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Novel *N*-hydroxybenzamide-based HDAC inhibitors with branched CAP group

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ABSTRACT

Ongoing effort to gather further knowledge about the structural requirements on histone deacetylase inhibitors led to the synthesis of novel *N*-hydroxybenzamide-based HDAC inhibitors **1a**–**o**, introducing branched hydrophobic groups at the capping group, and their inhibition activity against HDACs and anti-proliferation activity in four tumor cell lines were determined. Compounds **1j**–**o** were further tested against recombinant human HDAC1 and HDAC4 to evaluate their selectivity profile. This work further suggests that the chemical nature of the capping group is critical for subtle discrimination between the class I and the class II HDAC isoforms.

Histone deacetylases (HDACs) are enzymes that catalyze the deacetylation of lysine residues located at the N-ε terminal tails of core histones, which is associated with transcriptional silencing. Abnormity of histone deacetylation has been observed in human tumors, and inhibition of HDACs has emerged as a novel and validated therapeutic strategy against cancers.[1] Currently a small molecule inhibitor of HDACs, SAHA (vorinostat, Merck & Co.) has been approved for the treatment of advanced cutaneous T-cell lymphoma (CTCL),[2] and there are at least fourteen HDAC inhibitors under clinic evaluation.[3] Most of them are hydroxamic acid derivatives (e.g., PXD 101,[4] LBH 589[5] and ITF-2357[6]) (Fig. 1). However, these hydroxamic acid analogs exhibit nonspecifically inhibitory effects against all HDAC isoforms. In the past few years, tremendous effort has been dedicated to the research of class-selective and isoform-selective HDAC inhibitors.

Most of HDAC inhibitors have three common features, (i.e., capping group, hydrophobic space and zinc binding group).[7] Comparison of sequence alignments of HDACs indicates that main difference among the individual enzymes exists in the loops forming shallow grooves around the rim of enzyme pocket. Close examination of the corresponding crystal structures further indicates that these enzymes differ considerably in the rim near the surface of the active site;[8–11] which suggests that capping group of HDAC inhibitors means a lot for selectivity and that modification of capping group is a promising approach for the research of selective HDAC inhibitors.[12] Actually, the capping group region has been modified extensively towards the creation of selective HDAC inhibitors by several groups,[13–16] and several inhibitors with possible isoform selectivity were reported (e.g., compounds **2**, **3** and **4**) (Fig. 2). According to the selectivity data of the cyclic peptide and inhibitors with branched capping group, it seemed that the size of the capping group is likely important for governing class selectivity, although the elements account for selectivity are less understood.

Following our researches on HDAC inhibitors with the aim to gather further knowledge about the structural requirements on potent and selective histone deacetylase inhibitors, we designed and synthesized a new series of 5-*p*-tolylthiazol-2-amine compounds **1a–o**. Utilizing the *N*-hydroxybenzamide as a Zinc binding group, we maintained the tolylthiazol as one hydrophobic group and introduced another side chain at the amine N (represented by R group of Scheme 1) in order to explore the interaction with the shallow grooves near the rim of HDAC catalytic pockets.

The synthetic route was shown in Scheme 1. Thus, toluene was treated with chloroacetyl chloride to give *p*-methyl-α-chloroacetophone **2**, which was cyclized with thiourea to afford 5-*p*-tolylthiazol-2-amine **3**. The latter was formed the Schiff's base upon treatment with methyl 4-formylbenzoate in anhydrous toluene, which was further reduced with $NaBH_4$ to provide intermediate **4**. Compound **4** was alkylated with various alkyl bromides to generate **5**, which was subsequently reacted with hydroxylamine to give desired analogs **1a–o**.[17]

The pharmacological activities of these compounds were firstly examined by determination of inhibitory activities against HDACs using the commercially available Drug Discovery Kit AK500 (BIOMOL) and the results are summarized in Table 1. In general, analogs

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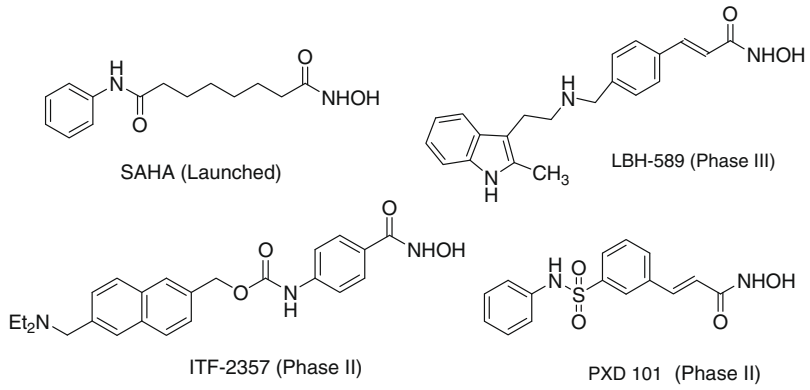


Figure 1. Examples of hydroxamate HDAC inhibitors.

