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New sulfurated derivatives of valproic acid with enhanced histone deacetylase inhibitory activity

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Abstract—One dithiolthione and two new methanethiosulfonate derivatives of valproic acid (**VPA**) were synthesized and tested in vitro as histone deacetylase (HDAC) inhibitors. The new molecules, as well as their sulfurated moieties, exhibited a much stronger inhibition of HDAC enzymatic and antiproliferative activities and histone hyperacetylation than **VPA**. **ACS 2** is the most interesting compound among the new **VPA** derivatives and its sulfurated moiety, 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione, also known to be a metabolite of anethole trithione, seems to contribute significantly to its activity. This is the first time that HDAC inhibitory activity is described for dithiolethiones and thiosulfonates.

Histone deacetylases (HDACs) are a family of enzymes that regulate chromatin remodelling and gene transcription[1] and there is a growing interest in inhibitors of HDAC as a promising class of anticancer agents.[2] Several classes of small-molecule HDAC inhibitors have been recognized and the most studied are short chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides.[3,4]

Short chain fatty acids as butyric and valproic acid (**VPA**, Fig. 1) were the first HDAC inhibitors discovered that can inhibit growth and induce apoptosis both in vitro and in vivo, but they exhibit a low potency, with IC_{50} in the millimolar range.[5]

Despite the weak in vitro activity, the anticancer characteristics of **VPA** have been investigated in preclinical models of skin, breast, colon, prostate and small cell lung cancer and currently the drug is in phase I–III clinical trials.[6] Unfortunately the doses of **VPA** necessary to achieve in vivo the concentrations exerting antineoplastic activity are very high and in such a case the side effects can become an important limitation to its therapeutic use.

VPA **Butyric acid**

Figure 1. Structures of valproic acid (**VPA**) and butyric acid.

Structure–activity studies have shown the importance of binding to a zinc ion in the catalytic domain of the enzyme, for preventing the deacetylation of histones.[7] Lu et al. rationalized that the weak potency of **VPA** was, in part, attributable to the inability to access the zinc cation in the HDAC active site pocket, which is pivotal to the deacetylation catalysis and demonstrated that coupling a Zn^{2+}-chelating moiety (hydroxamic acid and *o*-phenylenediamine) with valproic, butyric, phenylacetic and phenylbutyric acids, through aromatic ω-aminoacid linkers, a new class of HDAC inhibitors, much more powerful than the parent compounds, had been achieved.[8]

An increase of histone acetylation by organosulfur compounds, via inhibition of histone deacetylase[9,10] was also

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described. Interestingly, some dietary chemopreventive agents, as diallyl disulfide and sulforaphane have HDAC inhibitory activity.[11,12] Moreover, *S*-methyl methanethiosulfonate, isolated from cauliflower has been shown to inhibit colon tumour incidence when administered to rats during the post-initiation phase of carcinogenesis.[13]

Compounds with a free thiol group were described as potent HDAC inhibitors[14,15] but often their cellular activity was very weak.[16] Different head groups containing sulfur include thiocarboxylates,[17,18] thioglicolamides,[16,19–21] α-thio-substituted acetyl compounds[22] and trithiocarbonates.[23]

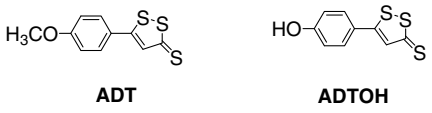
Recently we have demonstrated that an ester of **VPA** with 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (**ACS 2**) had significant antiangiogenic activity, inhibiting endothelial cell proliferation and vascular cell outgrowth and invasion of extracellular matrix under wound healing as well as tumour-driven conditions,[24] but the mechanism at the base of this activity has not yet been investigated. Although dithiolethiones (DTTs) are known to exert antiangiogenic activity[25] and anticancer effects,[26] their activity on HDAC has not been studied yet.

As it is recognized that DTTs are able to coordinate metal ions[27] such as Zn^{2+}, we decided to explore the activity on HDAC of **ACS 2** and of 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (**ADTOH**, Fig. 2) which could be released from the former by in vivo hydrolysis.

