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Identification and optimisation of a series of substituted 5-(1*H*-pyrazol-3-yl)-thiophene-2-hydroxamic acids as potent histone deacetylase (HDAC) inhibitors

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Abstract—Optimisation of ADS100380, a sub-micromolar HDAC inhibitor identified using a virtual screening approach, led to a series of substituted 5-(1*H*-pyrazol-3-yl)-thiophene-2-hydroxamic acids (**6a–i**), that possessed significant HDAC inhibitory activity. Subsequent functionalisation of the pendent phenyl group of compounds **6f** and **6g** provided analogues **6j–w** with further enhanced enzyme and anti-proliferative activity. Compound **6j** demonstrated efficacy in a mouse xenograft experiment.

The histone deacetylases (HDACs) belong to a family of zinc-dependent enzymes that cleave acetyl groups appended to the ε-amino side chain of lysine residues, such as those found in the amino-terminal tails of histone proteins. Deacetylated histones acquire a net positive charge that interacts strongly with the negatively charged DNA, which becomes tightly wound around the nucleosome core. Therefore, changing the acetylation status of the histone tails can affect chromatin structure, DNA accessibility to transcription factors, and ultimately gene expression. It is known that, in malignant cells, epigenetic events such as histone deacetylation can contribute to the transformed phenotype, and inhibition of HDAC enzymes has been shown to induce growth arrest, differentiation and apoptosis through the increased expression of tumour suppressor genes such as $p21^{WAF1/CIP1}$.[1]

Several different classes of HDAC inhibitors have entered clinical trials, some of which have demonstrated significant anti-proliferative activity against a number of solid and haematological tumours.[2] These classes of HDAC inhibitors include aliphatic acids, *o*-aminoanilides, electrophilic ketones and cyclic peptides.[3] However, the most extensively exemplified class of inhibitors is based upon the hydroxamic acid moiety, which is a widely recognized zinc-binding group.[4] Figure 1 illustrates some typical hydroxamic acid-containing HDAC inhibitors, including trichostatin A (TSA),[5] NVP-LAQ824,[6] PXD101,[7] and SAHA (Zolinza™, Vorinostat),[8] which has been recently approved by the FDA for once-daily oral treatment of advanced cutaneous T-cell lymphoma (CTCL).

At Argenta, we initiated a hit-finding exercise that utilised a virtual screening approach based on the published crystal structure (PDB code: 1C3R) of HDAC-like protein (HDLP).[9] A virtual library of

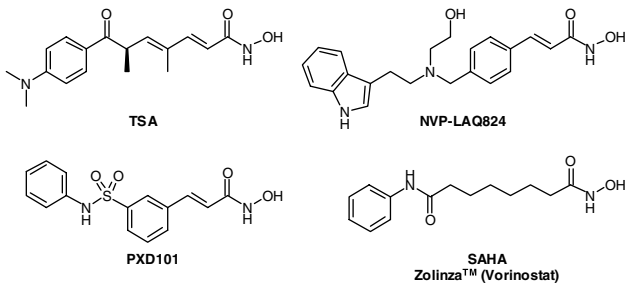

Figure 1. Typical hydroxamic acid-containing HDAC inhibitors.

Keywords: Histone deacetylase inhibitors; HDAC; Hydroxamic acids.
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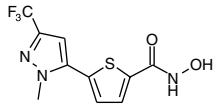
644 hydroxamic acids was generated from a database of carboxylic acids available in-house, followed by a docking and scoring exercise using the FlexX program.[10] Based on the virtual screening results, 75 compounds were synthesised and tested in an HDAC enzyme assay,[11] from which ADS100380 (Fig. 2) was identified as a sub-micromolar (IC_{50} = 0.75 μM, Table 1) HDAC inhibitor. ADS100380 also displayed weak functional activity (IC_{50} = 11.4 μM, Table 1) in a cell-based proliferation assay.[12] The corresponding carboxylic acid of ADS100380 was inactive.

The HDLP structure indicated that the catalytic zinc ion to which the hydroxamic acid group of ADS1000380 binds is located at the bottom of a tube-shaped pocket. Thus, optimisation of ADS100380 was designed to make additional interactions at the entrance to the HDAC active site by tethering hydrophobic aromatic groups to the pyrazole nitrogen (Scheme 1).[13]

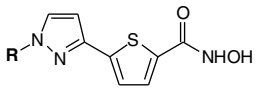


ADS100380

Figure 2. An HDAC inhibitor identified by virtual screening.

