Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 3456–3461
Bioorganic & Medicinal Chemistry Letters

2-Trifluoroacetylthiophenes, a novel series of potent and selective class II histone deacetylase inhibitors

Philip Jones,* Matthew J. Bottomley, Andrea Carfí, Ottavia Cecchetti, Federica Ferrigno, Paola Lo Surdo, Jesus M. Ontoria, Michael Rowley, Rita Scarpelli, Carsten Schultz-Fademrecht and Christian Steinkühler

IRBM/Merck Research Laboratories, Via Pontina km 30,600, 00040 Pomezia, Italy

Received 18 November 2007; revised 7 February 2008; accepted 9 February 2008
Available online 14 February 2008

Abstract—The identification of class II HDAC inhibitors has been hampered by lack of efficient enzyme assays, in the preceding paper two assays have been developed to improve the efficiency of these enzymes: mutating an active site histidine to tyrosine, or by the use of a trifluoroacetamide lysine substrate, allowing screening to identify class II HDAC inhibitors. Herein, 2-trifluoroacetylthiophenes have been demonstrated to inhibit class II HDACs, resulting in the development of a series of 5-(trifluoroacetyl)thiophene-2-carboxamides as novel, potent and selective class II HDAC inhibitors. X-ray crystal structures of the HDAC 4 catalytic domain with a bound inhibitor demonstrate these compounds are active site inhibitors and bind in their hydrated form.

The role of two counteracting enzyme families, the histone acetyl transferases (HATs) and the histone deacetylases (HDACs) in controlling the post-transcriptional acetylation status of lysine residues on histone tails, and a number of other proteins, is well-documented.[1,2] This equilibrium is a crucial determinant of chromatin structure, and hence gene transcription. The HDAC enzyme family has been implicated in a diverse array of processes, including: neoplasias, skeletal and muscle formation, cardiac hypertrophy, T-cell differentiation and neuronal survival.[2,3] However, the role of each of the individual HDAC isoforms or indeed classes in each of these biological processes is still being established.[3,4] Consequently, there is an urgent need for selective small molecule HDAC inhibitors (HDACi), both as research tools and ultimately as therapeutic agents.

The HDAC enzyme family can be divided into two classes: class I (HDACs 1, 2, 3 + 8), and class II, which can be further subdivided into class IIa (HDACs 4, 5, 7 + 9) and class IIb (HDACs 6 + 10). The class II HDACs differ from their class I counterparts in the fact that in addition to their catalytic domain, they contain a long N-terminal regulatory domain,[3–5] which has been implicated in gene regulation through protein–protein interactions.[3,5,6] The sub-division of the class II HDACs is the result of the presence of a second known or putative catalytic domain within class IIb HDACs. Aside from HDAC6 which has been demonstrated to function as a tubulin deacetylase,[7] there has been an extensive debate in the literature as to whether other class II HDACs, in particular class IIa enzymes, possess any intrinsic deacetylase activity.[5,6] In the preceding paper we have shown that pure class IIa HDACs do possess weak but measurable deacetylase activity,[8,9] and this activity can be enhanced and measured either by an H-Y mutation in the active site to give a 'gain of function' (GOF) enzyme, or by use of the wild-type (WT) enzyme with an 'unnatural' trifluoroacetamide lysine substrate. With this toolbox a program was initiated to identify selective class II HDAC inhibitors, targeting in particular HDAC4.

A focused screen of both in-house and commercial derivatives containing known zinc binding groups was initially carried out on both HDAC4 GOF[10] and 4WT.[11] Specific interest was given to trifluoromethyl ketone derivatives given that trifluoroacetamide lysine was a substrate for HDAC4WT, and that alkyl trifluoromethyl ketones had already been shown to inhibit both HDAC4 assays in the tens of nanomolar range.[9] These

Keywords: Histone deacetylase; HDAC; Trifluoroacetyl ketones; Hydrated ketones.
* Corresponding author. Tel.: +39 0691093559; fax: +39 0691093654; e-mail: philip_jones@merck.com