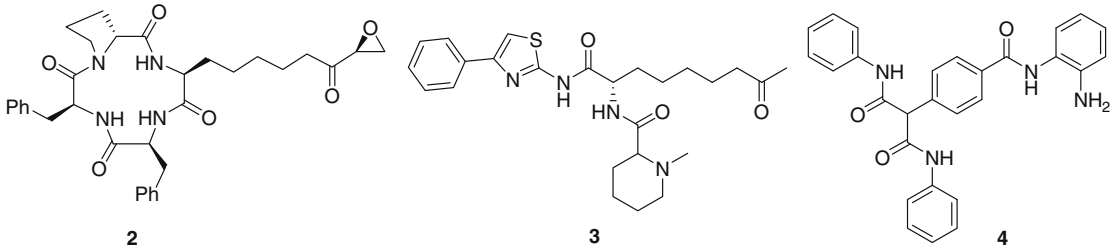
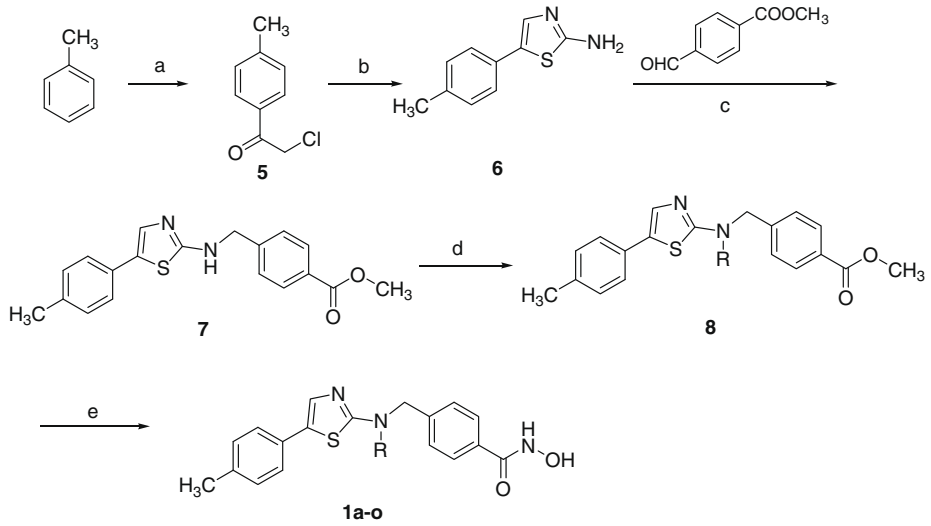


Figure 2. Examples of selective HDAC inhibitors with cyclic peptide or branched capping group.

bearing shorter alkyl substitutes (compounds **1a** and **1b**) displayed very good potency against the enzymes, comparable to the current clinical development compound SAHA. As the length of substitutes become longer, the activities diminished dramatically (compounds **1d**, **1f**, **1g** and **1h**). In addition, branching chain is unfavorable at neither α-position nor β-position, leading to a 3 to 57-fold (**1a** vs **1c** and **1i**) and 10-fold (**1b** vs **1e**) loss of activity, indicating a strict special restriction close to the rim of enzyme catalytic pockets.

To continue this SAR study, we next focused on the substitution of the non-branched alkyl moiety using an oxygen-based linker (Table 1). Interestingly, the absence or presence of a methyl group substitute at phenyl (compounds **1j**–**1o**) showed up to a 28-fold difference in HDACs activity, with compounds **1j** and **1n** being the most potent. Increasing the linker length of compound **1j** by two methylene groups, compound **1n** led to a minor decrease in HDACs activity, whereas increasing the linker length by one methylene units, compounds **1n** provided more than a fourfold decrease in activity. However, increasing the linker length of compounds **1k** with *p*-tolyloxy residue by one or two methylene moiety resulted in a 10-fold (**1m**) or a 16-fold (**1o**) of decrease in HDACs potency correspondingly.

