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New aryldithiolethione derivatives as potent histone deacetylase inhibitors

Valerio Tazzari [a], Graziella Cappelletti [b], Manolo Casagrande [c], Elena Perrino [a], Luigi Renzi [c], Piero Del Soldato [a], Anna Sparatore [a,c,*]

[a] Sulfidris s.r.l., Viale Gran Sasso 17, 20131 Milan, Italy
[b] Department of Biology, University of Milan, Via Celoria 26, 20133 Milan, Italy
[c] Department of Pharmaceutical Science 'Pietro Pratesi', Università degli Studi di Milano, Via Mangiagalli 25, 20133 Milan, Italy

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ABSTRACT

A series of dithiolethione derivatives was synthesized and the in vitro HDAC inhibitory activity was tested. The most active compounds, **1** and **2**, exhibited an IC_{50} in nM range with a strong hyperacetylation of histone H4 in A549 cells. The HDAC inhibitory activity comparable to that of SAHA and the inhibition of A549 cell proliferation suggest that these compounds are worthy of further studies as potential anticancer agents.

1. Introduction

Histone deacetylases (HDACs) are a family of enzymes which regulate chromatin remodelling and gene transcription. HDAC inhibitors represent a diverse group of compounds which interfere with the functions of HDAC enzymes and are emerging as a promising class of anticancer agents.[1,2] HDAC inhibitors have a potential role in the regulation of gene expression, inhibit the proliferation of tumor cells by induction of cell death, apoptosis etc. Several HDAC inhibitors have been studied in phase I, II, III clinical trials and two compounds, the hydroxamic acid SAHA (Vorinostat®, Merck)[3] and the natural depsipeptide romidepsin (FK228, Istodax®, Celgene)[4] have been approved for marketing by FDA in 2006 and 2009, respectively.[5]

One important aspect to be considered is related to cardiac toxicity (QTc prolongation) that is reported as a potential relevant side effect of HDAC inhibitors.[6]

We have recently found that 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (ADTOH) and its valproic acid ester (ACS 2) (Fig. 1) potently inhibited in vitro HDAC enzymatic activity as well as A549 cell proliferation with strong hyperacetylation of histone H4.[7–9]

We showed that ACS 2 significantly inhibited A549 or NCI-H1299 xenograft proliferation in nude mice and increased E-cadherin expression in NSCLC cells.[10]

Moreover, besides the chemopreventive effects by activation of the KEAP1/Nrf2/ARE pathway;[11] we found that dithiolethiones activated the tumor suppressor protein phosphatase 2A (PP2A) with antiproliferative effect in breast and lung cancer cell lines.[12]

In addition, there is evidence that dithiolethiones may exert cardioprotection increasing human and rat cardiomyocytes resistance to oxidative/electrophilic stress and doxorubicin toxicity[13,14] or protecting rat aortic smooth muscle A10 cells against peroxynitrite or acrolein-induced toxicity.[15,16] It has also been suggested by some of us that ADTOH and its hybrids may have cardioprotective activity due to the release of hydrogen sulfide (H_2S) from dithiolethione moiety.[17] Indeed, it has been shown that incubation of ADTOH or dithiolethione hybrids with rat plasma or liver homogenate caused a time-dependent release of H_2S. Incubation of the same compounds in phosphate buffer or boiled rat liver homogenate released a much lesser, although still detectable amount of H_2S, suggesting that the majority of the gas released from the parent molecule occurred as a result of a metabolic event. Increased plasma H_2S concentration was also observed when dithiolethione derivatives were administered ip or iv in rats.[18–20] It has been shown that H_2S may protect the heart mainly by activation of K_{ATP}

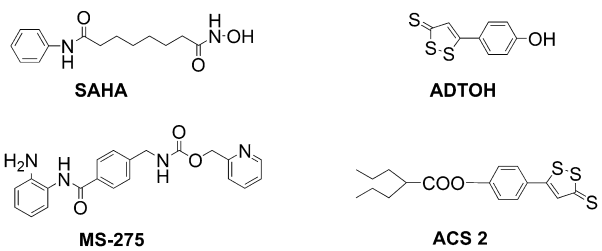

Figure 1. Chemical structures of some HDAC inhibitors.

* Corresponding author. Tel.: +39 02 50319365; fax: +39 02 50319359.
E-mail address: anna.sparatore@unimi.it (A. Sparatore).

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channels.[21] NaHS (a H_2S donor) in a myocardial ischemia-reperfusion model of isolated rat heart, provided a concentration-dependent reduction in myocardial infarct size.[22]

On this ground and with the aim to further explore the anticancer potentialities of dithiolethiones, we synthesized novel derivatives taking the chemical structures of the well known HDAC inhibitors SAHA and MS-275 (Fig. 1) as a model.[23–25]

As model inhibitors, the novel compounds conform to the pharmacophore formed by a zinc binding motif, a spacer with a polar cap group, which makes contacts with the rim of the tube-like enzymatic active site.

It is well known that dithiolethiones are able to coordinate several metal ions, including zinc.[26,27] Indeed the potent HDAC inhibitory activity of ADTOH may be related to the strong nucleophilic character acquired by the thione group as a consequence of the delocalization of the negative charge of phenoxide anion as shown in Figure 2.

In more or less stable O-substituted derivatives the nucleophilic character may be reduced with consequent decreased inhibitory activity, as observed in valproic acid ester ACS 2.[7] Nevertheless, the exchange of valproic residue with more suitable (more extended and functionalized) substituents on the phenolic oxygen may still give rise to compounds that will profitably occupy the tube-like active site of HDAC.

Figure 2. Resonance forms of deprotonated ADTOH.

Thus, a first subset of compounds is characterized by the replacement of either the anilide cap group or the hydroxamic zinc binding moiety of SAHA type inhibitors with a phenyldithiolethione moiety, joined to the polymethylene spacer by means of an ester or an ether linkage (**3**, **4** and **1**, **2**, **5**, **6**, respectively).

In compound **7** an acylated *o*-phenylenediamine moiety plays the role of zinc binding functionality, as it is seen in MS-275 (Fig. 1), CI 994 and other HDAC inhibitors.[28] Moreover, we investigated whether other kinds of dithiolethiones such as **8**, **9** and **10** (Fig. 3) share the HDAC inhibitory activity of ADTOH, even if not completely fulfilling the mentioned pharmacophore requirements.

On the basis that hydroxamic acids may bind to a wide range of metals, and in an attempt to prevent untimely binding to other metals outside the enzymatic active site, the hydroxamic acid function of SAHA was protected by O-acylation with the two acids **8** and **9** to give compounds **11** and **12**, respectively.

