore, we anticipated that such a strategy will allow examining further the SAR of different methylene chain lengths. Compounds **10a–d** resulting from *N*4-alkylation with allyl bromide were isolated in good yields together with only a small amount of *O*-alkylated products. Cross-metathesis between compounds **10a–d** and alkene **11**[21] in the presence of Grubbs II catalyst led to intermediates **12a–d** as a mixture of diastereoisomers. In all cases, the reaction did not reach completion. Compounds **12a** and **12b** were isolated after purification but compounds **12c** and **12d** were obtained as a mixture with starting material. Nevertheless, formation of these latter was confirmed by HRMS analysis. Hydrogenation of **12a–d** led to compounds **13a–d** and deprotection of the *tert*-butoxycarbonyl (Boc) group with trifluoroacetic acid gave the free hydroxamic acid of saturated analogues **BZD*Sa*–d**. In order to compare the nature of the side chain, similar Boc-deprotection was conducted on compounds **12a** and **12b** to give unsaturated analogues **BZD*Ia*** and **b**.

The benzodiazepinedione nucleus is quite rigid and adopts a 'cupped shape'.[17] ^1H NMR spectra of all compounds obtained after the *N*4-alkylation (**10a–d** to **BZD** analogues) showed clearly the presence of two conformers, not chromatographically separable, at a ratio of 60:40. This observation suggests that there is a ring inversion which is too rapid to allow the separation at room temperature, but slow enough for the detection of conformers by NMR. Performing ab initio B3-LYP/6-31G(d) calculations[22] for **10d**, we found two conformations, endo and exo, according to the way H3 pointed out in the seven-membered ring, with small energy difference (Fig. 2). To save computer time, barrier height for the interconversion of endo to exo was then evaluated on model A and



Scheme 1. Synthesis of BZD analogues. Reagents and conditions: (a) i—H_2O, TEA, rt, 24 h; ii—CH_3COOH, reflux, 48 h, 78%; (b) KF/Al_2O_3 (40% KF by weight), DMF, R–Br 1 equiv, rt, 48 h, 61–46%; (c) KF/Al_2O_3 (40% KF by weight), DME, allyl bromide 1.2 equiv, reflux, 48 h, 95–52%; (d) **10** 3×10^{-3} mol L^{-1}, **11** 3 equiv, 10% Grubbs II, reflux, 2 days, 58–42%; (e) H_2, Pd/C 10%, MeOH, rt, qtif; (f) TFA, DCM, 0 °C to rt, 3 h, 34–55%. [a]Yield not determined.

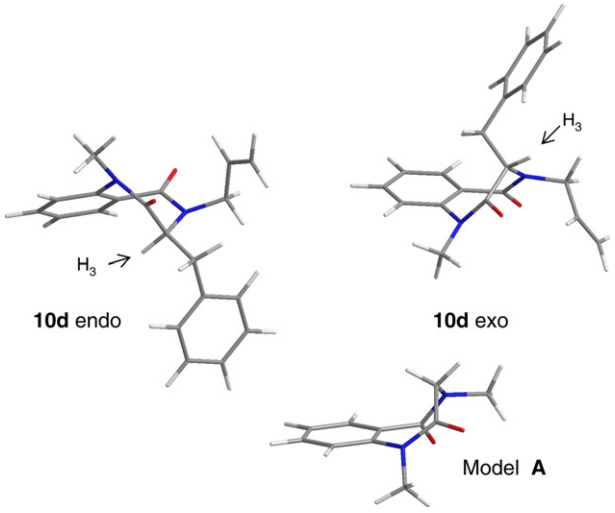


Figure 2. Conformational interconversion of **10d** and structure of model A.

found to be 14.8 kcal/mol. This relatively high value is consistent with previously reported data[20,23] and can explain the existence of two conformers at room temperature. Moreover, ^1H and ^{13}C NMR chemical shift calculations[24] revealed the same significant differences for conformers **10d** as the experimental ones.

