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N-(2-Amino-phenyl)-4-(heteroarylmethyl)-benzamides as new histone deacetylase inhibitors

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Dedicated to the memory of Naomy Bernstein, our colleague and friend.

Abstract—A variety of *N*-(2-amino-phenyl)-4-(heteroarylmethyl)-benzamides were designed and synthesized. These compounds were shown to inhibit recombinant human HDAC1 with IC_{50} values in the sub-micromolar range. In human cancer cells growing in culture these compounds induced hyperacetylation of histones, induced the expression of the tumor suppressor protein $p21^{WAF1/Cip1}$, and inhibited cellular proliferation. Certain compounds of this class also showed in vivo activity in various human tumor xenograft models in mice.

Histone acetylation/deacetylation is essential for chromatin remodeling and regulation of gene transcription in eukaryotic cells. Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are enzymes that catalyze the deacetylation (associated with transcriptional silencing) and acetylation (associated with transcriptional activation), respectively, of lysine residues located in the NH_2 terminal tails of core histones.[1] Perturbations of this balance in the form of histone deacetylation are often observed in human tumors.[2] Thus, inhibition of HDACs has emerged as a novel therapeutic strategy against cancer.[3] Small molecules of different classes such as MGCD0103 (**1**) (MethylGene Inc.),[4,5] CRA-024781 (**2**) (Celera Genomics),[6] PXD-101 (**3**) (Curagen/Topo-Target),[7] LBH-589 (**4**) (Novartis AG),[8] and MS-275 (**5**) (Syndax Pharmaceuticals/Schering AG)[9] are potent HDAC inhibitors, demonstrating in vivo antitumor efficacy and are currently undergoing clinical trials (Fig. 1).

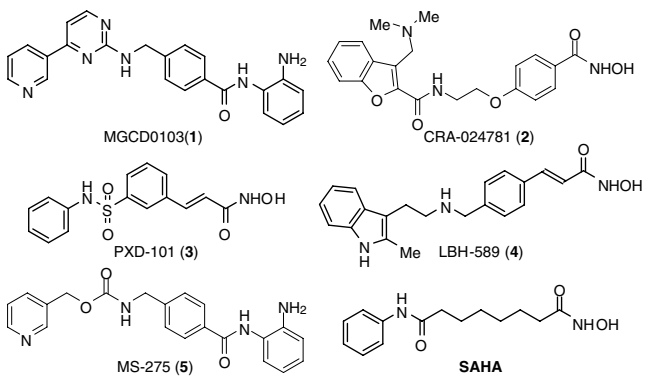

Figure 1.

Keywords: Histone deacetylase inhibitors; HDAC; Benzamides; Cancer cell proliferation.
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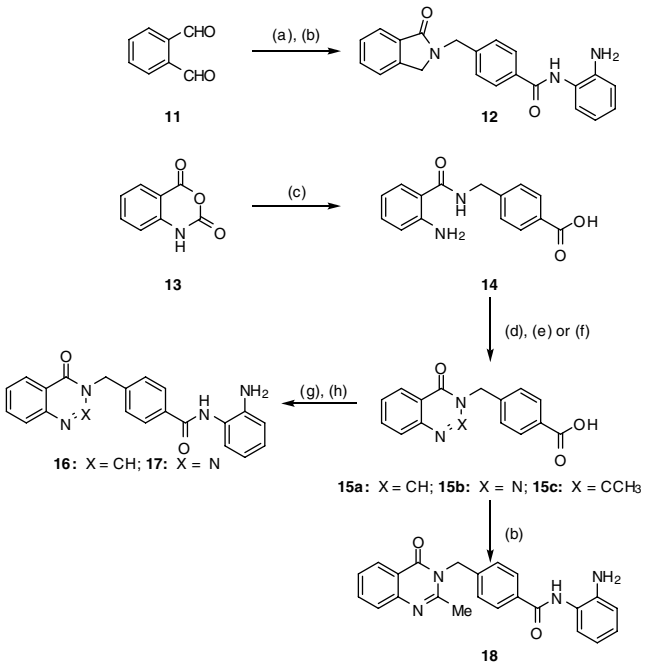
Suberoylanilide hydroxamic acid Zolinza(TM) (vorinostat, SAHA, Merck)[10] has recently been approved for the treatment of advanced cutaneous T-cell lymphoma (CTCL).

