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CONCISE ARTICLE

Developing novel non-hydroxamate histone deacetylase inhibitors: the chelidamic warhead†‡

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Herein we reported two novel series of histone deacetylase inhibitors bearing the pyridine-2,6-dicarboxylate moiety as a zinc binding group. Tested on U937 leukemia cell line at 50 μM, compounds **4a**, **4c** and **4d** showed cell cycle block in the S phase and apoptotic induction up to 50%, whereas compound **6h** was able to give granulocytic differentiation up to 40%. From these results, the chelidamic scaffold will be further investigated to find more potent compounds.

Introduction

Post-translational histone modifications regulate gene expression independently from DNA sequence, and are closely related to epigenetic mechanisms.[1] The most common mechanisms of epigenetic regulation are methylation of the CpG islands at the DNA level, and covalent modifications of amino acids in the *N*-terminal histone tails. Among these, reversible histone acetylation[2] plays a crucial role in chromatin packaging and control of gene expression, and so far it has been studied most extensively.

Histone acetyltransferases transfer acetyl moieties to lysines in the *N*-terminal histone tails leading to a more open form of chromatin, euchromatin, associated with gene activation. The acetyl groups are in turn cleaved off by histone deacetylases (HDACs) leading to a more condensed form of chromatin, heterochromatin, and gene silencing.[3] In correlation with these properties, in recent years, it became evident that HDACs are promising therapeutic targets with the potential to reverse aberrant epigenetic states associated with cancer as well as non-cancer diseases.[4–6] About their action in cancer, HDAC inhibitors (HDACi) are described to be able to induce apoptosis, differentiation, cell cycle arrest, inhibition of DNA repair, upregulation of tumor suppressors, downregulation of growth factors, oxidative stress, and autophagy.[7–9]

So far a lot of effort has been put into the development of small molecules able to inhibit HDAC activity and, among them, vorinostat (suberoylanilide hydroxamic acid, SAHA) and romidepsin (FK-228) have been approved by the FDA for the treatment of refractory cutaneous T-cell lymphoma (CTCL).[10,11] In addition, valproic acid (VPA), panobinostat (LBH589), belinostat (PDX101), givinostat (ITF2357), resminostat (4SC-201), entinostat (MS-275), and mocetinostat (MGCD0103) (Fig. 1) are

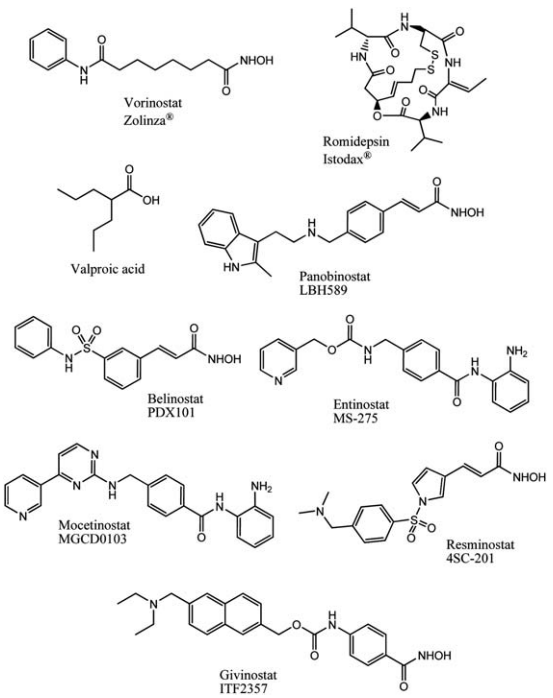

Fig. 1 HDACis approved by FDA and/or in clinical trials.

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† Electronic supplementary information (ESI) available: Chemical and physical data of compounds **4–7**, **9**, **13**, and **14**. Western Blot analyses of **4–7** for H3 histone and α-tubulin acetylation. Determination of apoptosis induction and cytodifferentiation activities on **4–7** at 50 μM in U937 cells. See DOI: 10.1039/c1md00249j

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into Phase II/III clinical trials for the therapy of hematological disorders as well as solid tumors.[9]

The pharmacophore model for HDACi includes a cap group (CAP) able to interact with the rim of the catalytic tunnel of the enzyme, a polar connection unit (CU) linked to a hydrophobic spacer (HS) which allows the molecule to lie into the tunnel, and a Zn-binding group (ZBG) able to complex the Zn^{2+} at the bottom of the cavity.[12,13]

To date a lot of different CAP, CU, HS and ZBG have been studied, and structurally diverse HDAC inhibitors have been recently reviewed.[14] However, among the ZBGs, the hydroxamic acid (HA) seems still to be the most efficient. In 2006, in an effort to identify potential non-HA ZBGs with both improved affinity and selectivity for the Zn^{2+} ion, Jacobsen *et al.* described several nitrogenous ligands, potentially selective for Zn^{2+} metalloproteins, as potent inhibitors of matrix metalloproteinases (MMPs).[15] All the reported compounds were more potent than acetohydroxamic acid (AHA) for MMP-1 and MMP-3.[15] Among these ligands, chelidamic acid **1** attracted our attention to design novel HDACi with different ZBGs from HA. In the last few years we published two series of HDACi, the uracil-based HAs (**2**, UBHAs)[16–19] and the *N*-hydroxy-(aroylaminophenyl)-acrylamides (**3**),[20,21] which showed high pro-apoptotic, cyto-differentiating and/or antiproliferative effects in cancer cells.[16,17,20–22] Thus we chose the CAP + CU moieties of **2** and **3** to develop novel small molecules with potential HDAC inhibition activity (compounds **4–6**, Fig. 2). In these new compounds, the HS is a differently sized oxypolimethylene chain, and the ZBG is represented by the chelidamic function. Furthermore, we also prepared the chelidamic–SAHA hybrid **7** (Fig. 2) for comparison purpose.

