Discovery of *N*-(2-Aminophenyl)-4-[(4-pyridin-3-ylpyrimidin-2-ylamino)methyl]benzamide (MGCD0103), an Orally Active Histone Deacetylase Inhibitor

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Abstract: The design, synthesis, and biological evaluation of *N*-(2-aminophenyl)-4-[(4-pyridin-3-ylpyrimidin-2-ylamino)methyl]benzamide **8** (MGCD0103) is described. Compound **8** is an isotype-selective small molecule histone deacetylase (HDAC) inhibitor that selectively inhibits HDACs 1−3 and 11 at submicromolar concentrations in vitro. **8** blocks cancer cell proliferation and induces histone acetylation, $p21^{cip/waf1}$ protein expression, cell-cycle arrest, and apoptosis. **8** is orally bioavailable, has significant antitumor activity in vivo, has entered clinical trials, and shows promise as an anticancer drug.

Histone acetylation/deacetylation is essential for chromatin remodeling and epigenetic regulation of gene transcription in eukaryotic cells. Histone acetyltransferases (HATs) are enzymes that catalyze histone acetylation, which is associated with transcriptional activation.[1] Histone deacetylases (HDACs) are enzymes that catalyze the deacetylation of lysine residues located in the NH_2 terminal tails of core histones, which is associated with transcriptional silencing.[1] There are 18 known human histone deacetylases, grouped into four classes based on the structure of their accessory domains. Class I (HDACs 1−3 and 8), II (HDACs 4−7, 9, and 10), and IV (HDAC 11) enzymes are Zn^{2+}-dependent enzymes and are called HDACs, while class III enzymes (also known as sirtuins) are defined by their dependency on NAD^+. Perturbations of histone deacetylation have been observed in human tumors, and inhibition of HDACs has emerged as a novel and validated therapeutic strategy against cancer.[1,2]

Small molecule inhibitors of HDACs belonging to different classes such as SAHA (**1**) (vorinostat, Merck & Co.),[3] recently approved for the treatment of advanced cutaneous T-cell lymphoma (CTCL), CRA-024781 (**2**) (Pharmacyclics),[4] PXD-101 (**3**) (CuraGen & TopoTarget),[5] LBH-589 (**4**) (Novartis AG),[6] R-306465 (**5**) (Janssen Pharmaceuticals),[7] and MS-275 (**6**) (Syndax Pharmaceuticals/Schering AG)[8] are in various stages of development and have demonstrated in vivo antitumor efficacy (Figure 1).

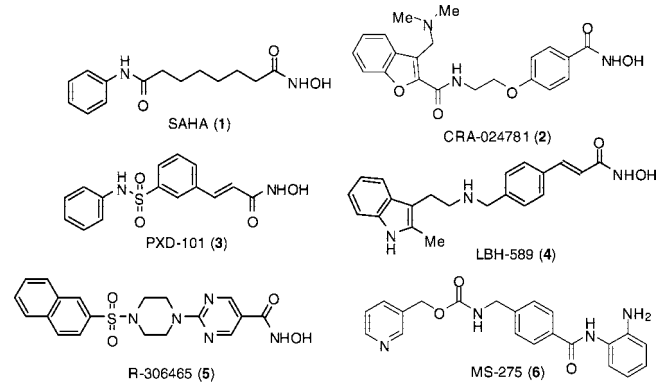

Figure 1. Small molecule HDAC inhibitors in clinical development.

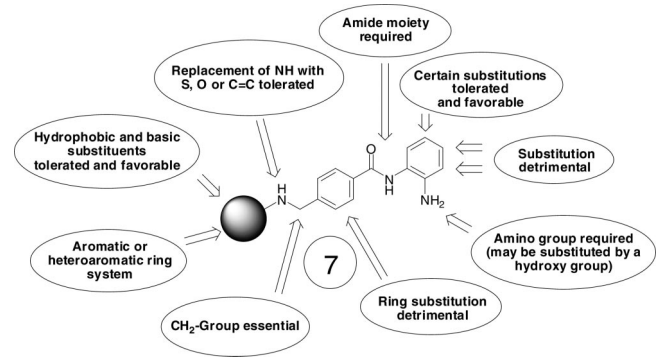

Figure 2. Pharmacophore model of aminophenyl benzamides **7**.