In our research program directed toward design, synthesis, and biological evaluation of novel HDAC inhibitors with adequate 'drug-like' properties, we identified several distinct classes of such molecules: arylsulfonamide-based hydroxamates exemplified by compound (**6**),[11] arylsulfonamide-based amino-anilides such as (**7**),[12] long-chain SAHA-like ω-substituted hydroxamic acids like (**8**),[13] and 2-amino-phenylamides of ω-substituted alkanoic acids such as (**9**).[14] All these classes of compounds while possessing good in vitro HDAC-inhibitory activity showed, however, marginal in vivo efficacy, which was attributed to their relatively short half-life and poor bioavailability (data not shown). Efforts to overcome these shortcomings led us to the novel class of *N*-(2-amino-phenyl)-4-(heteroarylmethyl)-benzamides (**10**) (Fig. 2), which not only retained high HDAC in vitro potency, but showed significant improvement in antitumor in vivo behavior.

The first series of target compounds representing the class of *N*-(2-amino-phenyl)-4-(heteroarylmethyl)-benzamides (**10**) was the set of compounds bearing a carbonyl group on the left-hand side heterocyclic moiety such as compounds **12**, **16**–**18**. Their synthesis is presented in Scheme 1, while the detailed procedures are described in Delorme et al.[15] It is worth mentioning that all these HDAC inhibitors as well as the ones described below bear the *N*-(2-amino-phenyl)-benzamide fragment which has been shown to be the pharmacophore.[9b] Removal of the amino group is detrimental to the HDAC-inhibitory activity. The amino group, however, can be replaced by an OH-group without the loss of potency and only a few other subtle substitutions in that fragment are allowed.[9b]

The second series of the synthesized target compounds was the series of molecules bearing two carbonyl groups on the left-hand side heterocyclic moiety such as compounds **20**, **22**, and **24**–**29** (Scheme 2). We also explored a replacement of the fused benzene ring in compounds **16**, **24**, and **26** for a thiophene thus preparing the corresponding thienopyrimidines **31**, **34**, and **36** (Scheme 3). To further investigate possible replacements within this class of molecules, we substituted the endocyclic nitrogen atom as an attachment point between heterocycles and the benzamide fragments, for a carbon atom, which resulted in the design and synthesis of the 'carbon analogues' **38**, **41**–**43** (Scheme 4). 4-(4-Oxo-chroman-3-ylidenemethyl)-benzoic acid methyl ester (**40**) turned out to be the key intermediate in the synthesis of target molecules **41**–**43**. Thus, the $RhCl_3$ mediated isomerization of its double bond[16] followed by hydrolysis and a coupling with 1,2-phenylenediamine afforded compound **41**. Reduction of the double bond using phenylsulfonyl hydrazine was the key procedure in the synthesis of the target **42**, while hydrogenation of **40** in the presence of palladium on charcoal ultimately allowed for the formation of the compound **43** devoid of the carbonyl group.



Scheme 1. Reagents and conditions: (a) *p*-aminomethylbenzoic acid, AcOH, 5 min, reflux, 49%; (b) 1,2-phenylenediamine, BOP, Et_3N, DMF, 31–49%; (c) *p*-aminomethylbenzoic acid, Et_3N, H_2O, 3 h, 40 °C 100%; (d) HCOOH, reflux, 6 h (X = CH), 96%; (e) $NaNO_2$, HCl, 0 °C (X = N), 96%; (f) Ac_2O, reflux, 1 h, then AcOH, reflux, 48 h (X = CCH_3), 43%; (g) (2-amino-phenyl)-carbamic acid *tert*-butyl ester, BOP, Et_3N, DMF (58%, X = CH; 62%, X = N); (h) TFA/CH_2Cl_2 (74%, X = CH; 19%, X = N).

