

Potent Antitumor Activity of N-Hydroxy-3-Benzimidazol-5-yl Acrylamides

A Novel Class of HDAC Inhibitors

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Abstract

A number of structural approaches have been undertaken to develop potent histone deacetylase (HDAC) inhibitors for the treatment of hematological and solid tumors. We herein describe N-Hydroxy-3-benzimidazol-5-yl acrylamides, a series of novel prototype HDAC inhibitors with improved *in vivo* properties as compared to other HDAC inhibitors in the clinic such as SAHA or PXD101. Out of this series SB623, SB624 and SB639 have been evaluated. These compounds inhibit histone deacetylase 1 (HDAC1) with K_i values of 29, 14 and 20 nM respectively.

These compounds show antiproliferative effects in human tumor cell lines, Colo205, HCT116, A2780 and PC3 with GI_{50} values (50% growth inhibition) in the range of 30 – 230 nM. No interaction with cytochrome P450 enzymes 2D6 and 3A4 was observed at submicromolar concentrations.

Under *in vivo* conditions, SB623, SB624 and SB639 were well tolerated with maximally tolerated doses above 50 mg/kg when given i.p. to mice over a period of 21 days. The anti-tumor activity of SB639 was studied in HCT116 bearing nude mice. SB639 was given q.d., over a period of 21 days, intraperitoneal administration (i.p.) at 100 and 130 mg/kg, and oral administration (p.o.) at 100 and 200 mg/kg. The tumor growth inhibition (TGI) at day 21 was 69%, 93%, 50% and 79%, respectively. Tumor growth delay (TGD), the time difference between the treatment group and the vehicle control on reaching the study endpoint (tumor volume of 1500 mm³), was significant ($p < 0.01$).

From the results of the present study it can be concluded that SB639, N-Hydroxy-3-[2-phenethyl-1-(2-pyrrolidin-1-yl-ethyl)-1H-benzimidazol-5-yl]-acrylamide, is a potent and effective anti-tumor drug and is the prototype of a new generation of histone deacetylase inhibitors suitable for the treatment of a variety of human tumors.

Introduction

Reversible acetylation of histones is a key component in the regulation of gene expression by altering the accessibility of transcription factors to DNA. In general, increased levels of histone acetylation are associated with increased transcriptional activity, whereas decreased levels of acetylation are associated with repression of gene expression.^{1,2} In normal cells, histone deacetylases (HDACs) and histone acetyltransferase (HAT) together control the level of acetylation of histones to maintain a balance.

Inhibition of HDACs results in the accumulation of acetylated histones, which trigger in a variety of cell type dependent cellular responses, such as apoptosis and inhibition of proliferation.

Inhibitors of HDAC have been studied for their therapeutic effects on cancer cells. For example, suberoylanilide hydroxamic acid (SAHA) is a potent inducer of differentiation and/or apoptosis in murine erythroleukemia, bladder, and myeloma cell lines.^{3,4} SAHA has been shown to suppress the growth of prostate cancer cells *in vitro* and *in vivo*.⁵ Recently, other small molecule HDAC inhibitors have become available for clinical evaluation.

The *in vivo* activity of such inhibitors can be directly monitored by their ability to increase the amount of acetylated histones in the biological sample.

At S*BIO, we have developed a series of novel, potent histone deacetylase (HDAC) inhibitors, N-Hydroxy-3-benzimidazol-5-yl acrylamides, with improved properties compared to the known HDAC inhibitors.

Materials and Methods

HDAC1 enzyme assay:

The HDAC1 cDNA was cloned and expressed in the insect cells. HDAC1 enzyme expressed in and purified from insect cells was used for screening of S*BIO compounds.

The Biomol HDAC1 fluorescent activity assay was applied and carried out in 96 well format (Biomol International, LP). The reaction mixture was composed of assay buffer, containing 25 mM Tris pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mg/ml BSA, tested compounds, 5 μ l of HDAC1 and 250 μ M *Flur de lys* generic substrate. The fluorescence was detected at the excitation wavelength 360 nm and emission wavelength 465 nm using Tecan Ultra Microplate detection System (Tecan Group Ltd, Switzerland).

Cell proliferation assay:

Human cancer cell lines, Colo205, PC3, HCT116 and A2780 were obtained from either ATCC or ECACC. They were cultivated according to instructions.

Cells were seeded in 96-wells plate. The plates were incubated at 37°C, 5%CO₂ for 24 h. Cells were then treated with compounds at various concentration for 96 h, after which the cell number was monitored using Cyquant™ cell proliferation assay (Invitrogen Pte Ltd).

