Class I Histone Deacetylase-Selective Novel Synthetic Inhibitors Potently Inhibit Human Tumor Proliferation

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ABSTRACT

We have developed previously a class of synthetic hybrid histone deacetylase (HDAC) inhibitors, which were built from hydroxamic acid of trichostatin A and pyridyl ring of MS-275. In this study we evaluated the antitumor effects of these novel hybrid synthetic HDAC inhibitors, SK-7041 and SK-7068, on human cancer cells. Both SK-7041 and SK-7068 effectively inhibited cellular HDAC activity at nanomolar concentrations and induced the time-dependent hyperacetylation of histones H3 and H4. These HDAC inhibitors preferentially inhibited the enzymatic activities of HDAC1 and HDAC2, as compared with the other HDAC isotypes, indicating that class I HDAC is the major target of SK-7041 and SK-7068. We found that these compounds exhibited potent antiproliferative activity against various human cancer cells in vitro. Growth inhibition effect of SK-7041 and SK-7068 was related with the induction of aberrant mitosis and apoptosis in human gastric cancer cells. Both compounds induced the accumulation of cells at mitosis after 6 h of treatment, which was demonstrated by accumulation of tetraploid cells, lack of G2 cyclin/cyclin-dependent kinase inactivation, and higher mitotic index. After 12 h of treatment, apoptotic cells were increased through mitochondrial and caspase-mediated pathway. Finally, in vivo experiment showed that SK-7041 or SK-7068 was found to reduce the growth of implanted human tumors in nude mice. Therefore, based on isotype specificity and antitumor activity, SK-7041 and SK-7068 HDAC inhibitors are expected to be promising anticancer therapeutic agents and need additional clinical development.

INTRODUCTION

Cancer cells contain multiple genetic defects and, thus, the targeting of single genetic abnormalities may be an ineffective approach to a cancer therapy. Epigenetic gene inactivation has emerged recently as an important step in tumorigenesis. It is known that multiple target genes, such as tumor suppressor genes, cell cycle genes, differentiation genes, and DNA repair genes are silenced by epigenetic transcriptional repression (1–7). Therefore, the targeting of epigenetic transcriptional regulation might be more powerful than the targeting of a single genetic pathway (3). Because of the linkage between transcriptional repression and histone deacetylase (HDAC) recruitment, HDAC inhibitors may reverse silenced genes (8–10) and induce cell-cycle arrest at G1 and/or at G2, differentiation, and apoptosis in vitro; moreover, they may have potent antitumor activity in vivo (11–18). Almost all of the HDAC inhibitors activate the transcription of the cyclin-dependent kinase inhibitor, p21WAF1, which results in G1 arrest (7). On the contrary, it is reported that some HDAC inhibitor such as azelaic bishydroxamic acid target G2 checkpoint and, thus, induce aberrant mitosis in G2 checkpoint-defective cancer cells (19). Moreover, trichostatin A (TSA) induced mitotic arrest by activating mitotic spindle checkpoint protein such as BubR1, or possibly by acetylation of α-tubulin (20, 21). Therefore, it is conceivable that the anti-tumor effects of HDAC inhibitors may be dependent on mitotic regulation as well as direct modulation of gene expression (22, 23).

Many structurally diverse compounds can bind to HDACs and inhibit HDAC enzymatic activity. These compounds are viewed as the first generation of HDAC inhibitors and fall into four classes, short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, and benzamides (3, 4). Each HDAC inhibitor is viewed as the first generation of HDAC inhibitors and fall into four classes, short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, and benzamides (3, 4). Each HDAC inhibitor is composed of a functional moiety, a linker, and a cap structure. The functional moiety of TSA binds to the zinc located in the catalytic pocket of HDACs and, thus, inhibits activity. The cap structure of HDAC inhibitors interacts with amino acids at the entrance rim of the N-acetyl lysine binding channel of HDAC, which determines the specificity of the inhibitor (24). Although TSA has a potent inhibitory effect upon HDACs, it also has the drawbacks of instability and low bioavailability. On the other hand, the recently developed benzamide derivative MS-275 has a pyridyl ring cap substructure, which has better physicochemical properties than TSA (24–26).

