A novel histone deacetylase inhibitor, CG0006, induces cell death through both extrinsic and intrinsic apoptotic pathways

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Histone deacetylase inhibitors (HDACIs) are potent anticancer drugs, and suberoylanilide hydroxamic acid is used for the treatment of cutaneous T-cell lymphoma patients. We synthesized a novel hydroxamate-based HDACI, CG0006, and assessed its antiproliferative effects on the NCI-60 cancer cell panel and cell lines from liver and stomach cancers that are common in Korea. Micromolar levels of CG0006 induced cell death in several breast, central nervous system, colon, hematopoietic, lung, melanoma, ovarian, prostatic, renal, and stomach cancer cell lines. We further analyzed cell death mechanisms activated by CG0006 in HCT116 (colon cancer) and K562 (leukemia) cells. First, to test the activity of CG0006, we analyzed acetylation of substrates of HDACs and effect on gene expression. CG0006 increased acetylation of histone 3, histone 4, and tubulin in a time-dependent and dose-dependent manner in both HCT116 and K562 cells. Moreover, CG0006 increased the mRNA level of p21 and decreased that of Bcl-xl efficiently in HCT116 cells. Cell cycle analysis showed G_2-M arrest, and increased apoptosis in populations of HCT116 and K562 cells treated with CG0006. Western blot analysis showed that CG0006 increased levels of p21 in HCT116 cells and of p21 and p27 in K562 cells. In addition, CG0006 activated caspase-9, caspase-3, and caspase-8. These results indicate that CG0006 induces death in HCT116 and K562 cells through both intrinsic and extrinsic apoptotic pathways. The HDACI CG0006 may be a potent anticancer drug for solid tumors and leukemia. ***Anti-Cancer Drugs*** **20:815–821 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Recently, epigenetic gene modulation has been postulated to be a key mechanism in cancer development and metastasis. Chromatin is composed of nucleosomes that contain 146-bp DNA repeats wrapped around core histone octamers. Each octamer is composed of two H2A, H2B, H3, and H4 proteins. The lysine-rich N-terminal tails of these proteins are posttranslationally modified by phosphorylation, acetylation, methylation, and/or ADP-ribosylation [1,2]. Of these modifications, acetylation is linked to chromatin structure changes and gene transcription [2,3]. Acetylation by histone acetyltransferases neutralizes charges of lysine tails, reduces DNA-binding properties, loosens nucleosome structure, and activates transcription [1,2]. In contrast, deacetylation of histone tails by histone deacetylases (HDACs) increases the affinity of histones for DNA and compacts nucleosomes, resulting in gene repression [1–3]. Increased HDAC activity represses transcription of many genes related to differentiation and tumor suppressor genes in cancer cells [4,5]. Thus, HDAC inhibitors (HDACIs) have been developed as new anticancer drugs. Increasing evidence supports the idea that HDACIs induce differentiation and/or apoptosis of cancer cells through several mechanisms. For example, HDACI increases acetylation of lysine residues on histone tails, resulting in expression of cyclin-dependent kinase inhibitor p21 or p27, death receptors, or death receptor ligands [6–11]. In addition, HDACI induces acetylation of many nonhistone proteins including heat-shock proteins and tubulin [12,13]. Acetylation of these proteins by HDACIs disrupts their physiological functions, resulting in death of cancer cells [6,12,14]. Other death mechanisms include generation of reactive oxygen species [11,15] and inhibition of the NFκB pathway [16,17]. HDACIs have been reported to show additive or synergistic effects when used in combination with other anticancer therapies including radiotherapy and chemotherapy, resulting in increased growth inhibition or apoptosis of cancer cells [18,19].