We have identified several distinct classes of novel HDAC inhibitors with development potential: arylsulfonamide-based hydroxamates,[9] long-chain ω-substituted hydroxamic acids,[10] arylsulfonamide-based aminoanilides,[11] 2-aminophenylamides of ω-substituted alkanoic acids,[12] chalcone type[13] or cinnamic acid[14] aminobenzamides. All of these classes of compounds inhibited HDAC enzyme activity in vitro and in many cases demonstrated significant antitumor efficacy in vivo. Further studies revealed the novel class of *N*-(2-aminophenyl)-4-(arylaminomethyl)benzamides (**7**) (Figure 2), which not only retained potent enzymatic and cellular inhibition of HDAC activity in vitro but also displayed significant improvements in pharmacokinetic properties and antitumor efficacy in vivo in human tumor xenograft models.[14,15]

Structure−activity relationships (SAR) of aminoanilides **7** were thoroughly investigated[14−17] and are summarized in Figure 2. Extensive optimization of the left-hand-side aromatic (heteroaromatic) ring of **7**, as well as the types and positions of substituents attached to that ring, resulted in the discovery of a potent HDAC inhibitor *N*-(2-aminophenyl)-4-[(4-pyridin-3-ylpyrimidin-2-ylamino)methyl]benzamide **8** (MGCD0103), which became the MethylGene clinical candidate. The aminophenylbenzamide **8** was obtained via a short reaction sequence[18] starting from commercially available 3-acetylpyridine (**9**), 4-aminomethylbenzoic acid methyl ester hydrochloride salt (**10**), and 1,2-phenylenediamine (**11**) (Scheme 1).

The reaction of **9** with *N,N*-dimethylformamide dimethyl acetal provided 3-dimethylamino-1-pyridin-3-ylpropenone (**12**) as the first synthetic intermediate. Treatment of the amino ester **10** with pyrazole-1-carboxamidine hydrochloride in a basic

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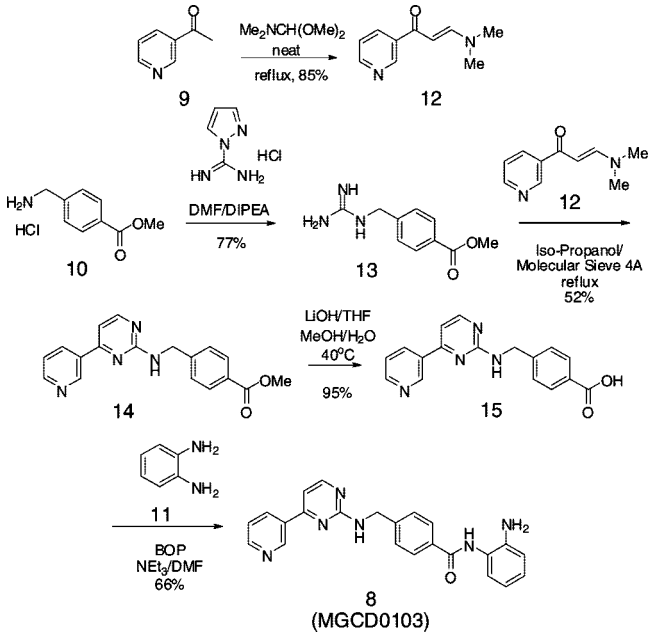
Scheme 1



medium produced 4-guanidinomethylbenzoic acid methyl ester (**13**). This second intermediate reacted with the enaminoketone **12** to form methyl 4-((4-(pyridin-3-yl)pyrimidin-2-ylamino)methyl)benzoate (**14**), which was hydrolyzed to provide 4-((4-(pyridin-3-yl)pyrimidin-2-ylamino)methyl)benzoic acid (**15**). Acid **15** underwent an amide coupling reaction with 1,2-phenylenediamine (**11**), forming **8**.

The aminophenyl benzamide **8** is an isotype selective HDAC inhibitor targeting HDACs 1−3 and 11 isoforms and is inactive against HDACs 4−8 (Table 1).

The biological activity of this molecule was explored by examining its effects on cancer cell proliferation, histone acetylation, the protein expression of $p21^{cip/waf1}$, and apoptosis (Table 2). Compound **8** differentiated between cancer and normal cells as exemplified by inhibition of cell proliferation in HCT116 human colon cancer cells, A549 human lung cancer cells, DU145 human prostate cancer cells, but not in HMEC human normal mammary epithelial cells. The aminophenyl benzamide **8** potently induced acetylation of H3 histones, $p21^{cip/waf1}$ protein expression, cell-cycle arrest at G2/M stage, and apoptosis.