In an attempt to enhance the pharmaceutical properties and the enzyme selectivity of HDAC inhibitors, we replaced the cap structure of TSA with a pyridyl ring. Furthermore, the incorporation of a 1,4-phenylene carboxamide linker and the modification of the 4-(pyrrolidi-L -yl)phenyl group of the cap ring gen-
erated the set of novel hydroxamic acid-based HDAC inhibitors (Fig. 1; Ref. 27). Of these novel inhibitors, SK-7041 and SK-7068 potently inhibited cancer cell proliferation. Both SK-7041 and SK-7068 also showed target enzyme preference for class I HDAC1 and HDAC2. Interestingly, SK compounds induced aberrant mitosis and apoptotic cell death in human cancer cells. More importantly, among various HDAC inhibitors, SK-7041 showed the most potent antitumor activity in an in vivo animal study. Therefore, the novel HDAC inhibitors SK-7041 and SK-7068 are regarded as promising anticancer drug candidates in view of their target specificities and antitumor activities.

MATERIALS AND METHODS

Reagents. Antibodies to caspase 3 (H-277), caspase 9 (H-83), cytochrome C (H-104), cyclin-dependent kinase (Cdk)2 (M2), Cdc2 (sc-54), Cdc25c (C-20), cyclin B1 (GNS1), Chk1 (FL-476), HDAC4 (H-92), HDAC5 (H74), and HDAC6 (H-300) were purchased from Santa Cruz Biotechnology, Inc. Antibodies to p21 (C24420) and p27 (K25020) were obtained from Transduction Laboratories. The antibody to poly(ADP-ribose) polymerase (7D3–6) was provided by PharMingen. Antibodies to Chk2 (07–126), HDAC1 (06–720), and HDAC3 (06–890); acetylated histones H3 (06–599) and H4 (06–866); and mitotic phosphoprotein monoclonal (MPM2; 05–211) were purchased from Upstate Biotechnology Inc. Antibody to HDAC2 (51–5100) was obtained from Zymed Laboratories Inc. and zVAD-fmk from Calbiochem-Novabiochem Corp. Histone H1 was purchased from Boehringer Mannheim. The novel synthetic HDAC inhibitors, referred to as SK compounds, were supplied by In2Gen Co. (Seoul, Korea). TSA was purchased from Sigma, and MS-275 and suberoylanilide hydroxamic acid were generous gifts from Dr. Dae-Kee Kim (In2Gen Co.).

Cell Culture. Human gastric adenocarcinoma cells (SNU-1, 5, 16, 601, and 638) were obtained from the Korea Cell Line Bank (28) and grown in RPMI 1640 supplemented with 10% fetal bovine serum and gentamicin (10 μg/ml). Human breast cancer cells (SKBR3) and lung cancer cells (A549) were obtained from American Type Culture Collection and grown in RPMI 1640 or DMEM supplemented with 10% fetal bovine serum and gentamicin (10 μg/ml). Nontransformed rat intestinal epithelial cells were grown in DMEM supplemented with 5% fetal bovine serum and gentamicin (10 μg/ml). All of the cells were incubated under standard culture condition (20% O2 and 5% CO2; 37°C).

Cell Growth Inhibition Assay. Cells were seeded on 96-well plates and incubated for 24 h at 37°C and treated with drugs (i.e., SK-7041, SK-7068, MS-275, and suberoylanilide hydroxamic acid) for 3 days at 37°C. After drug treatment, cells were washed thoroughly with PBS, and fresh medium with no SK-7068 or MS-275 was added back. Cells were then incubated additionally for 4 days.

