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Studies of the metabolic stability in cells of 5-(trifluoroacetyl)thiophene-2-carboxamides and identification of more stable class II histone deacetylase (HDAC) inhibitors

Rita Scarpelli *, Annalise Di Marco, Federica Ferrigno, Ralph Laufer, Isabella Marcucci, Ester Muraglia, Jesus M. Ontoria, Michael Rowley, Sergio Serafini, Christian Steinkühler, Philip Jones

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

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ABSTRACT

5-(Trifluoroacetyl)thiophene-2-carboxamides were found to be potent and selective class II HDAC inhibitors. This paper describes their further development and the investigation on the cause for the lack of cell-based activity. A rapid screening assay was set up which enabled the identification of more metabolic stable compounds as potent and selective class II HDAC inhibitors.

Histone deacetylases (HDACs) are a class of Zn^{2+} metalloenzymes involved in the deacetylation of histones, transcription factors, and other proteins in eukaryotic cells.[1] HDAC inhibition causes hyperacetylation of histones which leads to a more open chromatin structure, thereby facilitating gene transcription and ultimately leading to cell-growth arrest, differentiation, and apoptosis. The development of specific HDAC inhibitors (HDACi) represents a new strategy in cancer therapy.[2]

In human, Zn^{2+} dependent HDACs are divided in two major subclasses (class I and class II), based on sequence homology to the yeast HDACs. Class I HDACs include HDAC 1, 2, 3, and 8 which are related to yeast RPD3 deacetylase. Class II HDACs are related to yeast Hda1 and include HDAC 4, 5, 6, 7, 9, and 10. All these HDACs possess a highly conserved zinc-dependent catalytic domain.[3]

Both hydroxamic acid-based HDACi, for example, vorinostat (SAHA)[4] and PDX101[5] and non-hydroxamic acid-based HDACi, for example, the 2-aminophenylamide MS-275[6] and the dithiol FK228[7] are currently in the clinic. Most of them hit a subset of both class I and class II or, as in the case of the 2-aminophenylamides, show selectivity versus class I HDACs. All of them elicit similar adverse effects, mainly fatigue, nausea, vomiting, and diarrhea that become dose-limiting in clinical trials.

In an effort to develop a second generation HDACi with enhanced efficacy and larger therapeutic window, we were interested in targeting a more restricted set of HDAC isoforms. In particular, RNAi knock-down experiments were performed against all 11 class I and II HDACs in three different cancer cell lines (HeLa, HCT116, and A549)[8] where it was observed that knock-down of HDAC 4 was sufficient to inhibit cell proliferation. Consequently a program was initiated to develop HDAC 4 or selective class II HDAC inhibitors which would be expected to show growth inhibition in the same cancer cell lines.

As previously reported, a series of 5-(trifluoroacetyl)thiophene-2-carboxamides was identified as a novel class of potent and selective class II HDAC inhibitors.[9] These compounds typically display around 10-fold selectivity for class II HDACs (HDAC 4+6) over their class I HDACs (HDAC 1+3) counterparts (Table 1).[10,11]

Unfortunately, most of these derivatives lacked cell-based activity as they showed no antiproliferative effect against HeLa and A549 cell lines, and failed to show inhibition of either histone H3 or α-tubulin deacetylation in HCT116 cells, the cellular substrates of HDAC 1 and 6, respectively.[12]

In the light of these results, it was necessary to establish the cause for the lack of cellular activity. Several possibilities were considered: (a) the lack of cell penetration and/or efflux of these compounds from cells[13]; (b) high binding to plasma protein of the cell incubation medium; (c) metabolic degradation of compounds during incubation with cells; or (d) lack of activity on HDAC 1+3, assuming that these compounds can enter into cells and are stable,

* Corresponding author. Tel.: +39 0691093248; fax: +39 0691093654.
E-mail address: rita_scarpelli@merck.com (R. Scarpelli).

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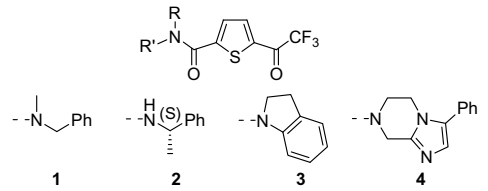
Table 1
Activity of 5-(trifluoroacetyl)thiophene-2-carboxamides on the various HDAC isoforms.