Table 1. Antiproliferative activities in H661 cells for **BZD** analogues and TSA

Entry	Compound	R	IC_{50} (μM)
1	**BZD*Sa***	Bn	6
2	**BZD*Sb***	3,5-CH_3–Bn	8
3	**BZD*Sc***	C-Naphth-1-CH_2	16
4	**BZD*Sd***	CH_3	No activity
5	**BZD*Ia***	Bn	8
6	**BZD*Ib***	3,5-CH_3–Bn	17
7	TSA		0.085

The antiproliferative study was performed with six different concentrations for each **BZD** analogue (1, 5, 10, 15, 20, 25 μM) and with seven different concentrations for TSA (0.025, 0.050, 0.1, 0.2, 0.3, 0.5, 1 μM). For each **BZD** analogue, three to four independent experiments were performed in triplicate and five for TSA. The IC_{50}values were determined from the curves as the concentration that is required for 50% inhibition of cell proliferation measured 48 h after treatment of H661 cells. For each compound, the curves are provided as Supplementary data.

Antiproliferative activities of **BZD** analogues were evaluated after 48 h treatment of non-small cell lung cancer H661 cells and compared to TSA and IC_{50}s were determined (Table 1). The presence of an aromatic substituent appears to be essential to achieve activity. Indeed, **BZD*Sd*** (entry 4) did not present any detectable antiproliferative activity for the tested concentrations from 1 to

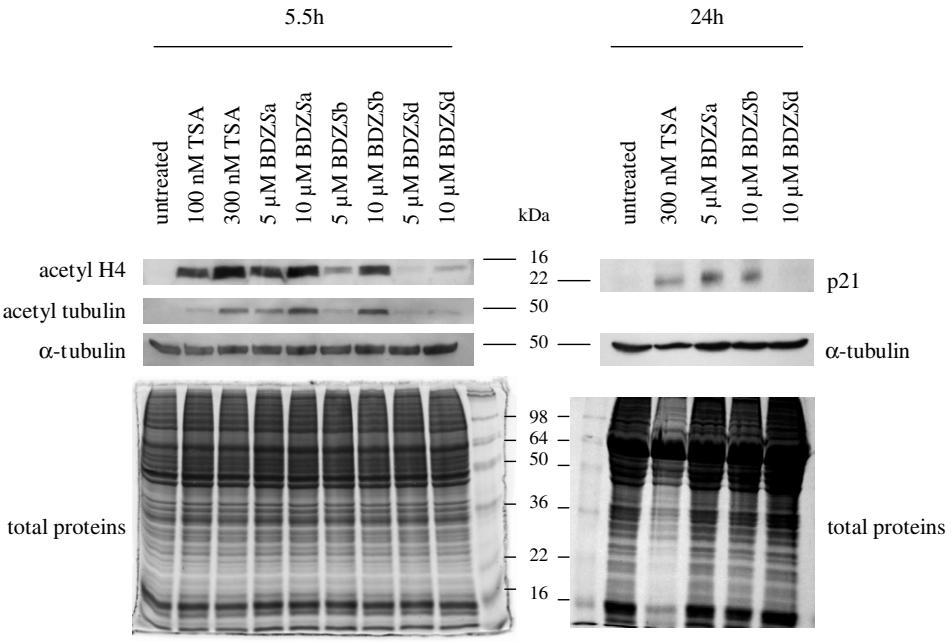


Figure 3. Western blot analysis for histone H4 and tubulin acetylation, and p21 expression. H661 cells were treated with the indicated compounds for 5.5 h (left) and 24 h (right). A 24-h treatment was necessary to detect p21 induction. Corresponding antibodies were used to detect histone H4 and tubulin acetylation and p21 induction. α-Tubulin and total protein extract stained by Coomassie blue are loading controls.