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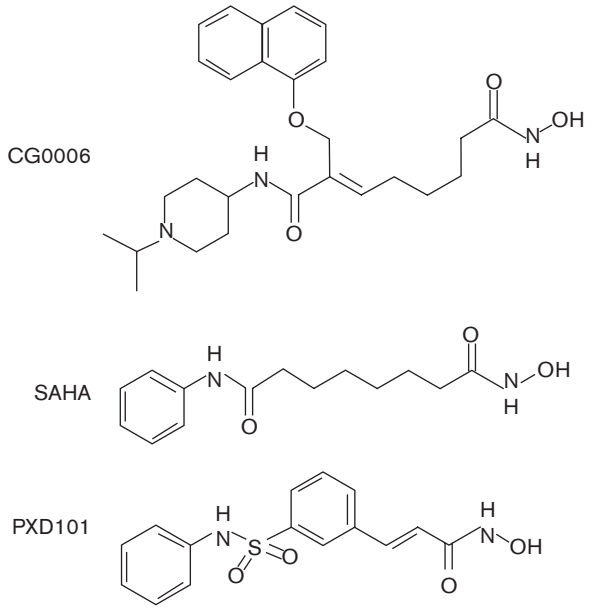
Fig. 1



Structures of histone deacetylase inhibitors.

There are several HDACI classes, including short-chain fatty acids (such as 4-phenylbutyrate and valproic acids); hydroxamic acids [such as suberoylanilide hydroxamic acid (SAHA), trichostatin A, cyclic hydroxamic-acid-containing peptide, LAQ-824, PXD101, and LBH 589]; cyclic tetrapeptides [such as trapoxin, apicidin, and depsipeptide (FK228)]; and benzamide (such as MS-275) [2,6,18,20]. Only SAHA has been approved by the FDA for treatment of cutaneous T-cell lymphoma and other HDACIs await permission for use against various types of cancers. We synthesized a novel hydroxamate-based pan-HDACI, CG0006 {2-(aryloxymethyl)-oct-2-enedioic acid 8-hydroxyamide 1-[(1-akyl-piperidin-4-yl)-amide]} (Fig. 1). The compound contains hydroxamic acid but belongs to a chemical class distinct from that of known HDACIs.

In this study, we tested the cytotoxicity of CG0006 against the NCI-60 cancer cell panel and cell lines from cancers, including stomach and liver cancers that are common in Korea, using cell proliferation assays and DNA and western-blot analyses. CG0006 inhibited growth of many cancer cell types, and induced both cell cycle arrest at G_2-M and apoptosis through activation of intrinsic (caspase-9 and caspase-3) and extrinsic (caspase-8) apoptotic pathways.

Materials and methods

Cell culture and treatment with HDACIs

NCI-60 and other cancer cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and penicillin/streptomycin (Invitrogen, Carlsbad, California, USA). Cells were treated with various concentrations of HDACIs, including SAHA, PXD101, and CG0006 (Crystal Genomics, Inc., Seoul, Korea) in 5% (v/v) serum-containing RPMI 1640 medium for the indicated times.

Cell proliferation assay: MTS assay

Cells were cultured in 96-well plates and treated with various concentrations of HDACIs for the indicated times. CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, Wisconsin, USA) was added to each well according to the manufacturer's instructions. After the cells had been incubated for 2 h, their viability was determined by measuring absorbance at 490 nm with an ELISA reader.

Cell cycle analysis

Cells were treated with HDACIs for the indicated times, fixed with 70% (v/v) ethanol, and stained with 60 μg/ml propidium iodide (Sigma, St. Louis, Missouri, USA) containing 10 U/ml RnaseA, for 30 min. The percentages of cells in different cell cycle phases were measured using a fluorescence-activated cell sorting flow cytometer (Becton Dickinson, San Jose, California, USA) and inbuilt software.

Western blotting

Antibodies to acetylated (on Lys18) histone 3, p21, p27, caspase-9, caspase-3, and caspase-8, tubulin (Cell Signaling, Beverly, Massachusetts, USA), actin, and acetylated tubulin (Sigma) were used. After rinsing with PBS, cells were suspended in lysis buffer containing 20 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, and a protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were centrifuged at 12 000*g* in a microcentrifuge for 10 min and supernatants were retained. Equal amounts of proteins were loaded onto and electrophoresed in sodium dodecyl sulfate–polyacrylamide electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts, USA). After blocking with 3% (w/v) non-fat dry milk powder for 1 h, membranes were incubated with various antibodies overnight at 4°C. Appropriate secondary antibodies, conjugated with horseradish peroxidase (Pierce, Rockford, Illinois, USA) were added. Immobilon Western ECL solution (Millipore) and a Kodak Image Station 4000MM (Kodak, Rochester, New York, USA) were used to visualize immunoreactive bands.