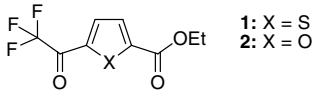
0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.02.026

findings suggested that there may be a larger binding pocket beneath the zinc in the active site of these class II enzymes that could be exploited. This focused screen identified ethyl 5-(trifluoroacetyl)thiophene-2-carboxylate (**1**)[12] to be a potent inhibitor of class II HDACs showing IC_{50} = 350 and 320 nM on HDAC4 GOF and WT assays, respectively (Fig. 1). This compound also inhibited HDAC6 with IC_{50} = 550 nM, whilst showed modest selectivity over the class I isoforms with IC_{50} = 5.7 and 3.5 μM against HDACs 1 and 3. Analysis of the ^{1}H NMR data of this compound showed that the trifluoromethyl ketone was readily hydrated with >85% in the hydrated form, suggesting that this compound may be bound in the active site as the hydrate rather than as the ketone. Interestingly, the corresponding furan derivative **2** was 10-fold less potent on HDAC4, revealing the importance of the thiophene moiety.

Given the potency and selectivity of **1**, and the presence of the ester group ready for functionalisation, a number of amide derivatives were prepared looking to improve both HDAC4 activity and selectivity (Fig. 2). Based on published X-ray crystal structure data,[13] it was assumed that these compounds would be active site inhibitors and that the thiophene moiety would sit in the narrow binding cleft, whilst the amides would be orientated to the surface on the enzyme where they would be able to engage in binding interactions with residues there.

Initial SAR on the amide moiety showed that a wide array of derivatives were tolerated, maintaining their activity on class II HDACs. Simple alkyl groups such as the ethyl amide **3a** lost activity, although this could be regained by increasing lipophilicity, such as cyclohexyl derivative **3g**. Both benzylic and homo-benzylic derivatives were tolerated, see **3f** and **3b**, as was alkylation of the amide, for instance see *N*-methyl analogue **3c**. These tertiary amides could be sterically constrained without losing activity, as seen with dihydroindolyl derivative **3h** which shows 130-300 nM activity on the class II HDACs and around 10-fold selectivity over class I isoforms. Substitution of the benzylic position was also tolerated in **3d** and **3e**, but significant differences were seen between the two enantiomers, with the *R*-isomer being a potent and unselective HDAC inhibitor, whereas the *S*-enantiomer maintained hundred nanomolar activity on class II HDACs with more than an order of magnitude selectivity. Heterocycles, such as the quinoxaline **3i**, were also accommodated, although with reduced selectivity for the class II HDACs.

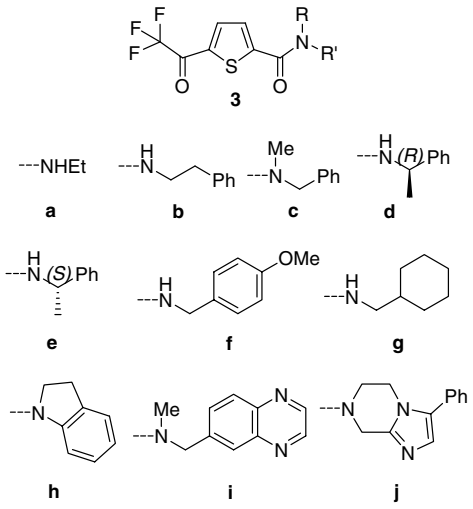
Unfortunately, the natural substrate of HDAC4 is unknown and accordingly there is no cellular target engagement marker for HDAC4. Given that these compounds also inhibit HDAC6, and knowing that α-tubulin is deacetylated by HDAC6,[7] it was possible to use inhibition of tubulin deacetylation as a surrogate marker of activity. Unfortunately, most of these derivatives lacked cell based activity and failed to show inhibition of either histone or tubulin deacetylation in HCT116 cells. However, **3i** showed inhibition of tubulin deacety-



Compds	HDAC IC_{50} (nM) 1	3	4GOF	4WT	6
1	5700	3500	350	320	550
2	48%inh at 10 μM	NA at 10 μM	3100	2500	550

[a]Values are means of >2 expts, std. dev. were < 30% of the IC_{50} values.

Figure 1. Activity of lead **1** on HDAC isoforms.



