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ScienceDirect
Biochemical and Biophysical Research Communications 356 (2007) 233–238
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Anti-tumor activity of *N*-hydroxy-7-(2-naphthylthio) heptanomide, a novel histone deacetylase inhibitor

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Received 21 February 2007
Available online 2 March 2007

Abstract

Histone deacetylase (HDAC), a key enzyme in gene expression and carcinogenesis, is considered an attractive target molecule for cancer therapy. Here, we report a new synthetic small molecule, *N*-hydroxy-7-(2-naphthylthio) heptanomide (HNHA), as a HDAC inhibitor with anti-tumor activity both *in vitro* and *in vivo*. The compound inhibited HDAC enzyme activity as well as proliferation of human fibrosarcoma cells (HT1080) *in vitro*. Treatment of cells with HNHA elicited histone hyperacetylation leading to an up-regulation of p21 transcription, cell cycle arrest, and an inhibition of HT1080 cell invasion. Moreover, HNHA effectively inhibited the growth of tumor tissue in a mouse xenograph assay *in vivo*. Together, these data demonstrate that this novel HDAC inhibitor could be developed as a potential anti-tumor agent targeting HDAC.

Keywords: Histone deacetylase; HDAC inhibitor; *N*-Hydroxy-7-(2-naphthylthio) heptanomide; Anti-tumor agent; Cell cycle arrest

Histone acetylation is regulated by two covalent modifying enzymes, acetylases and deacetylases, and plays a crucial role in gene expression of eukaryotes. Acetylated histones are found in transcriptionally active regions; whereas, transcriptionally inactive genes are associated with hypo-acetylated histones [1]. Numerous studies have shown that inappropriate histone acetylation results in dysregulation of cell growth/death, leading to neoplastic transformation [2].

Recent studies have revealed that histone deacetylase (HDAC) plays a key role in carcinogenesis [3]. HDAC is overexpressed in several tumor cells [4]. Many studies have demonstrated that the expression of tumor suppressors, such as p53, p21, and gelsolin, are repressed [5–7], whereas, tumor activators, such as hypoxia-induced factor-1 (HIF-1) and vascular endothelial growth factor (VEGF), are up-regulated in HDAC-overexpressed cells [8]. Moreover, it has been recently reported that hypo-acetylated tubulin, which is regulated by HDAC6, is present at high levels in patients with neurodegenerative disorders such as Alzheimer's disease [9,10]. Accordingly, inhibition of HDAC activity has been recognized as a promising strategy for treating cancer and other HDAC-related diseases. In fact, HDAC inhibition by specific inhibitors leads to several changes at the molecular and cellular level. Included among these changes are alterations in protein acetylation state and subsequent changes in expression of target genes as well as changes in cellular morphology, proliferation, and migration [11]. In the last 10 years, a number of HDAC inhibitors from natural sources and from chemical libraries have been used to study the function of post-translational modification in regard to acetylation in cells. Among them, trichostatin A (TSA), HC toxin, apicidin, and recently, FK228 have been isolated from microbial

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doi:10.1016/j.bbrc.2007.02.126

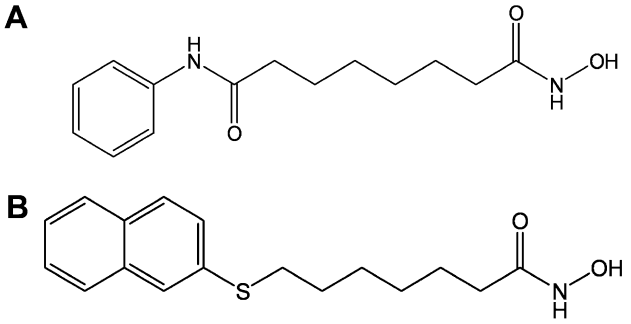

Fig. 1. Chemical structure of HDAC inhibitors. (A) Suberoylanilide hydroxamic acid (SAHA). (B) *N*-Hydroxy-7-(2-naphthylthio) heptanomide (HNHA), a novel HDAC inhibitor.