Finally, compound **13** is an hybrid between ADTOH and phenylbutyric acid, which has been shown to be active against refractory solid tumor[29] and whose weak HDAC inhibitory potency has been strongly enhanced by tethering it with 4-aminobenzohydroxamic acid.[30]

The structures of the investigated compounds are depicted in Figure 3; SAHA and ADTOH (Fig. 1) have been also tested as reference compounds.

2. Chemistry

The syntheses of the ether compounds **1**, **2**, **5**, **6** and **7** were carried out as indicated in Scheme 1, by coupling 5-[4-(3-thioxo-3*H*-1,2-

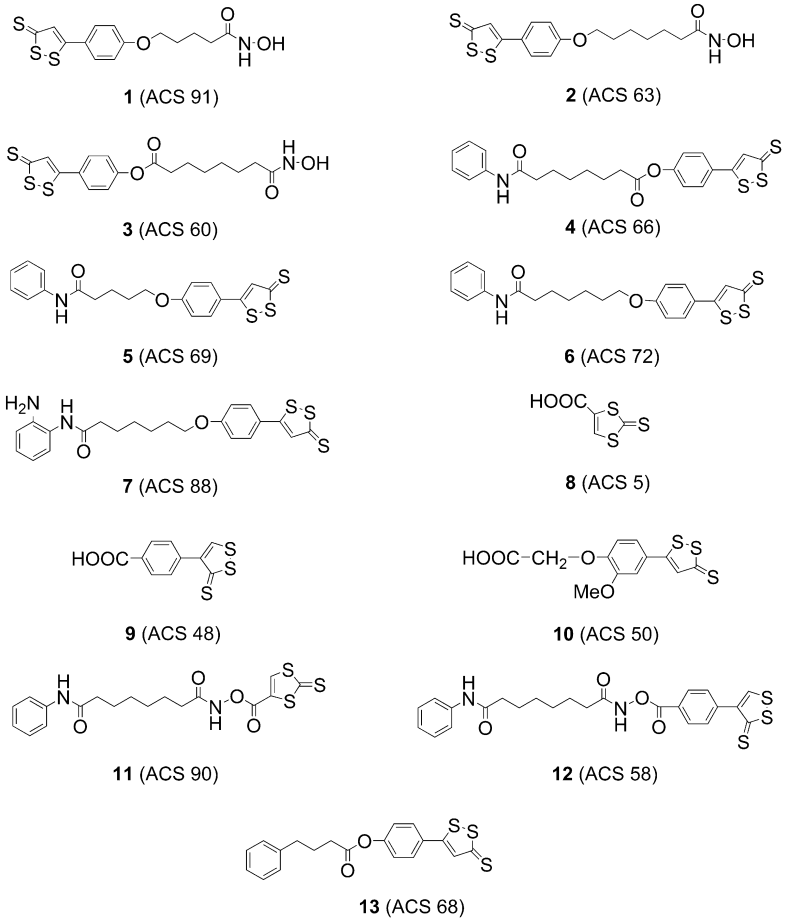
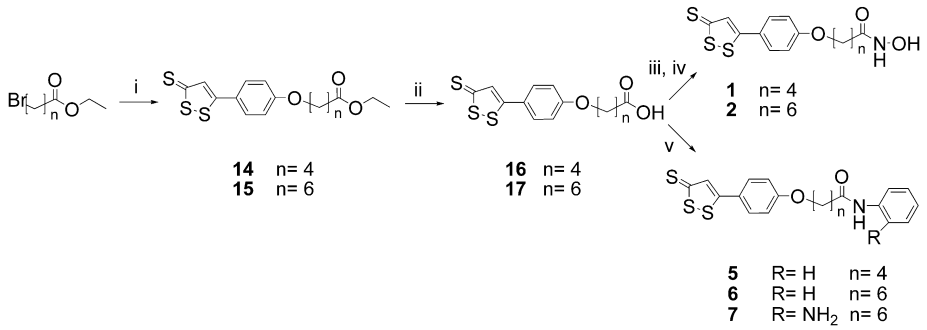
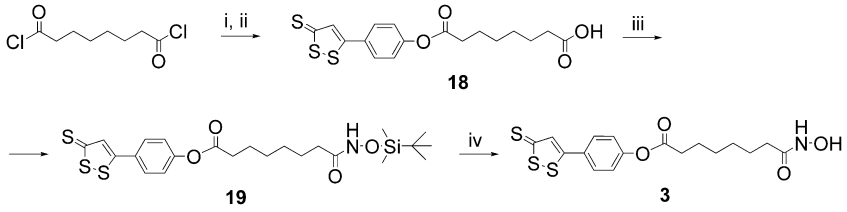


Figure 3. Chemical structures of compounds investigated as HDAC inhibitors.



Scheme 1. Reagents and conditions: (i) ADTOH, NaOH, EtOH, 80 °C; (ii) acetic acid, 50% H_2SO_4, 100 °C; (iii) $TBDMSONH_2$, EDAC, $CHCl_3$, rt; (iv) 1 N HCl; (v) aromatic amine, EDAC, suitable solvent, rt.



Scheme 2. Reagents and conditions: (i) ADTOH, pyridine, THF; (ii) H_2O; (iii) $TBDMSONH_2$, EDAC, DMAP, CH_2Cl_2, 3 h, N_2, rt; (iv) triethylsilane, TFA, CH_2Cl_2, 0 °C, 30 min.

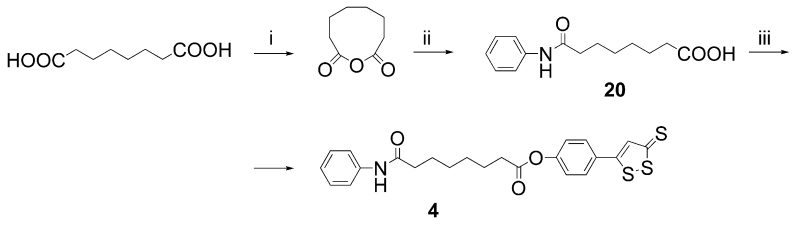
dithiol-5-yl)phenoxy]pentanoic acid (**16**) or 7-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]heptanoic acid (**17**) with the proper amine in presence of EDAC and DMAP (or HOBt and TEA for **7**). The starting acids were prepared through the hydrolysis of the corresponding ethyl esters (**14** and **15**) that were obtained by heating an ethanolic solution of NaOH and ADTOH with ethyl 5-bromopentanoate or ethyl 7-bromoheptanoate. To be noted that the yields of etherification were very low (14%), due to the low reactivity of the phenolate anion of ADTOH resonating with a thiolate form.[7] The substitution on the oxygen is proved by the identity of the UV–vis spectrum of **2** with that of anetholedithiolethione (ADT).[31]

The synthesis of compound **3** required first the preparation of 8-oxo-8-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]octanoic acid (**18**), obtained through the reaction of suberoyl chloride and ADTOH in anhydrous THF, and then its coupling with *O*-(*tert*-butyldimethylsilyl)hydroxylamine ($TBDMSONH_2$) in presence of EDAC and DMAP. In this case, deprotection of the silanated hydroxamic acid (**19**) required stirring the compound with trifluoroacetic acid and triethylsilane for 30 min at 0 °C (Scheme 2).