-NRR'	HDAC IC_{50} (nM) 1	3	4GOF	4WT	6
3a	7100	34%inh at 10 μM	1000	1700	310
3b	880	1700	180	330	210
3c	1800	1200	170	510	230
3d	320	230	160	76	360
3e	5300	9400	280	170	210
3f	210	120	70	180	240
3g	2900	3100	350	400	460
3h	2000	1700	300	130	200
3i	580	670	87	98	89
3j	5500	44%inh at 5 μM	370	320	310

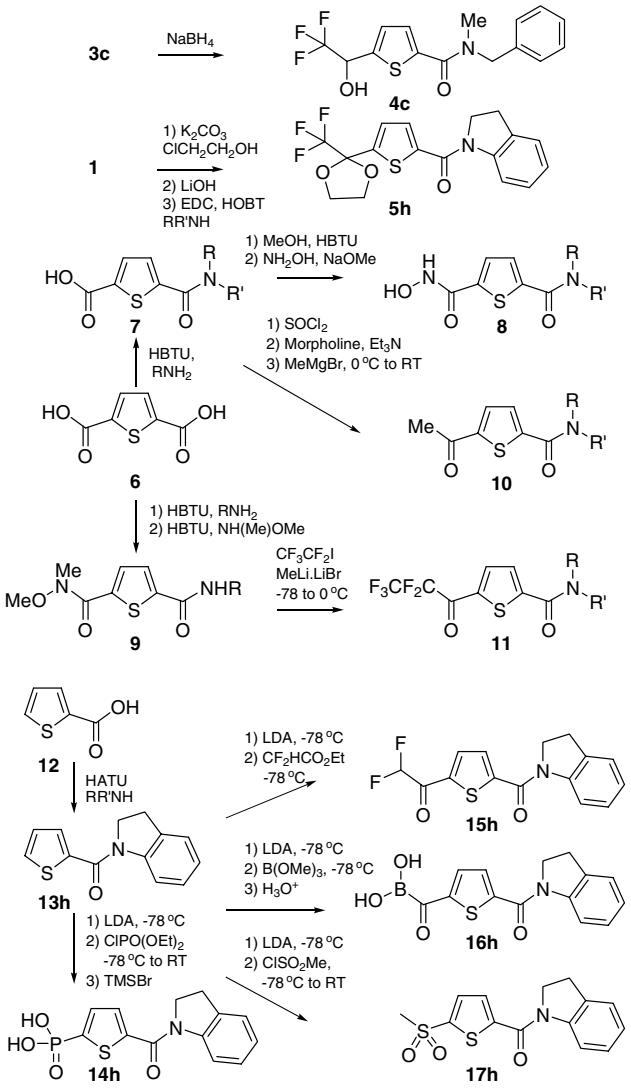
[a]Values are means of >2 expts, std. dev. were < 30% of the IC_{50} values.

Figure 2. Activity of 5-(trifluoroacetyl)thiophene-2-carboxamides **3** on HDAC isoforms.

lation with IC_{50} = 12 μM, whereas only 40% inhibition of histone H3 deacetylation was seen at 50 μM, thereby demonstrating that these compounds are selective class II HDACis in cells.

Having shown that potent class II HDACis could be developed, a more in depth understanding of the determinants of activity was undertaken, focusing on three regions of the inhibitors: the trifluoromethyl ketone zinc binding group, the central thiophene ring and the amide moiety.

The zinc binding group was explored as outlined in Scheme 1 and Table 1. Reduction of the trifluoromethyl ketone to alcohol **4c** with $NaBH_4$ resulted in a complete loss of activity. As did protection of the ketone as ketal **5h**, the formation of which required cyclization with chloroethanol under basic conditions to form the dioxolane. In contrast, the corresponding hydroxamic acid **8d**, readily prepared from thiophene-2,5-dicarboxylic acid (**6**) proved to be a potent but unselective HDACi, showing nanomolar activity against both class I and II HDACs.



Scheme 1. SAR exploration of the zinc-binding group.