Having confirmed the pan-HDAC inhibitory activity of these compounds, we select compounds **1j**–**o** to evaluate the possible class specificity by tested their inhibitory activity against human



Scheme 1. Synthesis of *N*-hydroxybenzamides **1a**–**o**. Reagents and conditions: (a) CH_3COCl, CH_2Cl_2, rt; (b) NH_2CSNH_2, EtOH, reflux; (c) (1) anhydrous toluene, reflux, 2 h; (2) CH_3OH, $NaBH_4$; (d) NaH, DMF, RX, rt; (e) NH_2OH, KOH, CH_3OH, rt.

Table 1
HDACs inhibitory activities of indicated compounds

Compd	R	HDACs IC_{50} (μM)
1a	Ethyl	0.054
1b	Propyl	0.052
1c	*iso*-Propyl	0.168
1d	Butyl	1.349
1e	*iso*-Butyl	0.494
1f	Amyl	4.328
1g	Hexyl	2.790
1h	Octyl	1.587
1i	Benzyl	3.108
1j	2-Phenoxyethyl	0.041
1k	2-(*p*-Tolyloxy)ethyl	0.101
1l	3-Phenoxypropyl	0.195
1m	3-(*p*-Tolyloxy)propyl	1.068
1n	4-Phenoxybutyl	0.057
1o	4-(*p*-Tolyloxy)butyl	1.671
SAHA	—	0.053

HDAC1 and HDAC4, for the two enzyme are considered as representative of class I and class IIa HDACs respectively. As demonstrated in Table 2, compounds **1j–o** were tested at 5 μM against human recombinant (hr) HDAC1 and HDAC4 enzymes, in comparison with SAHA. It turns out that all of the compounds **1j–o** are highly selective and potent inhibitors of HDAC1 but not against HDAC4 compared with pan-HDAC inhibitor SAHA. The structure–activity relationships are not consistent with the trend of activity observed against mixtures of HDAC members, confirming selectivity occur within certain subtype of HDACs. Compounds with longer side chains (**lm**, **ln** and **1o**) are more potent against HDAC1 as well as HDAC4. In addition, the methyl group substitute at phenyl of side chain gave a little increase (compare **1k** with **1j** and **1o** with **1n**) or significantly improved (compare **1m** with **1n**) of both the HDAC1and HDAC4 inhibiting capacity; compound with three methylene moiety (**1o**) was the most potent. According to the IC_{50} value of compounds **1j** and **1k**, they displayed moderate activity to human recombinant (hr) HDAC1, but were almost inactive against HDAC4 (Table 3). In conclusion, analogs **1j–o** are potent HDAC inhibitors with remarkable HDAC1 selectivity, validating the structural difference around the rim of the catalytic pockets between HDAC1 and HDAC4.

The effects of compounds **1j–n** (5 μM, 24 h) on histone H3 and α-tubulin acetylation levels, taken as markers of class I HDACs and HDAC6 inhibition, respectively, were tested in the human leukemia U937 cell line (Fig. 3). SAHA and MS-275 have been taken as reference compounds.

As seen by Western blot analysis, the five tested compounds showed moderate levels of acetyl-H3, although all of them being equally weaker than SAHA. Moreover, most of the tested compounds (**1k**, **1l**, **1m** and **1n**) demonstrated strong induction of α-tubulin acetylation with the exceptions of **1j**, which showed lower activity possibly due to poor cell permeability or differences in the kinetics of induction. Above all, these data confirmed the capability of such compounds to inhibit class I HDACs as well as HDAC6.

