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Histone deacetylase inhibitors derived from 1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrazine and related heterocycles selective for the HDAC6 isoform

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ABSTRACT

Acyl derivatives of 4-(aminomethyl)-*N*-hydroxybenzamide are potent sub-type selective HDAC6 inhibitors. Constrained heterocyclic analogs based on 1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrazine show further enhanced HDAC6 selectivity and inhibitory activity in cells. Homology models suggest that the heterocyclic spacer can more effectively access the wider catalytic channel of HDAC6 compared to other HDAC sub-types.

Histone deacetylase (HDAC) enzymes catalyze the hydrolysis of acetyl groups from the ε-amino group of lysine residues of protein substrates. The HDACs are sub-divided into classes I (types 1, 2, 3, 8), IIa (4, 5, 7, 9), IIb (6, 10), III and IV (11) of which the class I, II and IV enzymes are Zn^{2+} dependent metalloamidases.[1] HDAC6 resides predominantly in the cytosol and has two functional catalytic domains as well as a carboxy-terminal zinc-finger domain that binds ubiquitinated proteins.[2] Substrates for HDAC6 including α-tubulin, cortactin, and Hsp90[3,4] mediate a wide range of cellular functions which are important in tumorigenesis, tumor growth and metastasis.[5–8] HDAC6 has also been linked to autophagy, a pathway for protein degradation that compensates for reductions in the activity of the ubiquitin proteasome system arising from treatment with a proteasome inhibitor.[9–12] In this process, HDAC6 binds ubiquitin, or ubiquitin-like conjugated proteins which would otherwise induce proteotoxic stress and transports them as ternary complexes with dynein to the microtubule organizing center where they are incorporated into the aggresome[6] and degraded by fusion with lysosomes. Simultaneously targeting the proteasome and aggresome pathways in tumor cells would therefore be expected to cause greater accumulation of polyubiquitinated proteins leading to cell stress and apoptosis. In support of this hypothesis, the HDAC6 selective inhibitor Tubacin is reported to induce hyper-acetylation of α-tubulin but not histones in multiple myeloma cells[13–15] and to potentiate proteasome inhibitor-induced apoptosis.[16]

Many HDAC inhibitors have been reported[17] comprising a Zn^{2+} complexing agent, usually a hydroxamic acid, spacer, and capping group (Fig. 1). Pan HDAC or class I selective HDAC inhibitors are most common, such as suberoylanilide hydroxamic acid (SAHA), or vorinostat, which has been shown to have therapeutic benefits.[18–21] In order to investigate synergistic effects of HDAC6 and proteasome inhibitors, we required inhibitors with at least comparable potency and selectivity to Tubacin and with improved physical properties. Despite their metabolic liabilities, hydroxamic acids exhibit unique binding energetics.[22] Thus, the uncharged form predominates in aqueous solution and the conjugate base is formed in the active site of the enzyme resulting in a much smaller desolvation penalty on binding than species that are already ionized in the aqueous phase. In terms of HDAC6 inhibitory activity, the highly potent inhibitor NOC-7 with moderate sub-type selectivity[23] and Tubastatin A[24] with high HDAC6 selectivity are notable. We screened several hundred hydroxamic acids in our collection for inhibitory activity of HDAC6 and those enzymes present in HeLa nuclear extract, predominantly the class I HDACs 1 and 2 and identified a derivative of 4-aminomethylbenzoic acid (compound **1**) as a selective inhibitor of HDAC6.[25] Optimization of this hit in the case