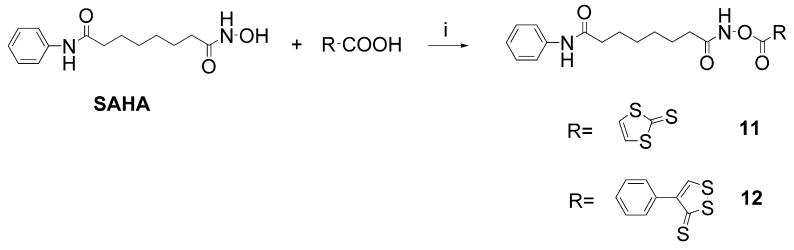
Compound **4** was obtained coupling ADTOH with the monoanilide of the suberic acid (**20**), which was synthesized as previously described[32] (Scheme 3).

Compounds **11** and **12** were synthesized coupling SAHA with 1,3-dithiole-2-thioxo-4-carboxylic acid (**8**) and 4-(3-thioxo-3*H*-1,2-dithiol-4-yl)benzoic acid (**9**), respectively, using EDAC and HOBt as coupling reagents (Scheme 4).

Compound **8** was synthesized as previously described by Dartigues et al.[33] and 4-(3-thioxo-3*H*-1,2-dithiol-4-yl)-benzoic acid (**9**) was obtained by acidic hydrolysis of the corresponding methyl ester prepared as described by Adelaere and Guemas.[34]



Scheme 3. Reagents and conditions: (i) acetic anhydride, reflux; (ii) aniline, THF, rt; (iii) ADTOH, EDAC, DMAP, $CHCl_3$, rt.



Scheme 4. Reagents and conditions: (i) EDAC, HOBt, DMF, rt.

[2-Methoxy-4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]acetic acid (**10**) was obtained by acidic hydrolysis of the corresponding methyl ester, which was synthesized as described by Lozac'h and Mollier.[35]

Finally compound **13** was synthesized with good yields by esterification of 4-phenylbutyric acid with ADTOH in presence of DCC and DMAP as coupling reagents (Scheme 5).

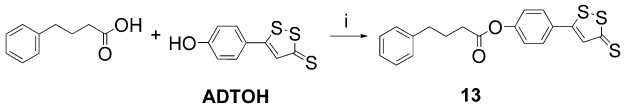
3. Results and discussion

All synthesized compounds exhibited variable degrees of HDAC inhibitory activity in vitro, and in some cases were comparable or even more potent than SAHA. Nine compounds showed inhibition of the proliferation of A549 cells with IC_{50} in the range from 3.4 to 66 μM and in most cases the antiproliferative activity was related to the HDAC inhibition, since significant histone H4 hyperacetylation was observed in the same cells (Table 1 and Fig. 4).

Results are reported in Table 1.

Compounds **2** and **1** exhibit HDAC inhibitory activity higher than SAHA, with IC_{50} = 0.01 μM and 0.08 μM versus 0.1 μM for the reference drug, but the antiproliferative activity was 7–8 times lower, suggesting that the determinants for the two activities are different. In these molecules it is assumed that the hydroxamic acid will prevail over the dithiolethione as zinc binding function. However, the more extended oxyphenyldithiolethione moiety may represent a better cap group than the anilide of SAHA, allowing a more efficient accommodation and binding of the molecule on the active site of HDAC with enhanced inhibitory potency. On the other hand, the lower antiproliferative activity in respect to SAHA, may be linked to a minor cellular uptake as well as to different compound stability in the cell environment.

Compound **3** differs from **2** only due to the presence of an additional carbonyl group in the spacer, but exhibits a strongly reduced



Scheme 5. Reagents and conditions: (i) DCC, DMAP, CH_2Cl_2, 3 h, rt.

Table 1
Effects of dithiolethione derivatives on inhibition of HDAC activity, inhibition of A549 cell proliferation and hyperacetylation of histone H4 in A549 cells[a]

Compound	HDAC IC_{50}[b] (μM)	A549 IC_{50}[b] (μM)	Hyperac. H4 (A549)
SAHA	0.1 ± 0.004	0.76 ± 0.005	++
ADTOH	0.45 ± 0.004	66 ± 4.2	+
1 (ACS 91)	0.08 ± 0.02	5.4 ± 0.1	++
2 (ACS 63)	0.01 ± 0.003	6.1 ± 0.04	++
3 (ACS 60)	10 ± 0.43	56 ± 1.5	+
4 (ACS 66)	100 ± 7.19	>1000	NT
5 (ACS 69)	50 ± 8.69	>1000	NT
6 (ACS 72)	918.7 ± 63.2	60.2 ± 3	+
7 (ACS 88)	32 ± 2.36	31.7 ± 6	+
8 (ACS 5)	46.4 ± 4.54	>1000	–
9 (ACS 48)	100 ± 1.13	102.9 ± 8.6	+
10 (ACS 50)	82.85 ± 18	879.8 ± 63	NT
11 (ACS 90)	0.25 ± 0.03	3.4 ± 0.8	++
12 (ACS 58)	1.19 ± 0.7	1.68 ± 0.23	++
13 (ACS 68)	179.4 ± 1.85	31.8 ± 0.007	+

NT, not tested.
+ and ++, a significant increase (++, at least twofold) in the level of hyperacetylated histone H4 was observed at concentrations equivalent to IC_{50} on cell proliferation.
–, no variation in the level of acetylated histone H4 were observed at concentrations equivalent to IC_{50} on cell proliferation.
[a] Values are means of at least three experiments.
[b] Values represent IC_{50} ± standard error as resulting from sigmoidal dose–response fitting.