The commercially available vinylogous amide **1** was treated with hydrazine hydrate to give the key cyanothiophene intermediate **2**. Compound **2** was converted to the corresponding carbomethoxythiophene (**3**), via basic hydrolysis of the nitrile group to provide the carboxylic acid, followed by esterification. Both intermediates **2** and **3** underwent regioselective[14] alkylation with alkyl halides to provide the 1-N-alkylated cyanothiophenes **4a**–**f**, and the 1-N-alkylated carbomethoxythiophenes **5** and **8**, respectively. Hydrolysis of compounds **4a**–**f** provided the corresponding carboxylic acids, which were then coupled with *O*-THP-protected hydroxylamine, and subsequent acidic deprotection liberated the desired final hydroxamic acids **6a**–**f**. Compound **5** was treated with trifluoroacetic acid in dichloromethane to liberate the terminal amino group, which was then subjected to reductive alkylation conditions with

Table 1. HDAC and anti-proliferative assay results for compounds **6a**–**i**



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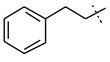
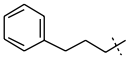
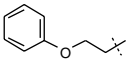
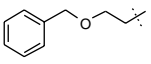
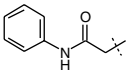
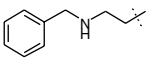
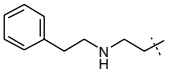
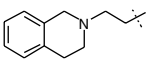
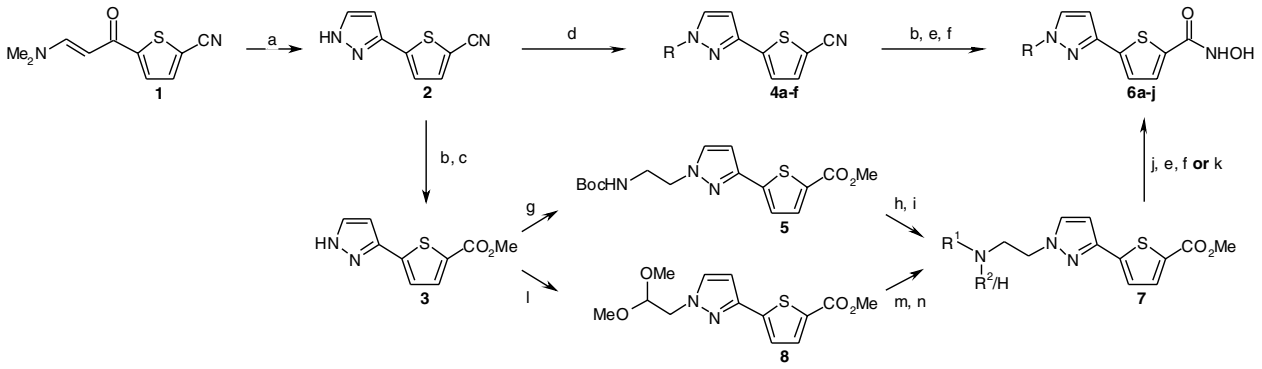
Compound	R	HDAC[a] (IC_{50}, μM)	Cell proliferation[a]	
			MCF-7 (IC_{50}, μM)	MDA-MB231 (IC_{50},μM)
ADS100380	—	0.750	11.4	31.5
6a		0.153	2.06	4.54
6b		0.100	1.24	2.91
6c		0.034	0.52	2.19
6d		0.022	0.50	1.50
6e		0.020	0.35	0.75
6f		0.021	0.12	0.84
6g		0.033	0.46	1.80
6h		0.069	0.47	1.50
6i		0.019	0.15	0.53

[a] Values are means of two experiments.



Scheme 1. Reagents and conditions: (a) EtOH, hydrazine hydrate (1.1 equiv), reflux, 16 h (89%); (b) 1 M NaOH, reflux, 2 h (52–97%); (c) MeOH, concd HCl, reflux, 16 h (93%); (d) RX (1.2 equiv), K_2CO_3, DMF, 70 °C, 16 h (43–90%); (e) $^{i}Pr_2NEt$ (2.5 equiv), DMF, H_2NOTHP (1.1 equiv), HATU (1.1 equiv) (60–95%); (f) *p*TSA, MeOH (23–85%); (g) $BocHN(CH_2)_2Br$ (1.1 equiv), K_2CO_3, DMF, 70 °C, 16 h (88%); (h) CF_3CO_2H, CH_2Cl_2; (i) R^1CHO (0.9 equiv), MeOH, 16 h, then $NaBH_4$, 2 h (50–73% two steps); (j) LiOH, H_2O, CH_3CN, 16 h (quant); (k) $NH_2OH{\cdot}HCl$ (5 equiv), KOH, MeOH, 16 h (25–65%); (l) $(MeO)_2CHCH_2Br$ (1.2 equiv), K_2CO_3, DMF, 90 °C, 16 h (60%); (m) 1 M HCl, THF, μW 150 °C, 100 s (80%); (n) R^1R^2NH (1.1 equiv), $Na(OAc)_3BH$, CH_2ClCH_2Cl, 16 h (65–80%).