Acid-Soluble Nuclear Protein Preparation. For histone preparation, cells were cultured as indicated, harvested, and washed with PBS. Cells were then pelleted and resuspended in 1 ml of ice-cold lysis buffer [10 mM Tris-HCl (pH 6.5), 50 mM sodium bisulfite, 1% Triton X-100, 10 mM MgCl2, and 8.6% sucrose] before being homogenized with two dounce strokes.

Fig. 1. Molecular structure of the novel synthetic histone deacetylase inhibitors. SK-7041 and SK-7068 are structurally based on hydroxamic acid and benzamide. R, substituted side chain; TSA, trichostatin A.
Nuclei were pelleted by centrifugation at 700 rpm for 5 min and washed three times with 1 ml of lysis buffer. The final wash was performed with 1 ml of Tris-EDTA solution [10 mM Tris-HCl (pH 7.4) and 13 mM EDTA]. Pelleted nuclei were resuspended in 100 μl of ice-cold water. Sulfuric acid was then added to the samples to a final concentration of 0.2 M, vortexed, incubated on ice for 1 h, centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant proteins so obtained were precipitated with 1 ml of acetic acid overnight at −20°C. The precipitated proteins were collected by centrifugation at 15,000 × g for 10 min at 4°C, air dried, and resuspended in 50 μl of water.

**HDAC Inhibition Assay.** SNU-16 cells were quickly cooled by placing the plates on ice and were then washed twice with ice-cold PBS. Collected cells were centrifuged and resuspended in five packed cell volumes of buffer A [20 mM Tris (pH 7.6), 10 mM KCl, 0.2 mM EDTA, 20% glycerol, 1.5 mM MgCl₂, 2 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM NaVO₄, and 2 μg/ml each of leupeptin, pepstatin, and aprotinin]. Nuclei were pelleted (2,500 × g; 10 min) and resuspended in two packed cell volumes of buffer B (identical to buffer A except that the KCl level was increased to 0.4 M). Nuclear debris was removed by centrifugation (15,000 × g; 20 min). The HDAC activity assay was performed using a HDAC fluorometric activity assay kit (BIOMOL Inc.), according to the manufacturer's recommendations. In brief, 5 μg of SNU-16 nuclear extract was added to the diluted HDAC inhibitor, and substrate was added. Samples were incubated for 10 min at 25°C, and the reaction was stopped by adding developer (30). Fluorescence was analyzed using a LS 55 luminescence spectrometer (Perkin-Elmer).

**Cell Cycle Analysis.** Cells were washed twice with PBS, fixed with 70% ethanol for 1 h, washed with PBS, and stained with 50 μg/ml propidium iodide containing 50 μg/ml RNase A. The DNA contents of the cells (10,000 cells/experimental group) were analyzed using a FACSCalibur flow cytometer (B&K) equipped with a ModFit LT program (Verity Software House Inc.). The percentages of cell populations in each cell cycle phase (G₁, S, or G₂-M) were calculated from DNA content histograms, excluding the population in the sub-G₁ phase (31).

**DNA Isolation and Ladder Formation Assay.** Cells were harvested by centrifugation and then suspended in a lysis buffer [0.5 M Tris-Cl (pH 8.0), 10 mM EDTA, and 0.5% laurylsarcosine] containing 50 μg/ml RNase A. After incubation at 37°C for 1 h, proteinase K was added to the cell lysate at 50 mg/ml and samples were additionally incubated at 55°C for 4 h. DNA was purified by phenol-chloroform extraction and precipitated with ethanol. The quantity and the quality of the DNA obtained were determined spectrophotometrically. Ten micrograms of the purified DNA was electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining (31).

