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Potent and selective HDAC6 inhibitory activity of *N*-(4-hydroxycarbamoylbenzyl)-1,2,4,9-tetrahydro-3-thia-9-azafluorenes as novel sulfur analogues of Tubastatin A†

Rob De Vreese,[a] Tom Verhaeghe,[b] Tom Desmet[b] and Matthias D'hooghe*[a]

Eight *N*-(4-hydroxycarbamoylbenzyl)-1,2,4,9-tetrahydro-3-thia-9-azafluorenes were efficiently prepared as sulfur analogues of Tubastatin A and thus evaluated as new HDAC6 inhibitors. All compounds exhibited potency against HDAC6, and four of them were active in the nanomolar range (IC_{50} = 1.9–22 nM). Further analysis revealed that the sulfone derivatives (designated as Tubathians) are superior to their non-oxidized sulfide analogues, and the two most active sulfones showed good to excellent HDAC6 selectivity compared to all other HDAC isoform classes.

The enzymatic addition and removal of acetyl groups at specific lysine residues comprise important biochemical reactions with a significant impact on many cellular processes.[1] The addition of acetyl groups within histone proteins, the chief protein components of chromatin, is catalyzed by histone acetyltransferases (HAT), and histone deacetylases (HDAC) mediate the corresponding deacetylation reactions. The inhibition of the latter group of deacetylases has become a hot topic in medicinal chemistry, and the use of HDAC inhibitors (HDACIs) has found many applications with regard to cancer and CNS disorder therapies.[2] In general, HDACIs act on 11 zinc-dependent HDAC isozymes, which are divided into four groups: class I (HDACs 1, 2, 3, 8), class IIa (HDACs 4, 5, 7, 9), class IIb (HDACs 6, 10), and class IV (HDAC11).[3] The majority of known HDACIs primarily inhibit the class I enzymes, making them excellent candidates for cancer therapy applications, but other than class I HDACIs are normally required for the pursuit of non-oncological applications.[4] Another important issue relates to the potential toxicity of compounds inhibiting multiple isozymes, as acetylation is involved in the control of many cellular processes and inhibition of some isozymes may cause undesirable side effects. Thus, the design and development of isozyme-selective inhibitors has emerged as an important challenge within the search for novel HDACIs.[5]

In recent years, HDAC6 has been acknowledged as an attractive target for drug development,[6] and an increasing number of research teams are currently involved in the quest for new compounds endowed with HDAC6 inhibitory activity.[7] In addition to the potential of HDAC6-selective inhibitors for applications in the treatment of CNS disorders and neurodegenerative diseases, these compounds seem to provoke fewer side effects, hence the growing interest in their preparation.[8] An important milestone in that respect concerns the identification of Tubacin as a selective HDAC6 inhibitor, although the application of this compound is hampered by its poor druglikeness and cumbrous synthesis.[9] Since then, considerable advances have been made with regard to the preparation of new HDAC6 inhibitors, leading to an array of different molecular entities with improved chemical and pharmacological properties. From a chemical viewpoint, many of these molecules comprise the typical HDACI basic structure accommodating an aromatic cap group (surface recognition domain), a linker and a zinc-binding hydroxamic acid unit. A major breakthrough was accomplished recently, involving the rational design and synthesis of Tubastatin A as a novel and selective HDAC6 inhibitor.[10] Elaborate studies in this direction showed that the HDAC6 isozyme tolerates modifications of the Tubastatin A chemical structure at the level of the cap group and, more specifically, that the introduction of structural diversity at the 2- and 8-position of the tetrahydropyrido[4,3-*b*]indole scaffold can be beneficial with regard to the overall bioactivity.[11]

Inspired by these recent SAR findings, and intrigued by the fact that several new HDAC6 inhibitors contain a sulfur atom in their molecular structure,[7c–e] efforts were made toward the preparation of a number of sulfur analogues (sulfides and sulfones) of Tubastatin A in the present study, supported by HDAC6 ligand docking. Furthermore, the replacement of a methylene group with a sulfone moiety in medicinally relevant compounds has been shown to induce a significant beneficial increase in stability,[12] suggesting this modification as a preferred change during compound optimization and thus providing an additional rationale for the work