metabolites [12–15]. In addition, sodium butyrate, suberoylanilide hydroxamic acid (SAHA), and benzamide derivatives (e.g., MS-27-275) are synthetic compounds [7,16–18]. Among these compounds, SAHA has been recently launched as the first clinical anti-tumor drug of this class of inhibitors and others such as FK228 and MS-27-275 are being investigated in clinical trials [19]. However, there is a continuous need to develop new HDAC inhibitors with better pharmacological efficacy and novel mode of action in comparison to known HDAC inhibitors.

Herein, we report the *N*-hydroxy-7-(2-naphthylthio) heptanomide (HNHA, Fig. 1) is a new HDAC inhibitor that potently suppresses histone hypoacetylation, down-regulation of target genes of HDAC, tumor cell invasion, and tumor growth via inhibition of the enzyme activity. These data demonstrate that HNHA could be developed as a new anti-tumor agent targeting HDAC function.

Materials and methods

Materials. AK-500 kit was obtained from Biomol (Butler Pike, PA). Dulbecco's modified Eagle's medium (DMEM), 1% antibiotics, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). Minimum essential medium was obtained from Invitrogen (Carlsbad, CA). PVDF membrane and chemiluminescence kit were purchased from Millipore (Bradford, MA) and Pierce (Rockford, IL), respectively. Acetylated histone and tubulin antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Matrigel and Transwell plate were from Collaborative Biomedical Products (Bedford, MA) and Corning Costar (Cambridge, MA), respectively. HNHA and its derivatives were synthesized in our laboratory and the procedure was documented at Korean Patent (KP 10-0620488).

In vitro HDAC assay. Nuclear fractions collected from cultured cells were used as a HDAC source. Nuclear fractions were lysed with 0.5% Triton X-100 containing phosphate buffer (pH 8.0) and centrifuged. HDAC activity was assayed from 10 μg of protein extract using a commercially available kit (AK-500). Assays were performed in a 96-well plate according to the manufacturer's instruction. Fluorescent, deacetylated substrate was detected with a FL600 Microplate Reader (Bio-Tek Instrument, Inc., Winooski, VT).

Cell proliferation assay. Human fibrosarcoma cells (HT1080) and gastric cancer cells (YCC-3) were maintained at 37 °C under a humidified, 5% CO_2 atmosphere in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% antibiotics. The stock solution of compounds were prepared immediately before use. The cells were seeded in 96-well plates, and 24 h later, the cells were treated with the newly developed chemicals at various concentrations for 72 h. Cellular proliferation was then measured using MTT assay, and morphology was observed using an optical microscopy at 100× magnification (Olympus Optical Inc., Melville, NY). The cells were cultured at 37 °C in 5% CO_2/95% air in MEM (minimum essential medium, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U penicillin.

Hyperacetylation of proteins. The cells were cultured in 100 mm dishes under the conditions described above. The compounds (10 μM) were treated with the cells for various time points from 0 to 16 h. After the treatment, the cells were washed and harvested by centrifugation. Histones were extracted from the nuclear fraction, and the isolated histones subjected to 15% SDS–PAGE and transferred to a PVDF membrane. Membrane was immunoblotted with antibodies against acetylated histone and tubulin and subsequently with α-rabbit or α-mouse antibodies. Acetylated proteins were detected using a chemiluminescence kit.

Induction of p21 gene. For reporter gene assays, the cells bearing human wild-type p21 promoter-luciferase fusion plasmid were plated onto 6-well plates and treated with various concentrations of compounds for 16 h. After the treatment, the cells were washed and harvested by centrifugation. Cells were lysed with 0.5% Triton X-100 containing phosphate buffer (pH 8.0), and lysates were clarified by centrifugation. Luciferase activity was measured with a FL600 Microplate Reader (Bio-Tek Instrument, Inc.) and was normalized for protein concentration.