The new compounds were screened against human recombinant HDAC1 and HDAC4 to assess their inhibitory activities and selectivity. Functional assays for testing the capability of compounds **4–7** to increase the acetylation levels of histone H3 and α-tubulin in human leukemia U937 cells were performed. Moreover, in U937 leukemia cells the effects of **4–7** on cell cycle, apoptosis induction and granulocytic differentiation were established. SAHA and MS-275 were prepared and included in the assays as reference drugs.

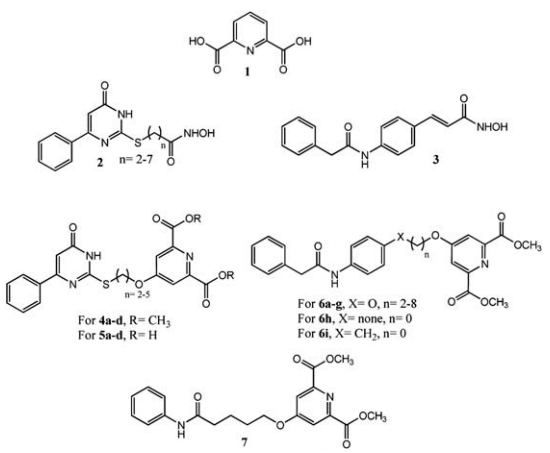


Fig. 2 Novel chelidamic-based HDAC inhibitors.

Chemistry

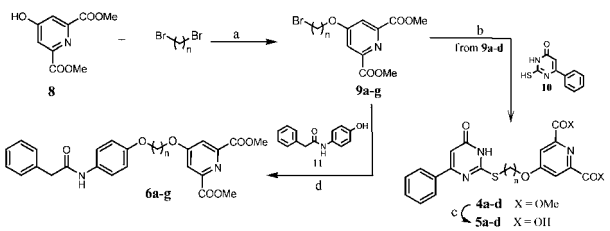
The chelidamic acid methyl esters **4a–d** and **6a–g** were prepared starting from the 4-hydroxychelidamic acid methyl ester **8**, which was treated with the opportune dibromoalkane in the presence of dry potassium carbonate to furnish the ω-bromoalkyloxy-chelidamic acid methyl esters **9a–g**. Alkylation of **9a–d** with the commercial 2-mercapto-6-phenylpyrimidin-4(3*H*)-one **10**, or alkylation of **9a–g** with the *N*-(4-hydroxyphenyl)-2-phenyl-acetamide **11**, in the presence of dry potassium carbonate yielded **4a–d** and **6a–g**, respectively (Scheme 1). Chelidamic acid derivatives **5a–d** were obtained by hydrolysis of **4a–d** with lithium hydroxide (Scheme 1). Compound **6h** was synthesized starting with the nucleophilic displacement of the 4-hydroxychelidamic acid methyl ester **8** on 4-fluoronitrobenzene **12** to afford the nitrophenyl-pyridyl ether **13**, which was in turn reduced into the corresponding amino derivative **14** by stannous chloride dehydrate, and 35% hydrochloric acid as a catalytic system. Further acylation of **14** with phenylacetyl chloride in the presence of triethylamine yielded the final compound **6h** (Scheme 2). Differently, the use of the Mitsunobu reaction between **8** and the commercial *N*-(4-(hydroxymethyl)phenyl)-2-phenylacetamide **15** with triphenylphosphine and diisopropylazodicarboxylate (DIAD) afforded the benzyl-pyridyl ether **6i** (Scheme 2). Finally, treating **8** with the 5-bromo-*N*-phenylpentanamide **16** and potassium carbonate the hybrid SAHA–chelidamic derivative **7** was obtained (Scheme 3).

Results and discussion

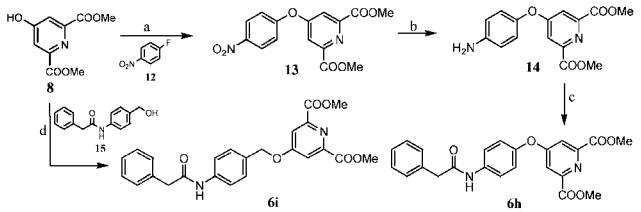
Human recombinant HDAC1 and HDAC4 assays

The novel chelidamic derivatives **4–7** were tested at 5 μM against human recombinant (hr) HDAC1 and HDAC4, using either the histone H3 or the non-histone trifluoroacetyl-lysine derivative[23] as substrates for HDAC1 or HDAC4, respectively. Table 1 shows the inhibitory activities of the tested chelidamic compounds against hrHDAC1 and hrHDAC4.