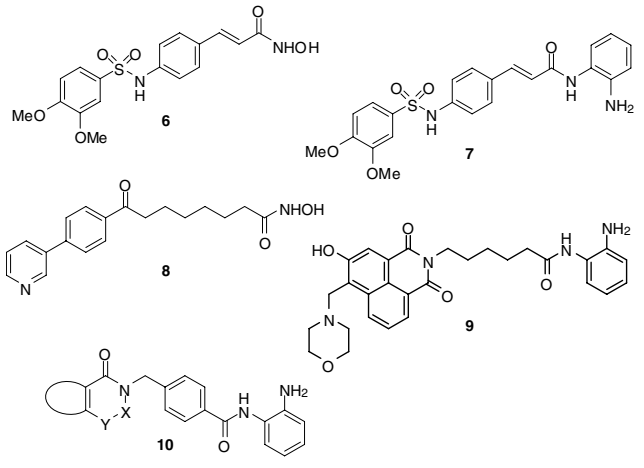
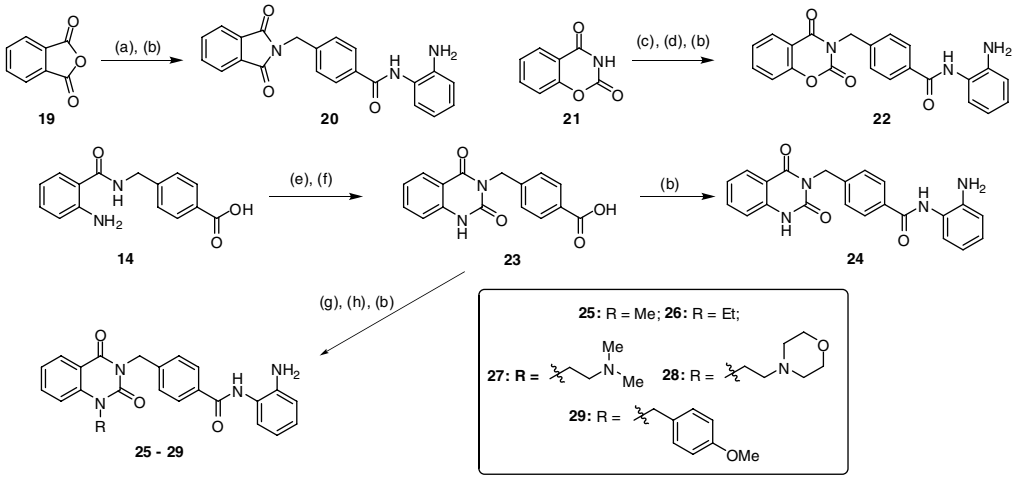
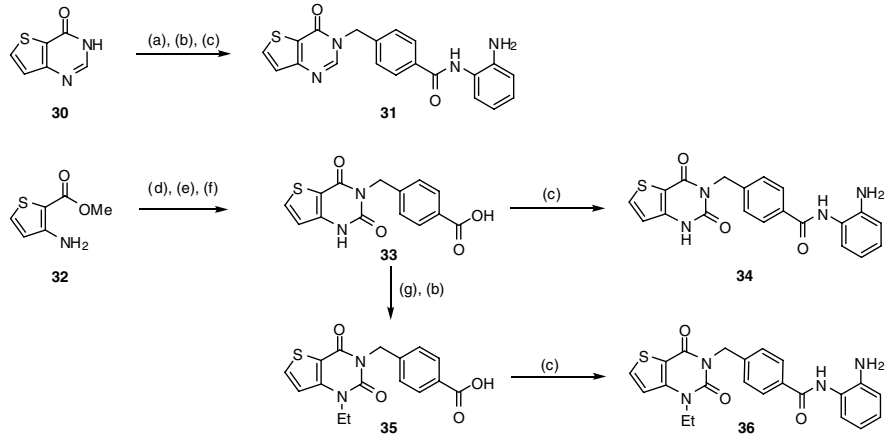


Figure 2.

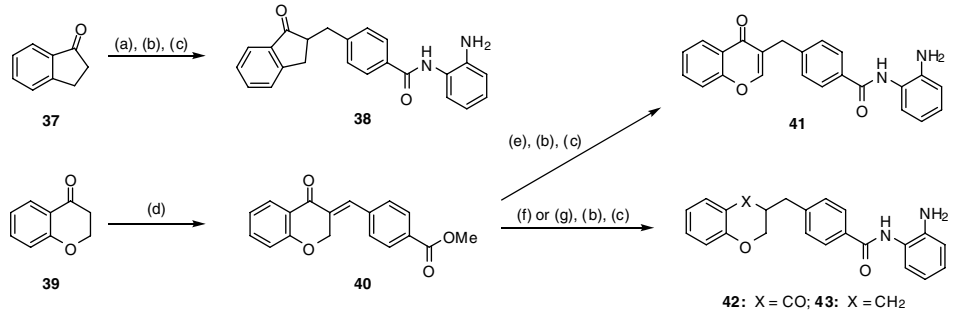
The data in Table 1 demonstrate the ability of these compounds to inhibit recombinant HDAC1 with an IC_{50} range of 0.1–1.0 μM (measured using BocLys(acetyl)AMC as substrate).[17] Also, these compounds demonstrated good in vitro antiproliferative potency (measured with MTT reagent) in the human colon cancer cell line (HCT116), with desirable selectivity (6- to >50-fold) over normal human mammary epithelial cells



