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Exploring the connection unit in the HDAC inhibitor pharmacophore model: Novel uracil-based hydroxamates

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Abstract—Starting from the pharmacophore model for HDAC inhibitor design, a novel series of hydroxamates bearing a uracil moiety as connecting unit (CU) has been prepared and tested. Almost all compounds exhibited HDAC inhibiting activity at low nanomolar concentrations, the *N*-hydroxy-6-(3,4-dihydro-4-oxo-6-benzyl- and -6-phenyl-2-pyrimidinylthio)hexanamides **1d** and **1l** being more potent than SAHA in enzymatic assays. Such compounds also caused hyperacetylation in NIH3T3 cell core histones and were endowed with interesting antiproliferative and cytodifferentiating effects in human leukemia (HL-60) cells.

Truly selective destruction of malignant neoplastic cells without killing normal cells remains a crucial and elusive goal for cancer chemotherapy in the 21st century. One recent and promising strategy[1] is the use of agents that can differentiate cancer cells to either a nonproliferating or normal phenotype, an approach that has the potential to be tissue-specific and avoid the side effects of currently used drugs. Most compounds that are presently known to differentiate cancer cells are histone deacetylase (HDAC) inhibitors.[2] The opposing functions of histone acetyltransferases (HATs) and HDACs in both activating and repressing transcription by controlling the tightness of nucleosomal integrity reflect the regulatory processes that are involved in turning genes on or off. In mammals, both these acetylating/deacetylating enzymes are components as catalytic subunits of multiprotein complexes containing other proteins known to function in transcriptional activation/repression.[3–6] Such multiprotein complexes are recruited to specific regions in the mammalian genome and generate a unique spectrum of expressed and silenced genes.

To date, 18 distinct human HDACs have been reported, classified into three classes (I, II, and III) depending on their primary homology to three *Saccharomyces cerevisiae* HDACs (RPD3, HDA1, and SIR2, respectively).[7,8]

Class I and II HDACs show some degree of homology in their catalytic domain and have a zinc ion-dependent mechanism of deacetylation, whereas class III HDACs (SIRT1-7, sirtuins) show no homology to class I/II enzymes and catalyze the deacetylation reaction through NAD^+ as a cofactor.[9,10]

It is now well documented that the aberrant transcription (i.e., epigenetic modulation) of genes that regulate cellular differentiation, cell cycle, and apoptosis is due to altered expression or mutation of genes that encode HATs, HDACs, or their binding and recruiting partners. Such histone modifications are key events in tumor onset and progression.[11,12] From these findings, compounds that are able to inhibit HDAC activity (i.e., trichostatin A (TSA)[13] or suberoylanilide hydroxamic acid (SAHA)[14]) can reverse inappropriate HDAC-mediated transcriptional repression and can induce re-expression of differentiation-inducing genes,[15] resulting in antiproliferative effects in vitro and antitumor effects in vivo.[16–18] However, most of

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HDAC inhibitors known to date are of low potency or suffer from low bioavailability, rapid metabolism, reversible differentiation, and no selectivity for cancer cells over normal cells,[19] so suggesting that more potent and selective differentiating agents should be studied.

Despite the variety of their structural characteristics (hydroxamates, carboxylates, benzamides, electrophilic ketones, thiols, α-ketoamides, and cyclic peptides),[18] the class I/II HDAC inhibitors can be broadly characterized by a common pharmacophore.[7,18] This pharmacophoric model consists in a cap (CAP) group able to interact with the rim space at the entrance of the catalytic tunnel of the enzyme,[20–22] linked to a hydrophobic spacer (HS) through a polar connection unit (CU). At the end of the hydrophobic spacer, a zinc-binding group (ZBG) assures the inhibition of enzyme activity (Fig. 1).[7] While CAP is an extremely variable moiety, ranging from a simple benzene ring to a more complex cyclic tetrapeptide, structural requirements for CU, HS, and ZBG are very restricted. CU is often a sp^2-hybridized group, such as ketone, amide, or sulfonamide, although some oxazole and thiazole rings have been reported.[23] HS can be represented by linear or cyclic structures, either saturated or unsaturated, and to date the most widely used ZBG is the hydroxamate. A comparison of amino acid sequences of the class I/II HDAC active site showed that its structural features are well-conserved across all the HDACs, except for the rim of the catalytic pocket.[20–22] Therefore, it has been reasoned that changes of CAP and/or CU, assumed to interact with the rim space at the entrance of the tubular catalytic pocket, could provide potent and possibly class I- or II-selective HDAC inhibitors.