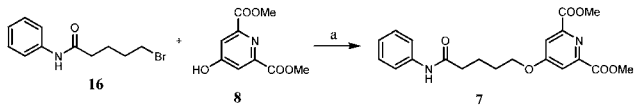
The uracil-based chelidamic derivatives **4** and **5** showed similar capability to inhibit both HDAC1 and HDAC4, with the tetra- and pentamethyleneoxy-chelidamic methyl esters **4c** and **4d** being the most potent derivatives against HDAC1. IC_{50} values determined for **4c** and **4d** against HDAC1 were 11.7 and 14.2 μM, respectively. Among the phenylacetamide series **6**, the majority of compounds inhibited more efficiently HDAC4 than HDAC1, showing the highest HDAC4-selectivity when their HSs were composed of 5 to 8 methylene units (**6d–g**). The complete elimination of the HS (**6h**) or the introduction of one methylene group without an oxygen atom (**6i**) led to compounds more



Scheme 1 *Reagents and conditions*: (a) K_2CO_3, CH_3CN, 80 °C, 3 h; (b): K_2CO_3, DMF, r.t., 2 h; (c): LiOH, H_2O, r.t., 30 min.



Scheme 2 *Reagents and conditions*: (a) K_2CO_3, CH_3CN, 80 °C, 3 h; (b): NaH, DMF, 90 °C, overnight; (c): $(Ph)_3P$, DIAD, dry THF, overnight; (d): $SnCl_2 \cdot 2H_2O$, 37% HCl, 0 °C, overnight; (e):TEA, CH_2Cl_2, 0 °C, 1.5 h.



Scheme 3 *Reagents and conditions*: (a) K_2CO_3, CH_3CN, 70 °C, 5 h.

Table 1 Human recombinant hrHDAC1 and hrHDAC4 inhibitory activity of compounds **4–7**[a]

			% of inhibition at 5 μM	
Cpd	X	*n*	hrHDAC1	hrHDAC4
4a	—	2	39	47
4b	—	3	29	45
4c	—	4	51	42
4d	—	5	55	28
5a	—	2	10	48
5b	—	3	39	41
5c	—	4	33	48
5d	—	5	48	45
6a	O	2	22	34
6b	O	3	31	43
6c	O	4	26	45
6d	O	5	0	38
6e	O	6	0	32
6f	O	7	0	28
6g	O	8	0	21
6h	None	0	46	37
6i	CH_2	0	33	25
7	—	—	42	11
SAHA			88	49

[a] Data represent mean values of at least three separate experiments.

potent against HDAC1 than against HDAC4. The hybrid SAHA–chelidamic derivative **7** was less active than SAHA against either HDAC1 or HDAC4, being 4-fold selective for HDAC1 over HDAC4.

Effects on the acetylation level of histone (histone H3) and non-histone (α-tubulin) substrates

As a functional test for HDAC inhibition we performed western blot analyses in human leukemia U937 cells with specific antibodies to determine the effects on the acetylation levels of histone H3 and α-tubulin for compounds **4–7**, tested at 50 μM for 24 h. SAHA and MS-275 were used at 5 μM as reference drugs. From these assays, only the uracil-based chelidamic esters **4a**, **4c** and **4d** showed an appreciable increase of histone H3 as well as α-tubulin acetylation (Fig. 3), while the other compounds displayed only faint signals (see ESI†).

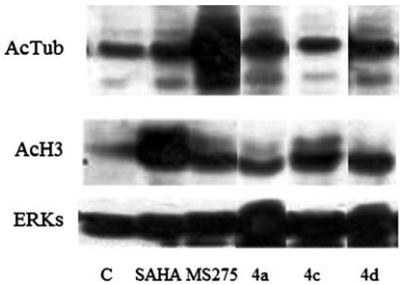


Fig. 3 Effects of compounds **4a**, **4c** and **4d** (50 μM, 24 h) on acetylation levels of histone H3 and α-tubulin, in U937 leukemia cells. Western blot analyses were performed with specific antibodies. ERKs were used for equal loading.

Cell cycle analysis, apoptosis induction, and granulocytic differentiation in human U937 leukemia cells

The described compounds **4–7** were tested at 50 μM for 30 h in human leukemia U937 cells to determine their effects on cell cycle, apoptosis induction, and granulocytic differentiation. SAHA and MS-275 (5 μM) were used as reference drugs.

About cell cycle effect (Fig. 4), compounds **4c** and **4d** as well as compounds **6a–i** and **7** displayed an arrest in the S phase, while compounds **5a–d** were ineffective, likely due to cell permeability defects.

The pro-apoptotic effects of these compounds were evaluated by the caspase 3–7 activation method. After treatment of U937 cells with compounds **4–7** at 50 μM for 30 h, only the uracil-based chelidamic esters **4a**, **4c** and **4d** showed high apoptosis induction (see ESI†), in accordance with the increased acetylation levels observed in the western blot analyses (see Fig. 3). In Fig. 5 the dose-dependent apoptosis induction by **4a**, **4c** and **4d** in U937 cells is reported.