**Immunoprecipitation and Kinase Assay.** Cells were collected after SK-7041 and SK-7068 for the indicated duration before harvesting, washed in PBS, and fixed in 70% ethanol. Fixed cells were resuspended in 100 μl of PBS containing 0.1% fetal bovine serum. MMP2 antibody recognizes a phosphorylated S/T-P epitope in a subset of mitotic phosphoproteins (32, 33). Of the MMP2 antigens that are identified, many play key mitotic roles regulated by phosphorylation (34–36), and nociodazole-arrested mitotic cells are strongly MMP2 reactive (37). MMP2 antibody was added at a dilution of 1:100, incubated for 1 h at room temperature, washed twice with PBS, and stained with FITC-conjugated antymouse IgG for 1 h in the dark. Stained cells were resuspended in 500 μl of PBS containing 40 μg/ml of propidium iodide, incubated at room temperature for 30 min, and then analyzed using FACSCalibur flow cytometer with Cell Quest software.

**In Vivo Antitumor Activity.** To test antitumor activities of SK-7041 and SK-7068 in vivo, the human gastric cancer cell line SNU-16 and the lung cancer cell line A-549 were xenografted s.c. into the right subaxillary region of nude mice and maintained by the serial s.c. transplantation of 3-mm³ fragments. Mice bearing a tumor xenograft of SNU-16 or A-549 were randomized into treated and control groups of 5 mice per group. Treatment was initiated at ∼3 weeks after transplantation, when tumors reached a weight of 100–200 mg. Compounds (SK-7041, SK-7068, MS-275, and suberoylanilide hydroxamic acid) were administered i.p. five times a week for 2 successive weeks at an indicated dose (determined previously as the maximum tolerated dose). Tumors were measured using a Vernier caliper, and tumor volume (V) was calculated daily.
using the equation \( V = \frac{1}{2}ab^2 \), where \( a \) and \( b \) represent the length and width, respectively (in millimeters). On day 17 after treatment, the mean tumor weight, relative tumor growth, and inhibition rate were determined. Relative tumor growth was calculated by dividing the tumor weight on day 17 by that on day 0. Inhibition rate was obtained from the equation \( (1 - \text{relative tumor growth in treated/relative tumor growth in control}) \times 100 \).

RESULTS

Effects of SK-7041 and SK-7068 on HDAC Inhibition and Histone Acetylation. We evaluated the abilities of SK-7041 and SK-7068 to inhibit partially purified HDACs from SNU-16 gastric cancer cells by measuring substrate HDAC fluorescence. Synthetic HDAC substrates were added into cell lysates including partially purified HDACs in the presence or absence of HDAC inhibitors and then developer was added after a 10-min reaction. Deacetylation of the substrate sensitizes it to the developer, which then generates a fluorophore. The fluorophore was excited with 360 nm light and the emitted light (460 nm) detected on a fluorometric plate reader. As shown in Fig. 2A, HDAC activity was inhibited in a concentration-dependent manner by hybrid compounds. The \( IC_{50} \) value of SK-7041 and SK-7068 for HDAC inhibition was 172 and 205 nM, respectively. For reference, the \( IC_{50} \) of TSA and MS-275 was 53 nM and 2,194 nM. On the basis of these results, we subjected SK-7041 and SK-7068, which were the two most potent analogs, to functional analysis. To confirm the ability of SK-7041 and SK-7068 to inhibit HDAC in human gastric cancer cells, we performed immunoblotting to determine their effects on the intracellular levels of acetylated H3 and H4. Cells were treated with 1 \( \mu \)M SK-7041 or 1 \( \mu \)M SK-7068 for various times, and the histones extracted from nuclei were then subjected to SDS-PAGE and immunoblot analysis using specific antibodies against acetylated H3 and H4 (Fig. 2B). SNU gastric cancer cells showed low basal levels of acetylated H3 and H4. However, treatment with SK-7041 or SK-7068 induced the hyperacetylation of H3 and H4 in a time-dependent manner. The cellular effect of SK-7041 or SK-7068 on nuclear histone acetylation correlated well with the cell-free in vitro effects of SK-7041 or SK-7068 on HDAC activity. The finding that treatment with SK-7041 or SK-7068 induces histone hyperacetylation reflects the potent inhibitory effects of SK-7041 and SK-7068 on the activity of intracellular HDACs. We next tested the reversibility of SK-7068 induced histone acetylation in vitro. As shown in Fig. 2C, withdrawal of MS-275 treatment resulted in

![Fig. 2](image-url)
histone deacetylation after 24 h. However, histone acetylation was maintained for at least 48 h after withdrawal of SK-7068 treatment, indicating that HDAC inhibitory effect by SK-7068 lasts longer but is reversible.