Replacement with a weaker zinc binding group, carboxylic acid **7i** abolished activity on all HDAC isoforms. In a similar manner, no inhibition of any of the HDAC isoforms was seen with the methyl ketone **10d**. To explore the importance of the trifluoromethyl group, the corresponding difluoromethyl **15h** and pentafluoroethyl **11f** derivatives were synthesized. The former by lithiation of the thiophene **13h** with LDA, followed by quenching with ethyl difluoroacetate, whereas the latter was obtained by addition of the lithium pentafluoroethyl onto the Weinreb amide **9f**. Both compounds showed substantial losses of activity on all HDAC isoforms. In particular, difluoroketone **15h** while potently inhibiting HDAC4GOF lost more than 10-fold activity on HDAC4WT and therefore was not pursued further. Finally, the boronic acid **16h**, methyl sulfone **17h**, and phosphonic acid **14h** derivatives were all inactive.

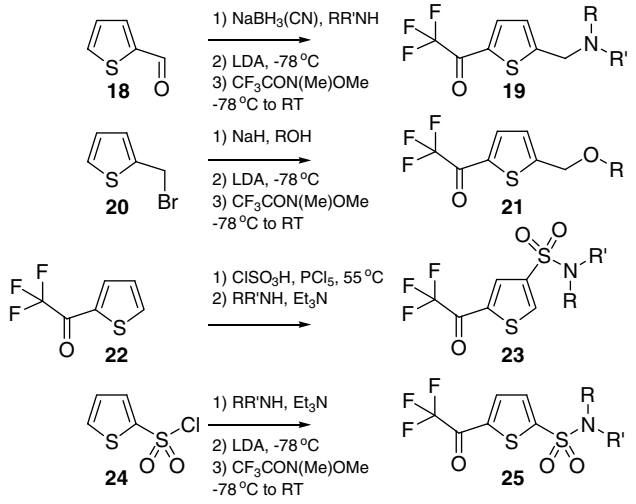
To enable SAR studies to look for alternatives to the 5-carboxamide group, chemistry had to be established to introduce the trifluoroacetyl group onto the thiophene as the ultimate synthetic step. Therefore simple functional group transformations were conducted on the 5-position of the thiophene and then the 2-position was deprotonated with LDA at −78 °C and the lithio derivative quenched with 2,2,2-trifluoro-*N*-methoxy-*N*-methylacetamide (Scheme 2). In this manner amines **19c** + **d**, ether **21f** and sulfonamides **25c** + **g** were investigated. The 4-position of the thiophene was also explored by sulfonylation with chlorosulfonic acid and PCl_5, followed by preparation of the sulfonamide **23c**. These alternative capping groups were all tolerated to some extent on the class II HDACs (Table 2), although a loss in HDAC4 activity was seen with all derivatives compared to the 5-carboxamides. For instance the ether **21f** resulted in a more than 30-fold loss in activity on HDAC4, although maintained HDAC6 activity. While whereas tertiary amine **19c** lost 2- to 6-fold activity on HDAC4, the secondary amines **19d** lost more than 20-fold activity. The isomeric sulfonamides **23c** and **25c** demonstrated the importance of the substitution pattern on the thiophene, as whilst the 5-substituted isomer **25c** remained a hundred nanomolar HDAC4 inhibitor, the region isomer **23c** lost almost all deacetylase activity.

Having established that the trifluoroacetyl group could be introduced as the ultimate synthetic step, this methodology was applied in the investigation of thiophene replacement (Scheme 3). In this manner, the 3-trifluoroacetylthiophene derivative **27c** was prepared from the 3-bromothiophene-5-carboxylic acid (**26**) by Br–Li exchange and quenching as previously. Similar chemistry, but using Mg–Br exchange, was used for the pyridine analogue **37**. The corresponding thiazoles **29c** + **g** could be prepared by direct lithiation of the thiazole with *n*-BuLi and quenching. Preparation of the corresponding pyrrole derivative **31h** necessitated sequential Br–Li exchanges on *N*-Boc 2,5-dibromopyrrole (**30**), firstly to allow introduction of the carboxylate group, and secondly the trifluoroacetyl group. In contrast, the analogous phenyl derivatives **34g** and **35g** were more straightforward, being readily prepared readily from carboxylic acids **32** and **33**.