[a] *SynBioC Research Group, Department of Sustainable Organic Chemistry and Technology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium. E-mail: matthias.dhooghe@UGent.be; Fax: +32-9-2646221; Tel: +32-9-2649394*

[b] *Centre for Industrial Biotechnology and Biocatalysis, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium*

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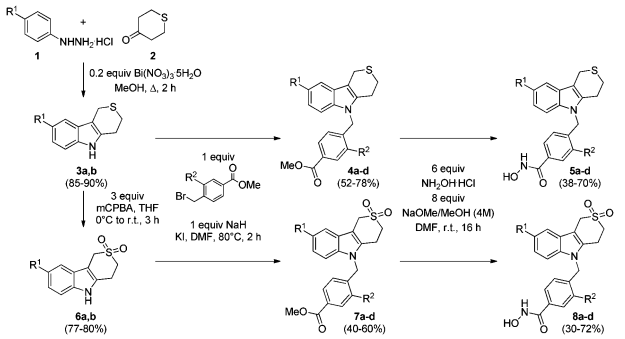

Scheme 1 Synthesis of *N*-(4-hydroxycarbamoylbenzyl)-1,2,4,9-tetrahydro-3-thia-9-azafluorenes **5** and their oxidized analogues **8** (R^1 = H, F; R^2 = H, MeO).

undertaken in this study. The results obtained point to the potential of sulfur analogues of Tubastatin A as new HDAC6 inhibitors, especially those containing a sulfone moiety in their structure.

The 1,2,4,9-tetrahydro-3-thia-9-azafluorene scaffold was prepared *via* a bismuth nitrate-promoted Fisher indole synthesis employing a phenylhydrazine hydrochloride **1** and tetrahydrothiopyran-4-one **2**, providing a convenient access to tetrahydro-3-thia-9-azafluorenes **3** in good yields (Scheme 1).[13] Subsequently, *N*-benzylation of compounds **3** was accomplished using a methyl 4-(bromomethyl)-benzoate in DMF in the presence of sodium hydride and potassium iodide, furnishing the corresponding *N*-(4-methoxycarbonylbenzyl)-1,2,4,9-tetrahydro-3-thia-9-azafluorenes **4**. The final step of the process comprised an ester to hydroxamic acid interconversion, which was realized utilizing an excess of hydroxylamine hydrochloride in the presence of methanolic sodium methoxide in DMF. In this way, the premised *N*-(4-hydroxycarbamoylbenzyl)-1,2,4,9-tetrahydro-3-thia-9-azafluorenes **5** were obtained in an efficient and straightforward approach.

Considering the presence of a (cyclic) sulfone moiety in several drugs and bioactive compounds,[12] the sulfide in systems **3** was oxidized to the corresponding sulfones **6** by means of *meta*-chloroperbenzoic acid treatment in tetrahydrofuran. The thus obtained sulfones **6** were taken further in the synthesis toward the contemplated hydroxamic acids **8** *via* esters **7** applying a similar strategy to that discussed above for the preparation of hydroxamic acids **5** (for structural details and reported yields of compounds **3–8**, see ESI†).

The binding of various ligands in the enzyme's active site was evaluated by means of automated docking. Since the crystal structure of HDAC6 is not available, a homology model was first generated following the example of Kozikowski[10] using the structure of HDAC isozymes as a template. Compounds that do not carry a methoxy group on their linker (**5a**, **5c**, **8a** and **8c**) were found to fit perfectly in the active site of HDAC6 (Fig. 1). In this case, the linker is positioned in the tubular access channel, with the carbonyl group of the hydroxamate moiety within chelating distance from the zinc ion at the bottom of the pocket. As the linker fills the access channel almost completely, very little space is left to accommodate a (bulky) substituent such as a methoxy group, which is in line with previous studies in that respect. In contrast, modifications of the tricyclic cap group do not seem to influence the binding mode very much, since the conformation and orientation of compounds **5a**, **5c**, **8a** and **8c** are nearly identical. However, oxidation of the sulfur atom results in additional interactions with the enzyme in the form of hydrogen bonds between the introduced oxygen atoms and the backbone nitrogen of residues Asp567 and Gly619 (Fig. 1b). The latter observation provided an interesting motive and an additional reason to experimentally assess the HDAC6 inhibitory activity of Tubastatin A analogues in which the NMe moiety is replaced by a sulfone unit.