From these evidences, pursuing our studies on hydroxamate-based HDAC inhibitors,[24–32] we performed the synthesis of compounds **1a**–**h** bearing a uracil moiety as novel CU, a benzyl group as CAP, and differently sized thioalkyl aliphatic chains as HS (Fig. 2).

In an effort to establish the preliminary structure–activity relationship (SAR), samples obtained by removal (**1i**) or replacement of the benzyl group at the pyrimidine-C6 position with small aliphatic moieties (**1j**,**k**), as well as with phenyl (**1l**) or 2-phenylethyl (**1m**) groups, were prepared (Fig. 2).

The reaction of (C6-substituted)-2-thiouracils **2a**–**f** (**2a**, 6-H; **2b**, 6-Me; **2c**, 6-*n*-Pr; **2d**, 6-Ph; **2e**, 6-Bz; **2f**, 6-phenethyl) with the appropriate ethyl ω-bromoalkanoates in alkaline medium afforded the corresponding

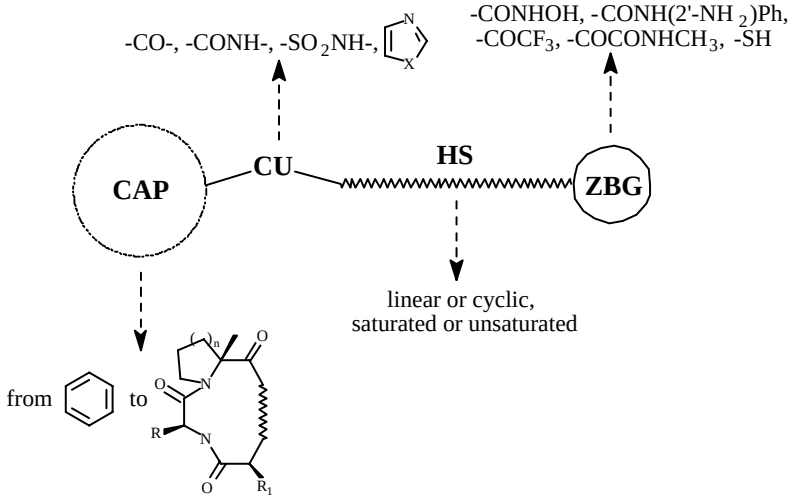
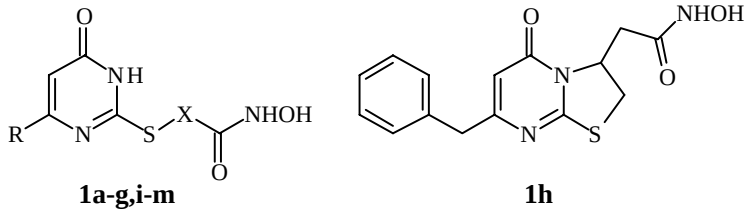


Figure 1. Pharmacophore model for HDAC inhibitors.



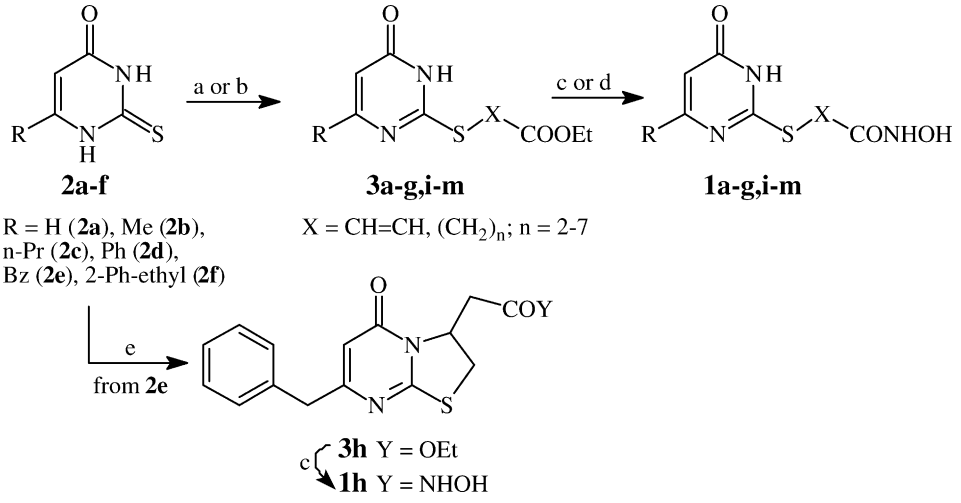
R = H, Me, n-Pr, Ph, Bz, 2-Ph-ethyl
X = CH=CH, $(CH_2)_n$; n = 2-7

Figure 2. Novel uracil-based hydroxyamides **1a**–**m**.