–NRR′	HDAC IC_{50} (nM)[a]			
	1	**3**	**4WT**[14]	**6**
1	890	560	510	230
2	5000	9400	170	150
3	1300	1400	150	130
4	4700	>5000	300	310

[a] IC_{50} is the concentration of drug required for 50% inhibition in vitro; values are means of >2 experiments with standard deviations <30%.

and that class II HDACs are not involved in the control of cell proliferation.

Consequently, a focused research was initiated to understand the causes for the lack of antiproliferative effects and remedy any untoward observations.

As a part of any lead optimization program routine counter screening for cytochrome P (CYP)450 inhibition is performed on all compounds synthesized. Five 5-(trifluoroacetyl)thiophene-2-carboxamides were identified that were relatively potent class II HDACi, but displayed little or no inhibition of α-tubulin deacetylation in cells (Table 2). These five compounds showed modest CYP2C9 inhibition in human liver microsomes (HLM) at 10 μM. Given this observation, the level of CYP2C9 inhibition in HLM was compared to that seen in human hepatocytes as any shifts in the IC_{50} between the sub-cellular microsome fractions compared to the whole cell system would suggest these class II HDACi suffer from poor cell permeability.

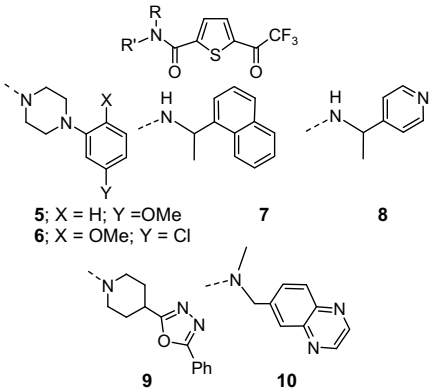
All five analogs were shown to be low micromolar inhibitors of CYP2C9 in microsomes, as determined by the release of tritium as tritiated water that occurs upon CYP2C9-mediated 4′-hydroxylation of diclofenac labeled with tritium in the 4′ position.[15]

When those compounds were tested in human hepatocytes similar IC_{50} were observed, for example, 5 μM in HLM and 8 μM in hepatocytes for compound **5** and 1.8 μM in both HLM and hepatocytes for compound **6**. The one compound that showed a modest shift was compound **7** with IC_{50} of 1.4 μM in HLM and 11 μM in hepatocytes. In order to exclude that the lack of cellular activity of these compounds was due to high plasma protein binding, the inhibition of CYP2C9 activity in hepatocytes was also determined in the presence of 10% fetal bovine serum (FBS), a component of the cell culture medium used for proliferation assays. The addition of serum to the cells caused no significant effect on IC_{50} (Table 2). Together these results suggested that the lack of activity in cell-based assays is not due to a cell permeability issue and/or high plasma protein binding, with the *caveat* that these data were generated in hepatocytes, whereas deacetylation and proliferation assays were carried out in HeLa, A549, and HCT116 cell lines.

We next focused our attention on the stability of these HDACi in cell culture. It has been previously documented that electrophilic ketones, such as trifluoromethyl ketones, α-keto heterocycles and α-keto esters, can be rapidly reduced by carbonyl reductases.[16] This phenomenon seems to be especially pronounced in straight-chain alkyl-linked compounds, both peptidic and non-peptidic.[17]

Representative 5-(trifluoroacetyl)thiophene-2-carboxamides were incubated with HCT116 cells at 1 and 5 μM in cell culture medium containing 10% FBS for 24 h and aliquots were taken at different times and analyzed by LCMS/MS; control incubation was also performed in cell culture medium without cells. All tested compounds were stable in culture medium, but in the presence of HCT116 cells, rapid degradation of compounds was observed, typically with $t_{1/2} < 2$ h. Degradation of tested compounds occurred in

Table 2
Effect on the CYP2C9 activity in human liver microsomes (HLM) versus human hepatocytes of 5-(trifluoroacetyl)thiophene-2-carboxamides.



