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Anti-Cancer Effect of IN-2001 in T47D Human Breast Cancer

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Abstract

Histone deacetylases (HDACs) are enzymes involved in the remodelling of chromatin, and have a key role in the epigenetic regulation of gene expression. Histone deacetylase (HDAC) inhibitors are emerging as an exciting new class of potential anti-cancer agents. In recent years, a number of structurally diverse HDAC inhibitors have been identified and these HDAC inhibitors induce growth arrest, differentiation and/or apoptosis of cancer cells *in vitro* and *in vivo*. However, the underlying molecular mechanisms remain unclear. This study aimed at investigating the anti-tumor activity of various HDAC inhibitors, IN-2001, using T47D human breast cancer cells. Moreover, the possible mechanism by which HDAC inhibitors exhibit anti-tumor activity was also explored. In estrogen receptor positive T47D cells, IN-2001, HDAC inhibitor showed anti-proliferative effects in dose-and time-dependent manner. In T47D human breast cancer cells showed anti-tumor activity of IN-2001 and the growth inhibitory effects of IN-2001 were related to the cell cycle arrest and induction of apoptosis. Flow cytometry studies revealed that IN-2001 showed accumulation of cells at G_2/M phase. At the same time, IN-2001 treatment time-dependently increased sub-G_1 population, representing apoptotic cells. IN-2001-mediated cell cycle arrest was associated with induction of cdk inhibitor expression. In T47D cells, IN-2001 as well as other HDAC inhibitors treatment significantly increased $p21^{WAF1}$ and $p27^{KIP1}$ expression. In addition, thymidylate synthase, an essential enzyme for DNA replication and repair, was down-regulated by IN-2001 and other HDAC inhibitors in the T47D human breast cancer cells. In summary, IN-2001 with a higher potency than other HDAC inhibitors induced growth inhibition, cell cycle arrest, and eventual apoptosis in human breast cancer possibly through modulation of cell cycle and apoptosis regulatory proteins, such as cdk inhibitors, cyclins, and thymidylate synthase.

Key Words: IN-2001, T47D, Hsitone deacetylase

INTRODUCTION

Current therapeutic approaches for human breast cancer include hormonal therapy with antiestrogenic compounds, as well as surgery, radiotherapy, hyperthermia, and chemotherapy (Hortobagyi, 1998). However, conventional strategies for treatment of breast cancer are yet unsatisfactory and limited. Therefore, there is an urgent need to develop more effective therapeutic approaches for prevention and treatment of breast cancer. In recent years, an increasing number of structurally diverse HDAC inhibitors have been identified as a promising new class of potential anticancer agents (Carron *et al.*, 1997; Butler *et al.*, 2000; Brown and Strathdee 2002; Bulavin *et al.*, 2004). Currently available HDAC inhibitors fall into four structural classes; short chain fatty acids, hydroxamic acids, cyclic tetrapeptides/epoxides, and benzamides (Drummond *et al.*, 2005). Short chain fatty acids such as phenylbutyrate, phenylacetate, and the antiepileptic drug valproic acid inhibit HDAC activity and affect the expression of numerous genes with disparate cellular functions (Saito *et al.*, 1999). Newer compounds such as cyclic hydroxamic acid containing peptides (CHAP) inhibit nanomolar concentrations and are synthetic hybrids of SAHA and the cyclic peptides (Furumai *et al.*, 2001; Komatsu *et al.*, 2001). The fungal metabolites trapoxin A, apicidin, and depsipeptide (FR901228) are cyclic tetrapeptides with potent HDAC inhibitory activities. The other class includes the synthetic benzamide derivatives such as MS-275 and CI-994 (Suzuki *et al.*, 1999). MS-275 is orally bioavailable and exerts antiproliferative effects at micromolar levels against a variety of cancer cell types (Saito *et al.*, 1999; Papeleu *et al.*, 2005). The result of HDAC inhibition is believed not to have a generalized effect on the genome but rather only effects the transcription of a small subset of the genome. Differential display analysis of transformed lymphoid cell lines revealed that the expression of only 2-5% of transcribed genes is changed significantly after treatment with HDAC inhibitor, TSA (Van *et al.*, 1996). Recent cDNA microarray studies have shown that treatment with HDAC inhibitors modulates the expression of

