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Design and synthesis of novel pyrimidine hydroxamic acid inhibitors of histone deacetylases

Alastair D. G. Donald [a,*], Vanessa L. Clark [a], Sanjay Patel [a], Francesca A. Day [a], Martin G. Rowlands [b], Judata Wibata [b], Lindsay Stimson [b], Anthea Hardcastle [b], Sue A. Eccles [b], Deborah McNamara [a], Lindsey A. Needham [a], Florence I. Raynaud [b], Wynne Aherne [b], David F. Moffat [a]

[a] Chroma Therapeutics, 93 Milton Park, Abingdon, Oxfordshire OX14 4RY, UK
[b] Cancer Research UK Cancer Therapeutics Unit, The Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5NG, UK

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ABSTRACT

Inhibition of histone deacetylase activity represents a promising new modality in the treatment of a number of cancers. A novel HDAC series demonstrating inhibitory activity in cell proliferation assays is described. Optimisation based on the introduction of basic amine linkers to effect good drug distribution to tumour led to the identification of a compound with oral activity in a human colon cancer xenograft study associated with increased histone H3 acetylation in tumour tissue.

Histone deacetylases (HDACs) are a family of epigenetic regulator enzymes whose inhibition leads to a reversal of tumour suppressor silencing, cell cycle arrest, differentiation, and/or apoptosis. Because of these effects, which manifest themselves most strikingly in transformed cells, HDAC inhibition has emerged as a strategy to reverse aberrant epigenetic changes associated with cancer.[1] Two such agents have been approved for use in the treatment of cutaneous T-cell lymphoma, Zolinza® (Vorinostat/SAHA, Merck),[2] and Istodax® (Romidepsin, Gloucester Pharmaceuticals/Celgene). A number of additional HDAC inhibitors have shown anti-tumour activity in xenograft models and are now in clinical trials.[3] A wealth of preclinical data suggests that HDAC inhibitors have potential against a wide range of additional tumour types either alone or in combination with other therapies.[4]

The HDACs comprise three separate classes of enzyme. Both class I and class II enzymes are metalloenzymes containing a catalytic zinc atom. There are thought to be at least 11 isoforms of the metalloenzyme HDAC proteins: class I HDACs (HDAC 1, 2, 3 and 8) are ubiquitously expressed and generally detected in the nucleus while class II HDACs (HDAC 4, 5, 6, 7, 9 and 10) can shuttle between the cytoplasm and nucleus.[5] HDAC 11 has a related catalytic domain but has only weak homology with the others. The class III enzymes are not metalloenzymes and, while relevant to certain human diseases, are not yet strongly implicated in cancer.[6]

The major objective of our discovery efforts towards therapeutically useful small molecule HDAC inhibitors was to achieve good intra-tumoural distribution of drug via oral administration.[7,8] Based on an analysis of all previously described HDAC inhibitor families, addressing their ability to meet our goals of robust physicochemical properties to facilitate improved cell permeability and oral dosing, and to provide potential for chemical novelty, we decided to utilise the pyrimidine hydroxamate moiety as the zinc-binding group (ZBG) in our approach. This seemed likely to be the most metabolically stable functionality that would be compatible with good HDAC inhibitory activity.[9,10] Such hydroxamic acids have been previously described as potent inhibitors of histone deacetylases (e.g., R306465/JNJ-16241199).[11]

Docking studies to an available structure of the class 1 isoform HDAC8, where the pyrimidine hydroxamate unit co-ordinated to the zinc ion at the base of the catalytic site were performed to identify linker groups that could potentially provide access to the hydrophobic region at the entrance of the active site.[12,13] These studies suggested fused bicyclic structures such as 2-(hexahydropyrrolo[3,4-*c*]pyrrol-2-(1*H*)-yl) substituted pyrimidines, as they possessed the required length to provide good interaction between aryl or heteroaryl substituents and the hydrophobic enzyme surface (Fig. 1).

After identification of potential groups for linking to the pyrimidine-based ZBG, our studies focused on exploring the hydrophobic cap group of the 2-(hexahydropyrrolo[3,4-*c*]pyrrol-2-(1*H*)-yl) system. Such compounds were readily accessible from a common intermediate **3**, which was prepared as shown in Scheme 1.

* Corresponding author. Tel.: +44 1235 829165.
E-mail address: adonald@chromatherapeutics.com (A.D.G. Donald).