a variety of aromatic aldehydes to yield tethered carbomethoxythiophenes **7**. Acidic deprotection of the dimethyl acetal **8** to liberate the corresponding aldehyde, followed by reductive amination conditions using tetrahydroisoquinoline, provided the desired intermediate carbomethoxythiophene **7** from which compound **6i** was obtained. Conversion of the carbomethoxythiophenes **7** to the desired hydroxamic acids **6g–j** was achieved by ester hydrolysis, followed by the same coupling and deprotection conditions used for the 1-N-alkylated cyanothiophenes **4a–f**, or directly by treating with basic hydroxylamine.

All compounds were initially tested in a primary HDAC enzyme assay,[11] and those possessing sub-micromolar activity were then simultaneously evaluated in MCF-7 and MDA-MB231 cell-based proliferation assays.[12]

The benzyl-tethered compound **6a** possessed a 5-fold increase in HDAC potency over ADS100380, both in the HDAC and the MCF-7 cell proliferation assays. Increasing the linker length of compound **6a** by a methylene group, compound **6b**, led to a minor increase in HDAC activity, whereas increasing the linker length by two methylene units, compound **6c**, provided more than a 4-fold increase in activity. Both compounds **6b** and **6c** display corresponding increases in activity in the MCF-7 cell proliferation assays. Replacement of a methylene group in the tether of compound **6c** with an oxygen atom, compound **6d**, had little effect on the activity. Homologation of the tether of compound **6d** with a methylene group, compound **6e**, provided no further increase in activity in the HDAC assay, but a 2-fold increase in anti-proliferative activity in the MDA-MB231 cell-based assay. Introducing an acetamide moiety between the phenyl and pyrazole ring systems, compound **6f**, afforded the most potent three-atom tether identified. Compound **6g** was designed to incorporate a basic amino group into a four-atom tether, a transformation that retained similar activity to the non-basic tethered analogue **6e** in the HDAC and MCF-7 assays. Homologation of the tether of compound **6g** by a methylene group to furnish the phenethylamino-tethered analogue **6h** resulted in reduced activity in the HDAC assay, but no loss of potency in the cellular assay. Cyclisation of the secondary amino group of compound **6g** onto the aromatic ring by an ethyl linker, compound **6i**, provided a marginal increase in activity in the HDAC assay, and a 3-fold increase in activity in both cell proliferation assays.

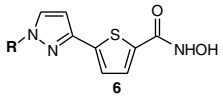
Encouraged by the promising activity of the unsubstituted phenyl-tethered analogues **6a–i**, we next focused our attention on investigating the effect of functionalising the phenyl rings of compounds **6g**, and **6f**, respectively. Table 2 includes a selection of compounds synthesised to investigate aromatic ring substitution SAR.

The aminoethyl-tethered compounds **6j–r** were produced using the existing synthetic methodology as outlined in Scheme 1. However, a modified synthetic route was required to make further acetamide-tethered hydroxamic acids that would enable the use of commercially available aromatic amines (Scheme 2).[13]

The carbomethoxythiophene **3** was alkylated with *tert*-butyl bromoacetate to provide compound **9**. The *tert*-butyl ester **9** was readily hydrolysed and the resulting acid was coupled with a variety of aromatic amines to give compounds of the generic structure **10**. Standard conditions were then used to convert the methyl carboxylate to the corresponding hydroxamic acids **6s–w**.