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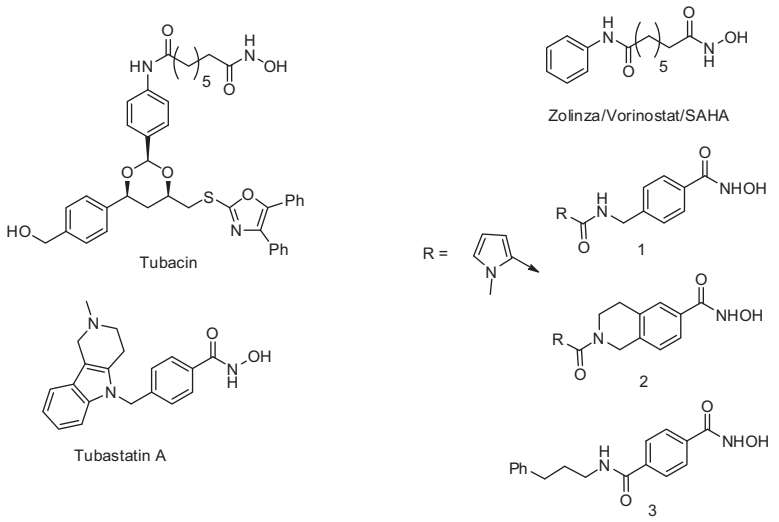


Figure 1. Examples of hydroxamic acid inhibitors of Histone deacetylases.

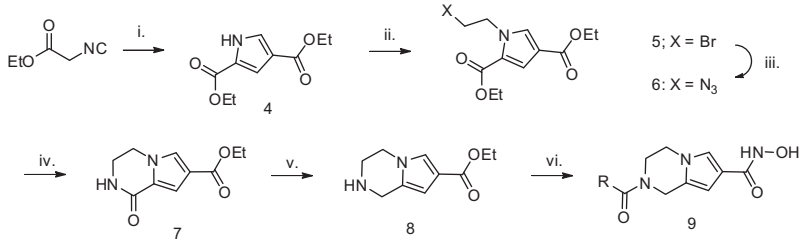
of fused ring heterocyclic analogs led to highly selective nanomolar HDAC6 inhibitors with cellular activity such as **2** (Fig. 1).[25] Similar observations on the HDAC6 selectivity of C-aryl hydroxamic acids, exemplified by compound **3**, have been made recently by Holson and co-workers.[26] Using homology modeling, we showed that the selectivity of compounds **1** and **2** may arise from the differing 'width' of the (benzylic) spacer as compared to long chain aliphatic series.[25] In order to investigate cyclic analogs further we designed three additional series based on 1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrazine, 4,5,6,7-tetrahydropyrazolopyrazine and 5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazines and report here their synthesis and biochemical characterization.

1,2,3,4-Tetrahydropyrrolo[1,2-*a*]pyrazines were prepared starting with the condensation of ethylpropiolate with ethyl isocyanoacetate in the presence of strong base to give pyrrole ester **4**.[27] which was alkylated with dibromopropane to give **5** and further transformed to the azide **6** (Scheme 1). Hydrogenation of azide **6** effected reductive ring closure to lactam **7** which was further reduced using diborane affording tetrahydropyrrolopyrazine ethyl ester (**8**). Acylation of **8** with a range of capping groups was followed by transformation to the corresponding hydroxamic acids **9** by reaction with hydroxylamine hydrochloride in the presence of base.

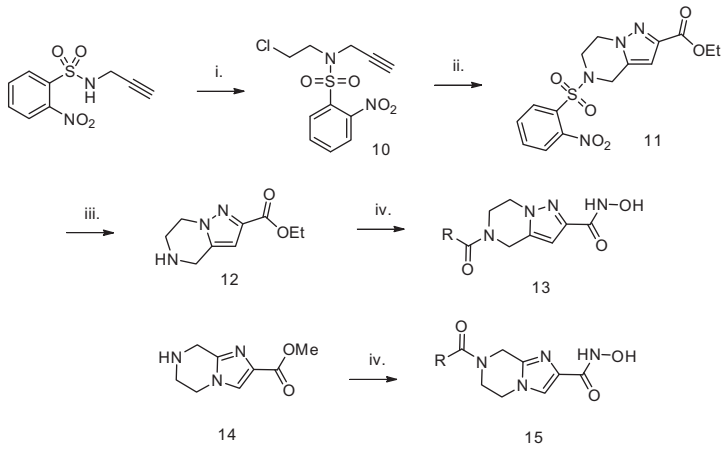
The tetrahydropyrazolopyrazines were accessed as shown in Scheme 2.[28] Thus, the nosyl derivative of propargylamine was alkylated with 1-bromo-2-chloroethane to give **10**. Cycloaddition of **10** with ethyldiazoacetate and ring closure in situ onto the chloroethyl group afforded **11** which was deprotected to give amine **12**. Reaction of **12** with a series of carboxylic acid capping groups followed by displacement of the ethoxy group by hydroxylamine afforded compounds **13**. Representative examples of the isomeric 5,6,7,8-tetrahydroimidazo[1,2-*a*]pyrazine series **15** were obtained by acylation of commercially available ester **14** followed by transformation to the corresponding hydroxamic acids **15** (Scheme 2).