Cell cycle assay:

Colo205 cells were plated in 6 well dish at 400,000 cells/well and incubated at 37°C for 24 h. Cells were treated with compounds at different concentrations. After 24 h treatment, cells were stained for DNA with propidium iodide, and analyzed on a BD Bioscience FACSCalibur cell sorter (BD Bioscience).

Apoptosis assay:

Colo205 cells were plated and treated as described in the cell cycle assay. After treatment, cells were stained with both Annexin V-FITC and PI (propidium iodide). The stained cells were analyzed on a BD Bioscience FACSCalibur cell sorter. The data from cell cycle and apoptosis assay were analysed using CellQuest software (BD Bioscience).

Hyperacetylation of histone H3:

ELISA plates were coated with mouse monoclonal antibody against histone H3. The plates were blocked and washed with PBST buffer.

Cells were cultivated in 96 well plates for 24 h and subsequently treated with HDAC inhibitors at various concentrations. After treatment for 24 h, cells were lysed and the protein concentration was determined.

The plates were washed and the protein lysates from treated cells were transferred to the ELISA plates. A secondary antibody was added to detect the acetylation of histone H3. The detection antibody conjugated with HRP (Pierce Pte Ltd) was applied to detect the secondary antibody. The absorbance was measured at OD₆₅₀ nm after addition of substrate or OD₄₅₀ nm after addition of stop solution (1M H₂SO₄).

In vivo efficacy study:

Female athymic nude mice, 10-12 weeks of age, were implanted subcutaneously in the flank with 5 x 10⁶ cells of HCT116 human colon carcinoma. When the tumor reached a size of 100 mm³, the mice were pair matched prior to treatment. Tumor size was measured every second day and the tumor volume calculated as follow:

$$\text{Tumor volume (mm}^3\text{)} = (w^2 \times l)/2, \text{ where } w = \text{width and } l = \text{length in mm.}$$

The selected compounds and a known HDAC inhibitor were dissolved in an appropriated vehicle for either intraperitoneal or oral administration. Drugs were orally administered everyday using a gavage for a period of 21 days.

Tumor growth inhibition (%TGI) was calculated according to

$$\%TGI = [(C_1 - T_1)/(C_1 - C_0)] \times 100$$

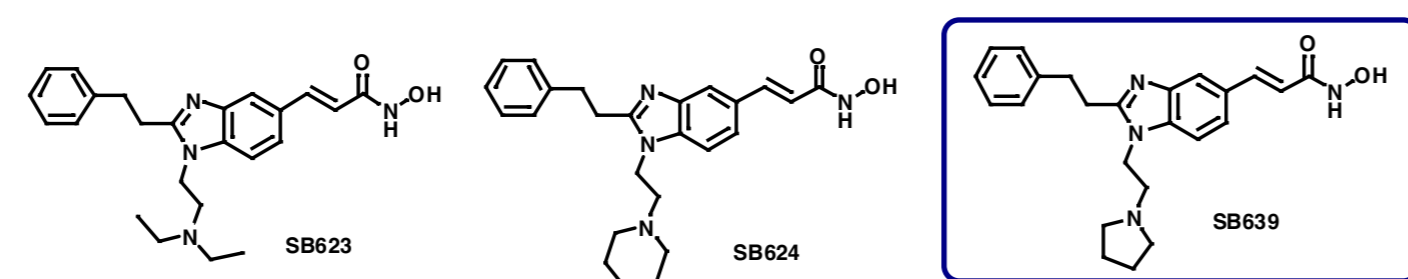
Where C_1 = the median tumor size of the vehicle control group at time t,

T_1 = the median tumor size of the treatment group at the time t,

C_0 = the median tumor size of the vehicle control group on the first day of treatment.

The tumor growth delay was defined as the increase in the median TTE (time to end point) for a treatment group compared to the control group. The Logrank test (Graph Pad Software Inc, USA) was used to determine the statistic significance of any difference in TTE between a treatment group and the control or vehicle control group.

Figure 1: Chemical structure of compounds from N-Hydroxy-3-benzimidazol-5-yl acrylamides series