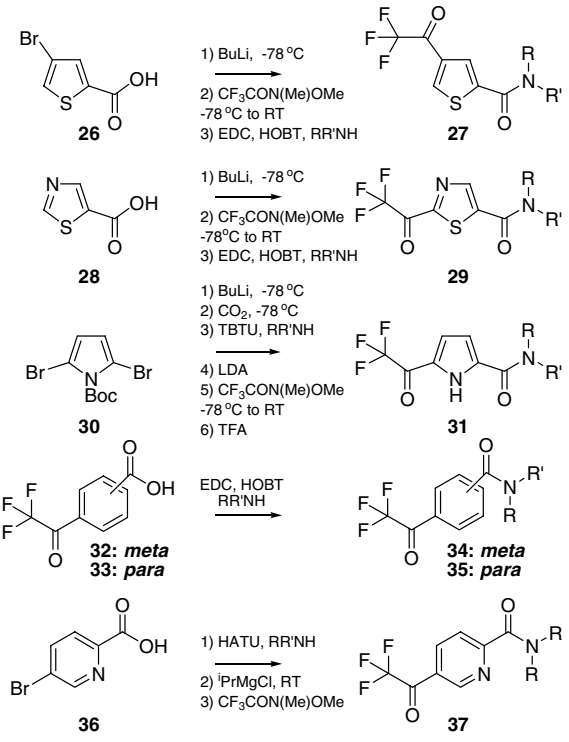
Table 1. Activity of 2-substituted thiophene-5-carboxamides with alternative zinc-binding groups

–NRR′	HDAC IC_{50} (nM)[a]				
	1	3	4GOF	4WT	6
4c	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM
5h	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM
7i	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM
8d	6	10	100	2700	94% inh. at 9 nM
10d	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM	6900
11f	4200	2900	NA at 10 μM	NA at 10 μM	NA at 10 μM
14h	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM
15h	9700	42% inh. at 10 μM	420	7600	15
16h	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM	42% inh. at 10 μM
17h	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM	47% inh. at 10 μM

[a] Values are means of >2 experiments, SD were <30% of the IC_{50} values.



Scheme 2. SAR exploration of capping group.



Scheme 3. SAR exploration of capping group.

These thiophene replacements were evaluated on the panel of HDAC isoforms as previously and data are shown in Table 3. The isomeric 3-(trifluoroacetyl)thiophene **27c** although maintaining some activity on the class II HDACs loses around 3-fold in activity on HDAC4WT and 15-fold on the GOF. This could be due to either an incorrect positioning on the substituent on the thiophene core or a weaker propensity to hydrate. Similarly, the thiazoles analogues **29c** + **g**, although displaying sub-micromolar activity on HDAC4WT lose around 10-fold potency on the H-Y mutated enzyme compared to the corresponding thiophene. In contrast, the pyrrole derivative **31h** maintains only activity on HDAC6 with an IC_{50} = 880 nM. More radical substitutions with replacement of the thiophene group either by phenyl or pyridyl groups are detrimental, as only very weak activity, if any, is seen on any HDAC isoform with **34g**, **35g** and **37**.

Table 2. Activity of 5-substituted 2-(trifluoroacetyl)thiophene derivatives

–NRR′	HDAC IC_{50} (nM)[a]				
	1	3	4GOF	4WT	6
19c	3100	4600	1100	970	490
19d	NA at 10 μM	NA at 10 μM	3500	3700	840
21f	NA at 10 μM	NA at 10 μM	2700	5500	410
23c	NA at 10 μM	NA at 10 μM	NA at 10 μM	9400	2500
25c	NA at 10 μM	NA at 10 μM	1200	340	730

[a] Values are means of >2 experiments, SD were <30% of the IC_{50} values.

Table 3. Activity of (trifluoroacetyl)heteroaryl carboxamides

–NRR′	HDAC IC_{50} (nM)[a]				
	1	3	4GOF	4WT	6
27c	NA at 10 μM	NA at 10 μM	2500	1700	640
27g	40% inh. at 10 μM	NA at 10 μM	2400	3300	2000
29c	46% inh. at 10 μM	7300	2200	760	1300
29g	NA at 10 μM	7600	2900	850	1500
31h	NA at 10 μM	NA at 10 μM	NA at 10 μM	47% inh. at 10 μM	880
34g	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM	4200
35g	NA at 10 μM	NA at 10 μM	31% inh. at 10 μM	32% inh. at 10 μM	2500
37	NA at 10 μM	NA at 10 μM	NA at 10 μM	NA at 10 μM	4200

[a] Values are means of >2 experiments, SD were <30% of the IC_{50} values.

