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Novel uracil-based 2-aminoanilide and 2-aminoanilide-like derivatives: Histone deacetylase inhibition and in-cell activities

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Abstract—A novel series of non-hydroxamate HDAC inhibitors (HDACi) showing a uracil group at the left and a 2-aminoanilide/2-aminoanilide-like portion at the right head have been reported. In particular, the new compounds incorporating a 2-aminoanilide moiety behaved as class I-selective HDACi. Compound **8**, the most potent and class I-selective, showed weak apoptosis (higher than MS-275) joined to cytodifferentiating activity on U937 cells. Surprisingly, the highest differentiation was observed with **13**, through an effect that seems to be unrelated to HDAC inhibition.

Chromatin remodelling is involved in the epigenetic regulation of gene expression.[1,2] This mechanism includes a growing number of families of enzymes that catalyse several covalent modifications (acetylation/deacetylation, methylation/demethylation, phophorylation, ubiquitilation, ADP-ribosylation, etc.) at the histone tails of nucleosomal histones H3 and H4.[3,4] In particular, histone deacetylases (HDACs) catalyse the removal of the *N*-acetyl lysine residues from the histone tails, thus changing the accessibility of transcription factors to DNA and switching the chromatin in its heterochromatin form, transcriptionally silent.[5–7] The inhibition of HDACs restores the recruitment of transcription factors to DNA and silences the growth of cancer cells by inducing pathways that lead to growth arrest, terminal differentiation and apoptosis, both in in vitro and in in vivo assays.[8–11] In humans, 18 HDACs have been identified and grouped into four classes according to their homology with the yeast deacetylases. Class I, II and IV HDACs are zinc-binding enzymes, whereas class III HDACs (sirtuins) have a NAD^+-dependent mechanism of deacetylation, do not share any homology with class I, II, IV HDACs, are not sensitive to class I, II, IV HDAC inhibitors (HDACi), and do not have histones as primary targets. Class I (HDAC1–3, 8) and class II (HDAC4–7, 9, 10) HDACs also deacetylate a growing number of non-histone proteins in addition to histones such as transcription factors and proteins related to cell proliferation, differentiation, migration and death.[8–11] Class I and II HDACs are generally considered valuable therapeutic targets for the treatment of leukemia and solid tumors,[8,11] and suberoylanilide hydroxamic acid (SAHA) (Fig. 1) is the first class I/II HDACi approved

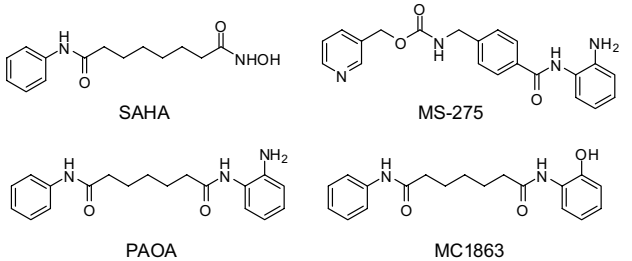

Figure 1. HDACi belonging to the hydroxamate (SAHA) or 2-aminoanilide/2-aminoanilide-like (MS-275, PAOA and MC1863) series.

