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SAR and biological evaluation of analogues of a small molecule histone deacetylase inhibitor *N*-(2-aminophenyl)-4-((4-(pyridin-3-yl)pyrimidin-2-ylamino)methyl)benzamide (MGCD0103)

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ABSTRACT

Analogues of the clinical compound **MGCD0103** (**A**) were designed and synthesized. These compounds inhibit recombinant human HDAC1 with IC_{50} values in the sub-micromolar range. In human cancer cells growing in culture these compounds induce hyperacetylation of histones, cause expression of the tumor suppressor protein $p21^{WAF1/CIP1}$, and inhibit cellular proliferation. Lead molecule of the series, compound **25** is metabolically stable, possesses favorable pharmacokinetic characteristics and is orally active in vivo in different mouse tumor xenograft models.

Histone deacetylase (HDAC) enzymes have emerged in the past decade as validated biological targets for the treatment of human cancer.[1] HDACs class I and II are zinc-dependent enzymes that catalyze the removal of the acetyl groups from the lysine residues of histone proteins.[2] Overexpression of class I HDACs with concomitant down-regulation and/or mutations of histone acetyltransferases (HATs) are associated with a condensed chromatin structure preventing the access of transcription factors to DNA during gene expression (e.g., tumor repressor genes) causing cancer cells survival and progression.[1] Selective inhibition of class I HDACs with small molecules has been shown to restore the acetylation of histones and the transcription of genes which induce cell cycle arrest and apoptosis of cancer cell lines.[3]

Recently, we reported on the synthesis and biological evaluation of *N*-(2-aminophenyl)-4-((4-(pyridin-3-yl)pyrimidin-2-ylamino)methyl)-benzamide (**MGCD0103**)[4] an isotype selective,[4,5] orally bioavailable, HDAC inhibitor currently undergoing clinical evaluation in solid tumors and hematological malignancies—both as a single agent and in combinations with approved drugs.[6] To further explore chemical space around this molecular entity, to better understand its SAR and to layout a foundation for potential next-generation HDAC inhibitors, we embarked on design and synthesis of analogues of **MGCD0103** (**A**).

Herewith we wish to report on the results obtained from the study which led to the identification of new potent HDAC class I selective inhibitors[7] demonstrating significant in vivo anti-tumor efficacy in tumor xenograft models in mice.

HDAC inhibitors are usually characterized by a general three-piece pharmacophore model, consisting of a zinc binding group and a surface-recognition domain attached to each other via a

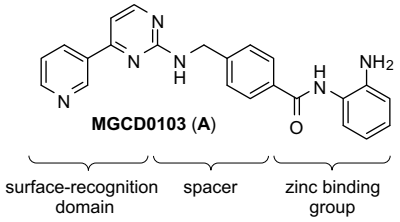

Figure 1. Pharmacophore model of **MGCD0103** (**A**).

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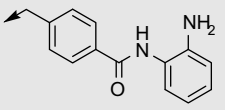
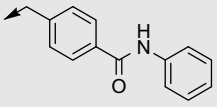
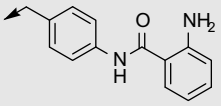
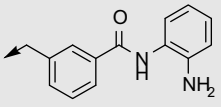
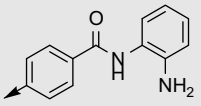
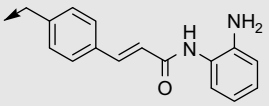
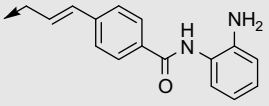
hydrophobic spacer.[3] Compound **A** could be represented in the same way (Fig. 1).

We first explored the zinc binding group and the spacer by keeping the 4-(pyridin-3-yl)pyrimidin-2-amino-fragment unchanged (Table 1). Thus, compound **A** devoid of the *ortho*-amino group on the phenyl ring turned out to be completely inactive against the HDAC1 enzyme. Compound **2**—a reverse amide—lost its activity as well. Detrimental to the HDAC inhibitory activity was a *meta* orientation of the linker phenylene group—compound **3**. Consistent with the loss of HDAC inhibitory activity, compounds 1–**3** did not show any anti-proliferative effects in the MTT assay (Table 1). Removal of the methylene group (compound **4**) or incorporation of a double bond between the phenylene spacer and the amide moiety (compound **5**) significantly decreased both HDAC1 enzymatic inhibitory activity and cytotoxicity, compared to the parent molecule **A** (Table 1). On the other hand, the insertion of a *trans*-double bond in the benzylic position allowed restoration of all the in vitro features: compound **6** was equipotent to **A**.