**SK-7041 and SK-7068 Preferentially Inhibit Class I HDAC1 and HDAC2.** Although a number of HDAC inhibitors have been identified, little is known about their target specificities on class I and II HDACs. It was reported recently that trapoxin and the related cyclic hydroxamic acid-containing peptide 1 preferentially inhibits HDAC1 and HDAC4 and that FK228 inhibits HDAC1 and HDAC2 (18, 38). To examine the target specificities of SK-7041 and SK-7068, we performed an *in vitro* HDAC inhibition assay on each HDAC isotype. SNU-16 cell lysates were immunoprecipitated with antibodies against each HDAC isotype. To test integrity and purity of immune complexes, collected immune complexes were subjected to Western blot analysis. As shown in Fig. 3A, we found that immunoprecipitations were successful, although collected immune complexes included some of other isotypes. Certified immune complexes were subjected to *in vitro* HDAC inhibition assays. As shown in Fig. 3B, the enzymatic activities of HDAC1 and HDAC2 were more significantly inhibited by SK-7041 or SK-7068 than the other HDAC isotypes examined (*P < 0.001*). TSA showed the same level of inhibition as SK-7041 and SK-7068. However, MS-275 did not significantly inhibit HDAC activity at 1 μM. These results suggest that SK-7041 and SK-7068 are isotype specific HDAC inhibitors, which preferentially target class I HDAC1 and HDAC2.

**SK-7041 and SK-7068 Show Potent Growth-Inhibitory Effects in Various Human Cancer Cells.** HDAC inhibitors have been shown to have potent antitumor activity in human cancer cells. Thus, we examined the effects of SK-7041 and SK-7068 on the growth of various human cancer cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. As shown in Fig. 4, cell growth was inhibited to various degrees in the presence of SK-7041 or SK-7068. Moreover, *IC*<sub>50</sub> values of SK-7041 and SK-7068 were found to be significantly lower than those of MS-275 and suberoylanilide hydroxamic acid. SK-7041 and SK-7068 have *IC*<sub>50</sub> values at nanomolar concentration in various cancer cell lines. However, SK-7041 and SK-7068 appeared to have 4-fold higher *IC*<sub>50</sub> values in nontransformed epithelial cells than those in cancer cells. These results indicate that SK-7041 and SK-7068 have potent antiproliferative activities against human cancer cells *in vitro* and

![Graphs showing HDAC inhibition](https://example.com/graph.png)

**Fig. 3** SK-7041 and SK-7068 preferentially inhibit histone deacetylase (HDAC)1 and HDAC2 in human gastric cancer cells. *In vitro* HDAC inhibition assay. A, 1 mg of SNU-16 cell lysates were immunoprecipitated with specific antibodies against each HDAC isotype. To test integrity and purity of immune complexes, precipitated lysates were immunoblotted using indicated antibodies. B, HDAC isotype immune complexes were used for the HDAC inhibition assay. Diluted HDAC inhibitor and substrate was added. Reactions were performed as described in “Materials and Methods.” Fluorescence was analyzed using a luminescence spectrometer. *Bars*, represent means ±SD. (*P* < 0.001; **P** < 0.01; ***P***, *P* < 0.05).
relatively nontoxic to nontransformed cells below submicromolar concentrations of the drugs.