Keywords: Chromatin remodelling; Histone deacetylase; Aminoanilide; Non-hydroxamate; Apoptosis; Cytodifferentiation.
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by the FDA in October 2006 for the treatment of cutaneous T-cell lymphoma (CTCL), and it is currently in clinical trial of Phase II or III (alone or in combination) for the treatment of a great number of tumors.[12,13] Class I HDACs are ubiquitary, primarily with nuclear localisation, and often act as transcriptional co-repressors, whereas class II HDACs have a tissue-specific expression and can shuttle between nucleus and cytoplasm. Mounting evidences suggest distinct roles for different classes of HDACs. Class I HDAC overexpression has been reported for human gastric, prostate (HDAC1) and colon tumors (HDAC3),[14–16] whereas the knockdown of HDAC1 and HDAC3 (but not HDAC4 and HDAC7) with siRNA in HeLa cells deeply inhibited proliferation and induced apoptosis.[17] Moreover, by combining the use of class-selective HDACi with the siRNA technique, Cohen et al. reported that inhibition of HDAC1 and HDAC2 but not HDAC3, HDAC6 and HDAC8 are primarily responsible for sensitisation to TRAIL-induced apoptosis in Jurkat, K562, U937, DU145 and CLL cell lines.[18] On the other hand, Altucci and co-workers clearly demonstrated that HDAC1 and HDAC2 reside on the TRAIL promoter in leukemia cells thus contributing with its aberrant silencing in tumors.[19,20] Finally, siRNA-mediated knockdown of HDAC1 or HDAC3 but not HDAC2 (unless the concomitant ablation of both HDAC1 and HDAC2 was made) led to an inhibition of cell proliferation in U2OS, MCF7 and MCF10A cancer cell lines.[21] Thus, the development of class I-selective HDACi seems to be a tool to evaluate if anticancer agents with improved efficacy and weaker toxicity profile compared to pan-HDACi could be obtained.[22]

MS-275 (Fig. 1) is a class I-selective HDACi from the Bayer Schering AG Pharma group, that inhibits tumor growth in vivo and promotes differentiation or apoptosis following the induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, the apoptotic TRAIL and the generation of ROS species.[23,24] In the U937 cell line, MS-275 showed an enzymatic inhibition profile deeply different from that of SAHA (Table 1).[25,26]

The main chemical difference between the two HDACi is the zinc-binding group,[27] which is the hydroxamate in SAHA[28] and probably the 2-aminoanilide portion in MS-275.[29] Thus, we prepared (see Supplementary material for chemical and physical data) and tested in both enzyme and cellular assays the pimeloylanilide orthoaminoanilide (PAOA) (Fig. 1), described by Wong et al.[30] as class-I selective HDACi, and its 2-hydroxyanilide analogue MC1863 (Fig. 1), as 2-aminoanilide/2-aminoanilide-like analogues of a SAHA homologue. In our hands, when tested in the U937 cells, PAOA and MC1863 at 5 μM shared a inhibiting behaviour similar to MS-275: they were active against HDAC1 [% inhibition: 28.9 (PAOA), 31.7 (MC1863), 47.8 (SAHA), 43.7 (MS-275)] but not against HDAC4 [% inhibition: 0 (PAOA), 7.9 (MC1863), 31.4 (SAHA), 0 (MS-275)], they both induced histone H3 and H4 (PAOA only) but not α-tubulin hyperacetylation (Fig. 2), and they were highly efficient cytodifferentiating agents [% CD11c positive cells: 18.3 (PAOA), 36.6 (MC1863), 6.6 (SAHA), 30.8 (MS-275)], but very weak if at all pro-apoptotic agents after 30 h of treatment (Fig. S1 in Supplementary material).

Recently we reported a new series of uracil-based hydroxamates (UBHAs) as novel HDACi.[25,31–34] Two of them (MC1641 and MC1751, **1d** and **1j** in Ref. 25, respectively) were active against HDAC1 but not against HDAC4, and were able to highly increase the levels of both acetyl-H3 and acetyl-α-tubulin. In cellular assays (U937 cells), they displayed some differentiation activity and low apoptosis.[25]

Following our researches on HDACi,[25,26,31–41] with the aim to increase the class-I selectivity of our uracil derivatives to obtain more efficient compounds in the cellular tests, we designed and synthesised a new series of uracil-based 2-aminoanilide and 2-aminoanilide-like derivatives **1**–**13** (Fig. 3), and we determined, in addition to the anti-HDAC activity, their effects on histone H3 and α-tubulin acetylation in the human leukemia U937 cell line. Moreover, $p21^{WAF1/CIP1}$ induction as well as the activities of the compounds **1**–**13** on cell cycle, apoptosis induction, and granulocytic differentiation in the U937 cells have been assayed. The 2-aminoanilide and 2-aminoanilide-like derivatives **1**–**13** were synthesised from the uracil-containing carboxylic acids **24**–**29** by treatment with benzotriazole-1-yl-oxy-tris-(dimethyl-