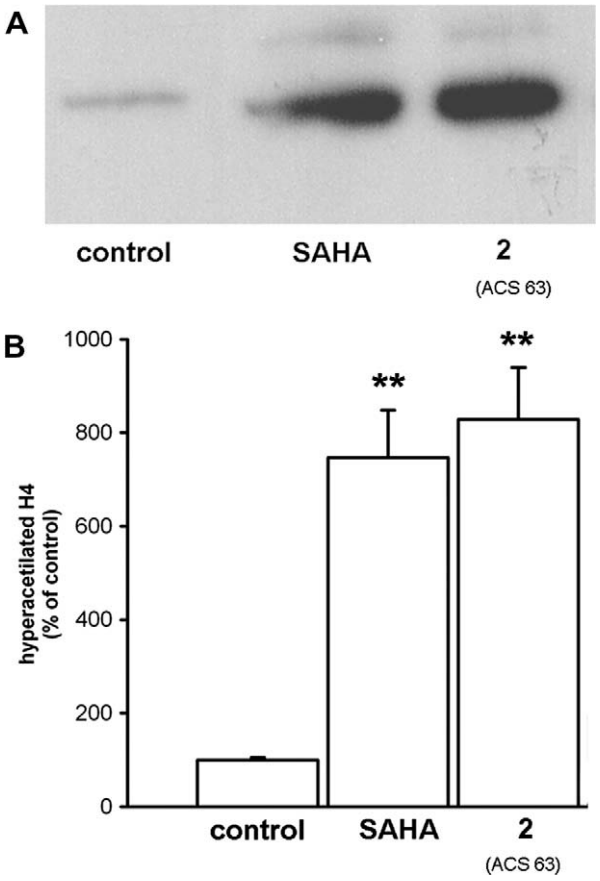


Figure 4. Histone hyperacetylation in A549 cells exposed to 0.76 μM SAHA and 6.1 μM compound **2** (ACS 63). Representative Western blot (A) and densitometric analysis (B) showing the levels of hyperacetylated histone H4. Data are expressed as means ± SE. **Significant difference versus control (P <0.001 according to ANOVA, Dunnet post-hoc test).

HDAC inhibitory activity (IC_{50} = 10 μM) and a 10-fold lower antiproliferative activity. The observed lower activities in the two assays may be inherent to the molecule itself, whose dimensions may exceed the optimal ones, but, they might also be due to the products generated by hydrolysis of the ester linkage, which may occur in different degrees in each assay, whose incubation time were 1 and 48 h, respectively. Indeed, after 1 and 48 h of incubation increasing degrees of hydrolytic cleavage of ester **3** were observed and a more accurate study of the kinetics of hydrolysis is needed to explain the observed results of HDAC inhibition and antiproliferation assays. Compounds **4**, **5**, **6** and **13**, devoid of the hydroxamic group, exhibited HDAC inhibitory activity that ranged from moderate (IC_{50} = 50 and 100 μM for compounds **5** and **4**, respectively) to rather poor (IC_{50} = 179 and 918 μM for **13** and **6**, respectively). If for the esters **4** and **13** the activity could be, even in part, related to the hydrolytic release of ADTOH, this possibility does not apply to the best compound **5**, whose activity must rely on the whole molecule, supporting that the zinc binding role may be accomplished by the dithiolethione moiety.

It is interesting to observe that compound **5** is 100-fold less potent than ADTOH, but it is >200-fold more potent than the simple methyl ether of the latter (ADT).[7] Therefore the etherification of the phenolic group of ADTOH may be exploited to form novel HDAC inhibitors, provided that the attached moieties are able to occupy the enzymatic channel and form both polar and hydrophobic bonds.

It is worth noting that, in this subset of compounds, the most (**5**) and the least (**6**) active ones differ only for two methylene groups in the spacer moiety. This confirms that the length of the spacer should be optimized in relation to the dimensions of the cap and

the zinc chelating groups. Indeed, while in this case the elongation of the spacer has deleterious effects on activity, the opposite is observed for the hydroxamic acids **2** and **1**, previously discussed.

Compound **13** shows close structural analogies with the previously studied[7] valproic ester of ADTOH (ACS 2, Fig. 1) and, indeed, they exhibit comparable activities as enzyme and cell proliferation inhibitor. Compound **7** is 29 times more potent than **6** but 3200 times less than **2**, from which it differs, respectively, for an additional *ortho*-amino group and for the replacement of the NHOH with an *o*-phenylenediamine moiety. The increase of potency produced by the insertion of an amino group in the anilide cap of **6** clearly indicates that the formed acylated *o*-phenylenediamine became a better zinc chelating motif than the dithiolethione moiety. On the other hand, the striking difference of potency between **2** and **7** may not be attributed exclusively to a better metal chelating capability of the hydroxamic function in respect to the acylated *o*-phenylenediamine, since the larger molecular size of **7** might also exceed the enzyme channel capacity.

The dithiolethione carboxylic acids **8**, **9** and **10** resulted endowed with moderate HDAC inhibitory activity (IC_{50} in the range from 46 to 100 μM), despite not fulfilling the requirement of the pharmacophore model for HDAC inhibition.

Even less potent than ADTOH, these acids present some interest for either the generation of extended molecules potentially more potent, or as protective group for the sensitive hydroxamic function of several HDAC inhibitors.

Thus, coupling the hydroxamic function of SAHA with the dithiolethione carboxylic acids **8** and **9** the N,O-diacylated hydroxylamines **11** and **12** were obtained. These compounds were endowed with strong HDAC inhibitory activity (IC_{50} = 0.25 μM and 1.19 μM, respectively) that was however 2.5-fold and 12-fold lower than that of SAHA. This decrease of activity may be related either to an intrinsic weaker zinc ion binding capacity or to the exceeding molecular dimension in respect to the enzyme active site channel. Indeed, the more extended **12** is about fivefold less potent than **11**. The observed activity could be also related to the hydrolytic release of SAHA and the difference between **12** and **11** may depend from the different rates of their hydrolytic cleavage.

Additional studies, that are beyond the scope of the present work, will be needed to explore the effects of masking the hydroxamic function of SAHA on its ADME characteristics, as well as on its safety profile.

As it is shown in Table 1, the inhibition of A549 cell proliferation paralleled, in most cases, the HDAC inhibitory activity, and significant histone H4 hyperacetylation was observed on the same cells.

However, for some compounds such a parallelism does not hold, since moderate enzyme inhibitors (**8**, **5**, **10** and **4**) are practically devoid of any antiproliferative activity, while, on the contrary, weaker HDAC inhibitors (**9**, **13** and **6**) still display a fair antiproliferative activity. For instance, compound **13** exhibited a IC_{50} = 31.8 μM for antiproliferative activity, while its IC_{50} for HDAC inhibition is as high as 179 μM.

Therefore, in these cases additional mechanisms may play a role in the inhibition of A549 cell proliferation.