Having demonstrated the importance of all three structural elements: the trifluoroacetyl group, the 2,5-disubstituted thiophene, and the carboxamide moiety, it was crucial to obtain structural information of one of these derivatives bound to a class II deacetylase in order to further improve the activity and selectivity of these HDACs. Although several X-ray crystal structures exist of several class I HDACs there were no information available on their class II counterparts. X-ray crystal structures were determined for both HDAC 4WT and 4GOF catalytic domains (Thr648–Thr1057), expressed and purified from *Escherichia coli*, with **3j** which was a modest inhibitor of HDAC4 (GOF/WT IC_{50}s = 370/320 nM).[14] Both structures, Figures 3 and 4, respectively, clearly show that these trifluoroacetyl thiophene derivative are active site binders with the hydrated trifluoromethyl ketone chelating the active site zinc in a bidentate manner. These oxygen-zinc bonds are in the range 2.04–2.4 Å. The requirement of the trifluoromethyl group is also explained by the crystal structures, as this group occupies a modest pocket at the bottom of the active site which is conserved in class II HDACs. The trifluoromethyl group fills this pocket entirely, comming within van der Waals contact distance of a proline-800 at the bottom of the cavity. The modest size of this pocket thereby explains the loss in activity of the more sterically demanding pentafluoroethyl group **11f**.

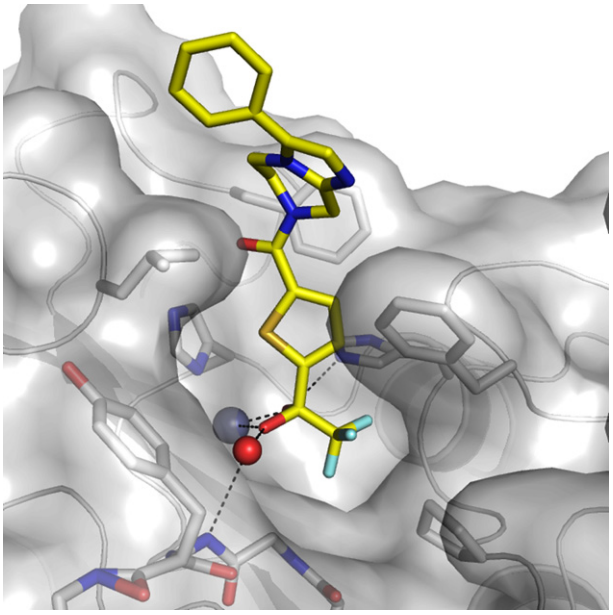


Figure 4. X-ray crystal structure of **3j** bound to HDAC4 H976Y 'Gain of Function' catalytic domain.[14]

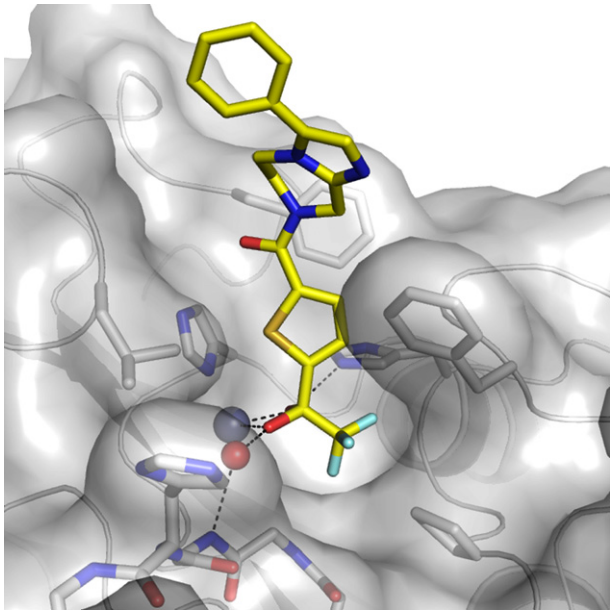


Figure 3. X-ray crystal structure of **3j** bound to HDAC4 WT catalytic domain.[14]

Interestingly, in both structures the H976 and the Y976 groups, the analogous group of which in class I enzymes make H bonds to the substrate carbonyl, are orientated away from the inhibitor. This may account for the low activity of HDAC4 to canonical acetylated lysines. However, in the HDAC4 structures an active site water molecule bridges between the inhibitor and active site Gly-975 and this water could be responsible for stabilization of the transition state during the deacetylation reaction.