The benzodioxole analogue **6j** was one of the first substituted phenyl-tethered analogues to be prepared and tested. Compound **6j** indicated that phenyl substitution was tolerated, and provided potent HDAC and cell proliferation IC_{50} values. The introduction of a *para*-methoxy substituent to compound **6g** afforded analogue **6k** that possessed a 3-fold increase in HDAC activity. However, the 3,4-bis-methoxy substituted phenyl compound **6l** lost almost an order of magnitude in potency in both HDAC and cellular proliferation assays. 5,6-Fused ring systems attached either by the five- or the six-membered

Table 2. HDAC and anti-proliferative assay results for compounds **6j–w**



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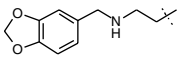
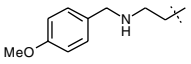
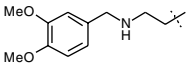
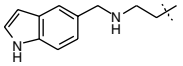
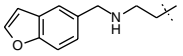
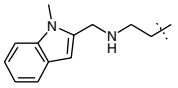
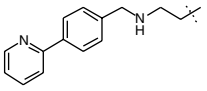
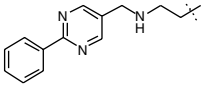
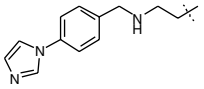
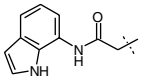
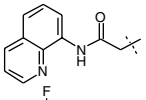
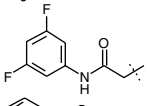
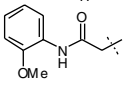
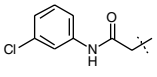
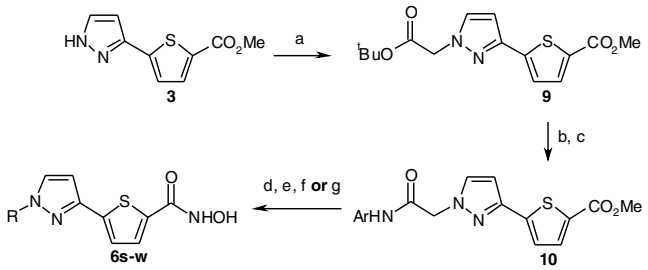
Compound	R	HDAC[a] (IC_{50}, μM)	Cell proliferation[a]	
			MCF-7 (IC_{50}, μM)	MDA-MB231 (IC_{50},μM)
6j		0.029	0.30	0.63
6k		0.013	0.20	0.55
6l		0.107	1.58	4.50
6m		0.005	0.08	0.28
6n		0.011	0.14	0.30
6o		0.006	0.17	0.37
6p		0.005	0.06	0.12
6q		0.048	0.80	1.51
6r		0.009	0.19	0.56
6s		0.012	0.37	2.00
6t		0.007	0.06	0.20
6u		0.008	0.14	0.70
6v		0.008	0.12	0.23
6w		0.006	0.10	0.30

[a] Values are means of two experiments.

ring system, compounds **6m–o**, provided very potent compounds in the HDAC assay. Two heterobiaryl ring systems, compounds **6p** and **6r**, possessing a terminal heteroaromatic ring, had excellent activity. However, the heterobiaryl ring system containing a terminal phenyl ring system, compound **6q**, displayed a much reduced level of activity.

For the acetamide-tethered analogues, fused heteroaryl ring systems once again provided significant activity in the HDAC assay, compounds **6s** and **6t**. Compounds **6u–w** illustrate that, as for the ethylamino-tethered series, substitution of the aromatic ring provides significant potency in both the HDAC enzyme and the cellular proliferation assays.

Further profiling of the substituted 5-(1*H*-pyrazol-3-yl)-thiophene-2-hydroxamic acid series of compounds was undertaken on one of the early phenyl substituted compounds, **6j**.



Scheme 2. Reagents and conditions: (a) $^{t}BuO_2CCH_2Br$ (1.1 equiv), K_2CO_3, DMF, 80 °C, 16 h (90%); (b) CF_3CO_2H, Et_3SiH, CH_2Cl_2 (75%); (c) $^{i}Pr_2Net$ (2.5 equiv), DMF, $ArNH_2$ (1.1 equiv), HATU (1.1 equiv) (17–74%); (d) 1 M NaOH, reflux, 2 h (66%); (e) $^{i}Pr_2Net$ (2.5 equiv), DMF, H_2NOTHP (1.1 equiv), HATU (1.1 equiv) (55%); (f) *p*-TSA, MeOH (44%); (g) $NH_2OH{\cdot}HCl$ (5 equiv), KOH, MeOH, 16 h (39–62%).

Table 3. HDAC and anti-proliferative assay results comparing compound **6j** and SAHA

Compound	HDAC[a] (IC_{50}, μM)	Cell proliferation[a] (IC_{50}, μM)			
		MCF-7	MDA-MB231	HCT116	PC3
SAHA	0.125	1.50	12.3	2.52	3.11
6j	0.029	0.30	0.63	0.58	0.40

[a] Values are means of two experiments.