Cell cycle analysis by flow cytometry. HT1080 cells were seeded in 6-well plates (10^5 cells/well) and incubated for 24 h. The cells were starved for 24 h in serum-free DMEM. After synchronization, the cells were treated with or without the indicated compounds, and the incubation was continued for 24 h in the presence of 10% serum. The cells were then harvested with trypsinization, fixed, and permeabilized in the presence of 70% ethanol. The cells were centrifuged and resuspended in phosphate-buffered saline (PBS, pH 7.4). To reduce background staining, RNase (80 μg/mL) was added. Specific DNA staining was then carried out using propidium iodide (50 μg/mL). The DNA histograms were determined using a Beckton–Dickenson FACS Vantage flow cytometer system (Beckton–Dickenson, San Jose, CA), and the cell cycle distribution was analyzed using Cell Quest software version 3.2 (Beckton–Dickenson).

In vitro invasion assay. The invasiveness of tumor cells was performed *in vitro* using a transwell chamber system with 8.0-μm pore-polycarbonate filter inserts (CorningCoster, Cambridge, MA). The lower side of the filter was coated with 10 μL gelatin (1 mg/mL), and the upper side was coated with 10 μL of the Matrigel. HT1080 cells (1×10^5) were placed in the upper part of the filter. The chamber was then incubated at 37 °C for 18 h. The cells were fixed with methanol and stained with hematoxylin/eosin. The invasiveness of tumor cells was determined by counting the total number of cells on the lower side of the filter at a 100× magnification.

Animal model. Female athymic BALB/C nu/nu mice aged 6 weeks and with a body weight of 20 g were obtained from the Department of Animal Experiment of Yonsei University Medical Research Center. Animals were maintained under specific pathogen free (SPF) conditions. All animal procedures were approved by the Korea Medical Experimental Animal Care Commission, prior to experimentation. The tumor cells in exponential growth phase were harvested using 0.25% trypsin–EDTA, washed, and suspended in normal saline. Only suspensions of single cells with a viability exceeding 95% were used. Approximately 2×10^7 cells (in 100 μL of normal saline) were injected subcutaneously into the upper left flank region of each mouse. Mice with tumors of 5 mm in diameter within proximity of the injection site (2 weeks after inoculation) were randomly allocated into the following four groups, with each group consisting of three mice: control group (DMSO), SAHA group (20 μM/mouse), and HNHA group (20 μM/mouse). Drugs were administered intraperitoneally every other day for 21 days. Mice were weighed three times weekly, and tumor size was measured by the caliper method three times weekly starting on day 1. Tumor volume was calculated using the following formula: $(\text{length} \times \text{width}^2)/2$. Survival time was also measured in each group.

Data analysis and statistics. Data were presented as means ± SD or as the percentage of control. The Student's *t* test was used to determine statistical significance between control and test groups. A p value of <0.05 was considered statistically significant.

Results and discussion

Inhibition of HDAC activity and cell growth by HNHA

The biological activity of newly synthesized HDAC inhibitors having a hydroxyamide-based pharmacophore as like SAHA and TSA was assessed using both HDAC enzyme assay and cell proliferation assay. The IC_{50} values of the compound are presented in Table 1. Among the compounds, HNHA exhibited potent HDAC-inhibitory activity with IC_{50} values of 0.1 μM. In contrast, other compounds containing substituted nitrogen or oxygen for sulfur atom and having different lengths of alkyl chain (C5 and C7) showed weaker activity than that of HNHA. Moreover, compounds containing different zinc-chelating motifs such as an aminophenyl group or a hydroxy-acetylamino group had a weaker HDAC-inhibitory activity (data not shown).