Results

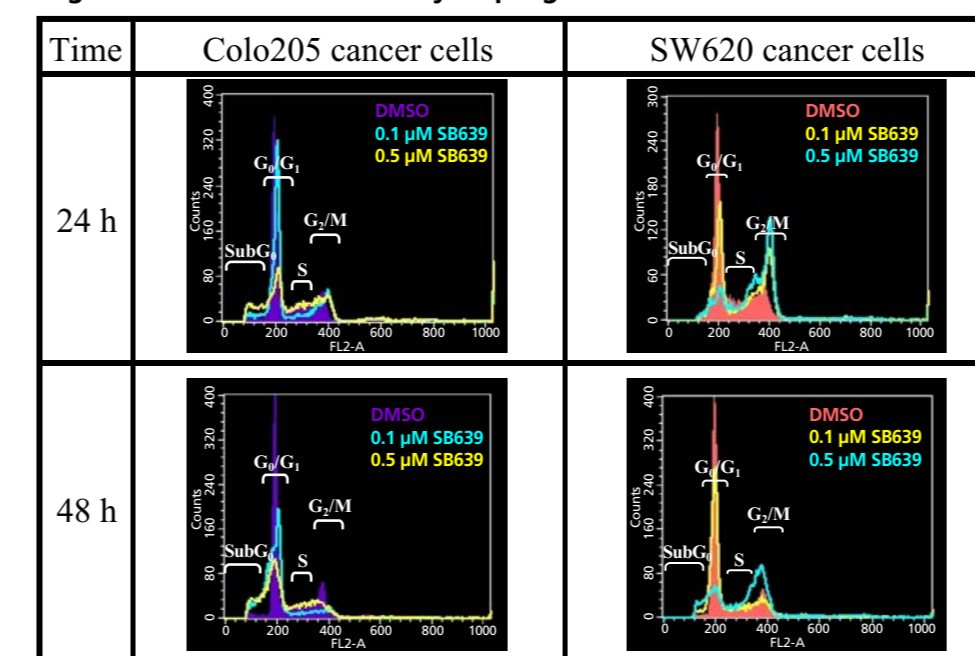
Figure 2: Inhibition of recombinant HDAC1 activity

Compounds	HDAC1 K_i (nM \pm SD; n \geq 2)
SB623	29 \pm 14
SB624	14 \pm 7
SB639	20 \pm 9
LBH589	2 \pm 0.1
MS275	284 \pm 4
PXD101	25 \pm 2
SAHA	61 \pm 13

Figure 3: Inhibition of proliferation of human cancer cell lines

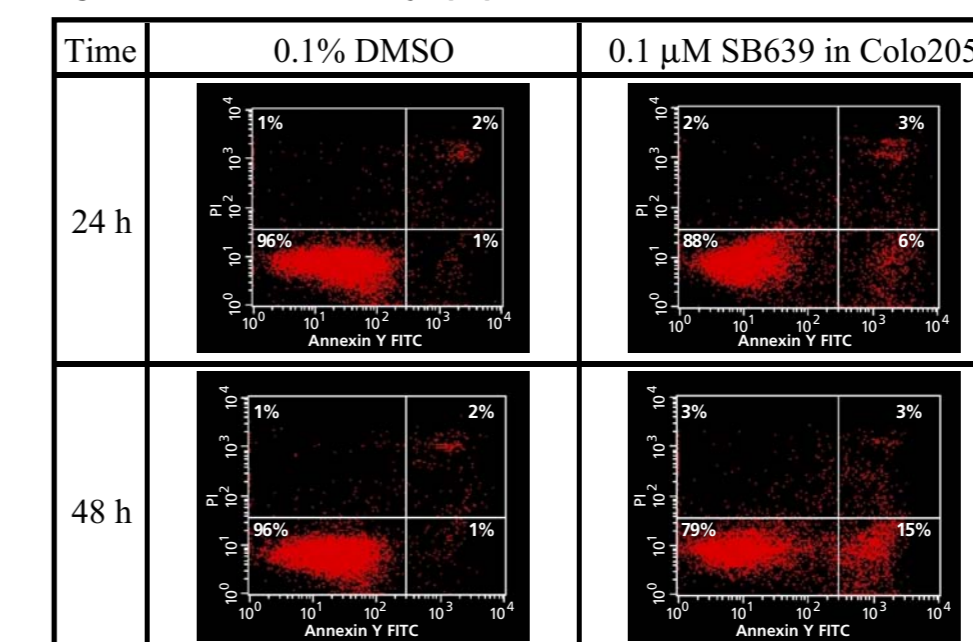
Compounds	GI_{50} (μ M \pm SD, n \geq 2)			
	Colon		Ovarian	Prostate
	Colo205	HCT116	A2780	PC3
SB623	0.21 \pm 0.10	0.23 \pm 0.09	0.09 \pm 0.00	0.20 \pm 0.09
SB624	0.09 \pm 0.06	0.10 \pm 0.03	0.03 \pm 0.00	0.12 \pm 0.04
SB639	0.13 \pm 0.04	0.20 \pm 0.10	0.15 \pm 0.09	0.15 \pm 0.08
LBH589	0.02 \pm 0.00	0.05 \pm 0.03	0.03 \pm 0.00	0.03 \pm 0.02
MS275	2.99 \pm 0.76	6.71 \pm 0.73	1.56 \pm 0.43	0.07 \pm 0.02
PXD101	0.60 \pm 0.34	0.58 \pm 0.11	0.42 \pm 0.12	0.42 \pm 0.23
SAHA	2.20 \pm 0.45	3.50 \pm 1.04	1.60 \pm 0.49	1.10 \pm 0.70

Figure 4: Prevention of cell cycle progression in cancer cells



The selected HDAC compound, SB639, arrested Colo205 cell at G₁/S phase and SW620 at G₂/M phase, and increased apoptotic cell death at subG₀ phase, in a dose and time dependent manner.