Comparison of compound **6j** with SAHA demonstrated that **6j** was 4-fold more potent than SAHA in the primary HDAC enzyme assay, and had a 5- to 20-fold improvement in anti-proliferative activity across a panel of four cell lines (Table 3).

Compounds **6j** and SAHA were subjected to rat (iv and po, Table 4), and mouse (iv, po and ip, Table 5) PK experiments. Compound **6j** demonstrated reduced clearance and increased half-life compared to SAHA in both species. Poor oral bioavailability was observed for both compounds in both species. However, excellent bioavailability and good plasma exposure were observed for compound **6j** in the mouse via an ip administration route.

Having achieved good plasma exposure for compound **6j** following ip dosing, we decided to undertake a xenograft efficacy study using HCT116 tumour cells in CD1 mice. Compound **6j** was dosed to female CD1 nude (nu/nu) athymic mice at doses of 5, 15 and 50 mg/kg, ip ($n = 12$ per group) for 21 days. The control vehicle was 10% DMSO in arachis oil. This study resulted in a statistically significant 33% ($T/C = 0.67$) tumour growth reduction when compound **6j** was dosed at 50 mg/kg by the ip route. The tumours were removed 6 h after the last dose of compound was administered. Significant levels of histone acetylation were measured in tumours treated with compound **6j** compared to vehicle, indicating that HDAC inhibition had occurred (data not shown).

In summary, following the identification of ADS100380, we have optimised a series of phenyl-tethered HDAC inhibitors that display significant potency in in vitro enzyme and cell proliferation assays. Functionalisation of the phenyl group of tethered 5-(1*H*-pyrazol-3-yl)-thiophene-2-hydroxamic acids **6g** and **6f** from this series has led to compounds with improved potency, the best of which possessed single digit nanomolar potency in the HDAC enzyme assay, and sub-60 nanomolar activity in the MCF-7 cell proliferation assay. One of the first phenyl-substituted compounds produced, **6j**, demonstrated an improved in vivo PK profile compared to SAHA, and was also shown to be efficacious in a mouse HCT116 xenograft study.

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Table 4. Rat PK profile for compound **6j** and SAHA after iv (1 mg/kg) and po (5 mg/kg) dosing

Compound	$t_{1/2}$ (h)	V_{dss} (L/kg)	CL (mL/min/kg)	$AUC_{0-\infty h}$ (μg h/mL)	*F* (%)
	iv	iv	iv	po	po
SAHA	0.7	1.6	67	0.4	16
6j	1.6	0.9	21	0.1	2

Table 5. Mouse PK profile for compound **6j** and SAHA after iv (1 mg/kg), po (5 mg/kg) and ip (5 mg/kg) dosing

Compound	$t_{1/2}$ (h)	V_{dss} (L/kg)	CL (mL/min/kg)	$AUC_{0-\infty h}$ (μg h/mL)		*F* (%)	
	iv	iv	iv	po	ip	po	ip
SAHA	0.8	2.1	78	0.2	0.6	14	61
6j	2.5	0.4	10	0.9	7.2	10	85

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.10.048.

References and notes

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11. The HDAC inhibitory activity of compounds was assessed using the commercially available HDAC Fluorescent Activity Assay Kit (Biomol, #AK-500) following the manufacturer's instructions, but with minor modifications: the reaction was carried out at 25 °C for 30 min in the presence of 116 μM substrate. Compounds were serially diluted in DMSO and the final concentration of the solvent in the assay was 1%.
12. The anti-proliferative activity of the compounds was tested on a panel of human cancer cell lines. Cells were seeded in 96-well plates at a density of 3000 cell/well and incubated with serially diluted compounds for 72 h. The final DMSO concentration in the assay was 0.1%. The final number of cells per well was assessed using the CyQuant DNA dye (Invitrogen, #C7026) following the manufacturer instructions.
13. Further experimental details can be found in: Archer, J. A.; Bordogna, W.; Bull, R. J.; Clark, D. E.; Dyke, H. J.; Gill, M. I. A.; Harris, N. V.; Van den Heuvel, M.; Price, S. WO2004013130.
14. Regioselective alkylation was determined by ^{1}H NMR NOE experiments; see supplementary data for examples **4a** and **4b**.