Table 2
Human recombinant HDAC1 and HDAC4 inhibitory activities of the compounds **1j–o** at 5 μM

Compd	% of residual activity	
	HDAC1	HDAC4
1j	6.47	89.31
1k	4.16	79.16
1l	6.54	92.65
1m	0.45	64.25
1n	−0.55	62.74
1o	−0.35	54.14
SAHA	1.68	0.49

Table 3
HDAC1 and HDAC4 inhibitory activities of **1j** and **1k**

	HDAC1 (IC_{50}, μM)	HDAC4 (IC_{50}, μM)
1j	2.3	>50
1k	1.05	>50

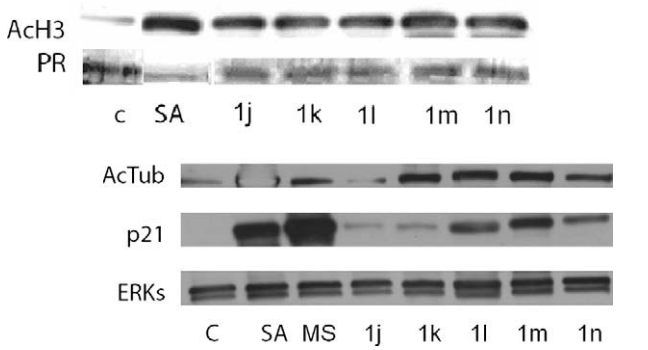


Figure 3. Western Blot analysis of $p21^{WAF1/CIP1}$ expression, α-tubulin and H3 acetylation in U937 cells after treatment with the indicated compounds (at 5 μM). SAHA and MS-275 (5 μM) were used for a comparison.

In addition, class I and pan-HDAC inhibitors usually induce over-expression of $p21^{WAF1/CIP1}$. Here, the capacity of these *N*-hydroxybenzamides based derivatives (**1j–n**) to induce over-expression of $p21^{WAF1/CIP1}$ was also tested (Fig. 3). The results showed that compound **1l** and **1m** induced expression of $p21^{WAF1/CIP1}$ moderately, while compounds **1j** and **1k** compounds were relatively weakly (compare to SAHA). The trend of potency in up-regulation of $p21^{WAF1/CIP1}$ of these compounds was quite consistent with their HDAC1 inhibitory activity.

We also analyzed the effect of these compounds on granulocytic differentiation, cell cycle and apoptosis induction. SAHA and MS-275 (5 μM) were used as reference drugs. Granulocytic differentiation was evaluated by measuring the CD11c expression level upon 30 h of stimulation with **1i–o** at 5 μM. Amongst the tested compounds **1j**, **1k** and **1o** showed a percent of CD11c positive PI negative cells higher than SAHA, although none of them being more potent than MS-275 as cytodifferentiating agent (Fig. 4A). At the tested conditions, **1j–n** were able to induce accumulation of the cells in the G1 phase, whereas **1o** gave a block of the cycle at the S phase (Fig. 4B). Apoptosis induction was measured with the Annexin V/propidium iodide (PI) double staining method. The results revealed that half of the tested compounds (**1j**, **1k** and **1o**) had higher apoptosis induction potency in the U937 cell line than MS-275, though the apoptosis induction potency of all of them was relatively weaker than SAHA in our assay. Taken together, our findings showed that compounds with higher levels of differentiation ability usually behaved better in apoptosis induction.

Finally, the anti-proliferative activity was evaluated against four human tumor cell lines with SAHA as controls. As shown in Table 4, the majority of the compounds displayed excellent anti-proliferative profiles compared with SAHA, especially in A549 and HEPG2 cells. Compounds **1n** and **1o** were particularly potent with respect to growth inhibition of HCT116, with IC_{50} of 0.68 and 0.58 μM, respectively, but the others are less potent than SAHA in the same cell line. The limited difference among the side chains of these compounds does affect their cytotoxicities against the four cancer cells, but no outstanding trends were observed here.

In summary, we have designed and synthesized a series of novel 5-*p*-tolylthiazol-2-amines HDAC inhibitors. Utilizing the *N*-hydroxybenzamide as a zinc binding group, we maintained the tolylthiazol as one hydrophobic group and surveyed substitutes with different