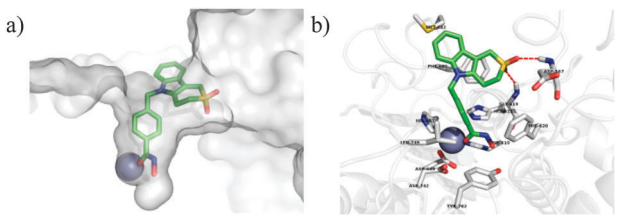

Fig. 1 Docking of compound **8a** in the active site of HDAC6. (a) View of the tubular access channel, and (b) additional interactions generated by the oxidation of the sulfur atom (green: carbon; blue: nitrogen; red: oxygen; yellow: sulfur; magenta: zinc ion).

In vitro pharmacology studies of novel hydroxamic acids **5a–d** and **8a–d** with regard to their HDAC1 and HDAC6 inhibitory activity revealed an interesting potency of these compounds as HDAC6 inhibitors (see ESI†). In particular, hydroxamic acids **5a**, **5c**, **8a** and **8c** showed complete inhibition at a test concentration of 10 μM, and also compounds **8b** and **8d** exhibited a good profile with an inhibition of 73% and 75%, respectively. In addition, these results pointed to a selectivity of the test compounds toward HDAC6 inhibition, with HDAC1 inhibition percentages ranging from 0% to a maximum of 53%. Furthermore, these data also indicate a detrimental effect of the introduction of a methoxy group in the linker moiety on the bioactivity (compounds **5b,d** and **8b,d**), as indicated by homology modeling. HDAC1 and HDAC6 were chosen for activity comparison in this preliminary test, as these two enzymes have a diverse phylogeny and are members of separate deacetylase classes.

The most promising molecules (those showing an inhibition of >70%) were then selected for determination of their IC_{50} values with respect to HDAC6 inhibition (Table 1). These assessments confirmed the presumption that molecules bearing a methoxy-substituted linker exhibit lower – but still moderate – activities, exemplified by compounds **8b** and **8d** (with IC_{50} values of 2.0 and 1.3 μM, respectively). Furthermore, sulfur oxidation indeed seems to be beneficial for bioactivity, as sulfones **8a** and **8c** show even more potent HDAC6 inhibition as compared to sulfides **5a** and **5c**. Overall, four compounds (**5a**, **5c**, **8a** and **8c**) can be considered to be promising lead templates for further elaborate studies. Sulfides **5a** and **5c** (with IC_{50} values of 15 and 22 nM, respectively) display HDAC6 inhibitory activities similar to the reference compound Trichostatin A and to Tubastatin A,[14]

Table 1 IC_{50} values for HDAC6 inhibition[a]

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
5a	0.015	**8b**	2.0
5c	0.022	**8c**	0.0037
8a	0.0019	**8d**	1.3

[a] Reference compound: Trichostatin A (IC_{50} = 0.012 μM).

Table 2 Comparison of HDAC selectivity

Compoud	HDAC1 IC_{50}[a] (μM)	HDAC4 IC_{50}[a] (μM)	HDAC6 IC_{50} (μM)	HDAC11 IC_{50}[b] (μM)	HDAC8 IC_{50}[a] (μM)
8a	11	1.6	0.0019	NC	1.7
8c	12	1.9	0.0037	NC	0.93

[a] Reference compound: Trichostatin A. [b] Reference compound: Scriptaid; NC = Not Calculable (concentration–response curve shows less than 25% effect at the highest validated testing concentration). **8a**: $R^1 = R^2 = H$; **8c**: $R^1 = F$, $R^2 = H$.

but sulfones **8a** and **8c** are even more potent than sulfides **5a** and **5c** with IC_{50} values of 1.9 and 3.7 nM, respectively.