–NRR′	HDAC IC_{50} [a] (nM)		Tub-Ac EC_{50}[b] (μM)	CYP2C9 in HLM IC_{50}[c] (μM)	CYP2C9 in hepatocytes IC_{50}[c] (μM)	
	4WT	**6**			No serum	10% FBS
5	480	170	50% inh at 50 μM	5(±0.9)	8(±1)	8(±4)
6	530	175	NA up to 10 μM	1.8(±0.4)	1.8(±0.3)	ND
7	170	360	50% inh at 25 μM	1.4(±0.2)	11(±3)	17(±4)
8	90	260	10μM	13(±4)	10(±2)	3(±1)
9	70	160	NA up to 25 μM	5(±0.3)	8(±0.8)	5(±0.5)

[a] Values are means of >2 experiments with standard deviation <30%.
[b] Results were calculated assuming the signal obtained treating cells with 10 μM of SAHA as the 100% activation signal. The activation of α-tubulin is measured after 24 h treatment; NA, not active.
[c] MTS assay showed no toxicity of these derivatives which were stable in the cells; standard deviation is given in parentheses; ND, not determinate.

a timeframe which was much shorter than the duration of proliferation and deacetylation assays, 72 h and 24 h, respectively. The corresponding inactive alcohol was identified as a major metabolite after incubation with HCT116 cells. Conversion seemed to be quantitative as shown for compound **10**, as a representative example, which was incubated with two different cell lines HCT116 and HeLa cells. The amount of inactive alcohol after 6 h of incubation with HCT116 cells was about equal to the initial amount of parent compound (Fig. 1).[18]

Similar results were obtained when compound **10** was incubated with HeLa cells in cell culture medium containing 10% FBS. In addition, no significant amount of the product of amide bond hydrolysis was detected. These results suggest that the lack of cellular activity of these compounds is due to metabolic instability.

Compound **9**, which was the most potent and selective class II HDAC inhibitor, unfortunately was very unstable in HCT116 cells with a $t_{1/2} < 2$ h (Table 3). This is in agreement with the lack of inhibition of either histone H3 or α-tubulin deacetylation in cell culture. On the other hand, compounds **8** and **10** which were the most stable compounds with $t_{1/2} = 5$ h and 3.5 h, respectively, resulted to be efficient inhibitors of α-tubulin acetylation with $IC_{50} = 10$ μM and 12 μM. Inhibition of histone H3 deacetylation was seen only at higher concentration.

In order to increase throughput of the HCT116 stability assay it was shown that the S9 sub-cellular fraction of HCT116 cells was suitable as enzyme source for a rapid screening of these trifluoromethyl ketones. The assay was validated using compound **10**, as representative compound, and other compounds and carried out in presence of NADPH which is the preferred cofactor for reduction of these trifluoromethyl ketones (Fig. 2).[19] Interestingly, we ob-

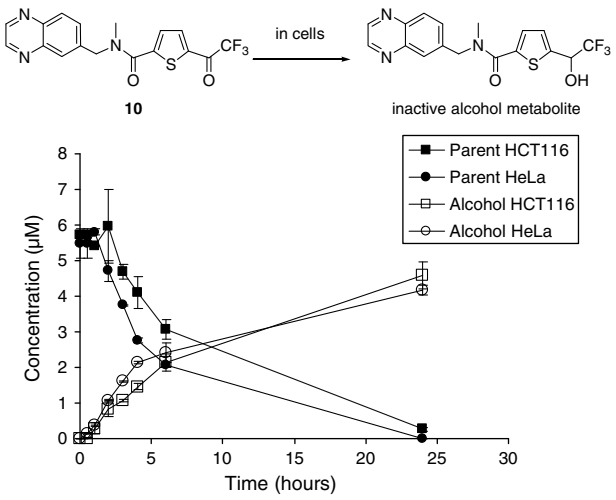


Figure 1. Stability of compound **10** and formation of the corresponding inactive alcohol in HCT116 and HeLa cells.

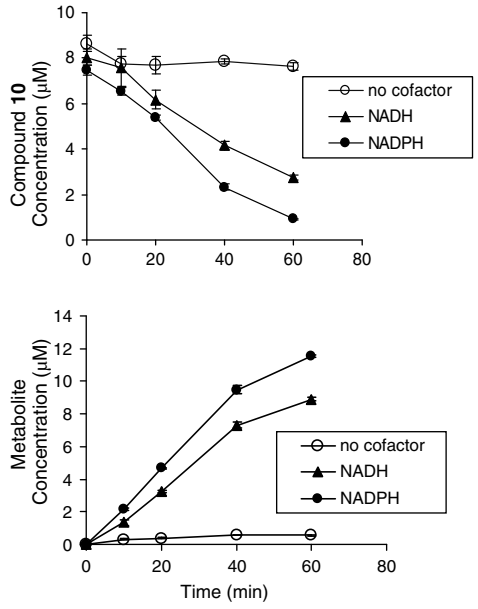


Figure 2. Conversion of compound **10** into alcohol metabolite in S9 fraction of HCT116 cells in the presence of NADH or NADPH.