In addition, following the hypothesis that the insertion of an organosulfur group can increase HDAC inhibition, we decided to synthesize new **VPA** derivatives bearing in their structures sulfurated moieties, selected among those known to be endowed with cancer chemopreventive activity, as we made for **ACS 2**.

Here we describe two new **VPA** derivatives (**ACS 33** and **ACS 43**, Fig. 3), bearing a thiosulfonate moiety linked to the parent compound by mean of an ester or an amide group. Also in these cases it is possible to anticipate a Zn^{2+} coordination either directly, or indirectly after liberation of the –SH group,[28] as it is required for deacetylation of the acetylated lysine substrate.

The compounds **ACS 2** and **ADTOH** were prepared as described previously.[24] The routes used for the synthesis of **ACS 33** and **ACS 43** are indicated in Scheme 1 and required firstly the preparation of the thiosulfonate intermediates 2-hydroxyethyl methanethiosulfonate[29] (**ACS 26**) and 2-aminoethyl methanethiosulfonate hydrobromide[30] (**ACS 42**), respectively. The new compounds were characterized by mp (Büchi), ^{1}H NMR (Varian Mercury 300VX) and HRMS (APEX II ICR-FTMS Bruker Daltonics, ESI).[31]



ADT ADTOH

Figure 2. Structures of ADT and ADTOH.

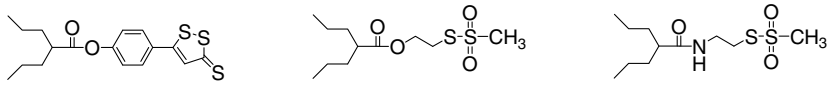
Next we investigated the HDAC activity of **VPA** derivatives **ACS 2**, **ACS 33** and **ACS 43** and of their corresponding sulfurated moieties using an in vitro assay.[32,33]

As shown in Table 1, all the three **VPA** derivatives exhibit a much higher HDAC inhibitory activity than the parent compound. Furthermore, the sulfurated moieties **ADTOH**, **ACS 26** and **ACS 42** were able to inhibit HDAC enzymes.

Indeed **ADTOH** is a potent HDAC inhibitor with an IC_{50} 0.45 μM. As **ADTOH** is known to be the main metabolite of anethole trithione (ADT, sulfarlem®),[34] a commercial drug used as a hepatoprotective and for the treatment of drug- and radiation-induced xerostomia, but also known to be endowed with cancer chemopreventive activity,[35] we decided to explore if also ADT is an inhibitor of HDAC enzymes. Surprisingly ADT in our hands showed a very low HDAC inhibition (46.7% at 10 mM, Table 1), more than four orders of magnitude lower than its demethylated metabolite. Therefore it is possible to speculate that one mechanism of cancer chemoprevention by ADT is likely related to the in vivo release of **ADTOH**.

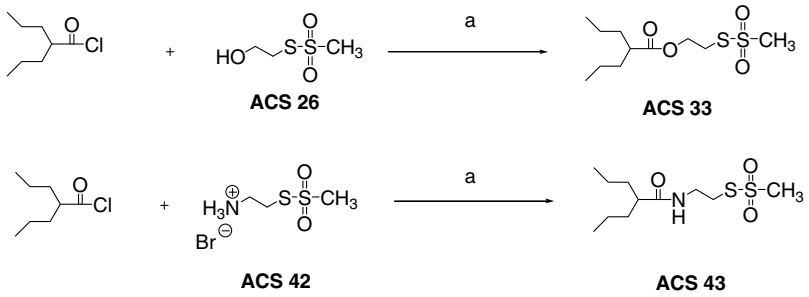
Moreover, we performed cellular assays using A549 cell line derived from human lung adenocarcinoma to investigate both antiproliferative activity and HDAC inhibition in cells.[36] As shown in Table 1, the new **VPA** derivatives and their corresponding sulfurated moieties exhibit a much higher antiproliferative activities than **VPA**. In addition, the increased levels of hyperacetylated histone H4-acetylation observed in A549 cells following exposure to the new compounds suggest their inhibitory activity on HDAC in cells (Fig. 4).