ethyl ω-(3,4-dihydro-4-oxo-6-(un)substituted-2-pyrimidinylthio)alkanoates **3a–f,i–m**. Treatment of 6-benzyl-2-thiouracil **2e** with ethyl propiolate in the presence of tetrabutylammonium fluoride (TBAF) under Michael conditions furnished the related propenoic ethyl ester **3g**, while by reaction between **2e** and ethyl 4-bromocrotonate the ethyl 7-benzyl-2,3-dihydro-5-oxo-5*H*-thiazolo-[3,2-*a*]pyrimidine-3-acetate (**3h**) was obtained via tandem S-alkylation/N3-Michael addition (Scheme 1). Some of these esters (**3a–f,h,l,m**) were converted into the corresponding *N*-hydroxyamides (**1a–f,h,l,m**) by reaction with hydroxylamine hydrochloride and potassium hydroxide. Differently, ethyl esters **3g,i–k** were hydrolyzed in alkaline medium to the carboxylic acid intermediates **4g,i–k** (see Supporting Information), which were then converted into the desired *N*-hydroxyamides **1g,i–k** by our reported one-pot, three-step procedure[31,32] (Scheme 1).

Uracil-based hydroxyamides (UBHAs) **1a–m** were evaluated for their ability to inhibit maize HDACs. The maize system offers the advantage that three different types of HDACs can be biochemically separated: class I (HD1-B)[33,34] and class II (HD1-A)[35,36] enzymes and the plant specific form HD2.[37] Despite HD2 showing an apparent structural diversity from class I/II enzymes, it exhibits very similar kinetic properties when compared to classical HDAC families. Furthermore, a good linear correlation between HD2 and HDAC1 inhibitory data has been recently described.[38]

The majority of tested compounds exhibited inhibiting activity at nanomolar concentrations (Table 1), the only exceptions being **1g,h** that showed too short HSs for efficient enzyme inhibition. The most active C6-benzyl derivative, **1d**, was 2.5–6 times more potent



Scheme 1. Reagents and conditions: (a) Br–X–COOEt, K_2CO_3, DMF, rt; (b) ethyl propiolate, TBAF, THF, 70 °C; (c) $NH_2OH{\cdot}HCl$, KOH, EtOH, rt; (d) 1—KOH, EtOH, H_2O, rt, 2—$ClCOOC_2H_5$, $(C_2H_5)_3N$, THF, 0 °C, then $NH_2OC(CH_3)_2OCH_3$, rt, 3—Amberlyst 15, CH_3OH, rt; (e) $BrCH_2CH{=}CHCOOEt$, K_2CO_3, DMF, rt.

Table 1. HDAC (maize HD2, HD1-B, and HD1-A) inhibiting activity of compounds **1a–m**[a]

Compound	R	X	IC_{50}, nM			SI[b]	
			HD2	HD1-B	HD1-A	Class I	Class II
1a	Bz	$(CH_2)_2$	38	41	126	3.1	
1b	Bz	$(CH_2)_3$	229	251	885	3.5	
1c	Bz	$(CH_2)_4$	125	130	181		
1d	Bz	$(CH_2)_5$	18	18	29		
1e	Bz	$(CH_2)_6$	37	76	53		
1f	Bz	$(CH_2)_7$	61	193	64		3.0
1g	Bz	CH = CH	9000	7400	13,300		
1h			31,800	30,200	38,200		
1i	H	$(CH_2)_5$	213	239	132		
1j	Me	$(CH_2)_5$	110	112	78		
1k	*n*-Pr	$(CH_2)_5$	135	116	66		
1l	Ph	$(CH_2)_5$	12	19	6		3.2
1m	2-Ph-ethyl	$(CH_2)_5$	35	48	25		2
TSA			7	0.4	0.8	2	
SAHA			50	28	178	6.3	

[a] Data represent mean values of at least three separate experiments.
[b] SI, selectivity index.

than SAHA and 2.6–45 times less potent than TSA (Table 1). On the whole, the inhibitory data indicate that in such novel series of derivatives the best activity is associated with a fully saturated, five carbon unit-containing HS. The removal (**1i**), as well as the replacement, of the C6-benzyl moiety with small aliphatic groups (**1j**, **k**) was detrimental to inhibitory activity, while the insertion of a phenyl ring at C6 led to a derivative (**1l**) that was 1.5 to 4.8 times more potent than **1d** in inhibiting maize enzymes. By increasing the distance between the phenyl ring and the C6 uracil position (**1m**, C6-phenethyl derivative), a slight decrease of activity was observed.