Pharmacokinetic studies of orally administered **8** (or its dihydrobromide salt) were carried out in female CD-1 mice, female Sprague−Dawley rats, and male beagle dogs. The pharmacokinetic parameters are shown in Table 3. The oral bioavailability was 12% in mice and 47% in rats. Pharmacokinetic parameters in mice and rats were linear with the dose administered and independent of vehicles used. In dogs, pharmacokinetic parameters were nonlinear with the dose and dependent on the vehicles used. A wide range of bioavailability (1−92%) was observed in dogs. The compound was quickly absorbed, with a short T_{max} across all species tested. The terminal phase elimination half-life for **8** administered iv was 0.6 h in mice, 0.7 h in rats, and 1.3 h in dogs. The clearance in the mice (4.3 (L/h)/kg) was high, but the clearance in rats (1.7 (L/h)/kg) and dogs (2.0 (L/h)/kg) was reasonable. Steady state volume of distribution was low in mice (0.34 L/kg) and higher in rats (0.91 L/kg) and dogs (0.80 L/kg).

The in vivo antitumor activity of orally administered **8** (or its dihydrobromide salt) was evaluated in several human tumor xenograft models. Compound **8** demonstrated significant antitumor activity in multiple models in a dose-dependent manner (Table 4). There were no significant effects on body weight at efficacious doses.

Table 1. Inhibitory Activity of Compound **8** against HDAC Enzymes[a]

enzyme	mean IC_{50} ± SEM (μM)
HDAC1	0.15±0.02
HDAC2	0.29±0.08
HDAC3	1.66±0.69
HDAC11	0.59±0.23

[a] IC_{50} values of **8** against HDACs 4−8 are greater than 10 μM.

Table 2. In Vitro Activity Profile of Compound **8**

assay	cell line	potency ($n = 4$)
inhibition of cell proliferation (IC_{50}, μM)	HCT116	0.29±0.02
	A549	0.9 ±0.04
	Du145	0.67±0.07
	HMEC	20 ± 2
H3Ac induction (EC_{50}, μM)[a]	T24	1.38±0.38
$p21^{cip/waf1}$ protein expression (EC_{50}, μM)[b]	HCT116	0.45±0.09
cell cycle arrest (G2/M) (EC_{50}, μM)	HCT116	<1
apoptosis induction (at 1 μM)	HCT116	+[c]

[a] H3Ac = histone H3 acetylation. Effective concentration of **8** for induction of histone H3 acetylation (relative to **6** at 1 μM). [b] Effective concentration of **8** for induction of $p21^{cip/waf1}$ (relative to **6** at 1 μM). [c] + = apoptosis positive.

Compound **8** has been evaluated in four single agent phase I trials. In 23 patients with leukemia or myelodysplastic syndrome (MDS), oral doses of **8** below 80 mg/m^2 given three times weekly were well-tolerated, and a complete marrow response was observed in three patients.[19] In patients with advanced solid tumors ($n = 28$), **8** was well-tolerated at doses of 12.5, 20, 27, 36, and 45 mg/m^2 given three times weekly for 2 of 3 weeks. Disease stabilization was noted in five patients. Dose-dependent pharmacodynamic activity and a dose-independent half-life were observed in this study.[20] Another phase I study in patients with leukemia or MDS ($n = 24$) demonstrated that **8** given twice weekly at oral doses (40, 53, 66, and 83 mg/m^2) was well-tolerated, and four patients experienced stable disease. Significant inhibition of total HDAC activity in peripheral blood mononuclear cells was observed in the majority of these patients.

The most prominent side effects of **8** are fatigue and gastrointestinal. Fatigue is usually low grade but is occasionally dose limiting. When necessary, fatigue is usually successfully managed by dose modification. Gastrointestinal side effects are also usually low grade and may include anorexia, nausea, vomiting, or diarrhea and can also often be managed successfully by supportive care or dose modification. Myelosuppression is generally rare but does occur in some patients with hematologic malignancies who have undergone significant prior chemotherapy.