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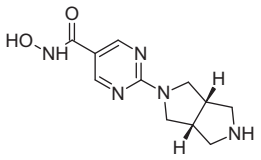
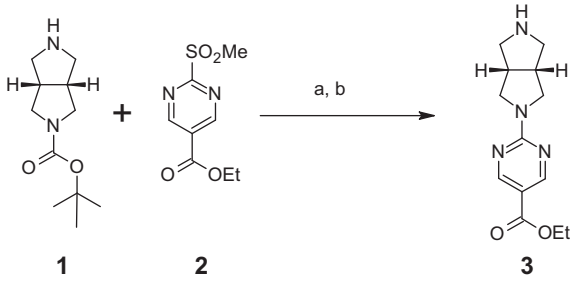


Figure 1. Fused bicyclic diamine scaffold identified as a potential core structure for further elaboration into novel inhibitors of HDACs.



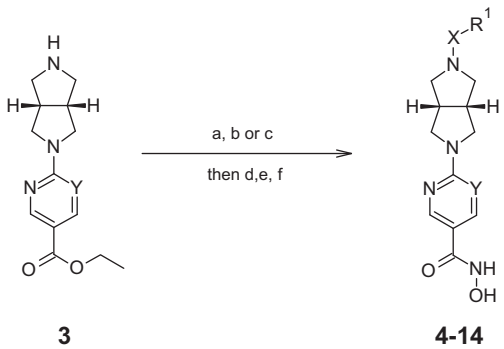
Scheme 1. Reagents and conditions: (a) K_2CO_3, acetonitrile, room temperature, 1 h (58%) and (b) 4 M HCl, dioxane, room temperature, 1.5 h (99%).

Condensation of the commercially available mono-protected diamine **1** with known pyrimidine **2**[14] followed by acidic deprotection of the Boc group yielded an intermediate **3** suitable for installation of a variety of hydrophobic cap groups. Reaction of **3** with a sulfonyl chloride, acid chloride or aldehyde yielded the required functionalized pyrimidine ester (Scheme 2).

Conversion of the ester into the hydroxamic acid was achieved by a three-step procedure—alkaline hydrolysis of the ester, coupling of the resultant acid with an O-protected hydroxylamine and acidic deprotection to yield the desired hydroxamic acids **4**–**14**.[15,16]

The HDAC inhibitory activities of these compounds are given in Table 1. Sulfonamide substituted compounds **4**–**6** showed encouraging activity in assays of inhibition of HDAC activity (comparable to SAHA), but showed modest to poor activity in cell-based assays of anti-proliferative activity (Table 2).[17]

Expansion of the SAR to examine the role of other linkers led to the synthesis of amide substituted compounds (**7** and **8**) and tertiary amine compounds (**9–13**). In the naphthalene substituted series of compounds (**4**, **7** and **13**), enzyme activity improved approximately fivefold whilst cellular activity improved by between 4 and 6-fold (dependent on cell line). A strong preference for large, lipophilic substituents is evident, with such compounds



Scheme 2. Reagents and conditions: (a) sulfonyl chloride, pyridine (51–66%); (b) acid chloride, pyridine (80–89%); (c) aldehyde, DCE, sodium triacetoxyborohydride (71–95%); (d) 6 M NaOH, ethanol, 80 °C (81–100%); (e) *O*-(1-isobutoxyethyl)hydroxylamine, EDC, HOBt, DIPEA, DCM; (f) TFA, DCM, MeOH (5–90%, two steps).

Table 1
Inhibition of HDAC activity (IC_{50}) for compounds **4**–**14**

Compd	X	Y	R	IC_{50}[a] (nM)
SAHA	—		—	87 ± 13
4	SO_2	N	2-Naphthyl	64
5	SO_2	N	3,5-Bis-(trifluoromethyl)phenyl	433
6	SO_2	N	4-Trifluoromethoxy-phenyl	123
7	CO	N	2-Naphthyl	12 ± 2
8	CO	N	4-Phenoxypropyl	122
9	—	N	5-Methoxyindan-1-yl	30 ± 2.9
10	CH_2	N	3,5-Difluorophenyl	50
11	CH_2	N	4-Chlorophenyl	42
12	CH_2	N	4-Methoxyphenyl	95
13	CH_2	N	2-Naphthyl	13 ± 1.9
14	CH_2	C	2-Naphthyl	45

[a] Values are mean of three experiments. HDAC activity was measured using Fluor De Lys reagents (Biomol) and a commercially available HeLa cell nuclear extract (Cilbiotech s.a.). Values are the mean of two independent determinations or mean ± SD for $n > 2$ determinations.