As a marker of granulocytic differentiation in U937 leukemia cells, the expression of the surface antigen CD11c was determined. The cells were treated with compounds **4–7** at 50 μM for 30 h, and then the percent values of CD11c positive/propidium iodide (PI) negative cells were determined (see ESI†). In this assay, only the compound **6h**, lacking any HS in its structure, displayed high, dose-dependent granulocytic differentiation (Fig. 6).

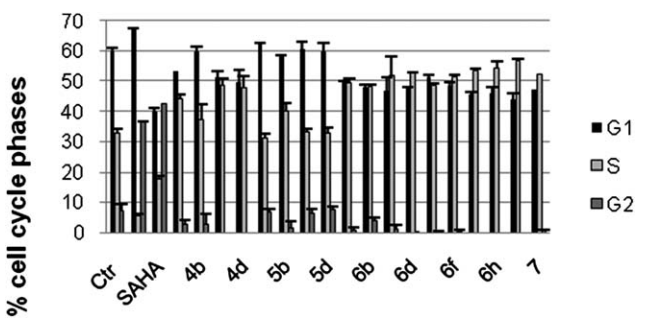


Fig. 4 Cell cycle analysis on U937 leukemia cells treated with compounds **4–7** at 50 μM for 30 h.

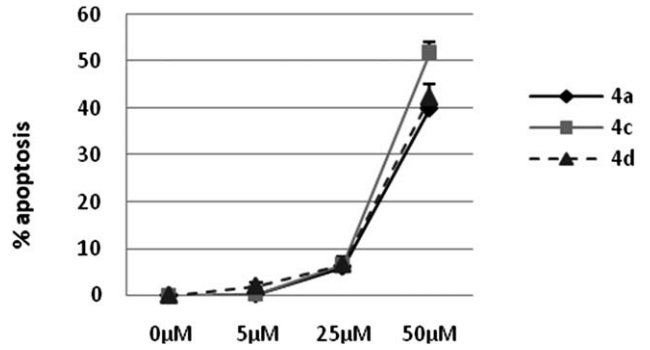


Fig. 5 Dose-dependent induction of apoptosis by **4a**, **4c** and **4d** in U937 cells for 30 h.

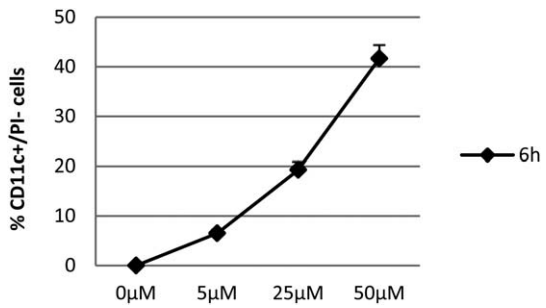


Fig. 6 Dose-dependent induction of cytodifferentiation by **6h** in U937 cells for 30 h.

Conclusions

In a search for new ZBGs different from hydroxamates and endowed with HDAC inhibiting activity, herein we described the synthesis and biological evaluation of novel dimethyl 4-(2-(6-oxo-4-phenyl-1,6-dihydropyrimidin-2-ylthio)alkoxy)pyridine-2,6-dicarboxylates **4a–d**, 4-(2-(6-oxo-4-phenyl-1,6-dihydropyrimidin-2-ylthio)ethoxy)pyridine-2,6-dicarboxylic acids **5a–d**, and dimethyl 4-(3-(4-(2-phenylacetamido)phenoxy)alkoxy)pyridine-2,6-dicarboxylates **6a–g** with their two shorter analogs dimethyl 4-(4-(2-phenylacetamido)phenoxy)pyridine-2,6-dicarboxylate **6h** and dimethyl 4-(4-(2-phenylacetamido)benzyloxy)pyridine-2,6-dicarboxylate **6i**. Furthermore, we prepared a SAHA analog **7** bearing a dimethyl 4-oxy-pyridine-2,6-dicarboxylate portion instead of the HA function.

The novel derivatives were tested against human recombinant HDAC1 and HDAC4 to evaluate their enzyme inhibiting activity. In addition, they were assayed in U937 leukemia cells to check their abilities to increase the level of histone H3 and α-tubulin acetylation, to block the cell cycle, and to induce apoptosis and granulocytic differentiation.

In enzyme assays, compounds **4c** and **4d** showed at 5 μM >50% inhibitory activity against HDAC1, **4d** being the most HDAC1-selective. Against HDAC4, the majority of the tested derivatives displayed >40% inhibitory activity, with **6d–g** being less potent (% inhibition of HDAC4 <40%) but selective for HDAC4 over HDAC1 (no activity at 5 μM against HDAC1, see Table 1). In histone H3 and α-tubulin acetylation assays, compounds **4a**, **4c** and **4d** showed increased acetylation levels. By the analysis of cell cycle in U937 cells, at 50 μM the uracil-based chelidamic esters **4c** and **4d** as well as the phenylacetamido derivatives **6** and the SAHA–chelidamic hybrid **7** gave an arrest in the S phase. Notably, chelidamic acids **5a–d**, despite their ability to inhibit HDAC1 and 4 activity in enzyme assays (see Table 1), did not show any effects when tested in cells, probably due to cell permeability problems.