Reverse transcription-PCR

Total RNA was prepared using TRIZOL (Invitrogen) according to the manufacturer's protocol and reverse-transcribed to cDNA using oligo-dT 20 primers (Bioneer, Daejeon, Korea). Specific cDNAs were amplified for 25–30 cycles. Primer sequences were as follows: forward 5′-GC GATGGAACTTCGACTTTGT-3′ and reverse 5′-GGGC TTCCTCTTGGAGAAGAT-3′ for p21 (352 bp); forward 5′-ATGTCTCAGAGCAACCGGG-3′ and reverse 5′-TTC ATTCACTACCTGTTCAAAGCT-3′ for Bcl-xl (387 bp);

and forward 5′-GAGTCAACGGATTTGGTCGTA-3′ and reverse 5′-AGTTGTCATGGATGACCTTGG-3′ for glyceraldehyde-3-phosphate dehydrogenase (491 bp).

Statistics

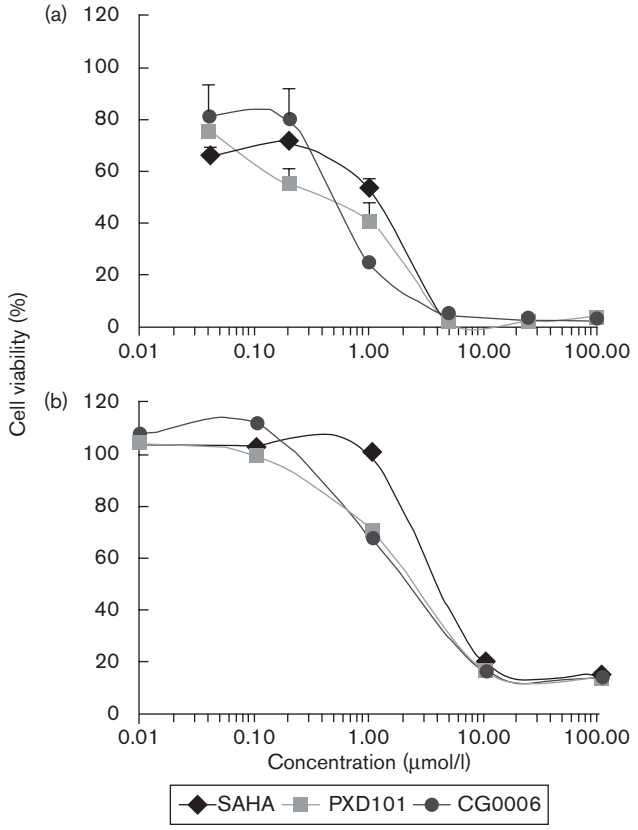
All data are presented as means ± SDs. Statistical significance ($P < 0.05$) was determined by one-way analysis of variance.

Results

Inhibitory effects of CG0006 on cancer cell proliferation

We synthesized a novel hydroxamate-based pan-HDACI, CG0006 {2-(aryloxymethyl)-oct-2-enedioic acid 8-hydroxyamide 1-[(1-akyl-piperidin-4-yl)-amide]} (Fig. 1). Like other inhibitors, for example SAHA and PXD101, CG0006 has the hydroxamic acid moiety to bind zinc at the bottom of catalytic pocket. The aliphatic chain of CG0006, which is presumed to be positioned at the narrow channel of pocket, is less flexible than that of SAHA and less rigid than that of PXD101. Of the other moieties, the naphthalene ring was adopted to improve water solubility. To compare the effects of CG0006 on cancer cell proliferation with SAHA and PXD101, the NCI-60 cancer cell panel, and liver, stomach, and cervical cancer cells, were exposed to 0.04, 0.2, 1, 5, 25, and 100 μmol/l of each HDACI for 48 h. Growth inhibitory potencies are presented as inhibitory concentration at 50% (IC_{50}) values. Table 1 shows the IC_{50} values of relatively CG0006-sensitive cell lines, and it is clear that CG0006 suppressed growth of a wide range of cancer cell lines including leukemia and solid tumor cells. Of all forms of cancer examined, one or more cell lines were CG0006-sensitive, with IC_{50} values less than 5 μmol/l. Figure 2 shows growth curves of the CG0006-sensitive cells HCT116 (Fig. 2a) and K562 (Fig. 2b) exposed to the indicated concentrations of CG0006, SAHA, or PXD101 for 48 h. The three HDACIs all showed dose-dependent effects on proliferation of HCT116 and K562 cells. The IC_{50} values of CG0006 were 0.5 and 2.1 μmol/l for HCT116 and K562 cells, respectively (Table 1), which are lower than those of SAHA (1.2 and 4.0 μmol/l) and similar to those of PXD101 (0.4 and 2.4 μmol/l). We used the HDACI-sensitive HCT116 and K562 cell lines in the following experiments to validate activities of CG0006 against solid and hematopoietic cancer cells.