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a selective subset of less that 10% of expressed genes in different cell types, with a near equal proportion of these being induced as repressed (Glaser *et al.*, 2003). The commonly up- and down-modulated gene transcripts identified in these expression microarray studies, as well as in numerous single-gene expression studies (Van *et al.*, 1996; Mariadason *et al.*, 2000; Suzuki *et al.*, 1999), are those encoding known tumor-associated proteins that mediate proliferation and cell cycle progression, survival factors, growth factor receptors, kinase and signaling transduction intermediates, DNA synthesis/repair enzymes, shuttling proteins, transcription factors, and proteases. $p21^{WAF1}$ mediates growth arrest in the G_1 phase of the cell cycle by inhibiting cyclin-dependent kinase complexes that regulate cell cycle progression (Gartel and Tyner, 1998; Blobel, 2000; Biswas *et al.*, 2006). All known HDAC inhibitors including butyrate (Nakano *et al.*, 1997; Archer *et al.*, 1998), TSA (Sowa *et al.*,1997), depsipeptide, oxamflatin (Kim *et al.*, 1999), MS-275 (Saito *et al.*, 1999), trapoxin (Sambucetti *et al.*, 1999), and SAHA (Richon *et al.*, 2000; Gui *et al.*, 2004), have been known to induce ^{WAF1} transcription. Increased transcription of the $p21^{WAF1}$ gene by HDAC inhibitors is associated with an increased level of histone acetylation at the $p21^{WAF1}$ gene promoter (Chan *et al.*, 2001; Gui *et al.*, 2004).

In order to develop a anti-cancer drug candidate, in this study, we tried to evaluate the anti-tumor effects of new HDAC inhibitor small molecule, IN-2001 on T47D human breast cancer. To examine the anti-tumor effect of IN-2001, we examined the effect of IN-2001 on the cell proliferation, cell cycle distribution, and apoptosis in T47D human breast cancer cells.

MATERIALS AND METHODS

Chemicals

HDAC inhibitors, such as Trichostatin A, IN2001, SAHA, and LAQ were generously provided from Dr. D. K. Kim (Ewha-Womans University, Seoul, South Korea). HC toxin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium pyrubate, penicillin-streptomycin, fetal bovine serum (FBS), trypsin-EDTA, minimum essential medium (MEM), and RPMI were acquired from GibcoBRL (Rockville, MD, USA). Antibodies were from Santa Crutz Biotechnology Inc. (Santa Crutz, CA, USA).

Cell lines and cell culture conditions

T47D cells were obtained from Korean Cell Line Bank (KCLB, Seoul, South Korea). T47D cells were maintained in RPMI1640 medium, supplemented with fetal bovine serum and penicillin-streptomycin. Cells were routinely maintained at 37°C and in 5% CO_2.

Cell proliferation assay

Cells were plated in 96 well plates at a density of 10^4 cells per well. The following day, the cells were treated with chemicals. The number of cells was measured based on the modified SRB assay. Cells were treated with cold 10% trichloroacetic acid (TCA) and incubated at 4°C for 30 min, then washed five times with tap water and left to dry. TCA-fixed cells were stained for 30 min with 0.4% (w/v) sulforhodamineB (SRB) dissolved in 1% acetic acid. Wells were washed with tap water and air dried. Bound dye was solubilized with 10 mM Tris base (pH 10.5) in a shaker for 30 min. Finally, optical intensity was read using ELISA reader (Bio-Rad, Hercules, CA, USA) at 570 nm.