Finally, the HDAC inhibition selectivity of the two most active compounds **8a** ($R^1 = R^2 = H$) and **8c** ($R^1 = F$, $R^2 = H$) against the other HDAC isoform classes was assessed and, to this end, a class I (HDAC1), a class IIa (HDAC4), a class IIb (HDAC6) and a class IV (HDAC11) isozyme was selected. Considering the fact that Tubastatin A has over 1000-fold selectivity against all HDAC isozymes except for HDAC8, where it has only a 57-fold selectivity, the HDAC8 inhibitory activity of compounds **8a** and **8c** was also evaluated.

The data in Table 2 point to a good to excellent HDAC6 selectivity of hydroxamic acids **8a** and **8c**, with the HDAC6 *versus* HDAC11 and HDAC1 selectivity being the most pronounced. The HDAC11 inhibitory effect of **8a,c** appeared to be very low and no IC_{50} values could be obtained. Furthermore, a 5789-fold and a 3243-fold selectivity against HDAC1 was determined for compounds **8a** and **8c**, respectively, which substantially exceeds the selectivity of Tubastatin A (1093-fold selectivity).[10] In addition, also a high HDAC6 *versus* HDAC4 selectivity was observed for sulfones **8a** and **8c** (842- and 513-fold, respectively). Finally, it is interesting to note that these compounds show a good HDAC6 *versus* HDAC8 selectivity, and both sulfone **8a** (895-fold) and sulfone **8c** (251-fold) exhibited a considerably higher selectivity in that respect as compared to Tubastatin A (57-fold).[10]

The experimental results listed in Tables 1 and 2 are in line with the structure–activity relationship insights provided by ligand docking. These data show that decoration of the *N*-(4-hydroxycarbamoylbenzyl)-1,2,4,9-tetrahydro-3-thia-9-azafluorene scaffold at the linker unit (*in casu* by a methoxy group) is unfavorable for HDAC6 inhibitory activity. On the other hand, introduction of a substituent (*in casu* a fluoro atom) at the cap group did not appear to have a significant effect on the activity profile. It should also be noted that replacement of the tertiary amine functionality (NMe moiety) in the tetrahydropyrido-[4,3-*b*]indole core structure of Tubastatin A by a sulfide unit results in compounds with a comparable HDAC6 inhibitory activity (at least as concerns the IC_{50} value), whereas replacement by a sulfone moiety (SO_2) affords even more potent HDAC6 inhibitors. The *in silico* observed occurrence of hydrogen bonds between the introduced oxygen atoms and the backbone nitrogen atom of residues Asp567 and Gly619 can account for the higher *in vitro* activity of these sulfone derivatives.

In addition to their promising biological potential and their straightforward and easy synthesis and purification, sulfones **8a** and **8c** (designated as Tubathian A and Tubathian B, respectively) also show an interesting profile for further evaluation based on their predicted druglikeness (M_W, *c*log*P*, solubility).

The findings described in this communication thus provide a platform for more elaborate studies with respect to the HDAC6 inhibitory activity of this new class of thiaheterocyclic compounds which, in combination with further optimization of drug-relevant molecular properties, might afford promising new lead structures.

In conclusion, *N*-(4-hydroxycarbamoylbenzyl)-1,2,4,9-tetrahydro-3-thia-9-azafluorenes were efficiently prepared and shown to be of interest as novel and selective HDAC6 inhibitors, culminating in the identification of two sulfone derivatives as interesting lead structures for further elaboration displaying potent and selective HDAC6 inhibition in the nanomolar range.

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14 Since an IC_{50} value is not a constant value and the experiments have not been performed in parallel, a comparison between the IC_{50} values for compounds **5** and **8** and the literature value for Tubastatin A should be made with care.