Growth-Inhibitory Effects of SK-7041 and SK-7068 Are Associated with the Induction of Aberrant Mitosis in Human Cancer Cells. The growth-inhibitory effects of HDAC inhibitors have been reported to induce G1 and/or G2-M arrest and/or apoptosis. Initially, we analyzed cell cycle profiles after treating SNU-16 gastric cancer cells with SK-7041 and SK-7068. When cells were treated with 1 μM of SK-7041 or SK-7068, they were accumulated predominantly at G2-M phase after 6 h and then sub-G1 population was increased rapidly after 12 h of treatment (Fig. 5). A slight increase in the number of cells at G1 was observed after 18 h. Cell cycle analyses showed that the growth-inhibitory effects of SK-7041 and SK-7068 are mainly associated with the accumulation of cells at G2-M phase and apoptosis and in part with later G1 arrest. To identify the molecules involved in SK-7068-induced cell cycle arrest, a panel of cell cycle regulatory proteins was examined by immunoblotting and kinase assay. The Cdk inhibitors p21WAF1 and p27 (upper band; native form, lower band; fragment; Ref. 31) were upregulated 6 h after treatment with SK-7068 (Fig. 6A), and the active form of Cdk2 (lower band) and its kinase activity were reduced after 18 h of SK-7068 treatment (Fig. 6B). This reduced Cdk2 kinase activity correlated well with an increase in the G1 phase after 18 h. However, p21 and p27 were induced much earlier than the Cdk2 kinase activity reduction. These results suggest that reduced Cdk2 kinase activity is independent of Cdk inhibitor induction and that this is related to reduced Cdk activating kinase activity, which is supported by an observed reduction in the active form of Cdk2 (Fig. 6, A and B). Taken together, these results are consistent with increased G1 arrest after 18 h of SK-7068 treatment. However, the expressions of both G1-S cyclins and protein retinoblastoma were not altered significantly by SK-7068 (data not shown). Next, we investigated molecules involved in the G2 checkpoint. The accumulation of tetraploid cells increased from 6 h after treatment with SK-7068, whereas Cdc2 kinase activity was maintained for up to 24 h after SK-7068 treatment. Cdc2 and cyclin B1 expressions remained unaltered, and Cdc25c, a downstream target of Cdc2, showed no remarkable expression changes until 24 h of treatment (Fig. 6C). We also checked other G2 checkpoint-related kinases, i.e., Chk1 and Chk2. However, no changes in their expression levels or changes in the activations of their kinase activities were observed after SK-7068 treatment (Fig. 6D). Taken together, the lack of G2 cyclins/cdk inactivation indicates that the G2 checkpoint is not involved in the accumulation of tetraploidy after treatment with SK-7068. SK-7041 produced similar results (data not shown).

It is also possible that SK-7068 treatment induces aberrant mitosis in human cancer cells. To examine this possibility, mitoses in SK-7041- or SK-7068-treated cells were examined by mitotic-specific MPM2 staining. MPM2 signals were observed from the G2-M phase and showed a 6.5-fold induction after SK-7041 or SK-7068 treatment in SNU-16 cells (Fig. 6E), indicating that SK-7041- and SK-7068-treated cells are accumulated in the mitotic phase. Although treatment with SK-7041...
or SK-7068 for 18 h caused cell cycle arrest in G₁ phase, in part, through the down-regulation of Cdk2 activity, and the accumulation of tetraploid cells, the MPM2 signal, and a lack of G₂ cyclin/cdk inactivation provided evidence of aberrant mitosis in cancer cells treated with SK-7041 and SK-7068. Mitochondrial and Caspase-Mediated Apoptosis by SK-7068. As shown in Fig. 5, the sub-G₁ population was increased slightly after 12 h of SK-7068 treatment and then increased dramatically, showing that SK-7068 induces apoptotic cell death in SNU-16 gastric cancer cells. We also confirmed apoptotic cell death by examining chromosomal ladder formation (Fig. 7A). When SNU-16 cells were treated with 1 μM of SK-7068, cytochrome c was released into the cytoplasm, and caspases 9 and 3 were then activated in a time-dependent manner (Fig. 7B). Native poly(ADP-ribose) polymerase was also cleaved into a typical 85 kDa apoptotic fragment by SK-7068 treatment (Fig. 7B). We could not observe any evidence of death receptor pathway activation (data not shown). These results indicate that mitochondria and caspase activation are involved in SK-7068-induced apoptosis in gastric cancer cells. Cell cycle analysis, as shown in Fig. 5, showed that SK-7068 caused both accumulation of cells at mitotic phase and apoptosis in gastric cancer cells. On the basis of the time sequence of the cell cycle analysis, we speculate that accumulated cells at mitotic phase exit mitosis abnormally and contribute to apoptotic cell death. To test this and to confirm the caspase dependence of apoptotic cell death by SK-7068, we examined the effect of the caspase inhibitor zVAD. Cells were treated with 1 μM of SK-7068 in the absence or in the presence of 50 μM of zVAD. Twenty-four h after treatment, cells were stained with propidium iodide and then analyzed by flow cytometry. In the presence of zVAD, apoptosis was dramatically decreased and,
in turn, cells accumulated at mitotic phase increased (Fig. 7C), confirming the caspase-dependent apoptosis by SK-7068. These results also suggest that SK-7068-induced apoptosis occurs after accumulation of cells at mitotic phase and that both events are mechanistically linked.