Among the tested compounds, **4a**, **4c** and **4d** displayed a dose-dependent apoptosis induction in U937 cells (see Fig. 5), in accordance with their increased histone H3 acetylation. In cytodifferentiation assay, the dimethyl 4-(4-(2-phenylacetamido) phenoxy)pyridine-2,6-dicarboxylate **6h** was the only compound, among the tested **4–7**, able to furnish a dose-dependent increase of the expression of the surface antigen CD11c (see Fig. 6).

From these data, the chelidamic scaffold seems to be able to replace the hydroxamate moiety in HDAC inhibitor design. Further chemical investigations must be performed to address this point.

Experimental section

Chemistry

Melting points were determined on a Buchi 530 melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz on a Bruker AC 400 spectrometer; chemical shifts are reported in δ (ppm) units relative to the internal reference tetramethylsilane (Me_4Si). EIMS spectra were recorded with a Fisons Trio 1000 spectrometer; only molecular ions (M^+) and base peaks are given. All compounds were routinely checked by TLC, ^{1}H NMR and ^{13}C NMR spectra. TLC was performed on aluminium-backed silica gel plates (Merck DC, Alufolien Kieselgel 60 F254) with spots visualized by UV light. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. The concentration of solutions after reactions and extractions involved the use of a rotary evaporator operating at a reduced pressure of *ca.* 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Analytical results are within ±0.40% of the theoretical values. All chemicals were purchased from Aldrich Chimica, Milan (Italy), or from Alfa Aesar, Milan (Italy), and were of the highest purity.

General procedure for the synthesis of the dimethyl 4-(2-bromoalkoxy)pyridine-2,6-dicarboxylates (9a–g). Example: synthesis of dimethyl 4-(3-bromopropoxy)pyridine-2,6-dicarboxylate (9b)

Dimethyl 4-hydroxypyridine-2,6-dicarboxylate **8** (3.67 mmol, 0.77 g) was added to a suspension of anhydrous potassium carbonate (7.34 mmol, 1.01 g) in acetonitrile (10 mL), and then 1,3-dibromopropane (18.35 mmol, 1.86 mL) was slowly added. The resulting mixture was stirred at 80 °C for 3 hours, the reaction was quenched with water (50 mL) and extracted with ethyl acetate (3 × 50 mL), the organic phases were washed with sodium carbonate 2 N solution (3 × 50 mL) and with sodium chloride solution (3 × 50 mL), dried with anhydrous sodium sulfate and concentrated. The oily residue was chromatographed on silica gel eluting with acetate/chloroform 1 : 5 to afford the pure **9b** as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz, δ; ppm) δ 2.45 (m, 2H, $CH_2CH_2CH_2$), 3.57 (t, 2H, $BrCH_2$), 3.98 (s, 6H,

COOCH_3), 4.28 (t, 2H, OCH_2), 7.80 (s, 2H, pyridine protons) ppm; ^{13}C NMR ($CDCl_3$, 400 MHz, δ; ppm) δ 29.9, 32.9, 51.5 (2C), 67.1, 112.8 (2C), 148.5 (2C), 161.9, 165.4 (2C) ppm; MS (EI): *m*/*z* $[M]^+$: 331.0055.

General procedure for the synthesis of the dimethyl 4-[4-(6-oxo-4-phenyl-1,6-dihydropyrimidin-2-ylthio)alkoxy]pyridine-2,6-dicarboxylates (4a–d). Example: synthesis of dimethyl 4-[4-(6-oxo-4-phenyl-1,6-dihydropyrimidin-2-ylthio)butoxy]pyridine-2,6-dicarboxylate (4c)

2-Mercapto-6-phenylpyrimidin-4-(3*H*)-one **10** (1.03 mmol, 0.21 g) was added to a suspension of anhydrous potassium carbonate (1.13 mmol, 0.16 g) in dry *N*,*N*-dimethylformamide (2 mL), and dimethyl 4-(4-bromobutoxy)pyridine-2,6-dicarboxylate **9c** (1.03 mmol, 0.36 g) was added to the stirring mixture. The resulting suspension was stirred at room temperature for 2 hours. The reaction was then quenched with water (50 mL) and the precipitate was filtered, washed with water (3 × 30 mL), and dried to give the pure **4c** as a white solid. ^{1}H NMR (DMSO-*d*6, 400 MHz, δ; ppm) δ 1.87 (m, 4H, $CH_2CH_2CH_2CH_2$), 3.25 (t, 2H, SCH_2), 3.85 (s, 6H, COOCH_3), 4.22 (t, 2H, OCH_2), 6.56 (s, 1H, pyrimidinone proton), 7.39 (m, 3H, benzene protons), 7.64 (s, 2H, pyridine protons), 7.98 (m, 2H, benzene protons) ppm; ^{13}C NMR ($CDCl_3$, 400 MHz, δ; ppm) δ 26.0, 26.7, 28.2, 51.5 (2C), 68.0, 110.5, 112.8 (2C), 126.4 (2C), 127.9, 128.6 (2C), 136.8, 148.5 (2C), 156.3, 160.8, 161.9, 165.4 (2C), 166.5 ppm; MS (EI): *m*/*z* $[M]^+$: 469.1308.