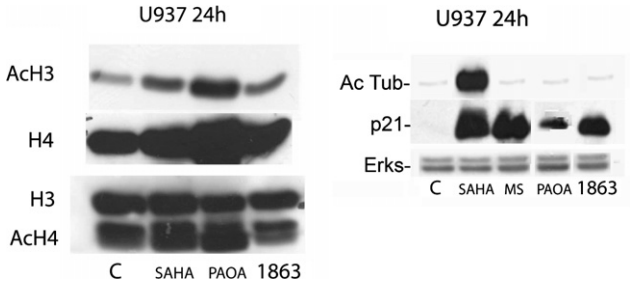


Figure 2. Effects of PAOA and MC1863 on histone H3/H4 and α-tubulin acetylation and on p21 induction.

Table 1. Biological properties of SAHA and MS-275 in the human leukemia U937 cell line[a]

Compound	HDAC1 inhbtn[b]	HDAC4 inhbtn[b]	Ac-H3	Ac-α-tubulin	p21 induct	Apoptosis induct[c]	Granuloc different[d]
SAHA	+++	++	+++	+++	+++	+++	+
MS-275	++	±	++	—	+++	+	+++

[a] See Refs. 25 and 26.
[b] Assay performed on immunoprecipitate (IP).
[c] Measured by the Annexin V/PI method.
[d] Measured by the CD11c expression.

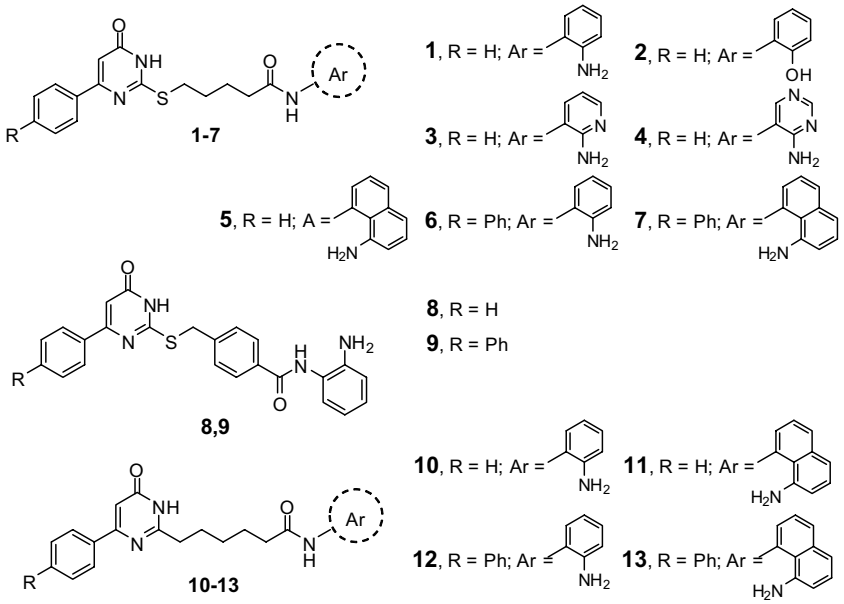
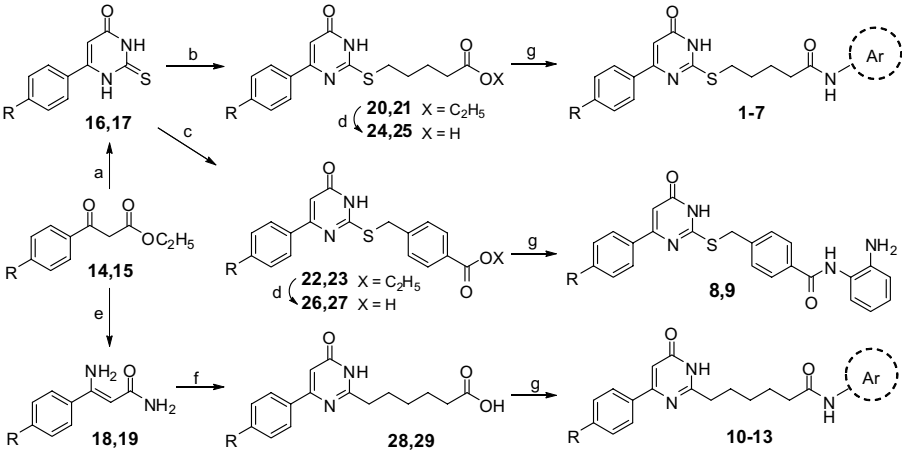