25 μM in opposition to **BZD*Sa*–c**. In the case of aromatic-substituted benzodiazepine-2,5-diones, **BZD*Sa*** was found to be the most active (entry 1 vs 2 and 3). **BZD*Sa*** (IC_{50} = 6 μM) seems slightly more active for antiproliferative activity than **BZD*Sb*** (IC_{50} = 8 μM) and **BZD*Ia*** (IC_{50} = 8 μM) even if their IC_{50} are not very different. Indeed, looking at the proliferation curves (Supplementary data), we can see that 5 μM **BZD*Sa*** induces a significant antiproliferative activity (70% to 40% proliferation of control), whereas at the same dose of **BZD*Sb*** or **BZD*Ia***, proliferation was around 80–95% of control.

Concerning the nature of the side chain, slightly better activities were found for the saturated analogues **BZD*Sa*** and **b**, compared to corresponding unsaturated, **BZD*Ia*** and **b** (entries 1 and 2 vs 5 and 6).

As histone H4 and α-tubulin acetylation are markers of HDACi activity, we performed western blot analysis to evaluate the HDACi potential of **BZD*Sa*** and **BZD*Sb*** active in proliferation test and inactive **BZD*Sd***, with TSA as reference (Fig. 3, left). As expected, TSA induced histone H4 acetylation, whereas **BZDSd** was barely active. Two doses around IC_{50} were tested (5 and 10 μM) for **BZD*Sa*** and **BZD*Sb***. **BZD*Sa*** was more active to acetylate histone H4 than **BZD*Sb*** after 5.5 h treatment. We also notice a dose–response in accordance with the antiproliferative XTT assay. Interestingly, with these two compounds, the level of tubulin acetylation is quite similar. We can hypothesize that HDAC6 that reverses the post-translational acetylation of tubulin[25] is similarly inhibited by these two compounds. p21 (the cyclin-dependent kinase inhibitor, WAF1), which is induced by HDACi, could be detected after 24 h treatment by these two compounds (**BZD*Sa*** and **BZD*Sb***) and TSA and not by **BZD*Sd*** (Fig. 3, right).

Biological assays showed a significantly reduced potency on **BZD** analogues compared to TSA. In the same way, **BZD** analogues are less potent compared with analogous cyclic tetrapeptide-like trapoxin and CHAPs.[26] However, it is worth mentioning that the comparison with trapoxin and CHAP does not concern the same cell line. Indeed Komatsu et al. described biological assays performed with B16/BL6 cells. Nevertheless, our results demonstrate the potential of 1,4-benzodiazepine-2,5-dione structures to play the capping group of new HDACi with a promising framework to develop isoform-selective HDACi.

In conclusion, in order to find new HDACi, we have designed and synthesized cyclic peptidomimetic hydroxamates by the mean of 1,4-benzodiazepine-2,5-dione central scaffold. Our synthetic strategy allows convenient preparation of these compounds and wide possibilities to create diverse cap structures. This preliminary work pointed out the need of an aromatic substituent attached to the *N*1-benzodiazepine nucleus to achieve an antiproliferative activity and the more promising compounds **BZD*Sa*** and **BZD*Sb*** were identified as HDACi. Complementary SAR studies are now needed to investigate different substituted cap groups and their ability to selectively inhibit single HDAC enzymes.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.06.067.

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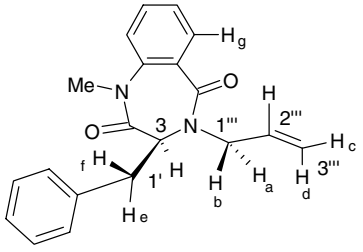

Position	**10d** exo		**10d** endo	
	δ_{calcd} (ppm)	δ_{obs} (ppm)	δ_{calcd} (ppm)	δ_{obs} (ppm)
H-1′f	2.38	2.33	3.22	3.11
H-1′e	2.41	2.52	3.32	3.41
H-1‴a	3.21	3.43	4.08	4.04
H-1‴b	4.62	4.04	3.84	4.04
H-2‴	6.06	5.41	6.4	5.79
H-g	8.82	7.88	8.44	7.77
C-3	61.8	66.62	54	57.31

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