General procedure for the synthesis of the 4-[4-(6-oxo-4-phenyl-1,6-dihydropyrimidin-2-ylthio)alkoxy]pyridine-2,6-dicarboxylic acids (5a–d). Example: synthesis of 4-[4-(6-oxo-4-phenyl-1,6-dihydropyrimidin-2-ylthio)pentoxy]pyridine-2,6-dicarboxylic acid (5d)

A lithium hydroxide (0.87 mmol, 0.036 g) solution in water (5 mL) was added to a solution of dimethyl 4-[4-(6-oxo-4-phenyl-1,6-dihydropyrimidin-2-ylthio)pentoxy]pyridine-2,6-dicarboxylate **4d** (0.29 mmol, 0.14 g) in tetrahydrofuran (5 mL), and the final solution was stirred at room temperature for 30 min. Then a 2 N HCl solution (10 mL) was added dropwise to the reaction and the precipitate was filtered, washed with water (3 × 30 mL), and dried to furnish the pure **5d**. ^{1}H NMR (DMSO-*d*6, 400 MHz, δ; ppm) δ 1.55 (m, 2H, $CH_2CH_2CH_2CH_2CH_2$), 1.77 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$), 3,24 (t, 2H, SCH_2), 4.18 (t, 2H, OCH_2), 6.63 (s, 1H, pyrimidinone proton), 7.44 (m, 3H, benzene protons), 7.63 (s, 2H, pyridine protons), 8.01 (m, 2H, benzene protons), 12.7 (bs, 2H, COO*H*) ppm; ^{13}C NMR (DMSO-*d*6, 400 MHz, δ; ppm) δ 24.5, 26.0, 28.9, 32.0, 68.7, 110.5, 112.8 (2C), 126.4 (2C), 127.9, 128.6 (2C), 136.8, 148.5 (2C), 156.3, 160.8, 161.9, 166.5, 168.3 (2C) ppm; MS (EI): *m*/*z* $[M]^+$: 455.1151.

General procedure for the synthesis of the dimethyl 4-[5-(4-(2-phenylacetylamino)phenoxy)alkyloxy]pyridine-2,6-dicarboxylates (6a–g). Example: synthesis of dimethyl 4-[5-(4-(2-phenylacetylamino)phenoxy)heptyloxy]pyridine-2,6-dicarboxylate (6f)

N-(4-Hydroxyphenyl)-2-phenylacetamide **11** (0.790 mmol, 0.192 g) was added to a suspension of anhydrous potassium carbonate (0.950 mmol, 0.131 g) and dimethyl 4-(7-bromoheptyloxy)pyridine-2,6-dicarboxylate **9f** (0.790 mmol, 0.320 g) in acetonitrile (10 mL), and the resulting mixture was stirred at 80 °C for 3 hours. The reaction was quenched with water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The organic phases were washed with sodium carbonate 2 N solution (3 × 50 mL) and sodium chloride solution (3 × 50 mL), dried with anhydrous sodium sulfate and concentrated. The residue was chromatographed on silica gel eluting with acetate/chloroform 1 : 5 to afford the pure **6f** as a white solid. ^{1}H NMR (DMSO-*d*6, 400 MHz, δ; ppm) δ 1.50–1.55 (m, 2H, $CH_2CH_2CH_2CH_2CH_2$), 1.74–1.84 (m, 4H, $CH_2CH_2CH_2CH_2CH_2$), 3.55 (s, 2H, PhCH_2CO), 3.86 (s, 6H, COOCH_3), 3.90 (t, 2H, PhOCH_2), 4.21 (t, 2H, PyrOCH_2), 6.82 (d, 2H, benzene protons), 7.23–7.29 (m, 5H, benzene protons), 7.44 (d, 2H, benzene protons), 7.69 (s, 2H, pyridine protons), 9.96 (s, 1H, N*H*) ppm; ^{13}C NMR (DMSO-*d*6, 400 MHz, δ; ppm) δ 25.6, 25.9 (2C), 29.6 (2C), 44.5, 51.5 (2C), 68.7 (2C), 112.8 (2C), 114.6 (2C), 122.2 (2C), 127.6, 129.2 (2C), 129.6 (2C), 130.1, 135.6, 148.5 (2C), 155.0, 161.9, 165.4 (2C), 168.9 ppm; MS (EI): *m*/*z* $[M]^+$: 534.2366.

Synthesis of dimethyl 4-[4-(2-phenylacetamido)benzyloxy]pyridine-2,6-dicarboxylate (6i)

A solution of **8** (1.36 mmol, 0.29 g), *N*-[4-(hydroxymethyl)phenyl]-2-phenylacetamide **15** (1.24 mmol, 0.3 g) and triphenylphosphine (1.36 mmol, 0.36 g) in dry tetrahydrofuran (10 mL) was cooled to 0 °C, and diisopropylazodicarboxylate (1.36 mmol, 0.25 mL) was slowly added. The resulting solution was stirred at room temperature for 12 h, then the reaction was quenched with water (100 mL) and extracted with ethyl acetate (3 × 50 mL). The collected organic phases were washed with saturated sodium chloride solution, dried over anhydrous sodium sulfate, filtered and concentrated under vacuum. The residue was purified by column chromatography on silica gel 60 eluting with ethyl acetate/*n*-hexane 1 : 4 to obtain the pure **6i** ^{1}H NMR (DMSO-*d*6, 400 MHz, δ; ppm) δ 3.65 (s, 2H, PhCH_2CONH), 3.91 (s, 6H, OCH_3), 5.30 (s, 2H, CH_2O) 7,25–7.26 (m, 1H, benzene proton), 7.33–7.34 (m, 4H, benzene protons), 7.41–7.43 (m, 2H benzene protons), 7.63–7.65 (m 2H benzene protons), 7.85 (s, 2H pyridine protons), 10.25 (s 1H $PhCH_2CONH$) ppm; ^{13}C NMR (DMSO-*d*6, 400 MHz, δ; ppm) δ 44.5, 51.5 (2C), 70.8, 112.8 (2C), 121.8 (2C), 127.6, 128.7 (2C), 129.2 (2C), 129.6 (2C), 132.3, 135.6, 137.4, 148.5 (2C), 161.9, 165.4 (2C), 168.9 ppm; MS (EI): *m*/*z* $[M]^+$: 434.1478.