The inhibitory activities of hydroxamic acids in series **9**, **13** and **15** on the rate of deacylation of the substrate Ac-Arg-Gly-Lys(Ac)-AMC (where AMC denotes 7-aminocoumarin) catalyzed by HDAC6 were measured and selectivity was assessed with respect to inhibition of the enzymes present in HeLa nuclear extract (NucEx), predominantly HDACs 1 and 2. In addition, compounds with an appropriate combination of inhibitory potency and selectivity were assayed in cells for their ability to induce hyperacetylation of tubulin (Table 1) and of nuclear histones.

We previously observed that C-arylhydroxamic acids are potent HDAC6 inhibitors with high selectivity with respect to inhibition of the other sub-types. For example, hydroxamic acid **1**, with an *N*-methylpyrrole carboxylate capping group, exhibited an IC_{50} value of 19 nM for HDAC6 inhibition as compared with 2.2 μM inhibition of the class I enzymes present in nuclear extract enzyme (Table 1). Cyclic analog **2** showed slightly reduced HDAC6 inhibitory activity but improved selectivity with respect to inhibition of nuclear extract enzymes.[25] Similarly, tetrahydropyrrolo[1,2-*a*]pyrazine **9a** has comparable potency to cyclic compound **2** though in this case the selectivity was somewhat more modest (190-fold). The cellular activity (Ac-Tub assay) of **9a** (IC_{50} = 69 nM) is, however, a marked improvement over both compounds **1** and **2**. In addition, **9a** and all other compounds tested in cellular assays (Table 1) showed selectivity with respect to inducing acetyl lysine accumulation in nuclear histones (<20% inhibition @ 20 μM). While other small aryl residues as capping groups led to potent and

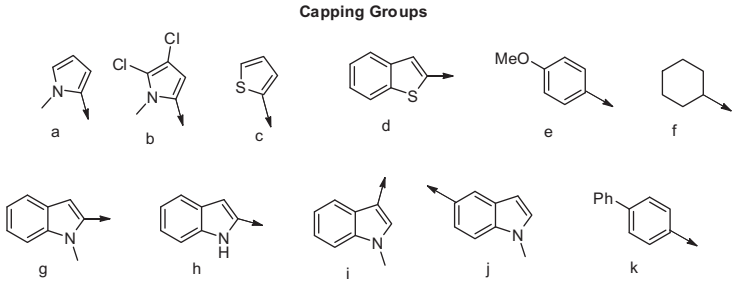


Scheme 1. Reagents and conditions: (i) ethyl propioloate, KH, MTBE; (ii) 1,2-dibromoethane, K_2CO_3, MeCN; (iii) NaN_3, DMF; (iv) Pd-C, H_2, MeOH; (v) BH_3, Me_2S, THF; (vi) (a) RCOOH, HATU, TEA; (b) $NH_2OH{\cdot}HCl$, KOH, MeOH.



Scheme 2. Reagents and conditions: (i), Cs_2CO_3, acetone; (ii) ethyldiazoacetate, toluene, 140 °C; (iii) PhSH, Cs_2CO_3; (iv). (a) RCOOH, HATU, TEA; (b) $NH_2OH{\cdot}HCl$, KOH, MeOH.