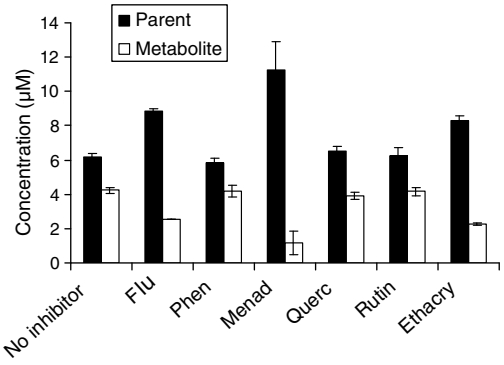
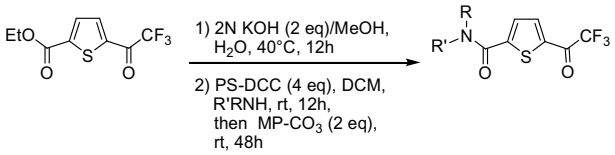


Figure 3. Effect of carbonyl reductase inhibitors on the reduction of compound **10** (10 μM) in S9 fraction of HCT116 cells.[20]



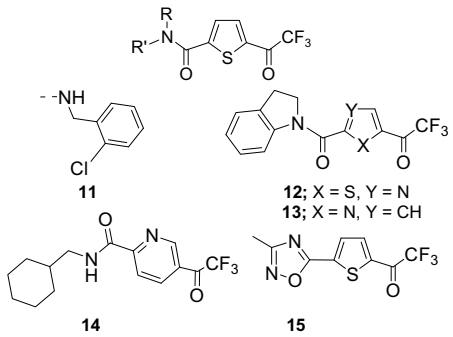
Scheme 1. Synthesis of 5-(trifluoroacetyl)thiophene-2-carboxamides.

Table 3
Stability of 5-(trifluoroacetyl)thiophene-2-carboxamides in HCT116 cells.

–NRR′	HDAC IC_{50}[a] (nM)				HCT116 Stability $t_{1/2}$ (h)	Tub-Ac EC_{50} (μM)	H3 EC_{50} (μM)
	1	**3**	**4WT**	**6**			
6	4400	>10 μM	530	175	2	NA up to 10 μM	NA up to 10 μM
7	830	840	170	360	3	50% inh at 25 μM	NA up to 25 μM
8	1600	1500	90	260	5	10 μM	>35 μM
9	1300	2800	70	160	2	NA up to 25 μM	NA up to 25 μM
10	580	670	100	90	3.5	12	40%inh at 50 μM

[a] Values are means of >2 experiments with standard deviations <30%; NA, not active.

Table 4
Metabolic stability in S9 fraction of HCT116 cells.



–NRR′	HDAC IC_{50}[a] (nM)			S9 Stability $t_{1/2}$ (min)	Tub-Ac EC_{50} (μM)
	1	**4WT**	**6**		
1	890	510	230	15	ND
10	580	100	90	20	12
11	550	100	190	15	NA up to 10 μM
12	1600	13	410	20	NA up to 10 μM
13	NA up to 10 μM	NA up to 10 μM	580	15	ND
14	NA up to 10 μM	NA up to 10 μM	3320	>120	ND
15	7600	75	650	>120	6.5

[a] Values are means of >2 experiments with standard deviations <30%; NA, not active; ND, not determinate.

served that in general the rank order with the selected compounds was the same with a good correlation between the two assays.

Given that selective class II selective inhibitors had been developed, a rapid way to assess the importance of class II HDACs for cell proliferation could be to stabilize those compounds using a reductase inhibitor. Studies were carried out on the effect of drugs that inhibit various classes of carbonyl reductase on the reduction of compound **10** in HCT116 S9 fractions. In particular, flufenamic acid and phenolphthalein were used as aldo-ketone reductase (ADK) inhibitors, while quercetin, menadione, rutin, and ethacrynic acid were used as short-chain dehydrogenase/reductase (SDR) inhibitors.[20] The choice of carbonyl reductase inhibitors was somewhat limited to avoid drugs known to have antiproliferative effects.