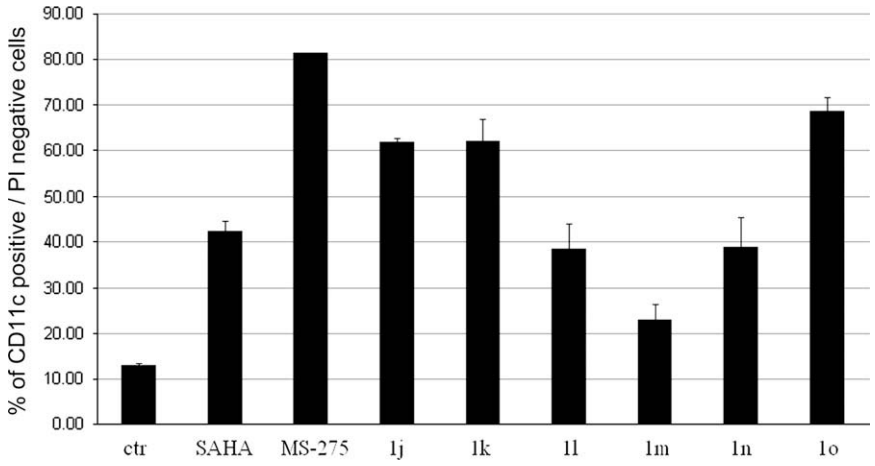


Figure 4A. Differentiation analysis in U937 cells after 30 h treatment with the indicated compounds at 5 μM. The data shown represent the media of independent quadruplicates.

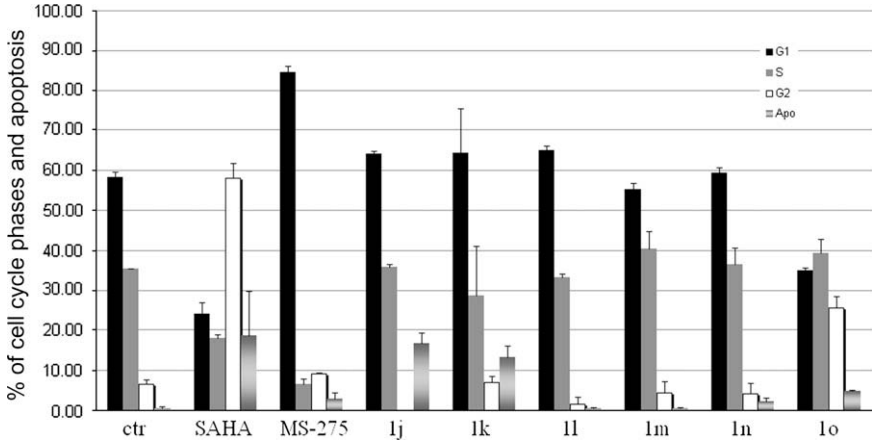


Figure 4B. Analysis of cell cycle and apoptosis in U937 cells treated with the indicated compounds for 30 h at 5 μM. The data represent the media of independent duplicates.

Table 4
Anti-proliferative activity of the indicated compounds in four tutor cell lines

Compd	R	PC3 GI_{50} (μM)	HCT116 GI_{50} (μM)	A549 GI_{50} (μM)	HEPG2 GI_{50} (μM)
1a	Ethyl	10.33	6.87	2.70	1.12
1b	Propyl	9.16	5.80	3.31	2.55
1c	*iso*-Propyl	1.15	7.80	3.83	2.18
1d	Butyl	6.41	8.78	4.78	4.30
1e	*iso*-Butyl	9.34	5.90	20.41	7.42
1f	Amyl	5.06	23.28	9.14	7.02
1g	Hexyl	5.27	4.85	3.71	7.42
1h	Octyl	13.26	6.50	4.63	8.76
1i	Benzyl	10.35	48.79	4.41	33.96
1j	2-Phenoxyethyl	7.86	8.81	14.01	12.03
1k	2-(*p*-Tolyloxy)ethyl	5.57	17.31	11.17	11.49
1l	3-Phenoxypropyl	17.53	4.14	2.53	23.44
1m	3-(*p*-Tolyloxy)propyl	9.33	12.55	32.48	12.18
1n	4-Phenoxybutyl	10.34	0.68	14.19	16.55
1o	4-(*p*-Tolyloxy)butyl	1.22	0.58	8.63	11.04
SAHA	—	8.17	2.69	>200	63.48

length of alkyl, phenoxyalkyl or tolyloxyalkyl to explore both the activity and selectivity. Compounds **1j**–**1o** demonstrated outstanding selectivity against HDAC1, and some of them were able to highly increase the levels of both acetyl-H3 and acetyl-α-tubulin. In cellular assays, they displayed some differentiation activity and moderate apoptosis. This work laid the ground for future development of selective HDAC inhibitors through modification of substitutes that could interact with the surface of enzyme catalytic pocket.

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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.2009.09.100.

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17. Chemical and physical data of compounds **1a–o** are reported in Supplementary data.