Table 1
HDAC6 inhibitory activity and selectivity of fused ring hydroxamic acids



Compd	HDAC6 IC_{50}/nM[a]	Selectivity NucEx[b]/HDAC6	Ac-Tub IC_{50} /nM[c]	Compd	HDAC6 IC_{50}/nM[a]	Selectivity NucEx[b]/HDAC6	Ac-Tub IC_{50} /nM[c]
9a	33	190	69	**9k**	32	44	ND
9b	33	100	150	**13a**	350	220	2200
9c	110	150	ND	**13e**	810	120	ND
9d	39	14	ND	**13g**	76	66	3500
9e	43	130	100	**15a**	130	770	ND
9f	100	250	730	**15d**	64	52	ND
9h	280	24	ND	**15k**	87	70	2100
9i	190	6.3	ND	**1**	19	115	460
9j	85	35	140	**2**	36	500	210

[a] Enzymatic data obtained with recombinant HDAC6 or HeLa nuclear extracts using Ac-Arg-Gly-Lys(Ac)-AMC as substrate with subsequent release of AMC by treatment with trypsin. IC_{50} values are means of two or more determinations. Standard deviation is within 20% of the IC_{50}.
[b] Nuclear extract (NucEx) expresses predominantly HDACs 1 and 2.
[c] Cellular data: Ac-tubulin accumulation was determined by immunofluorescence with an anti-Ac-tubulin antibody in cytosol. Ac-Lys accumulation in nuclear histones was measured by immunofluorescence in HeLa cells with all compounds showing <20% inh @ 20 μM. ND not determined.

selective compounds, larger groups gave more variable results. Thus, comparable potency was maintained for analogs **9b**, **9d**, **9e**, and **9k** but selectivity was adversely affected. The enzymatic HDAC6 inhibitory activities of pyrazolopyrazine analogs **13a**, **13e** and **13g** as well as tetrahydroimidazo[1,2-*a*]pyrazines **15a**, **15d**, and **15k** were invariably lower than for series **9** and could not be compensated by further changes to the capping groups.

In order to probe sub-type selectivity in more detail, the inhibitory activity of compound **9a** was assessed across the full panel of individual HDACs. These data are compared with previously reported HDAC6 selective compounds in Table 2 and confirm the significant improvement in selectivity of **9a** as compared to the acyclic analog **1** observed in the nuclear extract assay. Moreover, compound **9a** shows a better selectivity profile than those of vorinostat[21] and Tubacin[16,24] and comparable to that of Tubastatin,[24] with HDAC8 the only isoform for which any significant inhibitory activity was observed (ca. 100-fold selectivity). Significantly, **9a** is the most potent compound in the cellular acetyl tubulin assay. Other properties of compound **9a** appropriate for a biological probe and for potential further optimization include high aqueous solubility (>200 μM at pH 6.8), moderate Caco-2 permeability (4.2×10^{-6} cm s^{-1} at pH 7.4 with efflux ratio = 1.9) and relatively low in vitro clearance (human microsomal, E_h < 0.2; rat S9 E_h = 0.3).

Although the crystal structure of HDAC6 has not been reported, homology models of HDAC6 and HDAC1 have been developed to explain the selectivity of Tubastatin A[24] and tubacin[29] in terms of the interactions of capping groups with the rim of the catalytic channels. Our own homology models[25] of HDAC6 and HDAC7

Table 2
Comparison of HDAC isoform inhibitory activity of benzylic spacer derived inhibitors with reference compounds

Compd	IC_{50}[a] (μM) isoform										Ac-Tub[b] IC_{50} (μM)
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**10**	**11**	
Vorinostat[c]	0.119	0.164	0.106	NR	NR	0.090	NR	1.50	0.072	NR	NR
Tubacin[d,e]	1.40	6.27	1.27	17.3	3.35	0.004	9.70	1.27	3.71	3.79	2.9
Tubastatin[e]	16.4	>30	>30	>30	>30	0.015	>30	0.854	>30	>30	ca. 2.5
1	5.80	16.0	4.50	15.0	3.30	0.019	4.60	0.69	3.70	ND	0.460
2	45.0	>50	46.0	>50	>50	0.036	>50	2.10	>50	>50	0.21
9a	15	39	8.5	>50	29	0.033	35	3.3	41	29	0.069