Flow activated cell sorter (FACS) analysis

Cells were plated in 60 mm^2 dishes and exposed to chemicals. Treated cells were detached using trypsin-EDTA and fixed with 70% ethanol. After centrifugation, the cells were treated with RNase A (10 μg/ml) for 20 min at 37°C and stained with propidium iodide (2 μg/ml) for 30 min at 37°C in the dark. The DNA content per cell was evaluated in a FACScalibur (Becton Dickinson, San Diego, CA, USA).

RT-PCR) analysis

Cells were plated in 60 mm^2 dishes and exposed to chemicals for 24 hr. Total RNA was extracted using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA). Reverse transcription was carried out on 3 μg of total RNA diluted in a 22.5 μl mixture containing 1 μl random primer (0.5 μg/ml), 1 μl dNTP (1 mM), 2 μl DTT, 4 μl RT buffer (5X), 1 μl M-MLV reverse transcriptase (200 U/μl), 0.5 μlRNasin (40 U/μl), and H_2O. After incubation at 37°C for 1 hr, the reverse transcriptase was inactivated for 10 min at 95°C and cDNA was stored at −20°C or immediately used for PCR. 1 μl of the synthesized cDNA was subjected to PCR amplification with special primer in a 10 μl reaction containing dNTP and *Taq* polymerase. DNA was denatured at 95°C for 5 min and cycled immediately 25 times at 95°C for 30 sec with specific annealing temperature chosen by preliminary experiments; and extended at 72°C for 1 min. The PCR reaction ended with 5 min incubation at 72°C. Special primers (**GAPDH**; 5'ACATCgCTCAgACACCATgg3'; 5' gTAgTTgAggTCAATgAAggg3': **p21;**5'gAACTTCgACTTTgTCACCgAg3'; 5' CgTTTTCgACCCTgAgAgTCTC3': **Cyclin D1;**5'AgCCATggAACACCAgCTC3';5' gCACCTCCAgCATCCAggT3': **Cyclin D2;**5' TACTTCAAgTgCgTgCAgAAggAC3';5' TCCCACACTTCCAgTTgCgATCAT3') for PCR amplification and PCR products were analyzed on 2% agarose gels.

Western blot analysis

Cells were plated in 100 mm^2 dishes and then incubated with chemicals for 24 hr. Cells were collected and homogenized in a lysis buffer (Pro-prep protein extraction solution, INtRON; 20 mMTris, 160 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM NaF, 1 mMdithiothreitol (DTT), 1 mM sodium orthovanadate, pepstatin, leupeptin, and aprotinin) on ice for 10-20 min. Cell lysates were centrifuged at 14,000 g for 5 min at 4°C, divided into aliquots and stored at −80°C. Lysates containing 30-50 μg of total protein were separated by electrophoresis on 10%-15% SDS-acrylamide gels and then electrophoretically transferred to polyvinylidenedifluoride (PVDF) transfer membrane (Hybond-P; Amersham) at 200 mV for 2-3 hr. Membranes were blocked with 3% dry milk in PBST (PBS with 0.1% Tween) over night at 4°C and incubated with specific first antibodies for 1-2 hr at R.T. After membranes were washed and incubated with second antibodies conjugated to horse radish peroxidase for 2 hr at R.T., membranes were washed and air dried for ECL detection (ECL Plus; Amersham). Membranes were stripped in mild antibody stripping solution (Re-Blot Plus, Chemicon International, Temecular, CA) at R.T. for 30 min, washed in PBST, and reprobed.

RESULTS

IN-2001 causes dose-dependent growth inhibition

To determine the antiproliferative effect of IN-2001 (Fig. 1) on the T47D human breast cancer cells were treated with vehicle (0.1% DMSO) or various concentrations (0.001 μM-10 μM) of IN-2001 for 72 hr and then the number of cells was determined based on the SRB assay. As shown in Fig. 2, IN-2001 showed potent anti-proliferative effect in a dose-dependent manner. The IC_{50} values of IN-2001 and SAHA were 0.132 μM and 1.877 μM, respectively, in T47D cell lines (Table 1). This result showed that IN-2001 was more potent than SAHA which is a HDAC inhibitor anticancer drug in terms of inhibition of T47D cell proliferation