4. Conclusions

A series of dithiolethione derivatives was synthesized and found to display variable degrees of HDAC inhibitory activity, that in some cases is comparable or even higher than that of SAHA, used as reference drug.

The dithiolethione moiety may be either directly responsible for the enzyme inhibition, acting as zinc ion chelating group, or play a role as a more appropriate cap group (in comparison to the anilide group of SAHA), when a more effective chelating group is also present in the molecule.

Antiproliferative activity, with IC_{50} in the low μM range, was observed in nine compounds. Generally this activity is related to the HDAC inhibition, since increased levels of hyperacetylated histone H4 was observed in A549 cells.

Due to their potency as HDAC inhibitors and antiproliferative agents, compounds **2** and **1** deserve further investigation, particularly to explore if the peculiar biological characteristics of the sulfurated moieties are able to improve the safety profile of SAHA and its analogs. This is the object of an ongoing study.

These results support our hypothesis that the dithiolethione moiety is a suitable scaffold to build new potential anticancer agents.

5. Experimental

5.1. General

All commercially available solvents and reagents were used without further purification, unless otherwise stated. CC = flash column chromatography. Mps: Büchi apparatus, uncorrected. ^{1}H NMR spectra: Varian Mercury 300VX spectrometer; $CDCl_3$ or DMSO-d_6; δ in ppm, J in hertz. High resolution mass spectra (HRMS) on a LTQ ORBITRAP® XL mass spectrometer in positive electro spray ionization (ESI; Source: FINNIGAN ION MAX). UV–vis spectra: Varian DMS 80 spectrophotometer.

5.2. Ethyl 5-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]pentanoate (14)

5-(4-Hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (890 mg; 3.93 mmol) was added to a solution of NaOH (157 mg; 3.93 mmol) in ethanol (4 ml) and the mixture was stirred for 15 min, thereafter ethyl 5-bromopentanoate 98% (838 mg; 0.63 ml) and KI (65 mg; 0.1 equiv) were added and the mixture was refluxed for 4 h. The solvent was evaporated and the crude product was chromatographed on silica gel (cyclohexane/ethyl acetate; 85:15) and after washing with petrol ether, ethyl 5-(4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy)pentanoate as an orange solid was obtained. Mp 81–82 °C. Yield: 14%. ^{1}H NMR (DMSO-d_6): δ = 7.86 (d, J = 8.80 Hz, 2H); 7.75 (s, 1H); 7.05 (d, J = 9.09 Hz, 2H); 4.08–3.99 (m, 4H); 2.35 (t, J = 6.94 Hz, 2H); 1.73–1.66 (m, 4H); 1.16 (t, J = 7.03 Hz, 3H). HRMS (ESI) *m/z* calcd for $C_{16}H_{19}O_3S_3$ $[M+H]^+$: 355.04908; found: 355.04896.

5.3. Ethyl 7-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]heptanoate (15)

5-(4-Hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (400 mg; 1.76 mmol) was added to a solution of NaOH (70 mg, 1.76 mmol) in ethanol (2 ml) and the mixture was stirred at 45 °C for 10 min, then ethyl 7-bromo-eptanoate (0.34 ml; 1.76 mmol) was added and the mixture was stirred under nitrogen at 80 °C for 6 h. After evaporation of the solvent, the crude product was purified by CC (silica gel; cyclohexane/CH_2Cl_2; 6:4) as a red solid. Mp 70.3–70.9 °C. Yield: 14%. ^{1}H NMR (DMSO-d_6): δ = 7.85 (d, J = 8.50 Hz, 2H); 7.75 (s, 1H); 7.05 (d, J = 8.80 Hz, 2H); 4.06–3.98 (m, 4H); 2.27 (t, J = 7.33 Hz, 2H); 1.72–1.30 (m, 8H); 1.15 (t, J = 7.03 Hz, 3H). HRMS (ESI) *m/z* calcd for $C_{18}H_{23}O_3S_3$ $[M+H]^+$: 383.08038; found: 383.08029.

5.4. 5-[4-(3-Thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]pentanoic acid (16)

Ethyl 5-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]pentanoate (155 mg; 0.474 mmol) was suspended in a mixture of acetic acid (5.9 ml) and 50% sulfuric acid (0.9 ml) and heated, under stirring,

at 100 °C for 1.5 h. After cooling, the mixture was diluted with water and extracted with CH_2Cl_2. The organic phase was dried on anhydrous sodium sulfate, filtered and evaporated to dryness. The orange product was washed with ethyl ether. Mp 114.5–117 °C (dec.). Yield: 89% ^{1}H NMR (DMSO-d_6): δ = 12.05 (s,1H, collapses with D_2O); 7.85 (d, J = 8.80 Hz, 2H); 7.75 (s, 1H); 7.05 (d, J = 8.80 Hz, 2H); 4.06 (t, J = 5.23 Hz, 2H); 2.27 (t, J = 7.43 Hz, 2H); 1.75–1.60 (m, 4H). HRMS (ESI) m/z calcd for $C_{14}H_{15}O_3S_3$ $[M+H]^+$: 327.01778; found: 327.01793.

5.5. 7-[4-(3-Thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]heptanoic acid (17)

Ethyl 7-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]heptanoate (89 mg; 0.23 mmol) was suspended in a mixture of acetic acid (3 ml) and 50% sulfuric acid (0.48 ml) and the mixture was stirred at 100 °C, for 3 h. Water was added after cooling and the product was extracted with CH_2Cl_2. The organic solution was dried on anhydrous sodium sulfate and evaporated to dryness and the residue was washed with ethyl ether. A red solid was obtained. Mp 114.8–115.6 °C. Yield: 94%. ^{1}H NMR (DMSO-d_6): δ = 12.06 (s, 1H, collapses with D_2O); 7.92 (d, J = 8.50 Hz, 2H); 7.83 (s, 1H); 7.13 (d, J = 8.80 Hz, 2H); 4.12 (t, J = 6.15 Hz, 2H); 2.27 (t, J = 7.03 Hz, 2H); 1.81–1.29 (m, 8H). HRMS (ESI) m/z calcd for $C_{16}H_{19}O_3S_3$ $[M+H]^+$: 355.04908; found: 355.04914.