Fig. 2



Cytotoxicity of CG0006 for HCT116 and K562 cell lines. (a) HCT116 cells were exposed to 0.04, 0.2, 1, 5, 25, and 100 μmol/l of CG0006, SAHA, or PXD101 for 48 h. Cell viabilities were calculated as percentages of untreated cells (100% viability) using the MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay as described in the Materials and methods (means ± SDs, $n = 4$). (b) K562 cells were exposed to various concentrations of CG0006, SAHA, or PXD101 for 48 h. Cell viabilities were calculated as percentages of untreated cells (100% viability) using the MTS assay (means ± SDs, $n = 4$).

Effect of CG0006 on acetylation of histone and tubulin proteins, and on gene expression

To examine the inhibitory effect of CG0006 on HDACs, we measured acetylation levels of histone 3, histone 4, and a non-histone substrate of HDAC, tubulin. We exposed HCT116 (Fig. 3a) and K562 (Fig. 3b) cells to 1 and 10 μmol/l CG0006, SAHA, or PXD101 for the indicated times and performed western blot analyses using antiacetylated histone 3, antiacetylated histone 4, or antiacetylated tubulin antibodies. Acetylation of histone 3, histone 4, and tubulin was observed within 1 h of drug addition, increased gradually with time, and was sustained for 8 h in HCT116 cells treated with 1 μmol/l CG0006, SAHA, or PXD101. CG0006 at a concentration of 10 μmol/l was more effective in mediating acetylation of histones and tubulin than a concentration of 1 μmol/l. In K562 cells, both concentrations of CG0006 increased acetylation of histone 3, histone 4, and tubulin in a time-dependent and concentration-dependent manner. These results indicate that CG0006 effectively inhibits HDAC activities, to the same extent as SAHA or PXD101, resulting in acetylation of histones and tubulin.

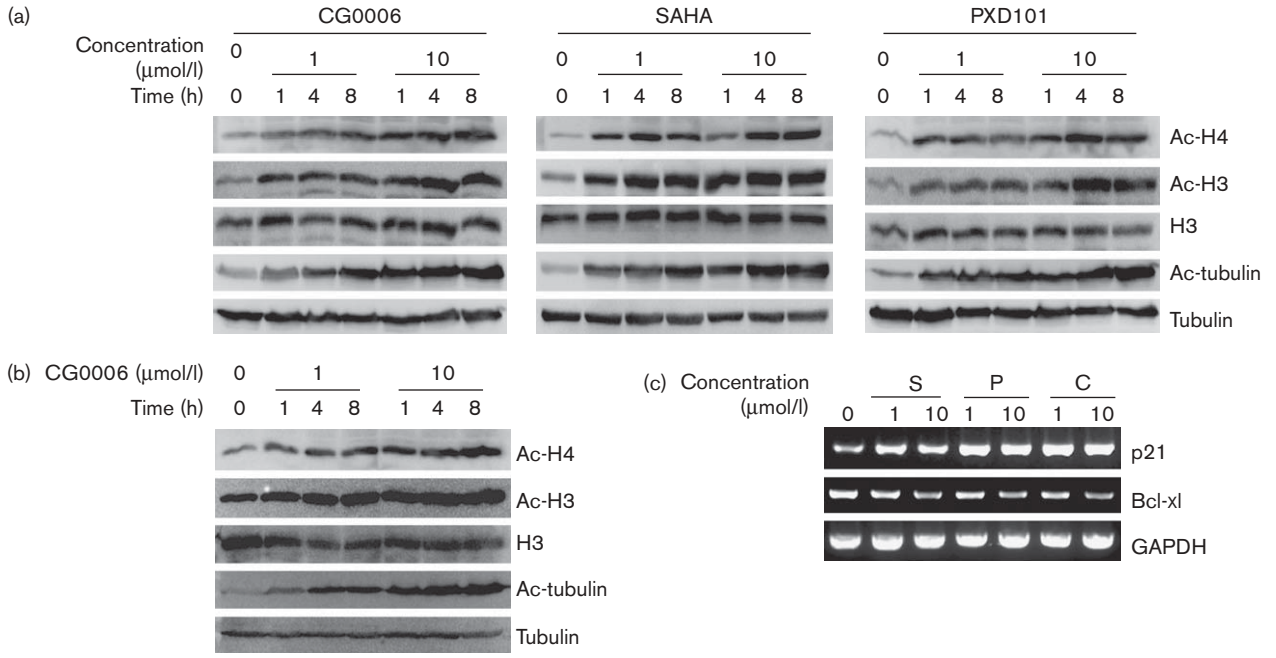
As HDACs control transcription of many genes, we examined the levels of mRNA of p21 and Bcl-xl, which are regulated by HDACIs. CG0006 increased p21 mRNA