We next turned our focus to the investigation of the surface-recognition domain by keeping the spacer and the zinc binding group of **A** unchanged. We studied first the replacement of the 2,4-pyrimidine fragment by a phenyl or other six-membered heteroaromatic rings (Table 2). Phenylene (**7**) or heteroarylene analogues (**8**–**11**) generally retained the potency against HDAC1, or even became slightly more potent (e.g., **7**, **10**, and **11**). Also, these compounds demonstrated good in vitro anti-proliferative activity (measured using the MTT reagent) in the human colon cancer cell line (HCT116), with desirable selectivity (>15-fold) over normal human mammary epithelial cells (HMEC). Compounds **7**–**11** induced H4 histone hyperacetylation in T24 human cancer cells ($EC_{50} \sim 1$ μM, similar to compound **A**) and consistent with their mechanism of action, induced the expression of the $p21^{WAF1/Cip1}$ protein (EC_{50} 0.2–0.6 μM, comparable to compound **A**). In addition, they caused apoptosis in HCT116 human colon cancer cell line (data not shown).

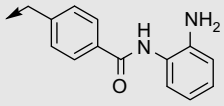
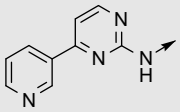
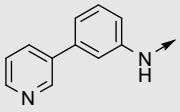
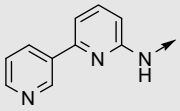
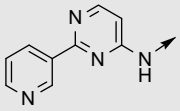
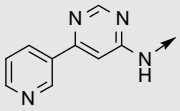
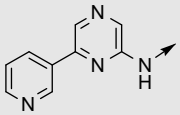
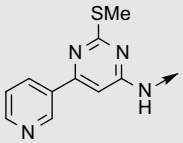
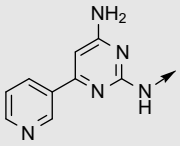
It turned out that the pyrimidine ring could accommodate a third substituent (compounds **12** and **13**) without significant decrease in potency against HDAC1 and retention of high cytotoxicity (and selectivity over HMEC cells), ability to induce H4 histone hyperacetylation and $p21^{WAF1/Cip1}$ protein (Table 2).

Table 1
Influence of the zinc binding groups and the spacers on the in vitro activities

Compound		HDAC1[a] IC_{50} (μM)	HCT116[b] IC_{50} (μM)
A		0.15	0.3
1		>25	>50
2		>20	25
3		>20	43
4		2	2
5		1.2	2
6		0.13	0.2

[a] Inhibition of recombinant HDAC1.
[b] MTT assay.

Table 2
2,4-Pyrimidine ring replacement in compound **A**

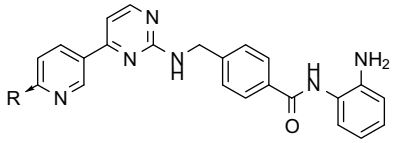
Compound		HDAC1[a] IC_{50} (μM)	HCT 116[b] IC_{50} (μM)	HMEC[b] IC_{50} (μM)	H4 Ac[c] (T24) EC_{50} (μM)	p21 Luciferease HCT 116[d] EC_{50} (μM)
						
A		0.15	0.3	21	<1	0.6
7		0.09	0.3	5	<1	0.6
8		0.18	0.6	>50	1	0.6
9		0.20	0.4	11	<1	0.6
10		0.07	<0.05	42	<1	0.5
11		0.11	0.3	16	1	0.4
12		0.25	0.1	13	1	0.6
13		0.17	0.2	14	1	0.2

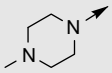
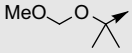
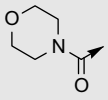
[a] Inhibition of recombinant HDAC1.
[b] MTT assays. Cytotoxicity/proliferation of human cancer HCT116 cells and human normal mammary epithelial (HMEC) cells.
[c] Relative effective concentration of compounds in induction of histone H4 acetylation in T24 human cancer cells, relative to pyridin-3-yl-methyl-4-(2-aminophenylcarbamoyl)benzylcarbamate (MS-275),[8] which was used as a positive control in these experiments 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 immunoblotting with antibodies specific for either H4 histones or acetylated H4 histones.
[d] Relative effective concentration of compounds in induction of $p21^{WAF1/Cip1}$ in HCT116 human cancer cells, relative to pyridin-3-yl-methyl-4-(2-aminophenylcarbamoyl)benzyl carbamate (MS-275),[8] which was used as a positive control in these experiments at 1 μM. Human cancer HCT116 cells were treated with compounds at 0, 1, 5, and 25 μM for 16 h before whole cell lysates were harvested and analyzed by Luciferase assay. Tubulin level was analyzed to reveal protein loading.