5.6. *N*-Hydroxy-5-(4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy) pentanamide (1)

5-[4-(3-Thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]pentanoic acid (125 mg; 0.38 mmol), EDAC (87 mg; 0.45 mmol) and a catalytic quantity of DMAP were dissolved in 6 ml of $CHCl_3$ and the solution was stirred under nitrogen for 20 min on an ice bath. Thereafter a solution of *O*-(*tert*-butyldimethylsilyl)hydroxylamine (59 mg, 0.38 mmol) in $CHCl_3$ was added and the reaction was stirred at room temperature for 3 h. The solution was diluted with chloroform and shaken first with 1 N HCl and then with water. The organic phase was dried on anhydrous sodium sulfate, filtered and evaporated to dryness. The solid orange product was washed with ethyl ether. Mp 101.5–103.5 °C. Yield: 65% ^{1}H NMR (DMSO-d_6): δ = 10.37 (s, 1H, collapses with D_2O); 8.69 (s, 1H, collapses with D_2O); 7.85 (d, J = 8.79 Hz, 2H, 2H); 7.75 (s, 1H,); 7.05 (d, J = 8.21 Hz, 2H); 4.05 (t, J = 5.91 Hz, 2H); 2.00 (t, J = 6.16 Hz, 2H); 1.75–1.57 (m, 4H). HRMS (ESI) m/z calcd for $C_{14}H_{15}NO_3S_3Na$ $[M+Na]^+$: 364.01063; found: 364.01035.

5.7. *N*-Hydroxy-7-(4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy) heptanamide (2)

A mixture of 7-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]heptanoic acid (66.7 mg; 0.19 mmol), EDAC (1.2 equiv 0.245 mmol; 46.8 mg) and DMAP (2.5 mg) in $CHCl_3$ (6 ml) was cooled on an ice bath, stirring under nitrogen for 20 min. A solution of *O*-(*tert*-butyldimethylsilyl)hydroxylamine (30 mg; 0.19 mmol) in $CHCl_3$ (1.2 ml) was added dropwise, then the ice bath was removed and the mixture was stirred for 3 h at rt. After addition of CH_2Cl_2, the mixture was shaken with 1 N HCl and then with cold water. The organic phase was dried on anhydrous sodium sulfate and evaporated to dryness. The product crystallized with CH_2Cl_2. Mp 110.6–111.9 °C. Yield: 69.5%. ^{1}H NMR (DMSO-d_6): δ = 10.32 (s,1H, collapses with D_2O); 8.65 (s,1H, collapses with D_2O); 7.85 (d, J = 8.20 Hz, 2H); 7.75 (s, 1H); 7.05 (d, J = 8.80 Hz, 2H); 4.04 (t, J = 6.32 Hz, 2H); 1.93 (t, J = 7.03 Hz, 2H); 1.70–1.27 (m, 8H). HRMS (ESI) m/z calcd for $C_{16}H_{19}NO_3S_3$ $[M+H]^+$: 392.04193; found: 392.04271. UV–vis (EtOH), λ_{max}, nm (log ε): 236 (4.10), 265 sh (3.82), 348 (4.31), 428 (4.06); λ_{min}: 288, 389.

5.8. *N*-Phenyl-5-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy] pentanamide (5)

A solution of 5-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]pentanoic acid (155 mg, 0.474 mmol), EDAC (109 mg, 0.57 mmol) and DMAP (6 mg) in 7 ml of CH_2Cl_2 was cooled on an ice bath and stirred under nitrogen for 20 min. A solution of aniline (44.1 mg, 0.474 mmol) in 3 ml of CH_2Cl_2 was added dropwise, then the ice bath was removed and the mixture was stirred under nitrogen for 2 h. The mixture was diluted with 20 ml of CH_2Cl_2 and extracted with cold 1 N HCl and then with cold water. The organic phase was dried on anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The crude product was chromatographed on silica gel (CH_2Cl_2/methanol; 99.8:0.2) and the obtained orange product was washed with ethyl ether. Mp 199.5–201.5 °C. Yield: 76%. ^{1}H NMR (DMSO-d_6): δ = 9.88 (s, 1H); 7.85 (d, J = 8.53 Hz, 2H); 7.75 (s, 1H); 7.57 (d, J = 8.50 Hz, 2H); 7.26 (t, J = 7.15 Hz, 2H); 7.07–7.00 (m, 3H); 4.09 (br s, 2H); 2.36 (br s, 2H); 1.76 (br s, 4H). HRMS (ESI) m/z calcd for $C_{20}H_{20}NO_2S_3$ $[M+H]^+$: 402.06507; found: 402.06480.

5.9. *N*-Phenyl-7-(4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy) heptanamide (6)

A solution of 7-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]heptanoic acid (150 mg; 0.423 mmol), EDAC (97 mg; 0.5 mmol), DMAP (5.2 mg) in 8 ml of $CHCl_3$ was cooled on an ice bath and stirred under nitrogen for 20′. A solution of aniline (39.4 mg; 0.423 mmol), in 2 ml of $CHCl_3$, was added dropwise, then the ice bath was removed and the mixture was stirred under nitrogen for 3 h. The mixture was diluted with 20 ml of CH_2Cl_2 and extracted first with cold HCl 1 N and then with cold water. The organic phase was dried on anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The product, after addition of CH_2Cl_2 crystallized. Mp 149–150 °C. Yield: 33%. ^{1}H NMR (DMSO-d_6): δ = 9.83 (s, 1H); 7.84 (d, J = 8.53 Hz, 2H); 7.74 (s, 1H); 7.56 (d, J = 8.25 Hz, 2H); 7.26 (t, J = 7.42 Hz, 2H); 7.06–6.97 (m, 3H); 4.05 (t, J = 6.32 Hz, 2H); 2.29 (t, J = 7.15 Hz, 2H); 1.73–1.39 (m, 8H). HRMS (ESI) m/z calcd for $C_{22}H_{24}NO_2S_3$ $[M+H]^+$: 430.09637; found: 430.09600.

5.10. *N*-(2-Aminophenyl)-7-(4-(3-thioxo-3*H*-1,2-dithiol-5-yl) phenoxy)heptanamide (7)

o-Phenylenediamine (39.65 mg, 0.37 mmol) was added to a solution of 7-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]heptanoic acid (130 mg, 0.36 mmol), EDAC (83 mg, 0.43 mmol), HOBt (66 mg, 0.43 mmol) and triethylamine (TEA, 0.05 ml) in anhydrous DMF (3 ml) and the mixture was stirred under nitrogen, at room temperature for 24 h. At the end of the reaction DMF was evaporated and the residue was taken up with CH_2Cl_2. The organic phase was extracted with water, dried on anhydrous sodium sulfate and evaporated to dryness. The crude product was purified by CC (silica gel; CH_2Cl_2/methanol; 99:1). The orange solid product was washed with ethyl ether. Mp 144–146 °C. Yield: 44%. ^{1}H NMR (DMSO-d_6): δ = 9.07 (s, 1H); 7.84 (d, J = 8.50 Hz, 2H,); 7.74 (s, 1H); 7.12 (d, J = 7.33 Hz, 1H); 7.05 (d, J = 8.80 Hz, 2H); 6.87 (t, J = 7.33 Hz, 1H); 6.70 (d, J = 7.62 Hz, 1H); 6.51 (t, J = 7.33 Hz, 1H); 4.79 (s, 2H, collapses with D_2O); 4.06 (t, J = 6.45 Hz, 2H); 2.30 (t, J = 7.04 Hz, 2H); 1.75–1.36 (m,8H). HRMS (ESI) m/z calcd for $C_{22}H_{25}NO_2S_3$ $[M+H]^+$: 445.10727; found: 445.10682.