IN-2001 causes time-dependent growth inhibition

In the next experiment, we carried out time-course experiment with 1 μ MIN-2001 or SAHA. As shown in Fig. 3, IN-2001 and SAHA decreased the proliferation of human breast cancer cells in a time-dependent manner. T47D cells showed significant growth inhibition when cells were exposed to IN-2001 and SAHA for more than 48 hr. T47D cells showed cytostatic effect with 24 hr treatment of IN-2001 and 48 hr treatment of IN-2001 and SAHA decreased cell proliferation by 20-60% in T47D cells compared to untreated cells.

IN-2001 induces cell cycle arrest

To investigate whether the growth inhibitory effect of IN-2001 and SAHA is related to cell cycle alteration, we analyzed the cell cycle distribution of IN-2001and SAHA treated breast cancer cells. ER positive T47D cells were treated with vehicle (0.1% DMSO) or 1 μM IN-2001 and SAHA for various time periods (12, 24, or 48 hr) and then analyzed cell cycle distribution by flow cytometric analysis after PI staining their DNA. Representative histograms and quantitative analysis data are shown in Fig. 4 and Table 2, respectively. As shown in Fig. 4, in T47D cells, IN-2001 and SAHA induced accumulation of cells at Sub-G_1 and G_2/M phase with reduction of cells in G_0/G_1 or S phase. However, G_2/M arrest and an increase in number of apoptotic cells were observed at the different time periods depending on the kinds of HDAC inhibitors. IN2001 treatment decreased number of cells in S phase at 12 hr and 24 hr and 48 hr treatments increased number of cells in Sub-G_1 phase. Also IN-2001 treatment for 48 hr increased the number of cells in G_2/M phase that appeared to be 28.7% compared to untreated control cells showed 19.7% of cells in G_2/M phase. In case of SAHA, 12 hr treatment increased number of cells in G_0/G_1 phase and 24 hr treatment of SAHA increased number of cells in G_2/M phase that appeared to be showed 26.7% of cells compared to untreated control cells showed 18.9% of cells in G_2/M phase. Also SAHA treatment increased number of cells in G_0/G_1 phase at 48 hr treatment and increased number of cells in Sub-G_1 phase at 24 and 48 hr (Table 2).

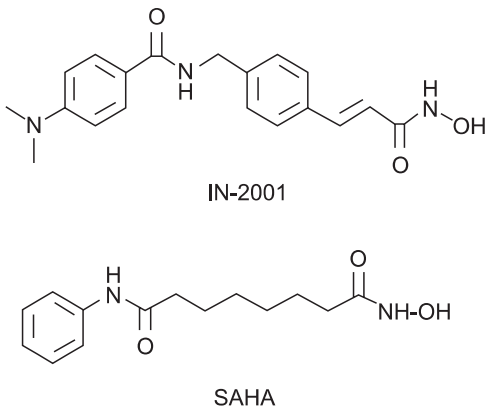

Fig. 1. Structure of newly synthesized HDAC inhibitor, IN-2001.

Table 1. 50% inhibitory concentration of IN-2001

HDAC inhibitors	IC^{50} (μM)
IN2001	0.132
SAHA	1.877

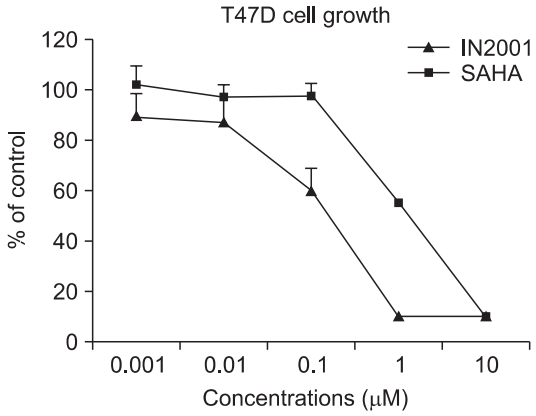

Fig. 2. Dose-dependent growth inhibition by IN-2001. Human breast cancer T47D cells were treated with vehicle (0.1% DMSO) or indicated concentrations (0.001-10 μM) of IN-2001 orSAHA for 72 hr. The number of cells was determined by SRB assay andcell proliferation was expressed as percent of control. Data present mean ± S.D (N=4).