Synthesis of dimethyl 4-(4-nitrophenoxy)pyridine-2,6-dicarboxylate (13)

A solution of **8** (5.68 mmol, 1.20 g) in dry *N*,*N*-dimethylformamide (10 mL) was slowly added to a suspension of sodium hydride 60% in mineral oil (8.52 mmol, 0.34 g) in dry *N*,*N*-dimethylformamide at 0 °C. After 15 min, 4-fluoro-nitrobenzene **12** (5.68 mmol, 0.60 mL) was added, and the mixture was placed into a sealed tube and stirred at 110 °C for 1 day, then the reaction was quenched by water (100 mL) and extracted with ethyl acetate (3 × 50 mL). The collected organic phases were washed with saturated sodium chloride solution, dried over anhydrous sodium sulfate, filtered and concentrated under

vacuum. The residue was purified by column chromatography on silica gel 60 eluting with ethyl acetate/chloroform 1/5 to afford the pure **13**. ^{1}H NMR (DMSO-*d*6, 400 MHz, *δ*; ppm) *δ* 3.91 (s, 6H, OCH_3), 7.51–7.53 (d, 2H, benzene protons), 7.84 (s, 2H pyridine protons), 8.36–8.39 (d, 2H, benzene protons) ppm; ^{13}C NMR (DMSO-*d*6, 400 MHz, *δ*; ppm) *δ* 51.5 (2C), 115.4 (2C), 120.0 (2C), 125.9 (2C), 143.8, 147.3 (2C), 161.3, 165.4 (2C), 167.1 ppm; MS (EI): *m*/*z* $[M]^+$: 332.0645.

Synthesis of dimethyl 4-(4-aminophenoxy)pyridine-2,6-dicarboxylate (14)

A suspension of **13** (1.5 mmol, 0.5 g), stannous chloride dihydrate (5.33 mmol, 1.2 g) and 37% hydrochloric acid (1 mL) in ethanol (5 mL) was stirred at room temperature overnight. The reaction was neutralized by 2 N sodium hydroxide solution till basic pH and extracted with ethyl acetate (3 × 20 mL). The collected organic solutions were washed with water (3 × 20 mL), dried with sodium sulfate and concentrated to obtain **14** as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz, *δ*; ppm) *δ* 3.85 (bs, 2H, NH_2), 4.00 (s, 6H, OCH_3), 6.73–6.75 (d, 2H, benzene protons), 6.90–6.92 (d, 2H, benzene protons), 7.79 (s, 2H, pyridine protons) ppm; ^{13}C NMR ($CDCl_3$, 400 MHz, *δ*; ppm) *δ* 51.5 (2C), 115.4 (2C), 120.0 (2C), 123.10 (2C), 142.4, 144.3, 147.3 (2C), 165.4 (2C), 167.1 ppm; MS (EI): *m*/*z* $[M]^+$: 302.0903.

Synthesis of dimethyl 4-[4-(2-phenylacetamido)phenoxy] pyridine-2,6-dicarboxylate (6h)

Phenylacetyl chloride (1.09 mmol, 0.14 mL) in dry dichloromethane (10 mL) was slowly added to a solution of **14** (0.99 mmol, 0.3 g) and triethylamine (1.19 mmol, 0.16 mL) in dry dichloromethane on an ice-bath. The final solution was stirred at room temperature for 1 h, then the reaction was quenched by water (50 mL) and extracted with dichloromethane (3 × 20 mL). The organic phases were washed with water (3 × 20 mL), dried with sodium sulfate and concentrated to furnish **6h** that was purified by column chromatography on silica gel 60 eluting with ethyl acetate/chloroform 1/3. ^{1}H NMR (DMSO-*d*6, 400 MHz, *δ*; ppm) *δ* 3.67 (s, 2H, $PhCH_2CO$), 3.89 (s, 6H, OCH_3), 7.15–7.35 (m, 7H, benzene protons), 7.64 (s, 2H, pyridine protons), 7.74–7.76 (d, 2H, benzene protons), 10.35 (s, 1H, N*H*) ppm; ^{13}C NMR (DMSO-*d*6, 400 MHz, *δ*; ppm) *δ* 44.5, 51.5 (2C), 115.4 (2C), 119.4 (2C), 122.6 (2C), 127.6, 129.2 (2C), 129.6 (2C), 134.4, 135.6, 147.3 (2C), 148.0, 165.4 (2C), 167.1, 168.9 ppm; MS (EI): *m*/*z* $[M]^+$: 420.1321.