Table 2
Growth inhibition GI_{50} for compounds **3–14** in human tumour cell lines

Compd	HCT116 GI_{50}[a] (nM)	HeLa GI_{50}[a] (nM)	U937 GI_{50}[a] (nM)	HUT78 GI_{50}[a] (nM)
SAHA	750	1460	390	300
4	740	1132	480	300
5	1700	42% @10 μM	1250	700
6	530	940	980	500
7	590	210	390	200
8	1800	3950	900	800
9	500	230	390	208
10	530	940	300	202
11	490	690	230	190
12	900	950	550	290
13	120	260	72	79
14	700	1260	450	340

[a] Values are means of three experiments. Growth inhibition was measured using 72 h SRB or MTT assays.

demonstrating enhanced potency compared to monocyclic aromatic systems such as **11**. The two methoxy substituted compounds (**9** and **12**) also demonstrate this trend; the additional ring of indane **9** confers a 2–3-fold potency advantage over the basic 4-methoxybenzyl system of **12**. Surprisingly we found that the pyridine hydroxamic acid **14** was significantly less active in both enzyme and cellular assays than its direct pyrimidine analogue **13**.[18]

Naphthyl substituted amine **13** emerged as a promising lead compound due to the desirable combination of excellent potency and drug-like molecular properties (molecular weight 389.4, *c* Log *P* 1.3, 6 H-bond acceptors, 2 H-bond donors, four rotatable bonds), and was selected for further in vitro and in vivo studies. We further hypothesised that the basic nature of the linker unit of **13** could aid our objective of enhanced drug distribution to tumour.

Incubations of **13** (10 μM) with human and mouse liver microsomes as previously described[19] indicated acceptable metabolic stability (72% and 45% of **13** remaining after 30 min, respectively). The disappointingly low cell permeability (P_{app} 0.5×10^{-6} cm/s), as assessed by PAMPA was identical to that of SAHA. There was no significant inhibition of cytochrome P450 isoforms 1A2, 2A6, 2C8, 2C19 and 3A4 at 50 μM but a moderate inhibition (IC_{50} = 10–50 μM) of CYP2D6 was observed.[20] Inhibition of the hERG channel by **13** was modest (4.1 μM).

Cassette PK studies in CD1 mice (3 mg/kg po, 1 mg/kg iv) indicated that **13** was orally available in mice (F = 15%) and distributed well into tissues (spleen C_{max} 218 ng/ml ≅ 0.56 μM, plasma C_{max}

31 ng/ml), and thus a mouse xenograft study was undertaken. Female CrTac:*NCr-Fox1(nu)* athymic mice ($n = 14$) with established bilateral HCT116 colon carcinoma xenografts were dosed orally, once-daily for 16 days with 50 mg/kg of **13** (Fig. 2).[21]

At this dose, **13** was well tolerated as evidenced by an increase in mean body weight of 7%, although inhibition of tumour growth was modest with a final tumour weight of 80% of control and a reduction in mean tumour volume of 28%. Measured peak tumour concentration of **13** 1 h post dosing was 215 ± 75 nmol/kg (SEM) (1.6 times cellular IC_{50}), whilst the plasma concentration at this time point was 14 nmol/L (Table 3). At 4 h, tumour concentrations of **13** were 3.4-fold greater than plasma concentrations, whereas spleen concentrations were 11-fold higher than plasma. For spleen, concentrations of **13** were approximately eightfold, sevenfold and threefold higher than tumour at 30 min, 1 h and 4 h, respectively. These data are consistent with concentrations of **13** found in the oral cassette PK study. The modest efficacy shown in this experiment may be a reflection of significant drug efflux as Caco-2 data was strongly suggestive of this, with permeability A → B below the limit of detection, whereas B → A was 9.4×10^{-6} cm s^{-1}.

Pharmacodynamic changes consistent with HDAC inhibition were observed in tumour tissue. A twofold increase in histone acetylation compared to DMSO controls was observed 1 and 4 h post final dose (Fig. 3) and paralleled tumour concentrations of **13**. Tumour concentrations of **13** were above cellular GI_{50} for 4 h. This is consistent with the sustained twofold histone hyperacetylation observed in tumour tissue at this time point (Fig. 4).

In summary we have identified a novel series of potent HDAC inhibitors based on a pyrimidine hydroxamic acid ZBG. One of the compounds identified, **13**, demonstrated activity in an HCT116 human colon carcinoma model when dosed orally once daily. Details of the further optimisation of pyrimidine hydroxamic acid HDAC inhibitors will be reported in due course.