**SK-7041 Effectively Represses the Growth of Tumor Xenografts in Nude Mice.** To evaluate the antitumor effect of SK-7041 and SK-7068 in vivo, we performed an animal study using a nude mouse model. We used human gastric cancer xenograft model (SNU-16; Fig. 8A) and human lung cancer xenograft model (A549; Fig. 8B) to assay in vivo antitumor activity of SK-7041 and SK-7068. The growth of tumors in mice treated with SK-7041 was inhibited by 49.5% (A549) and 61.4% (SNU-16) compared with that of control mice treated with only the vehicle (DMSO). For SK-7068, growth of tumors in treated mice was inhibited by 21.4% (A549) and 19% (SNU-16) compared with that of control mice treated with only the vehicle (DMSO). We found that the growth of tumors in mice treated with MS-275 was inhibited by 33.5% (A549) and 43.3% (SNU-16) compared with that of tumors of vehicle-treated animals. In this experiment, suberoylanilide hydroxamic acid did not show significant inhibition on the growth of tumors in mice (A549; 2.9%, SNU-16; 5.8%). Thus, SK-7041 has significant antitumor activity both in human gastric and lung cancer xenograft model in vivo.

**DISCUSSION**

HDAC inhibitors have been demonstrated to inhibit tumor growth in vitro and in vivo (1–4, 11–18). These compounds induce the hyperacetylation of chromatin, which, in turn, leads to the reactivation of transcriptionally silenced genes. HDAC
inhibitors act very selectively to alter the expressions of only 2–5% of genes in cultured transformed cells. These genes include tumor suppressors, cell cycle inhibitors, and differentiation and apoptosis-related genes. Moreover, inappropriate transcriptional repression induced by altered HDAC activity functions as a driving force in tumorigenesis, which is best demonstrated in acute promyelocytic leukemia (2, 3). Therefore, HDACs are recognized as a promising target for new anticancer drugs, and there is great demand to develop more potent and nontoxic selective HDAC inhibitors. More than 10 compounds are currently undergoing clinical trials (2–4).