Table 1 Growth inhibitory effects (IC_{50} values) of CG0006, SAHA, and PXD101 on sensitive cell lines in NCI-60 panel and other cancer cell lines

		IC_{50} of HDACIs (μmol/l)					IC_{50} of HDACIs (μmol/l)		
Origin	Cell line	CG0006	SAHA	PXD101	Origin	Cell line	CG0006	SAHA	PXD101
Breast	HS578T	1.8	2.6	2.9	Lung	A549	1.5	1.5	0.6
	MCF7	0.9	1.8	2.3		HOP92	1.8	3.0	2.1
	MDAMB231	0.7	2.6	1.0		NCI-H522	1.5	2.1	0.6
	T47D	2.0	7.0	3.0	Melanoma	MALME3M	3.2	3.3	1.9
CNS	SF268	2.0	3.2	1.7					
	SNB19	3.0	9.8	2.0	Ovarian	OVCAR5	1.7	1.1	0.8
	U251	2.0	2.7	0.6		SKOV3	1.9	2.9	0.7
Colon	COLO205	0.5	1.9	0.9	Prostate	DU145	1.6	2.5	0.7
	HCT116	0.5	1.2	0.4		PC3	3.5	6.6	1.2
	HT29	0.6	0.35	0.2	Renal	RXF393	4.3	3.0	1.0
Leukemia	HL60	0.4	3.6	1.0		SN12C	2.4	4.7	0.8
	K562	2.1	4.0	2.4	Stomach	SNU-1	0.7	2.0	1.6
	RPMI8226	2.0	2.0	1.1		SNU-5	2.6	7.0	2.4
	SR	0.3	1.0	0.3		SNU-638	2.5	3.5	1.4

CNS, central nervous system; HDACIs, histone deacetylase inhibitors.