We can hypothesize that **ADTOH** has stronger inhibitory activity than **ADT** because it can link more firmly the zinc ion. **ADTOH** is a quite acidic phenol with a pK_a 7.86;[37] therefore at physiological pH it is partially dissociated and the dissociation is further enhanced in our incubation conditions (pH 8). Moreover its anionic form **A** is in equilibrium with thiolate **B** which should be able to link Zn^{2+} even more strongly (Scheme 2). On the contrary ADT, as well as all the dithiolethiones without the free hydroxyl function in *para* position, can only



ACS 2 ACS 33 ACS 43

Figure 3. Structures of valproic acid derivatives **ACS 2**, **ACS 33** and **ACS 43**.



Scheme 1. Reagents and conditions: (a) N(Et)(*i*-Pr)$_2$, anhydrous THF, N_2, rt, 4 h, 40% for **ACS 33**, 63% for **ACS 43**.

Table 1. Inhibition of HDAC activity and A549 cell proliferation by valproic acid (**VPA**), sulfurated moieties and valproic acid derivatives[a]

Compound	% HDAC inhibition at 100 μM	HDAC IC_{50} (μM)	A549 IC_{50} (μM)
VPA	7.4	995	>1000[b]
ADT	31.8	>10,000[c]	NT[d]
ADTOH	83.6	0.45	66
ACS 2	53.4	56.4	89
ACS 26	48.7	102	161
ACS 33	92.4	9.6	154
ACS 42	21.5	198	154
ACS 43	80.0	17.5	ND[e]
VPA 100 μM + **ADTOH** 100 μM	99.0	NT	NT
VPA 100 μM + **ACS 26** 100 μM	92.9	NT	NT
VPA 100 μM + **ACS 42** 100 μM	49.9	NT	NT

[a] Values are means of at least three experiments.
[b] At 1 mM = 24% inhibition.
[c] At 10 mM = 46.7% inhibition.
[d] NT, not tested.
[e] ND, not detected because insoluble at 150 μM.

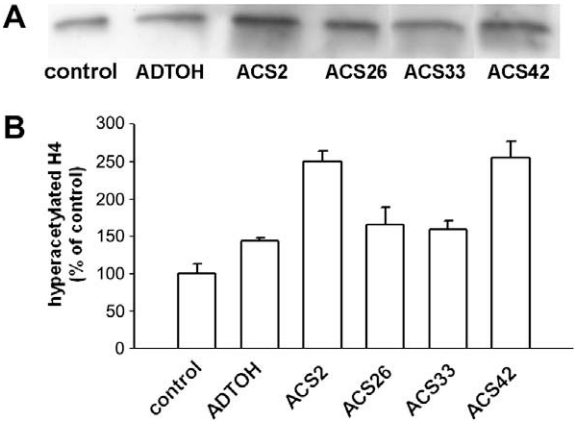
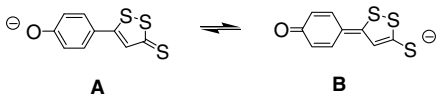


Figure 4. Histone hyperacetylation in A549 cells exposed to sulfurated moieties and valproic acid derivatives. Representative Western blot (A) and densitometric analysis (B) showing the levels of hyperacetylated histone H4.

coordinate Zn^{2+} with the free electron pair of sulfur atoms. The importance of the deprotonated forms of HDAC inhibitors has been stressed by Wang et al.[7]



Scheme 2. Equilibrium forms of deprotonated ADTOH.

This could also explain the lower in vitro HDAC inhibitory activity of **ACS 2** compared to **ADTOH**; indeed **ACS 2** is a quite stable ester and after 1–2 h of incubation in buffer at pH 8 and 37 °C, only a negligible amount of **ADTOH** (<3%) could be detected. However, in proper ex vivo conditions **ACS 2** showed a remarkable antiangiogenetic and antiproliferative activity[24] as it is confirmed also in our experiment on A549 cells.

Both **ACS 33** and **ACS 43** inhibit HDAC more potently than their parent compound **VPA** and also more than their sulfurated moieties **ACS 26** and **ACS 42**, respectively. Interestingly, **ACS 43** at 100 μM concentration seems to be more active (80% of inhibition) than the equimolar association of **VPA** and **ACS 42** (49.9% of inhibition).