The HDAC inhibitor classes differ in terms of functional moieties and cap structures, and it has been suggested that the functional moieties are responsible for enzyme inhibition. The cap structures of inhibitors have been shown to interact with an amino acid in the pocket of HDAC enzyme, and this interaction seems to be associated with target enzyme specificity and drug bioavailability (24, 38). We hypothesized that the chemical...
modulation of caps may allow the construction of HDAC inhibitors with enhanced bioavailability and enzyme specificity. Accordingly, we adopted a pyridyl ring as a cap structure for our HDAC inhibitors and also modified its amino acid. Our newly synthesized HDAC inhibitors might act in similar way to TSA in terms of HDAC inhibition, because their functional moiety is a hydroxamic acid group as in TSA. In the present study, we found that SK-7041 and SK-7068 inhibit HDAC activity at the nanomolar level, like TSA. Interestingly, we also observed that the modification of the cap structure of inhibitor resulted in the specific inhibition of HDAC isotypes. Specifically, SK-7041 and SK-7068 were found to be preferentially active against class I HDAC1 and HDAC2 rather than the other HDAC isotypes. Cyclic hydroxamic acid-containing peptide, a hybrid synthetic HDAC inhibitor constructed from TSA and tapoxin, was reported to be an isotype-specific inhibitor of HDAC1 and HDAC4. This compound has a hydroxamic acid functional group and a modified cap structure possessing a cyclic tetrapeptide (38). It was also expected that structural modification of cap may enhance bioavailability of these HDAC inhibitors. The finding that SK-7041 significantly reduced the growth of implanted tumor from \textit{in vivo} experiments is supporting this idea. Thus, this modification strategy might be useful in the development of more effective HDAC inhibitors.

In the present study, we showed that the antiproliferative effects of SK-7041 and SK-7068 on human cancer cells mainly involve aberrant mitosis and apoptosis. Tumor cells sensitive to SK-7041 or SK-7068 were found to enter the S phase and to be accumulated at mitosis. The absence of G2 kinase inactivation and an increased MPM-2 signal suggest that cells sensitive to SK-7041 or SK-7068 accumulate in the mitotic phase and that this leads to cell death. Moreover, the addition of the apoptotic inhibitor zVAD decreased apoptotic cell death and simultaneously increased the population of tetraploid cells in SNU-16 cells. Therefore, the antitumor activity of SK-7041 and SK-7068 seems to be closely related with the induction of aberrant mitosis. Similar to our observation, other investigators have also described that antitumor effect of some HDAC inhibitors can be explained by their mitotic effect (19–23). For instance, azelaic bishydroxamic acid induced mitotic arrest in cervical cancer cells with defective G2 checkpoint. The authors have shown that G2 checkpoint is lost in most cancer cells, resulting in aberrant mitosis and apoptosis (19). It has been also reported that TSA caused mitotic arrest and cell death and induced acetylation of tubulin, coinciding with G2-M arrest (20). Therefore, it is conceivable that HDAC inhibitors not only modulate gene transcription but also induce aberrant mitosis for antitumor effects. However, the mechanism of aberrant mitosis by HDAC inhibitor is not clearly understood yet. In contrast to previous reports (19), SK HDAC inhibitor-induced aberrant mitosis is independent on G2 checkpoint in gastric cancer cells (19). Regarding tubulin acetylation, TSA inhibits HDAC6, a microtubule-associated deacetylase, and causes acetylation of lysine 40 of \(\alpha\)-tubulin (39). Similarly, SK compounds were found to acetylate \(\alpha\)-tubulin (data not shown). However, whether the acetylation of \(\alpha\)-tubulin by HDAC inhibitor may be associated directly with aberrant mitosis and cell death remains a major challenge (39). An alternative explanation may be that these compounds may alter the expressions of genes involved in mitotic progression or directly induce hyperacetylation of centromeric histone during mitosis, leading to aberrant mitosis. Whereas HDAC inhibitors cause altered expression of specific genes, their effects on histone-regulated processes such as mitosis might be more important for the antitumor activity of HDAC inhibitor (23).

In conclusion, we describe two newly developed synthetic HDAC inhibitors, SK-7041 and SK-7068, which show antiproliferative activity \textit{in vitro} and \textit{in vivo}. In particular, these compounds both specifically target HDAC1 and HDAC2. We also show that these compounds induce aberrant mitosis in cancer cells and that this results in apoptotic cell death. On the basis of their target enzyme specificities and antitumor activities \textit{in vivo}, we believe that SK-7041 and SK-7068 present us with lead compounds for the development of the next generation of HDAC inhibitors.

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