Fig. 3



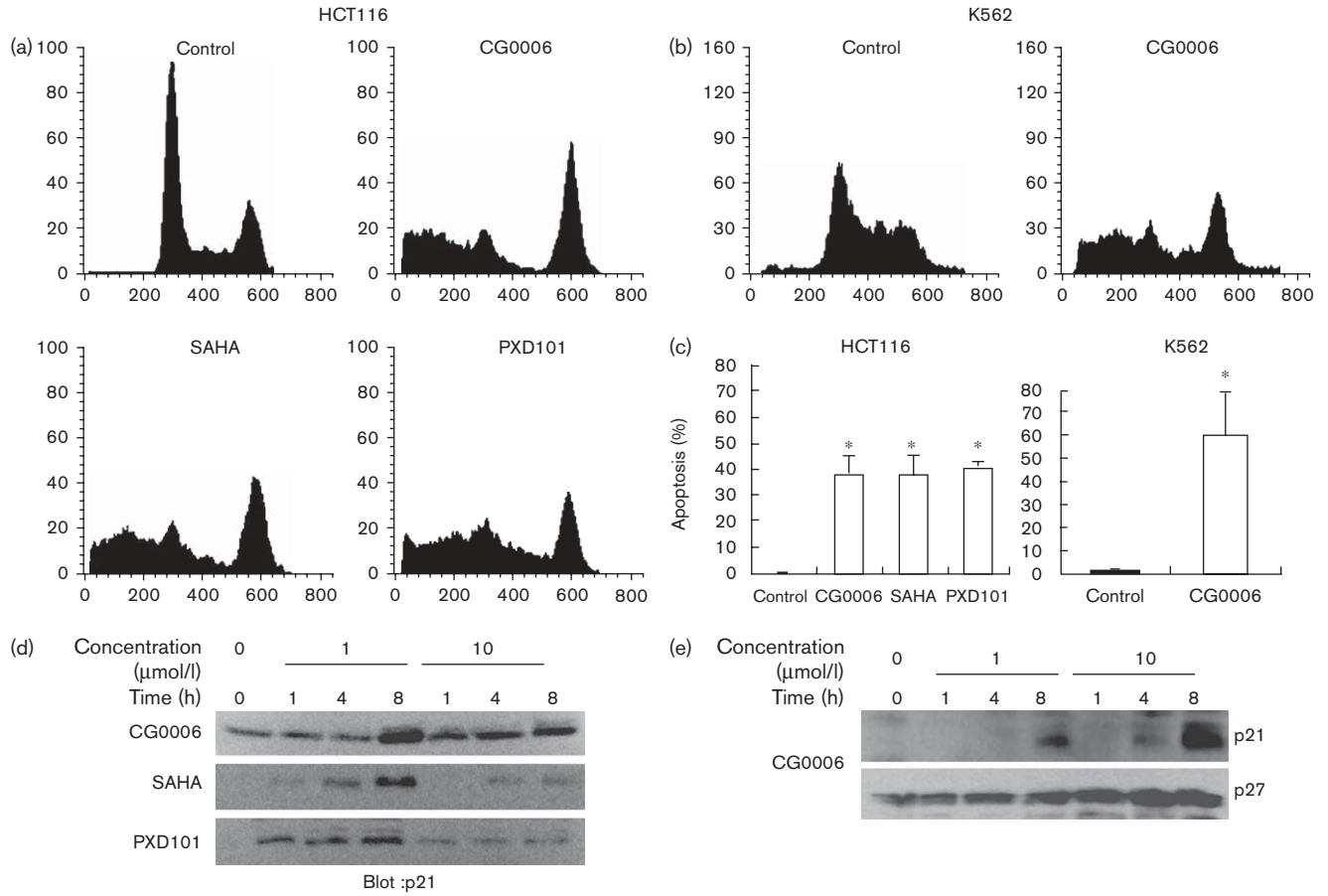
Effects of CG0006, SAHA, and PXD101 on acetylation of histone and tubulin proteins and on gene expression. (a) Western blotting detecting acetylated histone 3 (Ac-H3) and acetylated histone 4 (Ac-H4), histone 3 (H3), acetylated tubulin (Ac-tubulin), and tubulin, was performed. HCT116 cells were treated with 1 or 10 μmol/l of CG0006, SAHA, or PXD101 for the indicated times. Acetylation of histone 3, histone 4, and tubulin was increased by CG0006 to the extents similar to those observed when SAHA or PXD101 were used. (b) Western blotting detecting Ac-H3 and Ac-H4, H3, Ac-tubulin, and tubulin, was performed. K562 cells were treated with 1 or 10 μmol/l CG0006 for the indicated times. Acetylation of histone 3, histone 4, and tubulin increased in a dose-dependent and time-dependent manner. (c) Induction of p21 mRNA expression and inhibition of Bcl-xl mRNA by CG0006 were determined by reverse transcription-PCR in HCT116 cells treated with 1 or 10 μmol/l SAHA (S), PXD101 (P), or CG0006 (C) for 8 h. CG0006 increased p21 mRNA and decreased Bcl-xl mRNA.

and deceased Bcl-xl mRNA in HCT116 cell treated with 1 or 10 μmol/l CG0006, SAHA, or PXD101 for 8 h (Fig. 3c). Although CG0006 was most effective in p21 induction, it reduced Bcl-xl expression as efficiently as SAHA and PXD101.