The cellular effects of HNHA were further assessed in human fibrosarcoma cells (HT1080). Treatment of cells with HNHA resulted in a dose-dependent growth inhibition. In addition, cells treated with HNHA exhibited a more elongated shape than that of control cells; this is similar to the morphological changes seen in SAHA or other known HDAC inhibitor-treated cells (data not shown). The IC_{50} value was estimated to be 7.5 μM (Table 1). These data demonstrate that hydroxyamide as a zinc-chelating group and a sulfur atom as a linkage group in the compound are efficient as functional units of HDAC inhibition. This result also suggests that newly introduced sulfur linker in the compound may improve the pharmacokinetic properties or the HDAC-inhibitory potency of the compound via oxidation by monooxygenases in the cells. Considering HDAC role in solid tumors and other diseases such as Alzheimer disease (AD), it is important to note that HDAC inhibitors with high absorption potentials may have better pharmacological potentials than known HDAC inhibitors [20]. In this sense, we further investigated the HDAC-inhibitory activity of HNHA both in *in vitro* and *in vivo*.

Effect of HNHA on HDAC activity in cells

Many studies have demonstrated that inhibition of HDAC activity by specific inhibitors induces hyperacetylation of histones in drug-treated cells. In addition, there is emerging evidence that HDAC6 is involved in stability of dynamic microtubules through the regulation of tubulin acetylation status [21–23]. To investigate whether HNHA inhibits HDAC activity in cells, the levels of hyperacetylated histone and tubulin were measured using antibodies specific for acetylated H3-histone and tubulin, respectively. As shown in Fig. 2A, HNHA induced the accumulation of acetylated H3-histone and tubulin, as compared to that of SAHA. Interestingly, the hyperacetylation induced by HNHA peaks at earlier time compared to that in SAHA-treated cells.

Table 1
HDAC-inhibitory activity of SAHA and HNHA

Compound	IC_{50} values (±SD, μM)	
	HDAC activity	Cell proliferation
SAHA	0.05 ± 0.005	3 ± 0.2
HNHA	0.10 ± 0.010	7.5 ± 0.8

Next, the changes of HDAC target gene expression as a consequence of inhibition of HDAC activity by HNHA were also investigated. It is well established that hyperacetylation of histones induces the expression of tumor suppressors such as a $p21^{WAF1}$. An inhibitor of cyclin-CDK, $p21^{WAF1}$, is essentially required for the HDAC inhibitor-induced cell cycle arrest in a variety of tumor cells [14,24,25]. The well-known HDAC inhibitors such as TSA, SAHA, and FK228 induce the expression of $p21^{WAF1}$. To examine the effect of HNHA on expression of $p21^{WAF1}$ gene, SAHA and HNHA was treated to Mv1Lu cells that were stably transfected with luciferase reporter construct under control of the $p21^{WAF1}$ promoter. The ability of HNHA to induce reporter expression was largely paralleled with the pattern of SAHA (Fig. 2B). These data clearly demonstrate that HNHA can inhibit HDAC activity in the cells.

Effect of HNHA on the cell cycle progression of tumor cells

Suppression of $p21^{WAF1}$ expression in transformed cells is alleviated by HDAC inhibitors, such as phenylbutyrate, TSA, and SAHA, leading to cell cycle arrest [14,26,27]. Indeed, FACS analysis of HT1080 DNA content revealed that HNHA inhibited cell cycle progression in a manner identical to SAHA (Fig. 2C). S phase was decreased by 20%, whereas G_0–G_1 phase was increased approximately 10%. These cell cycle changes induced by HNHA were paralleled with an increase of $p21^{WAF1}$ expression in HNHA-treated cells, implying that inhibition of cellular HDAC activity by HNHA resulted in the change of cell cycle regulation.

Inhibition of tumor cell invasion by HNHA

Several studies have shown that invasion of tumor cells is a crucial step for the spreading and migration of cells during metastasis and the formation of new blood vessel [8]. Accordingly, the ability to inhibit this step is considered to be a key feature of anti-metastasis agents [28]. Based on this idea, we next investigated the effect of HNHA on tumor cell invasion *in vitro*. Indeed, HNHA potently inhibited HT1080 cell invasion in a dose-dependent manner without showing any toxicity to the cells (Fig. 3). Quantitative data from the tumor cell invasion assay are presented in C.