However, partial inhibition of reduction of compound **10** was observed with only flufenamic acid at 10 μM or menadione and ethacrynic acid at high concentration 500 μM (Fig. 3).[20] Unfortunately, the partial effects of these drugs was insufficient to test the effects of our HDACi in long term cellular assays and we needed a way to further stabilize this series of compounds.

In the light of these results, being unable to stabilize the trifluoromethyl ketones with carbonyl reductase inhibitors, we needed to further explore this series and design new compounds that could show improved stability in order to demonstrate if inhibitors of class II HDACs have any antiproliferative effects.

An extensive SAR exploration was performed on the carboxamide moiety of our inhibitors using solid phase supported coupling reagents, which allowed for the parallel synthesis of a large number of amides in a short period of time, avoiding the need of HPLC purification.

The optimized procedure used PS-carbodiimide and excess of carboxylic acid followed by treatment with MP-carbonate as scavenger resin enabled the preparation of compounds with more than 90% purity by LC/MS analysis (Scheme 1).

Unfortunately, simple modification in the amide substituent failed to improve metabolic stability of our inhibitors. The most selective compounds were unstable in S9 fraction of HCT116 cells with $t_{1/2} < 20$ min.

Attempts to modulate this rapid reduction via heterocycle variation such as thiazoles and pyrroles offered modest but not sufficient improvements in stability (Table 4, compounds **12** and **13**). In general, those compounds that showed an improved stability suffered from poor class II HDAC activity. We concluded that we were unable to identify suitable compounds in the amide series.

A more substantial structural change was made by the modification of the amide bond to an oxadiazole ring which led to the identification of compound **15** which, although a weaker HDAC 4 and 6 inhibitor, showed improved metabolic stability.

In summary, we have identified the cause for the lack of cellular activity of the 5-(trifluoroacetyl)thiophene-2-carboxamides as being rapid metabolism in cells by carbonyl reductases. A rapid screening assay was set up in S9 sub-cellular fraction of HCT116 cells in order to identify rapidly more stable compounds. Compound **15** was discovered as a valid lead. Further development of this finding will be the topic of the accompanying paper.[21]

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13. The antiproliferative activity of selected compounds in HCT116 cells was not affected by inhibitors of MDR1 or MRP (data not shown), suggesting that lack of cellular activity is not due to active efflux mechanisms.
14. Compounds were incubated for 10 min with His-tagged HDAC4 CD (653-1084) from *Escherichia coli* in assay buffer (25 mM Tris/HCl, pH 8, 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. The fluorescence was measured at ex.360 nM/em.460 nM.
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18. In the absence of cell cultural medium the amount of alcohol was less than 0.1 μM (limit of quantification).
19. HCT116 cells were homogenized on ice at 4 °C in 20 mM Tris–HCl, pH 7.5, 150 mM KCl, 250 mM sucrose, 1 mM EDTA, and 1 mM DTT. Cell homogenate was centrifuged at 10,000*g*, for 30 min at 4 °C and the supernatant (S9 fraction) was frozen in liquid nitrogen and used for stability experiments. Similar results were obtained in fresh S9 fraction (data not shown). The reaction was carried out using S9 fraction (1 mg/ml) in the presence of 1 mM NADPH and a NADPH regenerating system. The experiment was run in 96-well conical plates, in a final assay volume of 200 μl, at 37 °C, under low shaking. The reaction was stopped by addition of 20 μl 1N HCl, followed by centrifugation for 10 min at 1690*g*, and aliquots of the supernatant were diluted with 1 volume of acetonitrile containing an analytical internal standard and analyzed by LCMS/MS.
20. Compound **10** (5 μM) was incubated for 30 min with 1 mg/ml of S9 fraction of HCT116 cells, in the presence or absence of the indicated reductase inhibitors: 10 μM flufenamic acid (FLU), 50 μM phenolphthalein (Phen) (AKR family), 500 μM menadione (Menad), quercetin (Querc), rutin and ethacrynic acid (Ethacry) (SDR family).
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