NR not reported. ND not determined.
[a] Enzymatic data obtained with recombinant HDAC6 using Ac-Arg-Gly-Lys(Ac)-AMC as substrate with subsequent release of AMC by treatment with trypsin. See Supplementary information for details of assays for the other sub-types.
[b] Cellular data: Ac-tubulin accumulation was determined by immunofluorescence with an anti-Ac-tubulin antibody in cytosol.
[c] Data from Yannini et al.[21] and Kozikowski et al.[23]
[d] Data from Schrieber et al.[16]
[e] Data from Kozikowski et al.[24]

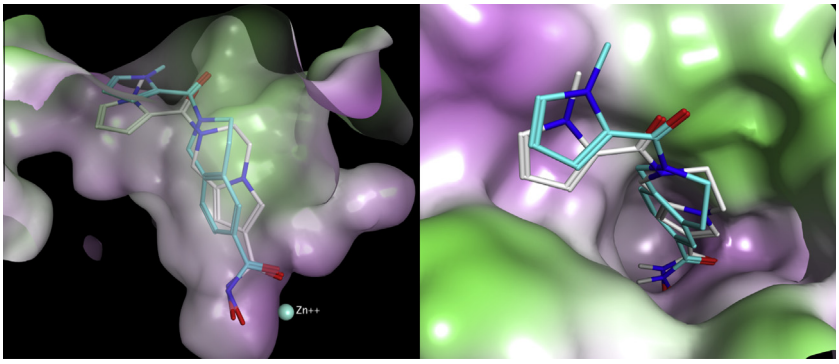


Figure 2. Compounds **2** (blue) and **9a** (grey) docked into the active site of HDAC6 from a homology model; side view (left) showing interaction with the catalytic zinc; top view (right) looking down the 11 Angstrom tunnel. Figure constructed using Moe pocket; color coding is purple polar and green hydrophobic.

(obtained from the published crystal structure)[30] focused on differences in the dimensions of the channels of the HDAC sub-types. Thus, while the HDACs are highly conserved,[29] differences in the loop regions[31] cause the channel of HDAC6 leading to the catalytic Zn^{2+} to be wider and shallower than that of the other sub-types. This is illustrated in Fig. 2 in which hydroxamates **2** and **9a** are docked into the active site where they bind to zinc in a bidentate fashion, with two Asp and one His residue completing the primary coordination sphere. The spacers project along the 11 Angstrom deep channel[32] with the pyrrole capping groups participating in additional binding interactions with the rim of the active site of the protein. While HDAC6 can readily accommodate hydroxamic acids with heterocyclic spacers such as compounds **2** and **9a**, significant steric clashes are expected with the narrower channels of the other sub-types as shown by docking into the crystal structure of HDAC7.[25,33] In contrast, the flexibility of hydroxamic acids derived from long chain aliphatic spacers facilitates access to the active site channels of all the sub-types potentially accounting for the pan HDAC inhibitory activity of vorinostat and related compounds.

In summary, we have shown that 1,2,3,4-tetrahydropyrrolo [1,2-*a*]pyrazine hydroxamic acid **9a** is a potent HDAC6 inhibitor with approximately 200 fold selectivity vs nuclear extract, 100 fold selectivity versus HDAC8 and even greater selectivity with respect to inhibition of the other sub-types. In cells these properties are reflected in an IC_{50} value of 69 nM for inducing tubulin acetylation selective over histone acetylation. Selectivity may be attributed to the fused ring spacer more effectively accessing the wider channel of HDAC6 compared to other HDAC sub-types as well as hydrophobic capping groups interacting with the protein surface near the rim of the active site as shown by homology modeling. These biological tools are of interest for studying the function of HDAC6 in proteotoxic stress and its interactions between the ubiquitin proteasome system and aggresome pathways for protein degradation.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.10.022.

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33. Molecular modeling suggests that compound **9a** can adopt low energy poses, inaccessible to compound **2**, that encounter less steric repulsion between the heterocyclic spacer and the walls of the HDAC7 channel (as depicted in Supplementary Fig. 1). This may account for the somewhat lower selectivity of **9a** and related compounds as compared to compounds in the tetrahydroisoquinoline series.