Anti-tumor activity of HNHA in mouse xenograft assay

Several HDAC inhibitors, including MS-27-275 and a hydroxyamide-based HDAC inhibitors such as SAHA,

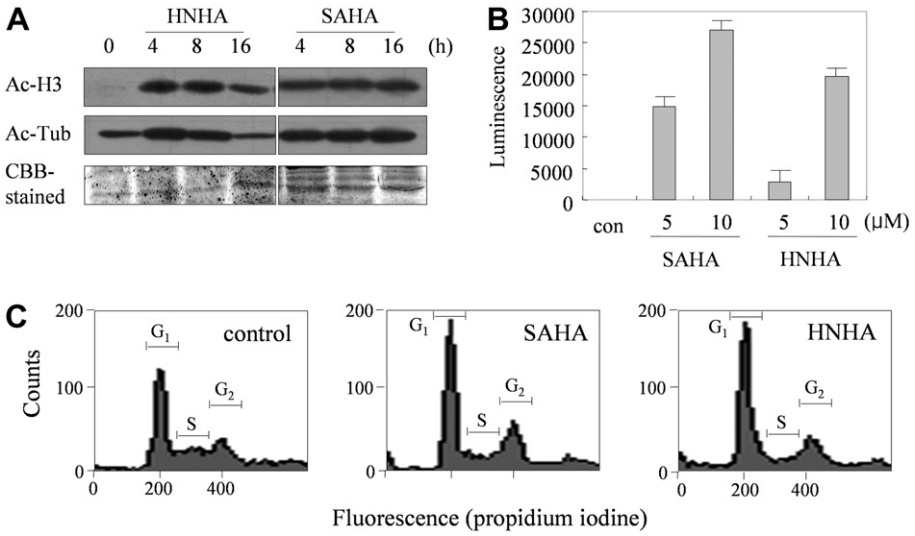

Fig. 2. Effect of HNHA on HDAC activity in HT1080 cells. Cells were treated with the indicated HDAC inhibitor, and lysates were subjected to Western blot analysis of acetylated histones (Ac-H3) and tubulin (Ac-Tub). Tubulin was stained with Coomassie brilliant blue (CBB) as a loading control. (B) Quantitative analysis of activation of p21 reporter gene expression by HNHA and SAHA. (C) Cells were treated with the indicated HDAC inhibitor at a concentration of 10 μM, and FACS analysis of DNA content was performed.

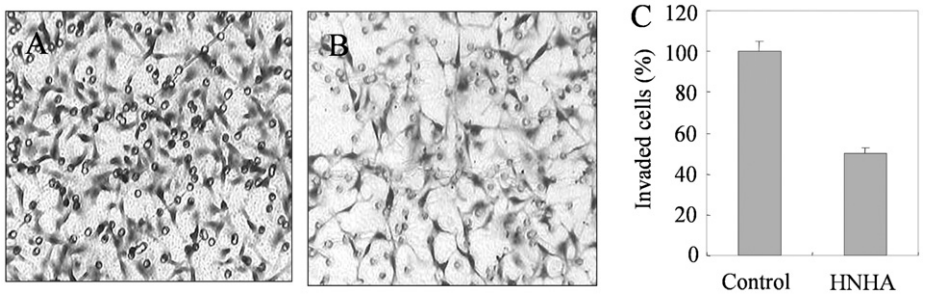

Fig. 3. Effect of HNHA on invasion of HT1080 cells. An *in vitro* tumor cell invasion assay was performed in (A) control cells and in cells treated with (B) 10 μM of HNHA. Quantitative analysis of cell invasion is presented in (C). Figures were selected as representative images from three independent experiments.