Figure 3. Novel uracil-based 2-aminoanilide and 2-aminoanilide-like derivatives **1**–**13**.

amino)-phosphonium hexafluorophosphate (Bop-reagent) and the appropriate aromatic amine in the presence of triethylamine. Alkaline hydrolysis of the ethyl 5-(6-oxo-1,6-dihydropyrimidin-2-ylthio)pentanoates **20**[25] and **21**, and of the ethyl 4-((6-oxo-1,6-dihydropyrimidin-2-ylthio)methyl)benzoates **22** and **23** yielded the acids **24**,[25] **25**, **26** and **27**, respectively. Condensation of the 3-amino-3-phenylacrylamides **18** and **19**, readily obtained by the reaction of β-oxoesters **14** [25] and **15** with ammonium hydroxide, with diethyl pimelate in the presence of sodium ethoxide furnished the 6-(6-oxo-1,6-dihydropyrimidin-2-yl)hexanoic acids **28** and **29**. The ethyl esters **20**–**23** were prepared by reaction the of ethyl ω-bromovalerate or ethyl 4-(bromomethyl)benzoate and the 6-substituted-2-thiouracils **16**[25] and **17**, in turn obtained by the condensation of the appropriate β–oxoesters **14** and **15** with thiourea (Scheme 1).[42]

Compounds **1**–**13** were tested at 5 μM against human recombinant (hr) HDAC1 and HDAC4 enzymes, in comparison with MS-275 and SAHA at 5 μM (Table 2). By the analysis of the data reported in Table 2, we can draw the following structure-activity relationships (SAR): (i) amongst the aromatic amines linked at the right head of the uracil-based molecules, only the 1,2-phenylenediamine and 2-hydroxyaniline were able to elicit the HDAC inhibiting effect of the compounds (**1**, **2**, **6**, **8**–**10**, **12**), whereas the introduction at the same position of 2,3-diaminopyridine, 4,5-diaminopyrimidine, or naphthalene-1,8-diamine was not effective (see compounds **3**–**5**, **7**, **11** and **13**). In addition, (ii) the replacement of the phenyl group at the C6 position of the uracil ring with a 4-biphenyl moiety did not influence (compare **1** with **6** and **8** with **9**) or gave a little decrease (compare **10** and **12**) of the HDAC1 inhibiting capacity,



Scheme 1. Reagents and conditions; (a) EtONa, thiourea, EtOH, reflux, 5–6 h, 70–80%; (b) Ethyl ω-bromovalerate, K_2CO_3, DMF, rt, 1 h, 50%; (c) Ethyl 4-(bromomethyl)benzoate, K_2CO_3, DMF, rt, 1 h, 51–55%; (d) 2 N KOH, EtOH, H_2O, rt, 18 h, 90–94%; (e) NH_4OH 28%, 120 °C, 2–3 h, 66–70%; (f) diethyl pimelate, EtONa, EtOH, reflux, 6 h, 90–95%; (g) i—BOP-reagent, Et_3N, dry DMF, N_2, rt, 0.5 h; ii—aromatic amine, rt, 0.5–1 h, 40–60%.