In conclusion, we designed, synthesized, and evaluated the biological activity of novel HDAC inhibitors, which resulted in the discovery of *N*-(2-aminophenyl)-4-[(4-pyridin-3-ylpyrimidin-2-ylamino)methyl]benzamide (**8**). Out of 11 known non-$NADH^+$-dependent HDAC isotypes, **8** selectively inhibits HDACs 1−3 and 11 at submicromolar concentrations in vitro. It also potently blocks the proliferation of cancer cells and does not affect the proliferation of the normal cells. At submicromolar concentrations, aminophenylbenzamide **8** induces hyperacetylation of histones, cell-cycle arrest, expression of $p21^{cip/waf1}$ protein, and apoptosis. Compound **8** has favorable pharmacokinetic parameters across a number of species (mouse, rat, and dog) and demonstrates antitumor efficacy in human tumor xenograft models in mice. Compound **8** has entered clinical trials

Table 3. Pharmacokinetic Parameters of Compound **8** in Mouse, Rat, and Dog[a]

parameter	dose iv (mg/kg)	dose po (mg/kg)	$T_{1/2}$ iv (hr)	CL (L/(kg·h))	V_{ss} (L/kg)	T_{max}, po (h)	C_{max}, po (μM/(mg/kg))	AUC, po (μM·h/(mg/kg))	F (%)
mouse	2.5−15	2.5−15	0.6	4.3	0.35	0.25	0.086	0.071	12
rat	2.5−5	2.5−50	0.7	1.7	0.91	0.59	0.30	0.63	47
dog	10	7−30	1.3	2.0	0.80	0.29−2.0	0.0029−1.0	0.011−1.2	1−92

[a] The dosing vehicles in mice were 0.05−0.1 N HCl in saline (both iv and po); in rats were 0.02−0.1 N HCl in saline (iv) and 0.02−0.1 N HCl in saline with 0.5% hydroxypropylmethylcellulose and 0.01% Tween-80, or 0.5% carboxymethylcellulose buffered with 50 mM acetate buffer at pH 4.0 (po); and in dogs were 0.2−0.3 N HCl in saline with PEG400 (40% v/v) (iv), 0.1−0.2 N HCl in saline, 0.1−0.2 N HCl in saline with PEG400 (40% v/v), or 0.5% hydroxyethylcellulose buffered at pH 4.0 with 50 mM acetate (po).

Table 4. In Vivo Antitumor Activity of **8** against Human Tumor Xenografts in Nude Mice (Oral Dosing)

tumor model	tumor type	**8** ((mg/kg)/day)	TGI[b] (%)
SW-48	colon carcinoma	60[a]	64
		90[a]	70
		120[a]	93
A431	vulval carcinoma	60	30
		90	64
A549	NSCLC	40	56
		60	73
DU145	prostate carcinoma	40	4
		70	47
		80	82

[a] Dihydrobromide salt. [b] Tumor growth inhibition.

and shows promise as an anticancer drug in combinations and as a single agent in hematological and solid malignances.

For cell culture experiments, human mammary epithelial cells (HMEC) were obtained from BioWhittaker (Walkersville, Maryland). All other cell lines were from American Type Culture Collection (Manassas, Virginia). Human normal and cancer cells were cultured following the vendor's instructions.

The HDAC enzyme in vitro assay was based on a homogeneous fluorescence release assay.[21] Purified recombinant HDAC enzymes were incubated with compounds diluted in various concentrations for 10 min in assay buffer (25 mM HEPES, pH 8.0, 137 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl) at room temperature. The fluorescent substrate Boc-Lys(ε-Ac)-AMC (Bachem, Torrance CA, I-1875) was added to the mixture for further incubation at 37 °C. The concentration of the substrate and the incubation time varied for different isotypes of HDAC enzymes. A 20 min trypsin incubation at room temperature allowed the release of the fluorophore from the deacetylated substrate. The fluorescent signal was detected by fluorometer (Molecular Devices, Sunnyvale, CA, GeminiXS): 360 nm excitation, 470 nm emission, 435 nm cutoff. The IC_{50} values of the compounds were determined by analyzing dose−response inhibition curves from 6−21 independent experiments.