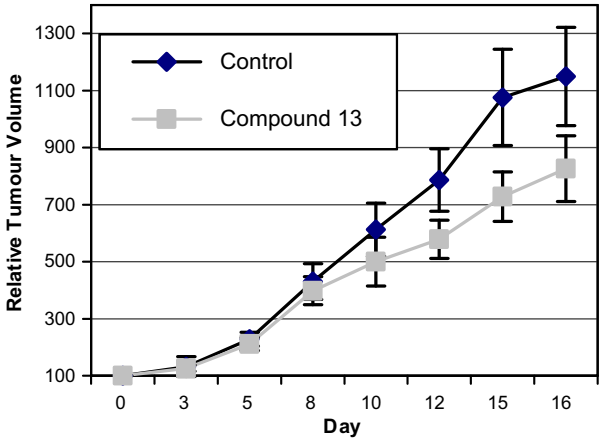


Figure 2. Relative tumour volumes in HCT116 tumour xenograft study for compound **13** (50 mg/kg, po, qd).

Table 3
Concentrations of **13** in in vivo study

Time[a] (h)	[Plasma][b]	[Tumour][c]	[Spleen][c]
0.5	<LOD[d]	80	620
1	14	215	1260
4	25	154	500

[a] 1 h timepoint values are the means of three samples, 0.5 and 4 h timepoints are means of two samples.
[b] Plasma concentration quoted in nM.
[c] Tumour and spleen concentrations quoted in nmol/kg.
[d] Below limit of detection.

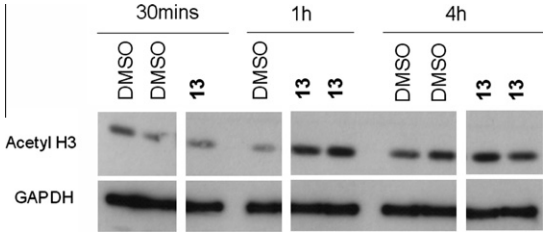


Figure 3. Increase in histone H3 acetylation observed in tumour tissue measured by Western blot using an anti-acetylated H3 antibody (Upstate). Intensity of bands was measured by densitometry and normalised to GAPDH. Change in acetylation was calculated as % DMSO controls.

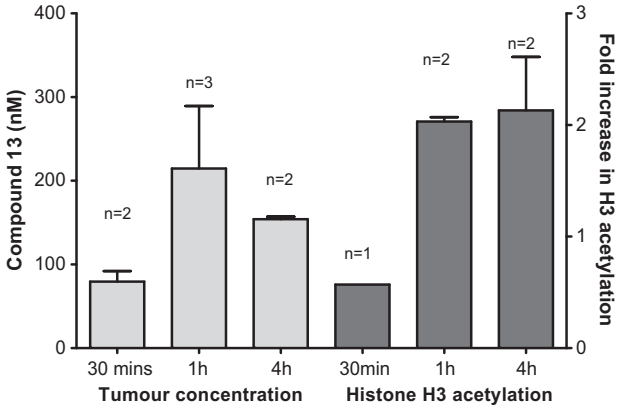


Figure 4. Quantitation of tumour concentrations (columns 1–3) and effects on histone H3 acetylation (columns 4–6) of compound **13**. Error bars represent value for $n = 1$, mean and range for $n = 2$, mean and SEM for $n = 3$. Each value is one animal. Changes in H3 acetylation are expressed as fold difference compared to DMSO controls.

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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.2010.09.016.

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16. Representative Procedure—the preparation of Compound **4** is detailed in Supplementary data (additional characterisation data for compounds **5**–**14** is available in Ref. 15).
17. HDAC activity was determined using HeLa cell nuclear extract (Cat. No. CC-01-20-50) obtained from Cilbiotech s.a., Mons, Belgium and the Fluor De Lys™ System (Biomol International, Exeter, UK). The fluorimetric assay was carried out as described in the Biomol technical manual using 40 μg HeLa extract protein for 15 min at room temperature. For IC_{50} determinations, each compound was assayed at eight concentrations in duplicate and the data analysed in GraphPad Prism.
18. Compound **14** was prepared from the product obtained from reaction of ethyl 6-chloronicotinate and amine **1** (72 h, 85 °C, 1,4-dioxane, 76%). Subsequent steps were performed in a fashion analogous to that described for compound **4** (see Refs. 15,16).
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