Table 2. Human recombinant HDAC1 and HDAC4 inhibitory activities of the new uracil-based compounds **1**–**13** at 5 μM[a]

Compound	% Residual activity	
	HDAC1	HDAC4
1	47.2	97.0
2	74.3	50.7
3	99.3	99.4
4	96.7	110.7
5	84.7	97.0
6	44.3	57.5
7	89.2	92.5
8	16.0	88.8
9	12.3	33.8
10	17.7	59.2
11	91.1	100.8
12	32.6	48.5
13	92.1	96.5
SAHA	6.7	61.7
MS-275	7.3	87.6

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

but significantly increased the HDAC4 inhibiting action of the derivatives (the effect is particularly clear with **1** → **6** and **8** → **9**, and less evident with **10** → **12**), thus abating the class I-selectivity of the compounds. Finally, the use of the 4-methylbenzamide moiety (compounds **8** and **9**) instead of the pentanamide chain (compounds **1** and **6**) as a spacer connecting the uracil portion with the 2-aminoaniline group highly increased the anti-HDAC1 activity of the derivatives with no or little influence on HDAC4, whereas the replacement of the sulphur atom (in **1** and **6**) with the isoster methylene unit (in **10** and **12**) improved the HDAC1 as well as the HDAC4 inhibiting action of the compounds, thus lowering the class I-selectivity. In conclusion, the most active and class I-selective derivative amongst those prepared in this study was the *N*-(2-aminophenyl)-4-((6-oxo-4-phenyl-1,6-dihydropyrimidin-2-ylthio)methyl)-benzamide **8**, which showed an activity/selectivity similar to MS-275 [IC_{50}s (μM) against HDAC1: 0.2 (**8**) and 0.3 (MS-275); IC_{50}s (μM) against HDAC4: 125 (**8**) and 142 (MS-275)].

In the U937 cells, we have determined the effects of **1**–**13** (at 5 μM for 24 h) on histone H3 and α-tubulin acetylation levels taken as markers of class I HDACs and HDAC6 inhibiting activities, respectively (Fig. 4). The induction of $p21^{WAF1/CIP1}$ for the tested compounds was assessed too (Fig. 4). SAHA and MS-275 have been taken as reference compounds. In the acetylation assays, the majority of the tested compounds showed high acetyl-H3 levels and no α-tubulin acetylation, with the exceptions of **2** and **9** which gave a modest signal on H3 acetylation, and **6** and **12** which weakly increased α-tubulin acetylation. Some of the tested compounds (**1**, **5**, **8**, **10** and **12**) showed high p21 induction. Compared with the hydroxamates MC1641 and MC1751, which behaved as class I/IIb HDACi,[25] the new 2-aminoanilide-like derivatives displayed a higher level of class I selectivity, the majority of them being unable to hyperacetylate α-tubulin.

Compounds **1**–**13** were then tested in the U937 cell line (at 5 μM for 30 h) to evaluate effects on cell cycle, apoptosis induction, and granulocytic differentiation. SAHA and MS-275 (5 μM) were used as reference drugs. At the tested conditions, **1**, **3**, **4**, and **5** showed a weak arrest of the cell cycle in the S phase, and **8** gave a block of the cycle at the G2 phase (Fig. S2 in Supplementary material). Apoptosis induction was measured with the An-