The third point of interest was the evaluation of substitution at position 6 of the pyridinyl ring of **A**, the supposed solvent-exposed area (Table 3). Electron-donating groups improved potency of inhibitors against HDAC1 (e.g., **A** vs **14–16**). The cyano compound **17** was equipotent to **A**, while the chemical entities with bulkier substituents (compounds **18–21**) became less potent against HDAC1. However, slight decrease in ability to induce H4 histone hyperacetylation in T24 human cancer cells (up to 2 μM) and expression of the $p21^{WAF1/Cip1}$ protein (up to 2 μM) were observed. Compounds **14–21** remained cytotoxic with needed selectivity (>20-fold) over normal human mammary epithelial cells (HMEC); and caused apoptosis in HCT116 human colon cancer cell line (data not shown).

Finally, we investigated the 3-pyridine replacement in **A** (Table 4) either by another heteroaryl ring (compounds **22–26**) or a substituted phenyl ring (compounds **27–31**). The latter compounds bearing basic substituents were made to increase aqueous solubility and potentially improve oral bioavailability. Compounds **22–31** were equipotent to **A**, thus indicating that the area contiguous to the pyrimidine ring is probably the solvent-exposed area. Com-

Table 3
6-Substituted pyridine analogues of **A**



Compound	R	HDAC1[a] IC_{50} (μM)	HCT 116[b] IC_{50} (μM)	HMEC[b] IC_{50} (μM)	H4 Ac[c] (T24) EC_{50} (μM)	p21 Luciferease HCT116[d] EC_{50} (μM)
A	H	0.15	0.3	21	<1	0.6
14		0.05	<0.05	5	<1	0.4
15		0.08	0.2	5	n.a.	0.2
16	MeS	0.09	0.3	6	1	0.3
17	NC	0.16	0.4	>50	1	0.7
18		0.25	0.1	49	1	1
19		0.23	0.1	38	2	0.6
20		0.42	0.3	>50	2	0.9
21		0.25	1	37	n.a.	2

See footnotes in Table 2.

pounds **22–31** also showed high cytotoxicity with excellent selectivity (>30-fold) over normal human mammary epithelial cells (HMEC) with the other parameters (induction of H4 histone hyperacetylation in T24 human cancer cells, and the expression of the $p21^{WAF1/Cip1}$ protein) comparable to the ones of **A**.

Despite the fact that most of the **MGCD0103** analogues were either equipotent or better than the parent compound when evaluated in vitro they failed to meet the requisite characteristics in the efficacy studies in tumor xenograft models in mice, due to (data not shown) poor p*K* profiles (e.g., compounds **8**, **11**, **15**, **20**, and **27**–**31**), narrow therapeutic safety window (e.g., compounds **7**, **15**, and **16**), improper physicochemical characteristics, low efficacy in vivo (e.g., compounds **22** and **27**), and/or toxicity problems (e.g., compounds **6**, **9**, **10**, **14**, **15**, and **24**). Nevertheless, few of them were found to be favorable from the efficacy standpoint with the most attractive being the compound **25** which was further evaluated in a greater detail.

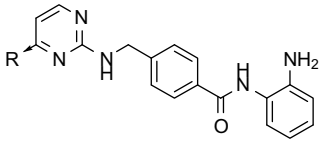
Thus the compound **25** was profiled against different HDAC isoforms with the results—representative for this class of molecules[9]—shown in Table 5. Compound **25** showed potent activity against HDAC1, 2, and 3 in the sub-micromolar range and were inactive against both HDAC8 and the class II HDAC's (HDAC5–7). Compound **25** showed improved selectivity profile for HDAC class I compared to the reference compound **A**.[4a]

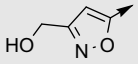
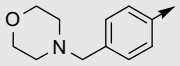
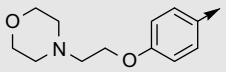
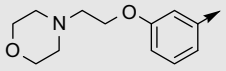
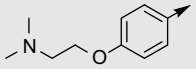
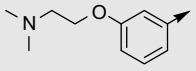
A pharmacokinetic study of **25** was carried out in female Sprague–Dawley rats. The pharmacokinetic parameters are shown in Table 6. The terminal phase half-life after iv dosing was 1.2 h in rats. The clearance was found to be low at 0.55 L/h/kg and the steady state volume of distribution was found to be low as well at 0.13 L/kg. The compound was quickly absorbed after oral dosing, with a T_{max} of 0.5 h. The oral bioavailability was found to be 16% in rats. The compound was also found metabolically stable in vitro (Microsomal assay) in three different species. The biotransformation rates (nmol/mg/min) were quite low: human (0.010), mouse (0.054), and rat (0.043). The solubility of compound **25** was found to be >1000 μg/ml (pH 1.2) and 77 μg/ml (pH 6.8).