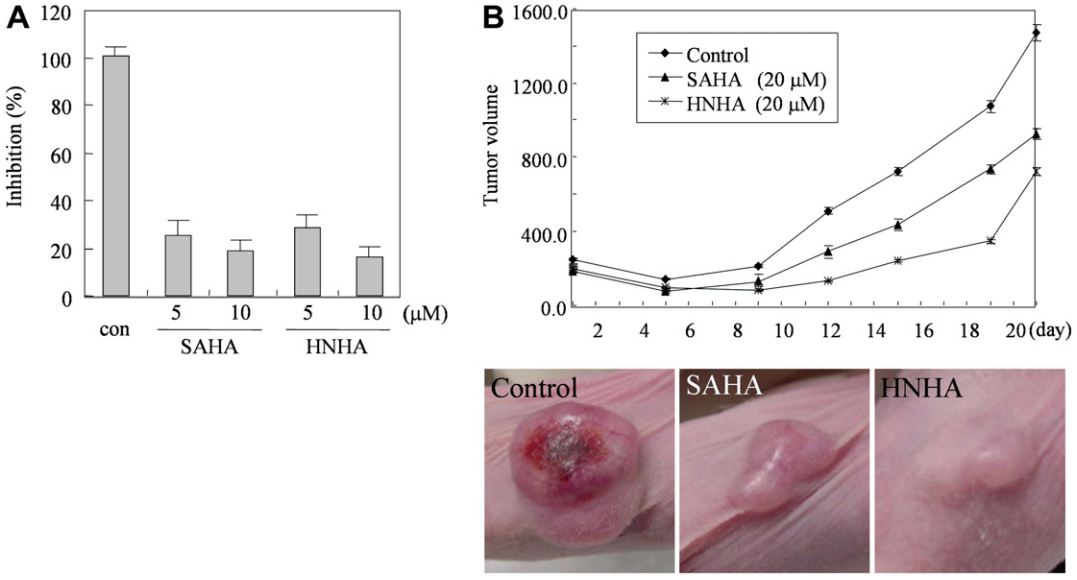

Fig. 4. Effect of HNHA in mice bearing human gastric cancer tissue. (A) Tumor volume was determined as described in Materials and methods. (♦), control; (▲), 20 μM of SAHA; (×), 20 μM of HNHA. (B) Reduction of tumor volume in the compound-treated mice after 21 days.

have been investigated extensively and shown to inhibit tumor growth *in vivo* [7,29]. Among them, SAHA elicits a dramatic reduction in tumor volume and incidence without side effects in experimental animals [29,30]. To test whether HNHA exhibits similar effects *in vivo*, VEGF production in serum prepared from cancer patients was measured after treatment with several concentrations of SAHA and HNHA (Fig. 4A). VEGF stimulates angiogenesis via promotion of endothelial cell proliferation, and its expression is known to be regulated by HDAC activity [8]. VEGF production was inhibited by HNHA to the same degree as SAHA. We next examined the effects of HNHA on human cancer cells bearing xenograph mice (Fig. 4B). As expected, HNHA dramatically reduced tumor volume as to (vehicle) control. Moreover, HNHA exhibited a stronger potency than that of SAHA. These data imply that the pharmacological potency of HNHA is better than SAHA *in vivo*.

In conclusion, HNHA is newly developed based on chemistry of a known HDAC inhibitor, SAHA and TSA, with an aim to develop a novel HDAC inhibitor having better pharmacological potentials. HNHA exhibited potent-inhibitory activity *in vitro* (HDAC enzyme assay) and *in vivo* (cellular proliferation and invasion assay). Indeed, the compound induced hyperacetylation of histones that leads to expression of tumor suppressor, $p21^{WAF1}$, and inhibition of tumor cell invasion. Notably, HNHA markedly decreased tumor volume in human cancer bearing xenograph mice and exhibited stronger potency than SAHA. Taken together, our data demonstrate that HNHA can be developed as a new anti-tumor agent targeting HDAC activity.

Acknowledgments

This study was supported by grants from the National R&D Program for Cancer Control, Ministry of Health & Welfare (0620360-1), and from the Brain Korea 21 Project, Republic of Korea.

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