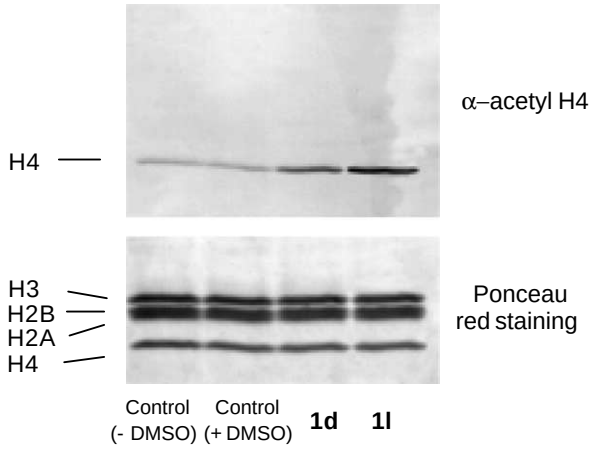


Figure 3. Hyperacetylation caused by **1d** and **1l** in NIH3T3 cell core histones.

Because HD1-B and HD1-A enzymes are homologues of mammalian class I and class II HDACs, the class I or class II selectivity ratio for **1a**–**m** is calculated. In general, the novel UBHAs showed no or little selectivity toward class I (SIs: **1a**, 3.1; **1b**, 3.5) or class II (SIs: **1f**, 3.0; **1l**, 3.2) HDACs (Table 1).

Compounds **1d** and **1l** were the most potent members of this series, and their hyperacetylation effect on core histones in NIH3T3 cells was analyzed by immunoblotting.

Exponentially growing cells were incubated for 8 h with **1d** and **1l** at final concentrations of 6 and 2 μM, respectively. After isolation of nuclei, equal amounts of nuclear extracts were subjected to SDS 14%–PAGE and blotted onto nitrocellulose membranes. Membrane strips were incubated with antibodies against acetylated histone H4. As control, cells were also grown in the presence and absence of DMSO (±DMSO) (0.1% v/v). Position of acetylated H4 is indicated and the Ponceau red-stained blot is shown. Immunodetection was performed with alkaline phosphatase-conjugated secondary antibody.

Treatment of cells with **1d** and **1l** leads to highly increased acetylation levels of H4 as compared to control histones, **1d** and **1l** giving significant immunoreactions, whereas in control only a very faint signal is visible (Fig. 3).

Moreover, the capability of **1d** and **1l** to induce antiproliferative and cytodifferentiating effects in human leukemia HL-60 cells in comparison with TSA (30 nM) and SAHA (1.2 μM) has been investigated. As shown in Figure 4A, **1d** and **1l** showed significant

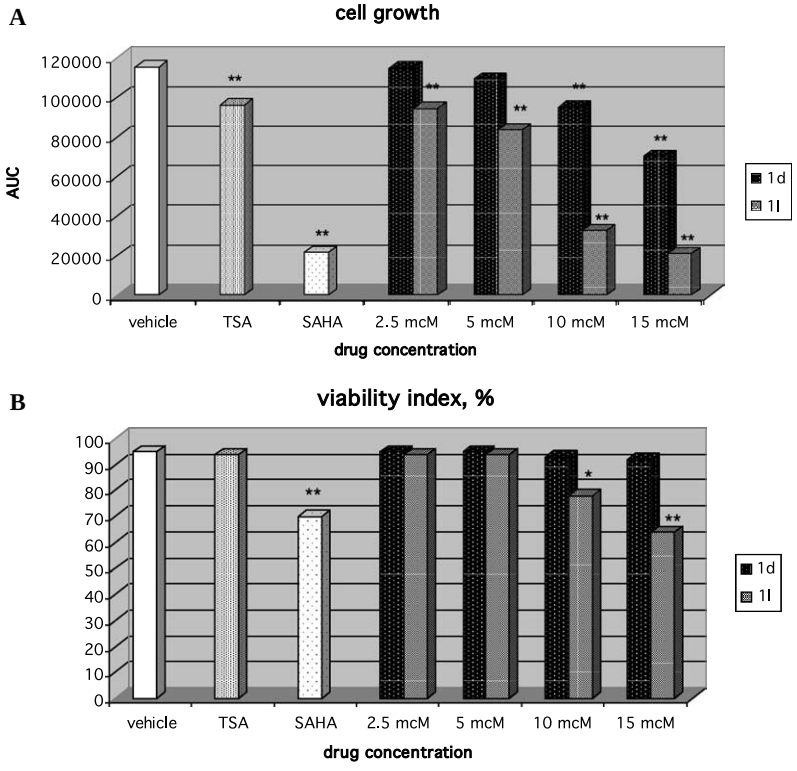


Figure 4. Antiproliferative (**A**) and cytotoxic (**B**) effects of **1d** and **1l** on HL-60 cells.