As it was mentioned above, compound **25** displayed significant in vivo efficacy in tumor xenograft model in mice when administered po at doses ranging from 5 to 20 mg/kg once daily for 12 days against different cancer cell lines (Table 7). No adverse effects or signs of toxicity were observed.

In conclusion, a novel class of selective HDAC class I inhibitors based on the close analogues of the clinical compound **MGCD0103** was designed and synthesized. The three fragments of the pharmacophore were explored in order to elucidate the SAR. From the present study, the zinc binding group [*N*-(2-aminophenyl)benzamide] and the spacer (aminobenzyl) are two pieces of the pharmacophore which are sensitive to changes and substitutions. On the

Table 4
3-Pyridinyl ring replacement in compound **A**



Compound	R	HDAC1[a] IC_{50} (μM)	HCT 116[b] IC_{50} (μM)	HMEC[b] IC_{50} (μM)	H4 Ac[c] (T24) EC_{50} (μM)	p21 Luciferease HCT 116[d] EC_{50} (μM)
A		0.15	0.3	21	<1	0.6
22		0.19	0.3	23	1	0.6
23		0.07	0.07	7	3	0.3
24		0.14	n.a.	14	<1	0.5
25		0.14	0.3	24	1	n.a.
26		0.21	0.8	>50	1	n.a.
27		0.09	0.1	27	1	0.5
28		0.08	0.1	8	<1	0.1
29		0.14	0.3	41	1	0.4
30		0.12	0.09	10	<1	0.1
31		0.13	0.3	11	<1	n.a.

See footnotes in Table 2.

other hand, the 4-(pyridin-3-yl)pyrimidin-2-amino-fragment, a supposed solvent-exposed section can accommodate a diversity of substituents and a variety of side-chains causing in many cases an increase in inhibitory activity against HDAC1 enzyme and the cytotoxicity against human colon cancer cells (HCT116) with desirable selectivity over human mammary epithelial cells (HMEC). These new selective HDAC class I inhibitors also induce H4 histone hyperacetylation in T24 human cancer cells and trigger the expression of $p21^{WAF1/Cip1}$ tumor suppressor protein. Few analogues of **MGCD0103** were found efficacious when tested in human tumor xenograft models in mice in vivo. Among them, compound **25** displays good in vitro profile, possesses favorable pharmacokinetic characteristics and physicochemical properties. Compound **25** is orally active in vivo at low doses in different tumor xenograft models in mice without signs of adverse effects. These results represent an important step toward the development of selective HDAC class I inhibitors with favorable drug-like characteristics in the fight against human cancers.

Table 5
HDAC isoforms profile of compounds **25** and **A**

HDAC's	1	2	3	5	6	7	8
25 IC_{50} (μM)	0.14	0.14	0.32	>10	>20	>20	>10
A IC_{50} (μM)	0.15	0.29	1.66	>10	>10	>10	>10

Table 6
Rat plasma PK summary of compounds **25**

Compound	$T_{1/2}$ iv (h)	Cl iv (L/kg h)	V_{ss} iv (L/kg)	T_{max} po (h)	N C_{max} po μM/(mg/kg)	N AUC po μM h/(mg/kg)	F (%)
25	1.2	0.55	0.13	0.5	0.45	0.76	16

Dose iv, 2.5 mg/kg; dose po, 5 mg/kg.
For pharmacokinetic study, blood was collected from animals at various time points up to 6 h and plasma samples were analyzed using an Agilent 1100 HPLC system coupled with MDS Sciex API2000 triple quadrupole mass spectrometer.
A solution of 0.05 N HCl in saline was used as the vehicle for both iv and po dosing.

Table 7
Anti-tumor activity of compound **25** in different cancer cell lines

Cancer cell lines	HCT15 (colon)	AZ-521 (gastric)	TSU-Pr1 (prostate)	H460 (lung)
T/C in % (dose in mg/kg)	79 (5) 63 (10)	43 (5) 53 (10)	61 (10) 48 (20)	34 (20)

Vehicle: T/C = 100%.

References and notes

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