Figure 5: Induction of early apoptotic cell death in cancer cells



At a fixed concentration of 0.1 μ M, treatment using SB639 resulted in an increase in early apoptotic cell death from 6% at 24 h to 15% at 48 h.

Figure 6: In vitro hyperacetylation of histone H3

Compounds	EC ₅₀ (μ M \pm SD, n \geq 2)
SB623	0.47 \pm 0.13
SB624	0.14 \pm 0.11
SB639	0.26 \pm 0.22
LBH589	0.08 \pm 0.04
PXD101	2.0 \pm 0.24
SAHA	> 7

After treatment of Colo205 cancer cells with different concentrations of the HDAC inhibitor, accumulation of cellular acetylation histone H3 was observed.

ADME attributes of N-Hydroxy-3-benzimidazol-5-yl acrylamides

- Aqueous solubility > 25 mg/ml
- No P450 inhibition potential with major drug metabolizing isozymes
- Compounds exhibit good metabolic stability in the human liver microsomes
- Highly permeable in a Caco-2 model
- Oral bioavailability
- Good correlation of PK/PD in tumor bearing mice

Conclusions

The compound, SB639, described herein, is a promising candidate in a series of N-Hydroxy-3-benzimidazol-5-yl acrylamides comprising the following attributes:

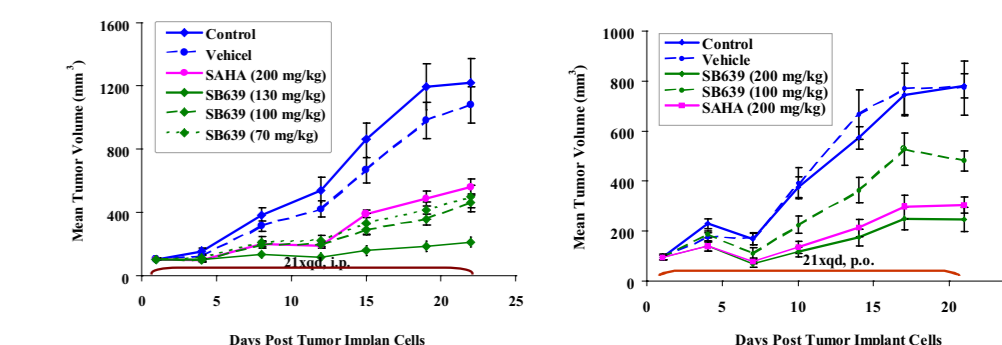
- 1) Drug like physico-chemical properties.
- 2) Inhibition of proliferation of numerous cancer cell lines.
- 3) Inhibition of cell cycle progression and induction of apoptosis
- 4) Good pharmacokinetic and ADME properties.
- 5) Long lasting inhibition of cellular HDAC enzyme activity with accumulation of acetylated histone H3.
- 6) Good *in vivo* anti-tumor activity, resulting in significant tumor growth delay, in xenografts compared to SAHA.

Figure 7: In vivo hyperacetylation of histone H3

p.o.	Vehicle	SB639	SAHA
Dose (mg/kg)		300	200
Time (h)	1 3 6 16 24	1 3 6 16 24	1 3 6 16 24
AcH3			
Actin			

- The *in vivo* biological effects of SB639 was investigated in HCT116 bearing nude mice and were compared to SAHA.
- Acetylation of histone H3 in the tumor tissue samples was detected by western-blot.
- SB639 increased the level of acetylation of histone H3 in the tumor tissue for up to 24 h after oral administration at 300 mg/kg respectively whereas hyperacetylation of histone H3 by SAHA was observed only up to 6 h after oral administration at 200 mg/kg.

Figure 8: Evaluation of anti-tumor activity of SB639 in HCT116 xenograft model via different routes and at different doses



- SB639 exhibited a dose response in the treated HCT116 xenograft via different routes.
- The parenteral anti-tumor activity of SB639 ranged from 67–93% while the oral anti-tumor activity of SB639 ranged from 50–79%.
- Significant tumor growth delay ($p < 0.01$) was observed.

References

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