5.11. 8-Oxo-8-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]octanoic acid (18)

5-(4-Hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (ADTOH, 226 mg, 1 mmol) and anhydrous pyridine (79 mg; 1 mmol) were added

to a solution of suberoyl chloride (211 mg; 1 mmol) in anhydrous tetrahydrofuran (THF). After stirring for 1 h at rt, THF was evaporated to dryness and the residue dissolved in CH_2Cl_2. The dichloromethane solution was thoroughly washed with cold water, then was dried on anhydrous sodium sulfate and evaporated to dryness. After chromatographic separation (silica; CH_2Cl_2/methanol; 99:1) an orange solid was obtained which was characterized only by ^{1}H NMR and used for the preparation of **3** without any further characterization. Yield: 40%. ^{1}H NMR (DMSO-d_6): δ = 12.00 (s, 1H, collapses with D_2O); 7.95 (d, J = 8.53 Hz, 2H); 7.80 (s, 1H); 7.30 (d, J = 8.53 Hz, 2H); 2.57 (t, J = 6.87 Hz, 2H); 2.20 (t, J = 6.87 Hz, 2H); 1.60–1.28 (m, 8H).

5.12. 4-(3-Thioxo-3*H*-1,2-dithiol-5-yl)phenyl 8-(hydroxyamino)-8-oxooctanoate (3)

A dichloromethane solution of *O*-(*tert*-butyldimethylsilyl)hydroxylamine (TBDMSONH$_2$, 49.3 mg; 0.335 mmol) was added dropwise to a solution of 8-oxo-8-[4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy]octanoic acid (**18**) (130 mg; 0.335 mmol), 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDAC; 77 mg; 0.40 mmol) and 4-(dimethylamino)pyridine (DMAP; 4 mg) dissolved in CH_2Cl_2 (10 ml, previously filtered through basic alumina). The reaction mixture was stirred at rt, under nitrogen for 3 h. The solution, after washing with 1 N HCl and cold water, was dried on anhydrous sodium sulfate and evaporated to dryness. The crude compound was chromatographed on silica gel CC (CH_2Cl_2/CH_3OH; 99:1). The obtained compound (**19**; yield: 62.5%) still contain the silyl protective group.

A part of the obtained compound (50 mg; 0.097 mmol) was dissolved in CH_2Cl_2 (2 ml), and after cooling at 0 °C, 0.02 ml of triethylsilane and 0.4 ml of trifluoroacetic acid were added. The reaction mixture was stirred for 30 min at 0 °C, then the solvent was evaporated and after addition of few drops of CH_2Cl_2 the product, kept in refrigerator overnight, crystallized. Crystals were washed with a CH_2Cl_2/ether (1:1) mixture. Mp 93–97 °C. Yield: 63.6%. ^{1}H NMR (DMSO-d_6): δ = 10.32 (s, 1H, collapses with D_2O); 8.66 (s,1H, collapses with D_2O); 7.95 (d, J = 8.53 Hz, 2H); 7.75 (s, 1H); 7.30 (d, J = 8.53 Hz, 2H); 2.59 (t, J = 6.87 Hz, 2H); 1.91 (t, J = 6.87 Hz, 2H); 1.53–1.28 (m, 8H). HRMS (ESI) m/z calcd for $C_{17}H_{20}NO_4S_3$ $[M+H]^+$: 398.05490; found: 398.05479.

5.13. 4-(3-Thioxo-3*H*-1,2-dithiol-5-yl)phenyl 8-oxo-8-(phenylamino) octanoate (4)

A mixture of the monoanilide of suberoyl acid prepared as previous described[32] (199.5 mg; 0.8 mmol), EDAC (193.6 mg 1.0 mmol) and DMAP (10.3 mg) in 12 ml of $CHCl_3$ were stirred for 20 min on an ice bath under nitrogen. 5-(4-Hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (ADTOH, 181 mg; 0.8 mmol) was added and the reaction mixture was stirred at rt for 2 h. At the end of the reaction, the organic phase was extracted with water and then with a solution of $NaHCO_3$, dried on anhydrous sodium sulfate and evaporated to dryness. The obtained residue was washed with CH_2Cl_2. Mp 177.5–179.5 °C. Yield: 66%. ^{1}H NMR (DMSO-d_6): δ = 9.85 (s, 1H); 7.94 (d, J = 8.53 Hz, 2H); 7.80 (s, 1H); 7.57 (d, J = 7.43 Hz, 2H); 7.30–7.23 (m, 4H); 7.00 (t, J = 6.87 Hz, 1H); 2.60 (t, J = 7.15 Hz, 2H); 2.29 (t, J = 6.88 Hz, 2H); 1.64–1.35 (m, 8H). HRMS (ESI) m/z calcd for $C_{23}H_{24}NO_3S_3$ $[M+H]^+$: 458.09128; found: 458.09096.

5.14. 4-(3-Thioxo-3*H*-1,2-dithiol-4-yl)benzoic acid (9)

A suspension of methyl 4-(3-thioxo-3*H*-1,2-dithiol-4-yl)benzoate[34] (100 mg, 0.35 mmol) in 4.5 ml of acetic acid and 0.72 ml of 50% (v/v) H_2SO_4 was stirred at 100 °C for 4 h. After cooling, the solution was diluted with water and extracted with a mixture of CH_2Cl_2/methanol (9:1). The organic phase was dried on anhydrous sodium sulfate and evaporated to dryness and the residue was washed with ether and CH_2Cl_2 to obtain a yellow-orange solid. Mp 240–245 °C. Yield: 93%. ^{1}H NMR (DMSO-d_6): δ = 13.03 (s, 1H, collapses with D_2O); 9.23 (s, 1H); 7.97 (d, J = 7.63 Hz, 2H); 7.69 (d, J = 7.63 Hz, 2H).