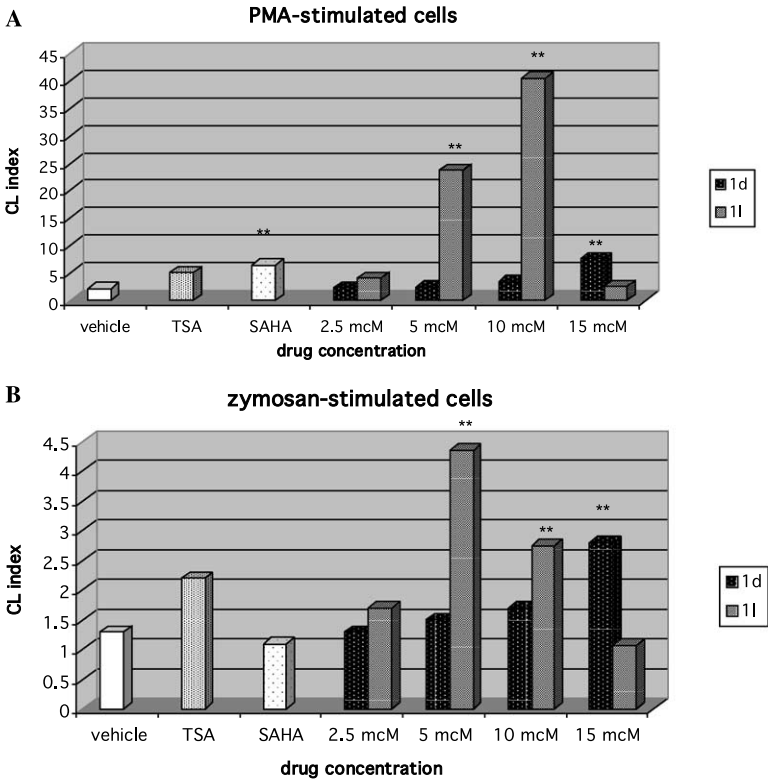


Figure 5. Cytodifferentiating effects of **1d** and **1l** on PMA- (**A**) or zymosan-stimulated (**B**) HL-60 cells.

dose-dependent inhibitory effects on the growth rate of HL-60 cells, cultured for 96 h. These inhibitory effects do not seem to depend on a direct cytotoxic action of the inhibitors, the viability indexes (VIs) appearing significantly modified only by **1l** at 10 and 15 μM concentrations (VIs = 78% and 64%, respectively) (Fig. 4B).

The restoration of so-called respiratory burst was evaluated as fundamental functional marker of differentiation in human leukemia cell lines. This aspect of phagocyte oxidative metabolism has been analyzed by chemiluminescence (CL)[39] in HL-60 cells, incubated with different concentrations of **1d** and **1l** for 96 h, and stimulated by phorbol-12-myristate-13-acetate (PMA) and zymosan. TSA (30 nM) and SAHA (1.2 μM) have been used as reference drugs. Results clearly showed a dose-dependent recovery of reactive oxygen species (ROS) metabolism when the cells were stimulated by PMA or zymosan, reaching the maximum CL activity ($p < 0.01$) with 5 μM of **1l** or with 15 μM of **1d** (Fig. 5).

It could be interesting to underline the fact that in human myeloid tumor cell lines, restoration of the respiratory burst permitted us to study a lot of differentiating agents (i.e., all-*trans*-retinoic acid, 13-*cis*-retinoic acid, 1α,25-dihydroxyvitamin D_3, hexamethylene bisacetamide, but also interferon, interleukin-6, and so on).[40] PMA- and zymosan-stimulated ROS metabolism, essentially due to the activity of NADPH oxydase system, analyzed by CL represents a sensible, specific, and reproducible method to qualitatively and quantitatively evaluate leukocytic oxidative burst.[41] In particular, PMA-stimulated ROS metabolism, by directly acting on protein kinase c, showed the correct assembly of the NADPH oxidase system, while opsonized zymosan-stimulated ROS metabolism attests the presence of a functional Fc receptor on the leukocyte membrane, thereby pointing to the correct assembly of the signal transduction pathway involved in the activation of the leukocytic oxidative burst.[39,41]

Further molecular modelling, synthetic, and biological studies are in progress to optimize the potency and selectivity of UBHAs against HDAC enzymes.

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Supplementary data

Supporting Information: Experimental procedures and chemical and physical data for compounds **1a**–**m**. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2005.07.081.

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