In conclusion, the linkage of valproic acid with a sulfurated moiety such as 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (**ADTOH**) or 2-hydroxyethylmethanethiosulfonate (**ACS 26**) and 2-aminoethylmethanethiosulfonate (**ACS 42**), by mean of an ester or an amide linkage, has allowed to obtain new compounds with a HDAC inhibitory and antiproliferative activities in a micromolar range.

In addition we have demonstrated, for the first time to our knowledge, that dithiolethiones such as **ADTOH** and thiosulfonates such as **ACS 26** and **ACS 42** have HDAC inhibitory activity. On this basis we are studying these compounds as chemical scaffolds for preparing novel HDAC inhibitory agents through the usual SAR studies.

The antiproliferative activity of the novel compounds on different tumour cell lines as well as on some in vivo models is under study. The growth arrest in PC3 and DU-145 cells and the significant up regulation of histone H3 and H4 acetylation in prostate cancer cell lines by **ACS 2** and **ACS 33** is reported elsewhere.[38] Tested in vivo, **ACS 33** inhibited the growth of PC3 in subcutaneous xenografts.[38]

Our findings suggest that the novel **VPA**-derivatives **ACS 2** and **ACS 33** which are more active in in vitro HDCA inhibition, H_4 histone hyperacetylation and antiproliferation tests than **VPA** itself, are worthy of further study as potential anticancer agents.

Acknowledgment

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31. **ACS 33**: oil. ^{1}H NMR ($CDCl_3$): δ = 4.38 (t, J = 6.23 Hz, 2H, –O–CH_2–), 3.42 (t, J = 6.23 Hz, 2H, –CH_2–S–), 3.37 (s, 3 H, CH_3–SO_2–), 2.37 (m, 1H, –CH–), 1.71–1.12 (m, 8H, 2CH_2–CH_2–), 0.9 (t, J = 7.22 Hz, 6H, 2CH_3–). HRMS (ESI) *m/z* calcd for $[M(C_{11}H_{22}O_4S_2) + Na]^+$ 305.08517; found 305.08512.
ACS 43: mp 74.4–75.4 °C. ^{1}H NMR ($CDCl_3$): δ = 5.96 (s, 1H, NH collapses with D_2O), 3.64 (t, J = 6.29 Hz, 2H, –NH–CH_2–), 3.37 (s, 3H, CH_3–SO_2–), 3.32 (t, J = 6.29 Hz, 2H, –CH_2–S–), 2.06 (m, 1H, CH), 1.68–1.14 (m, 8H, 2–CH_2–CH_2–), 0.9 (t, J = 7.15 Hz, 6H, 2CH_3–). HRMS (ESI) *m/z* calcd for $[M(C_{11}H_{23}NO_3S_2) + Na]^+$ 304.10116; found 304.10104.
32. HDAC activity was measured by using a fluorescence activity assay kit (Cayman Chemical, USA) as previously reported.[33] 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 **ADTOH**, **ACS 2** and **ADT** still exhibit a low absorbtion 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, **ADTOH**, **ACS 2**, or **ADT** were added. After 15 min, the fluorescence was measured. The inhibition of HDAC activity by **ADTOH**, **ACS 2**, or **ADT** was calculated taking this value as the 100% of activity. All experimental data were fitted and analysed by computer using a sigmoidal dose–response function (Sigma Plot, Jandel, CA).
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36. For cell proliferation assay, A549 cells (50,000 cell/well) were seeded onto 24-well tissue culture plates, incubated for 3 days in the absence or presence of increasing concentrations of the compounds, harvested and counted. For histone hyperacetylation analyses, A549 cells (approx. 100,000 cell/well) were seeded onto 12-well tissue culture plates and exposed to the compounds at a concentration of 150 μM for 24 h. At the end of

incubation, cells were lysed and analysed by Western blot using polyclonal anti-hyperacetylated histone H4 as previously described.[33] Data are normalized on the levels of total proteins and expressed as a percentage of control.

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