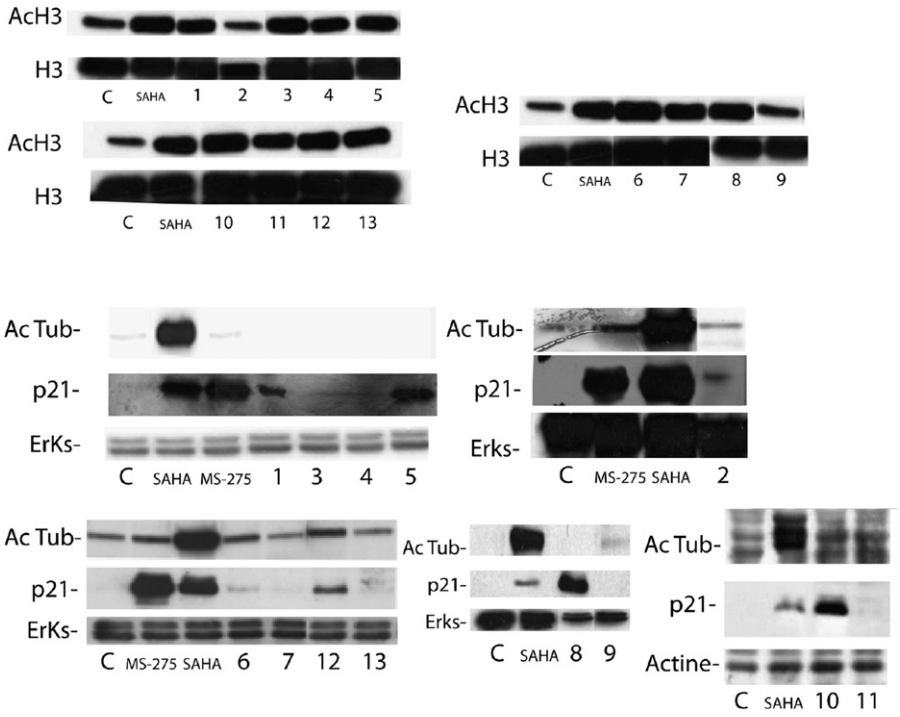


Figure 4. Western blot analyses on U937 cells performed on **1**–**13** (at 5 μM) to determine their effects on histone H3 and α-tubulin acetylation, and on p21 induction. SAHA and MS-275 (5 μM) were used for a comparison.

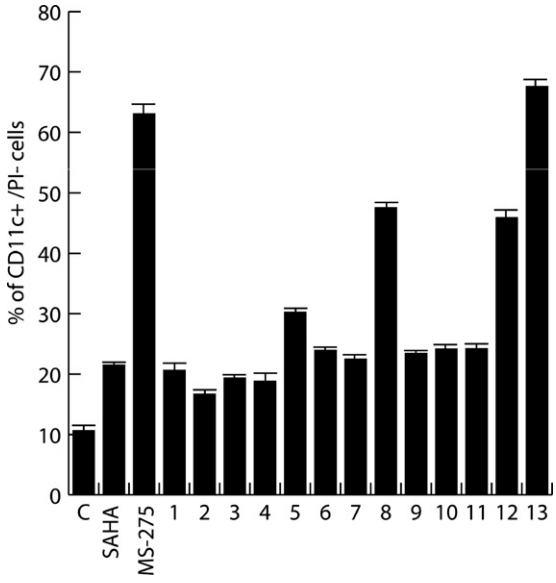


Figure 5. Granulocytic differentiation activity displayed by **1**–**13** (at 5 μM for 30 h) on the human leukemia U937 cell line.

nexin V/propidium iodide (PI) double staining method: in this test, compound **8** and, to a lower extent, **10** and **12** displayed 4.8% to 8.4% of apoptosis induction, they being less active than SAHA (16.8%) and more potent than MS-275 (3.5%) (Fig. S3 in Supplementary material). Granulocytic differentiation was evaluated by measuring the CD11c expression level upon 30 h of stimulation with **1**–**13** at 5 μM. Amongst the tested compounds, **5**, **8**, **12** and **13** showed a percent of CD11c positive PI negative cells higher than SAHA (**5**: 30.3; **8**: 47.6; **12**: 45.9; **13**: 67.6; SAHA: 21.5), **13** being even more potent than MS-275 (% CD11c+/PI- cells: 63.1) as cytodifferentiating agent (Fig. 5).

In conclusion, we reported here a new series of uracil-based HDACi **1**–**13** bearing a 2-aminoanilide or 2-aminoanilide-like moiety at the right head of the molecules. Such compounds were in general more efficient in inhibiting hrHDAC1 than hrHDAC4, and were able to increase the H3 but not α-tubulin acetylation levels in the U937 cells. The highest class I-selectivity was displayed by the uracils bearing at C6 a phenyl ring, at C2 a sulphur atom, and showing a 4-methylbenzamide moiety as a spacer between the uracil ring and the 2-aminoanilide group. Compound **8**, the most potent and class I-selective HDACi for this series, yielded together with **12** a high level of CD11c positive/PI negative cells in the cytodifferentiation assay (U937 cells, 5 μM, 30 h). Surprisingly, compound **13**, which failed in inhibiting HDACs, showed in the same assay a cytodifferentiating effect higher than MS-275. Studies are in progress to probe this issue.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.03.055.

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42. Chemical and physical data of compounds **1**–**29** are reported in Supplementary Material.