Cell cycle analysis

To investigate the effects of CG0006 on the cell cycle, we analyzed DNA contents by flow cytometry analysis. Treatment of HT116 cells with 10 μmol/l CG0006, SAHA, or PXD101 for 24 h increased both G_2-M and

Fig. 4



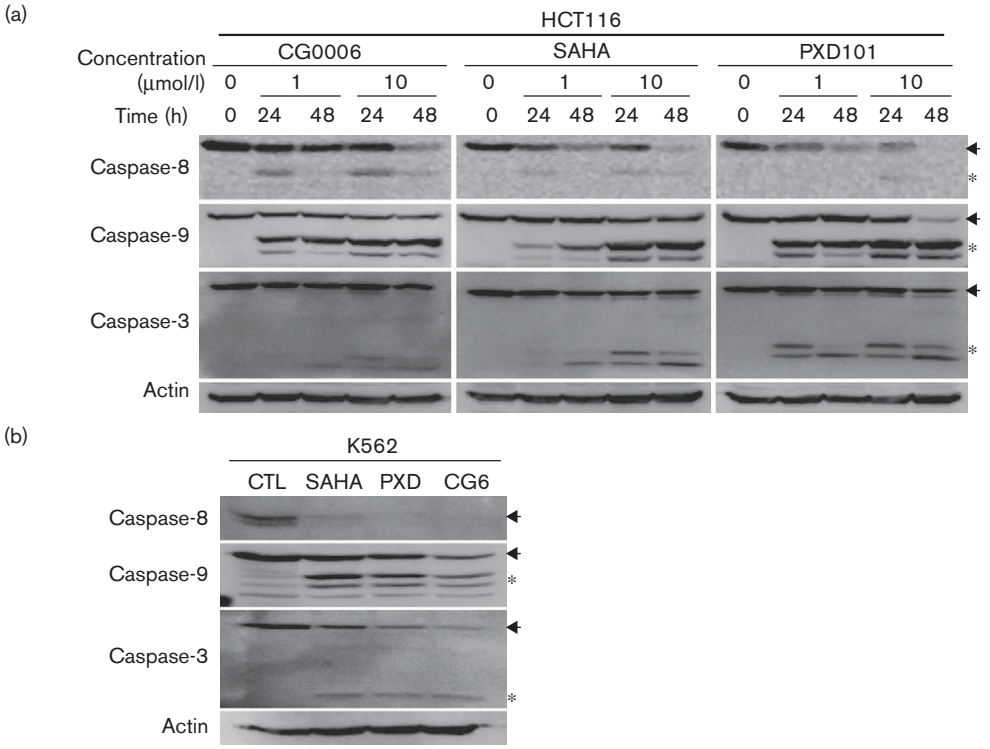
Cell cycle analysis of HCT116 and K562 cells treated with histone deacetylase inhibitors (HDACIs). (a) Flow cytometry profiles showing cell cycle distributions of HCT116 cells treated with 10 μmol/l CG0006, SAHA, or PXD101, over 24 h. HDACIs increased apoptotic populations (sub-G_1 cells) and induced G_2-M arrest. (b) Histogram of cell cycle analysis data from K562 cells treated with 10 μmol/l CG0006 over 24 h. (c) Bar graphs showing sub-G_1 populations of HCT116 (left) or K562 (right) cells treated with 10 μmol/l HDACIs over 24 h (means ± SDs, $n=2$, $^*P<0.05$ compared with control, one-way analysis of variance). (d) Western blotting to detect p21 of HCT116 cells treated with 1 or 10 μmol/l CG0006, SAHA, or PXD101, for the indicated times. The membrane of Fig. 3a was stripped and reblotted using anti-p21 antibodies. (e) Western blotting detecting p21 and p27 in K562 cells treated with 10 μmol/l CG0006 for the indicated times. The membrane of Fig. 3b was stripped and reblotted using anti-p21 and anti-p27 antibodies.

hypodiploid populations (sub-G_1) (Fig. 4a and c, left panel). The percentage increase in the apoptotic sub-G_1 population mediated by CG0006 (38.3 ± 6.6%) was similar to that mediated by SAHA (38.1 ± 8.0%) or PXD101 (40.4 ± 2.1%). In K562 cells treated with 10 μmol/l CG0006 for 24 h, CG0006 also induced G_2-M arrest and apoptosis (59.9 ± 19.0%) (Fig. 4b and c, right panel). These results show that CG0006 arrested cells in G_2-M phase, finally leading to apoptosis.