For histone extraction, cultured cells or frozen pieces of tumor (after crunching) were lysed in lysis buffer [10 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 5 mM $MgCl_2$, 0.5% NP-40; protease inhibitors pepstatin (1 μ g/mL), leupeptin (2 μ g/mL), aprotinin (2 μ g/mL), TLCK(5 μ g/mL), PMSF (5 μ g/mL), DTT (5 μ g/mL), and 5 mM Na Butyrate] on ice for 10 min. Samples were centrifuged at 350*g* for 15 min, washed in the same lysis buffer once more, and resuspended in cold water. Non-histone proteins were precipitated with 3.3% H_2SO_4 for 1 h. To extract histones, samples were centrifuged at maximum speed for 5 min and the supernatant was added to 10 volumes of acetone and incubated overnight at −20 °C. Histones were then precipitated by centrifugation at maximum speed for 5 min, the supernatant was removed, and the pellet was resuspended in distilled water. Protein concentrations of histones were determined using the BioRad protein assay reagent, and equal quantities from each sample were analyzed by enzyme-linked immunosorbant assay (ELISA).

For ELISA analysis, the levels of histone acetylation were evaluated by ELISA: black plates (Nunc 437111) were coated with antihistone antibodies (Chemicon, Millipore, Billerica MA, product H11-4) and blocked with 1% BSA + 0.1% TritonX-100 in PBS. To detect H3Ac, an amount of 2 μg of purified histones was incubated in the coated plate with rabbit antiacetyl-H3 (Upstate, Millipore, Billerica MA, product 06-599, 1:1000). The signals were normalized with total H3: 0.5 μg of purified histones was mixed in separate wells with rabbit anti-H3 (Abcam, Cambridge MA, product ab1791, 1:5000). Binding to H3Ac or H3 was revealed with HRP-coupled goat antirabbit (Sigma, Oakville Ontario, Canada, product A-0545) using Amplex-Red (Molecular Probes, Invitrogen, Burlington Ontario, Canada, product A12222) as a substrate.

For flow cytometric cell cycle analysis, cells were treated with inhibitors for 16 h, harvested, and fixed with 70% ethanol at −20 °C. Nucleic acids from fixed cells were treated with RNaseIII and stained with propidium iodide (50 μ g/mL).[21] DNA content was measured by using a fluorescence-activated cell cytometer (FACScan, Becton-Dickinson, Franklin Lakes, NJ) and analyzed using CellQuest Pro software. The MTT cytotoxic assay was performed as described.[21] Compounds at various concentrations were added to cells in 96-well plates. Cells were incubated for 72 h at 37 °C in 5% CO_2. MTT (Sigma) was added at a final concentration of 0.5 mg/mL and incubated with the cells for 4 h before an equal volume of solubilization buffer (50% *N*,*N*-dimethylformamide, 20% SDS, pH 4.7) was added onto cultured cells. After overnight incubation, solubilized dye was quantified by colorimetric reading at 570 nm using a reference at 630 nm. OD values were converted to cell numbers according to a standard growth curve of the relevant cell line. The concentration that reduced cell numbers to 50% of those of DMSO-treated cells was determined as MTT IC_{50}. All experiments were performed at least three times independently.

Apoptosis of cancer cell lines was analyzed by the "Cell Death Detection ELISA Plus" kit (Roche).[21] Cells were treated with antisense oligonucleotides for 4 h at 37 °C, after which cells were washed with PBS and returned to serum-containing culture medium. Transfection was repeated every 24 h. After 48 h after the initial transfection with antisense oligonucleotides, cells were harvested and ELISA was performed following manufacturer's protocol. Results were read in SPECTRAMAX 190 (Molecular Devices) at 405 nm. Reference was set at 490 nm.

For in vivo antitumor efficacy and drug pharmacokinetic studies,[21] approximately 2 million human cancer cells were injected subcutaneously (sc) in the flank of female CD-1 nude mice (aged 8−10 weeks, Charles River Laboratories, Wilmington, MA) and were allowed to form solid tumors. Tumor fragments were serially passaged a minimum of three times before use in the experiments described. Tumor fragments (about 30 mg) were implanted sc through a small surgical incision on the flank of the mice while under general anesthesia. HDAC inhibitors were dissolved in vehicles and

dosed as solution (PBS acidified with 0.1 N HCl for oral dosing). Vehicles and inhibitors were administered daily, and tumor volumes and body weight were monitored three times weekly for ≥2 weeks. Each experimental group contained six to eight animals. At the end of each experiment, blood was collected and white blood cell counts were performed. Student's *t*-test was used for statistical analysis. For pharmacokinetic study, blood was collected from animals at various time points and plasma samples were analyzed in-house using an Agilent 1100 HPLC system coupled with an MDS Sciex API2000 triple quadrupole mass spectrometer.

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