Scheme 2. Reagents and conditions: (a) *p*-aminomethylbenzoic acid, AcOH, reflux, 68%; (b) 1,2-phenylenediamine, BOP, Et_3N, DMF, 10–27%; (c) methyl 4-(bromomethyl)benzoate, Cs_2CO_3, DMF, 40%; (d) AcOH, HCl, reflux, 91%; (e) $ClCO_2Et$, Pyr, 0 °C; (f) NaOH, MeOH, H_2O, reflux, 90% (over two steps); (g) MeI, K_2CO_3, DMF, 85%; (h) NaOH, THF, H_2O, 96%. Compounds **26**–**29** were obtained similarly to the compound **25** using in the step (g) ethyl iodide, (2-chloroethyl)-dimethylamine, 4-(2-chloroethyl)-morpholine or 1-bromomethyl-4-methoxy-benzene, respectively, instead of methyl iodide.



Scheme 3. Reagents and conditions: (a) methyl 4-(aminomethyl)benzoate, K_2CO_3, DMF, 100%; (b) LiOH, THF, H_2O, 80–87%; (c) 1,2-phenylenediamine, BOP, Et_3N, DMF, 31–75%; (d) triphosgene, Et_3N, DCM, −78 °C to rt; (e) methyl 4-(aminomethyl)benzoate, Et_3N, DCM, 91% (two steps); (f) NaOH, MeOH, reflux, then H_2O, reflux, 95%; (g) EtI, K_2CO_3, DMF, 94%.



Scheme 4. Reagents and conditions: (a) methyl-4-bromomethylbenzoate, LDA 2 M, THF, 17%; (b) LiOH, THF, H_2O, 61–98%; (c) 1,2-phenylenediamine, BOP, Et_3N, DMF, 10–69%; (d) methyl-4-formylbenzoate, AcOH, H_2SO_4, 82%; (e) $RhCl_3{\cdot}H_2O$, EtOH, 16 h, 70 °C, 59%; (f) $PhSO_2NHNH_2$, DMF, 100 °C, 42% (X = CO); (g) Pd/C, H_2, MeOH, DMA, 59% (X = CH_2).

(HMEC). Consistent with inhibiting cellular HDACs, these inhibitors induced histone hyperacetylation in T24 human cancer cells ($EC_{50} \sim 1$ μM), in a dose-dependent fashion. Also consistent with their mechanism of action, these compounds induced the expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$

Table 1. In vitro profile of histone deacetylase inhibitors

Compound	HDAC1 IC_{50} (μM)	MTT HCT116 IC_{50} (μM)	MTT HMEC IC_{50} (μM)	H4-Ac EC_{50} (μM)	p21 induction EC_{50} (μM)
12	0.1	0.5	37	1	2
16	0.1	0.4	28	<1	1
17	0.2	0.6	17	1	1
18	0.08	0.6	>50	1	2
20	0.2	5	>50	5	3
22	0.1	0.3	21	1	2
24	0.06	0.8	18	1	2
25	0.1	0.7	>42	1	1
26	0.2	0.4	25	1	0.6
27	0.3	1	>25	1	1
28	0.4	1	>25	3	5
29	0.1	0.8	>25	1	3
31	0.2	0.7	>50	1	1
34	0.06	0.4	29	1	1
36	0.3	0.8	>50	1	3
38	0.1	0.6	8	<1	2
41	0.03	0.3	9	1	1
42	0.09	0.9	6	2	1
43	1	2	23	3	3

HDAC1: Inhibition of recombinant HDAC1.
MTT: Cytotoxicity/proliferation of human cancer HCT116 cells and human normal mammary epithelial (HMEC) cells.
H4-Ac: Relative effective concentration of compounds in induction of histone H4 acetylation in T24 human cancer cells, relative to MS-275 at 1 μM. Human T24 cells were treated with compounds at 0, 1, 5, and 25 μM for 16 h. Cells were harvested and histones were acid-extracted. Histones were analyzed by SDS–PAGE and immunoblotted with antibodies specific for either H4 histones or acetylated H4 histones.
$p21^{WAF1/Cip1}$: Relative effective concentration of compounds in induction of $p21^{WAF1/Cip1}$ in T24 human cancer cells, relative to MS-275 at 1 μM. Human cancer T24 cells were treated with compounds at concentrations at 0, 1, 5, and 25 μM or DMSO alone for 16 h before whole cell lysates were harvested and analyzed by Western blot using $p21^{WAF1/Cip1}$ antibody. Tubulin level was analyzed to reveal protein loading.