Activation of caspases

To further characterize the mechanisms of apoptosis induced by CG0006 in HCT116 or K562 cells, we assessed expression levels of p21 and p27, and activation of caspases using western blot analysis. The expression level of p21 was increased in HCT116 cells exposed to 1 and 10 μmol/l CG0006, SAHA, or PXD101 (Fig. 4d). The levels of p27 were not changed by CG0006, SAHA, or PXD101 treatment (data not shown). In K562 cells, exposure to 10 μmol/l CG0006 for 8 h induced expression of both p21 and p27 (Fig. 4e). Then, we examined whether CG0006 activated extrinsic apoptosis. Caspase-8 was activated by 1 and 10 μmol/l CG0006, SAHA, or PXD101 in HCT116 cells (Fig. 5a, left panel). Moreover, the two intrinsic apoptotic caspases, caspase-9 and caspase-3, were also activated by both levels of CG0006, SAHA, or PXD101 in a time-dependent and dose-dependent manner (Fig. 5a, middle and right, respectively). In leukemic K562 cells, 5 μmol/l CG0006 also activated caspase-8, caspase-9, and caspase-3 as much as SAHA or PXD101 (Fig. 5b). Collectively, the data show that CG0006 kills cancer cells by inducing both extrinsic and intrinsic apoptosis, similar to SAHA and PXD101.

Fig. 5



Activation of caspase-8, caspase-9, and caspase-3 in HCT116 and K562 cells treated with CG0006, SAHA, or PXD101. (a) Western blotting detecting caspase-8, caspase-9, caspase-3, and actin, in HCT116 cells treated with 1 or 10 μmol/l CG0006, SAHA, or PXD101, for the indicated times. Histone deacetylase inhibitors activated caspase-8, caspase-9, and caspase-3, indicating induction of caspase-dependent apoptosis (procaspases and active caspases are presented as arrows and asterisks, respectively). (b) Western blotting detecting caspase-8, caspase-9, and caspase-3 in K562 cells treated with 5 μmol/l of CG0006 (CG6), SAHA, or PXD101 (PXD), for 48 h. CTL, control.

Discussion

There is increasing evidence that epigenetic changes are important in cancer development as well as other diseases, and regulation of gene expression, together with chemotherapy or radiotherapy, has been suggested as an anticancer strategy. There are two ways to regulate gene expression. One is to regulate methylation of DNA by modulating DNA methyltransferase activity, and another is to control acetylation of histone proteins using HDACIs. Among several HDACIs, SAHA is an FDA-approved HDACI used in the treatment of cutaneous T-cell lymphoma patients. Many clinical trials are currently under way with a view to extending the applications of HDACIs. We synthesized a new hydroxamate-based HDACI, 2-(aryloxymethyl)-oct-2-enedioic acid 8-hydroxyamide 1-[(1-akyl-piperidin-4-yl)-amide, termed CG0006, and assessed the effects of the drug on the NCI-60 cancer cell panel and cell lines from cancers that are common in Korea, including liver and stomach cancers. CG0006 inhibited growth of many kinds of cells (Table 1), and increased acetylation of histone 3, histone 4, and tubulin in the HCT116 colon cancer cell line and the K562 leukemia cell line by as much as SAHA or PXD101 (Fig. 3a and b). It also controls the expression p21 and Bcl-xl as well as SAHA and PXD101 (Fig. 3c). These results show that CG0006 effectively inhibited HDAC activity in these cell lines. The results of cell cycle analyses showed that CG0006 increased apoptosis of HCT116 and K562 cell lines by arresting cells in G_2-M phase similar to SAHA and PXD101 (Fig. 4). The levels of p21 and p27 were increased by CG0006. CG0006 activated caspase-8, caspase-9, and caspase-3, indicating that the drug causes apoptosis through both extrinsic and intrinsic pathways (Fig. 5). In summary, we developed a novel HDACI, CG0006, which is a potential therapeutic agent for the treatment of leukemia and solid tumors, such as breast cancer, colon cancer, and stomach cancer; however, preclinical studies on toxicity and efficacy *in vivo* are planned.

Acknowledgements

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