In summary, a novel series of 5-(trifluoroacetyl)thiophene-2-carboxamides has been developed as potent and selective class II HDAC inhibitors. The compounds show around 10-fold selectivity for HDACs 4 + 6 over their class I counterparts, HDACs 1 + 3. In cells, selective inhibition of tubulin deacetylation is observed, demonstrating their selectivity of HDAC6 over the class I

isoforms. X-ray crystal structures of both HDAC 4GOF and 4WT catalytic domain have been obtained with a bound inhibitor, demonstrating that these compounds are active site inhibitors and bind in their hydrated form.

References and notes

1. Cheung, W. L.; Briggs, S. D.; Allis, C. D. *Curr. Opin. Cell Biol.* **2000**, *12*, 326.
2. Minucci, S.; Pelicci, P. G. *Nat. Rev. Cancer* **2006**, *6*, 38.
3. Martin, M.; Kettmann, R.; Dequiedt, F. *Oncogene* **2007**, *26*, 5450.
4. De Ruijter, A. J. M.; van Gennip, A. H.; Caron, H. N.; Kemp, S.; van Kuilenburg, A. B. P. *Biochem. J.* **2003**, *370*, 737.
5. Verdin, E.; Dequiedt, F.; Kasler, H. G. *Trends Genet.* **2003**, *19*, 286.
6. Yang, X. J.; Gregoire, S. *Mol. Cell. Biol.* **2005**, *25*, 2873.
7. Hubbert, C.; Guardiola, A.; Shao, R.; Kawaguchi, Y.; Ito, A.; Nixon, A.; Yoshida, M.; Wang, X. F.; Yao, T. P. *Nature* **2002**, *417*, 455.
8. Lahm, A.; Paolini, C.; Pallaoro, M.; Nardi, M. C.; Jones, P.; Neddermann, P.; Sambucini, S.; Bottomley, M. J.; Lo Surdo, P.; Carfí, A.; Koch, U.; De Francesco, R.; Steinkühler, C.; Gallinari, P. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 17335.
9. Jones, P.; Altamura, S.; De Francesco, R.; Gallinari, P.; Lahm, A.; Neddermann, P.; Rowley, M.; Serafini, S.; Steinkühler, C. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1814.
10. DMSO/compound solution were incubated for 10 min with His-tagged HDAC4GOF (653–1084, H976Y) from *E. coli* in assay buffer (20 mM Hepes, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.1 mg/ml BSA), *Fluor-de-Lys* substrate solution was added and left for 1 h at 37 °C and the reaction stopped by adding developer/TSA solution. Measure the fluorescence at ex. 360 nM/em. 460 nM.
11. DMSO/compound solution were incubated for 10 min with His-tagged HDAC4 CD(653–1084) from *E. coli* in assay buffer (25 mM Tris/HCl pH8, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.1 mg/ml BSA), trifluoacetamide substrate solution was added and left for 1 h at 37 °C and the reaction stopped by adding developer/TSA solution. Measure the fluorescence at ex. 360 nM/em. 460 nM.
12. Compound **1** was purchased from Rieke Metals, Lincoln, USA.
13. Finnin, M. S.; Donigian, J. R.; Cohen, A.; Richon, V. M.; Rifkind, R. A.; Marks, P. A.; Breslow, R.; Pavletich, N. P. *Nature* **1999**, *401*, 188.
14. Bottomley, M.J.; Lo Surdo, P.; Di Giovine, P.; Cirillo, A.; Scarpelli, R.; Ferrigno, F.; Jones, P.; Neddermann, P.; De Francesco, R.; Steinkühler, C.; Gallinari, P.; Carfí, A. *Structure*, submitted for publication.