($EC_{50} \sim 2$ μM). In addition, they caused apoptosis and G2/M cell cycle arrest in HCT116 human colon cancer cell line (data not shown).

The first compound from the mono-carbonyl series, compound **12**, already exhibited an in vitro profile similar to the one of MS-275, which was used as a positive control in our experiments. Changing the ring from 5- to 6-membered (proceeding from compound **12** to compounds **16** and **17**) or adding a methyl group to the carbon between the pyrimidine nitrogens (compound **18**) did not affect the enzymatic or cellular activities.

The first compound from the di-carbonyl series, compound **20**, although only 2-fold less potent against the HD1 enzyme, showed, however, weaker cytotoxic activity and was inferior in terms of induction of histone acetylation and $p21^{WAF1/Cip1}$ when compared with its mono-carbonyl counterpart, compound **12**. The cytotoxicity of the HDAC inhibitors and the ability to induce acetylation of H4 histones have been improved when the 5-membered ring (in **20**) was replaced by the 6-membered one (compounds **22** and **24**). Alkylation of the nitrogen in **24** generally did not affect the in vitro parameters (compounds **25**–**29**); their enzymatic and cellular potencies remained micro- or sub-micromolar with comparable numbers for H4-acetylation or $p21^{WAF1/Cip1}$ induction. Thienopyrimidine based HDAC inhibitors **31**, **34**, and **36** showed similar overall in vitro profiles when compared to their benzo annelated analogues (compounds **16**, **24**, and **26**) with the most attractive being **34**. The carbon analogues **38**, **41**–**43** did not look superior relative to the best compounds with a C–N linker (**16**, **25**–**26**, or **34**): the IC_{50} values in cytotoxicity assay against human normal mammary epithelial cells (HMEC) were lower, indicating the potential for toxicity by these molecules. In addition to that, compound **43** (lacking the carbonyl group on the left-hand side) exhibited only micromolar potencies in all the assays (Table 1).

Selected compounds underwent pharmacokinetic evaluation in female Sprague–Dawley rats. The compounds were dosed at 2.5 mg/kg (iv) or 5.0 mg/kg (po) with the dosing vehicles being acidified saline or acidified saline/PEG400. Compounds **27**, **31**, **34**, and **36** turned out to be reasonable with respect to their half-lives and oral availability (Table 2).

Compounds **24**, **31**, and **34** were further evaluated in vivo in several human tumor xenograft models [A549 (non-small cell lung), DU145 (prostate), and SW48, HCT116 (colon)] in nude mice (6 animals per group). Compounds were administered once daily for 14 days by either intraperitoneal (ip) injection or orally (po). The compounds demonstrated significant antitumor activity in all models tested (% tumor growth inhibition vs vehicle treated control animals) by either dosing route at well tolerated doses (Table 3).

We designed and synthesized a novel class of HDAC inhibitors, the *N*-(2-amino-phenyl)-4-(heteroarylmethyl)-benzamides. These compounds exhibit in vitro antiproliferative activities in numerous human cancer cells, but not normal cells, induce $p21^{WAF1/Cip1}$ expression

Table 2. Pharmacokinetic profile of selected compounds

Compound	$T_{\frac{1}{2}}$ iv (h)	*F*%
12	0.6	34
18	0.9	16
27	2.1	38
31	1.4	47
34	1.3	47
36	1.2	64

Table 3. In vivo antitumor activity of **24**, **31**, and **34**

Compound	Dose (mg/kg)	% tumor growth inhibition
24	20 (ip)	51% (HCT116)
24	40 (ip)	59% (HCT116)
31	80 (po)	42% (A549), 45% (DU145)
34	40 (ip)	61% (HCT116)
34	80 (po)	55% (A549), 67% (DU145)

and hyperacetylation of core histones, and cause cell cycle arrest and apoptosis of human cancer cells.

The most promising HDAC inhibitor of this series was found to be compound **34**. It has an excellent in vitro profile and shows significant antitumor activity in vivo in several human cancer xenograft models. Compound **34** is metabolically stable, does not show any major binding to receptors, channels or enzymes that can predict potential toxicity liabilities (data not shown), and possesses acceptable physiochemical and pharmacokinetic characteristics.

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