Synthesis of dimethyl 4-[5-oxo-5-(phenylamino)pentyloxy] pyridine-2,6-dicarboxylate (7)

5-Bromo-*N*-phenylpentanamide **16** (1.37 mmol, 0.350 mg) was added to a suspension of anhydrous potassium carbonate (1.64 mmol, 0.227 mg) and dimethyl 4-hydroxypyridine-2,6-dicarboxylate **8** (1.37 mmol, 0.290 mg) in acetonitrile (10 mL). The resulting mixture was stirred at 70 °C for 5 hours. Afterwards, water (50 mL) was added to the reaction, the aqueous phase was extracted with ethyl acetate (3 × 50 mL), and the organic phases were washed with sodium carbonate 2 N solution (3 × 50 mL) and with sodium chloride solution (3 × 50 mL), dried with anhydrous sodium sulfate, and concentrated. The residue was chromatographed on silica gel eluting with acetate/chloroform 1 : 1 to afford the pure **7** as a white solid. ^{1}H NMR (DMSO-*d*6, 400 MHz, *δ*; ppm) *δ* 1.72–1.78 (m, 4H, $CH_2CH_2CH_2CH_2$), 2.35 (t, 2H, $COCH_2$), 3.86 (s, 6H, $COOCH_3$), 4.22 (t, 2H, $PyROCH_2$), 6.99 (t, 1H, benzene protons), 7.23 (t, 2H, benzene protons), 7.53 (d, 2H, benzene protons), 7.70 (s, 2H, pyridine protons), 9.80 (s, 1H, N*H*) ppm; ^{13}C NMR (DMSO-*d*6, 400 MHz, *δ*; ppm) *δ* 21.9, 27.6, 38.3, 51.5 (2C), 68.0, 112.8 (2C), 121.6 (2C), 128.0, 128.9 (2C), 138.5, 148.5 (2C), 161.9, 165.4 (2C), 179.8 ppm; MS (EI): *m*/*z* $[M]^+$: 386.1478.

Fluorimetric human recombinant HDAC1 and HDAC4 assays

HDAC1 and 4 assays. The HDAC Fluorescent Activity Assay for HDAC1 and HDAC4 is based on the Fluor de Lys Substrate and Developer combination (BioMol), and has been carried out according to supplier's instructions and as previously reported. First, the inhibitors and purified recombinant HDAC1 or HDAC4 enzymes were pre-incubated at room temperature for 15 minutes before substrate addition, the Fluor de Lys Substrate, which comprises an acetylated lysine side chain. For HDAC4 assay, the HDAC4-selective, non-histone substrate reported by Lahm *et al.*[23] has been used. Full length HDAC1 and HDAC4 with the *C*-terminal His tag were expressed using baculovirus systems. Deacetylation sensitizes the substrates that, in the second step, treated with the developer produce a fluorophore. Fluorescence has been quantified with a TECAN Infinite M200 station.

Cellular assays. cell lines and cultures. U937 cell line was cultured in RPMI with 10% fetal calf serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 250 ng mL^{-1} amphotericin-B, 10 mM HEPES and 2 mM glutamine. U937 cells were kept at the constant concentration of 200 000 cells per millilitre of culture medium.

Histone H3 and α-tubulin acetylation in U937 cells. For quantification of histone H3 acetylation, 5 μg of total histone extracts were separated on a 15% polyacrylamide gel and blotted as described.[24] Western blots were shown for acetylated histone H3 (Upstate), and ERKs (Santa Cruz) were used to normalise for equal loading. For determination of α-tubulin acetylation, 25 μg of total protein extracts were separated on a 10% polyacrylamide gel and blotted as described.[25] Western blots were shown for acetylated α-tubulin (Sigma) and total ERKs (Santa Cruz) were used to normalize for equal loading.

Cell cycle analysis on U937 cells. 2.5×10^5 cells were collected and resuspended in 500 μL of a hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate, 50 μg mL^{-1} propidium iodide (PI), RNAse A). Cells were incubated in the dark for 30 min. Samples were acquired on a FACS-Calibur flow cytometer using the Cell Quest software (Becton Dickinson) and analysed with standard procedures using the Cell Quest software (Becton Dickinson) and the ModFit LT version 3 Software (Verity) as previously reported.[25] All the experiments were performed 3 times.

FACS analysis of apoptosis on U937 cells. Apoptosis was measured with the caspase 3–7 detection (B-Bridge) method;

samples were analysed by FACS with Cell Quest technology (Becton Dickinson) as previously reported.[26]

Granulocytic differentiation on U937 cells. Granulocytic differentiation was carried out as previously described.[26] Briefly, U937 cells were harvested and resuspended in 10 μL phycoerythrine-conjugated CD11c (CD11c-PE). Control samples were incubated with 10 μL PE conjugated mouse IgG1, incubated for 30 min at 4 °C in the dark, washed in PBS and resuspended in 500 μL PBS containing PI (0.25 μg mL^{-1}). Samples were analysed by FACS with Cell Quest technology (Becton Dickinson). PI positive cells have been excluded from the analysis.

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