5.15. [2-Methoxy-4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy] acetic acid (10)

Methyl [2-methoxy-4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy] acetate[35] was hydrolyzed as above described for the preparation of compound **9**. Mp 198–200 °C. Yield: 92%. ^{1}H NMR (DMSO-d_6): δ = 7.86 (s, 1H); 7.45–7.42 (m, 2H); 6.96 (d, J = 9.09 Hz, 1H); 4.78 (s, 2H); 3.86 (s, 3H).

5.16. N^1-Phenyl-N^8-(2-thioxo-1,3-dithiole-4-carbonyloxy) octanediamide (11)

A solution of N^8-hydroxy-N^1-phenyloctanediamide (SAHA, 100 mg; 0.38 mmol) in anhydrous DMF (1 ml) was added to a solution of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDAC, 86.8 mg, 0.45 mmol), HOBt (69.5; 0.45 mmol) and 2-thioxo-1,3-dithiole-4-carboxylic acid[33] (**8**, 60 mg; 0.38 mmol) in anhydrous DMF (2 ml) and the mixture was stirred under nitrogen, at room temperature, for 24 h. At the end of the reaction, DMF was evaporated and the residue dissolved in ethyl acetate. The organic phase was washed with water, dried on anhydrous sodium sulfate and evaporated to dryness. The product was washed with a little of ethyl acetate and then with ether and crystallized from THF. Mp 138–141 °C (dec.). Yield: 31%. ^{1}H NMR (DMSO-d_6): δ = 12.15 (s, 1H, collapses with D_2O); 9.82 (s, 1H, collapses with D_2O); 8.69 (s, 1H); 7.60 (d, J = 7.91 Hz, 2H); 7.26 (t, J = 7.92 Hz, 2H); 6.99 (t, J = 7.32 Hz, 1H); 2.27 (t, J = 7.33 Hz, 2H); 2.16 (t, J = 7.33 Hz, 2H). HRMS (ESI) m/z calcd for $C_{18}H_{21}N_2O_4S_3$ $[M+H]^+$: 425.06580; found: 425.06540.

5.17. N^1-Phenyl-N^8-(4-(3-thioxo-3*H*-1,2-dithiol-4-yl)benzoyloxy) octanediamide (12)

A solution of N^8-hydroxy-N^1-phenyloctanediamide (SAHA) (100 mg; 0.38 mmol) in 1 ml of dimethylformamide (DMF) was added to a solution of **9** (96 mg; 0.38 mmol), 1-hydroxybenzotriazole hydrate (HOBt, 64 mg; 0.418 mmol), EDAC (80 mg; 0.418 mmol) in 2 ml of anhydrous DMF. The reaction was stirred under nitrogen at room temperature for 24 h. At the end of the reaction DMF was evaporated and the crude product was taken up in water, filtered and washed with CH_2Cl_2 and then with THF. The obtained product has a melting point of 139.5–140 °C. Yield: 66%. ^{1}H NMR (DMSO-d_6): δ = 11.90 (s, 1H, collapses with D_2O); 9.85 (s, 1H); 9.25 (s, 1H); 8.05 (d, J = 7.91 Hz, 2H); 7.76 (d, J = 8.21 Hz, 2H); 7.55 (d, J = 7.62 Hz, 2H); 7.25 (t, J = 7.91 Hz, 2H); 6.99 (t, J = 6.88 Hz, 1H); 2.28 (t, J = 7.03 Hz, 2H); 2.18 (t, J = 6.74 Hz, 2H); 1.60–1.25 (m, 8H). HRMS (ESI) m/z calcd for $C_{24}H_{25}N_2O_4S_3$ $[M+H]^+$: 501.09710; found: 501.09666.

5.18. 4-(3-Thioxo-3*H*-1,2-dithiol-5-yl)phenyl 4-phenylbutanoate (13)

A dichloromethane solution of dicyclohexylcarbodiimide (DCC) (755 mg; 1.2 equiv) was added dropwise at room temperature to a suspension of 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (680 mg; 3 mmol), 4-phenylbutyric acid (500 mg; 3 mmol) and DMAP (18.5 mg) in CH_2Cl_2 (25 ml). The reaction was stirred for 3 h at rt. At the end of the reaction the dicyclohexylurea (DCU) was filtered and the solution was chromatographed on SiO_2 (cyclohexane/

CH_2Cl_2; 7:3). The product crystallized with ethyl ether. Mp 82.5–83 °C. Yield: 80%. ^{1}H NMR (DMSO-d_6): δ = 7.95 (d, J = 8.50 Hz, 2H); 7.81 (s, 1H); 7.31–7.16 (m, 6H); 2.69–2.58 (m, 4H); 1.98–1.88 (m, 2H). HRMS (ESI) m/z calcd for $C_{19}H_{17}O_2S_3$ $[M+H]^+$: 373.03852; found: 373.03822.

5.19. HDAC activity measurement

HDAC activity was measured by using a fluorescence activity assay kit (Cayman Chemical, USA) according to the manufacturer's instructions. Briefly, HeLa nuclear extracts were incubated with acetylated fluorogenic substrate (100 μM) for 60 min at 37 °C in the presence of test compound dissolved in DMSO or DMSO alone. The deacetylated reaction was stopped by addition of 40 μl of HDAC developer containing 5 μM trichostatin A. After 15 min, fluorescence activity was measured with a Fluorocount™ reader (Packard BioScience, USA) at 360 nm excitation and 465 nm emission. As the majority of test compounds still exhibit a low absorption at the wavelength of fluorescence emission, appropriate controls were prepared as follows. HeLa nuclear extracts were incubated for 60 min at 37 °C in the absence of these compounds, the reaction was stopped by addition of developer, and, immediately, the compounds were added. After 15 min, the fluorescence was measured. The inhibition of HDAC activity by test compounds was calculated taking this value as the 100% of activity.

All experimental data were fitted and analyzed by computer using a sigmoidal dose–response function (Sigma Plot, Jandel, CA).

5.20. Cell proliferation

For cell proliferation assay, A549 cells (50,000 cell/well) were seeded onto 24-well tissue culture plates, incubated for 48 h in the absence or presence of increasing concentrations of the compounds, harvested and counted. All experimental data were fitted and analyzed by computer using a sigmoidal dose–response function (Sigma Plot, Jandel, CA).

5.21. Histone hyperacetylation analysis

For histone hyperacetylation analyses, A549 cells (approx. 100,000 cell/well) were seeded onto 12-well tissue culture plates and exposed for 24 h to the compounds at concentration equivalent to IC_{50} on cell proliferation. At the end of incubation, cells were lysed and analyzed by Western blot using polyclonal anti-hyperacetylated histone H4 as previously described.[36]

Data are normalized on the levels of total proteins.

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