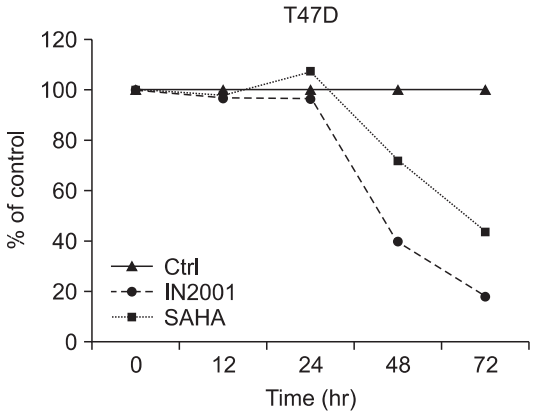

Fig. 3. Time-dependent growth inhibition by IN-2001. Human breast cancer T47D cells were treated with vehicle (0.1% DMSO) or 1 μM IN-2001 or SAHA for various exposure time (0-72 hr). The number of cells was determined by SRB assay and cell proliferation was expressed as percent of control. Data present mean ± S.D. (N=4).

HDAC inhibitors increases $p21^{WAF1}$ and $p27^{KIP1}$ expression

T47D cells were treated with vehicle (0.1% DMSO) or 1 μM IN-2001 for 24 hr. And then the expression of cdk inhibitors, such as $p21^{WAF1}$ and $p27^{KIP1}$ was examined by RT-PCR and western blot analysis. As shown in Fig. 5, in T47D cells, all kinds of HDAC inhibitors, such as TSA, HC toxin, IN2001, SAHA and LAQ increased $p21^{WAF1}$ expression in both mRNA level and protein level. $p21^{WAF1}$ mRNA level was slightly increased to 1.4-fold-2-fold compared to control level. However, interstingly, the induction of $p21^{WAF1}$ protein level was extremely greater than that of $p21^{WAF1}$ mRNA level. LAQ treatment most efficiently increased in $p21^{WAF1}$ protein level by 30-fold. TSA and IN2001 treatment resulted in 12-fold induction of $p21^{WAF1}$ protein level. HC toxin and SAHA increased $p21^{WAF1}$ protein level by 7-fold and 3.4-fold, respectively. These results suggested that HDAC inhibitors may up-regulate $p21^{WAF1}$ expression through post-transcriptional regulation, such as protein stabilization and inhibition of protein degradation as well as transcriptional regulation. In addition, $p27^{KIP1}$ protein level was increased by HDAC inhibitors with less extent than $p21^{WAF1}$ (Fig. 6). $p27^{KIP1}$ protein level was increased by 1.7-fold, 2.3-fold, 1.7-fold, 1.3-fold and 1.8-fold with TSA, HC toxin, IN2001, SAHA, and LAQ treatment, respectively.

In summary, HDAC inhibitiors increased the expression of cdk inhibitors, $p21^{WAF1}$ and $p27^{KIP1}$ in both ER positive and ER negative breast cancer cells. These results suggested that the HDAC inhibitor-induced up-regulation of cdk inhibitor may lead to cell cycle arrest, ultimately resulting in growth inhibition. In interest, the main target of HDAC inhibitor, which related with cell cycle regulation, seemed to be cell-type specific. In the T47D cells, $p21^{WAF1}$ more dramatically increased by HDAC inhibitors than $p27^{KIP1}$. In contrast, MDA-MB-231 cells showed similar extent of increase in both $p21^{WAF1}$ and $p27^{KIP1}$

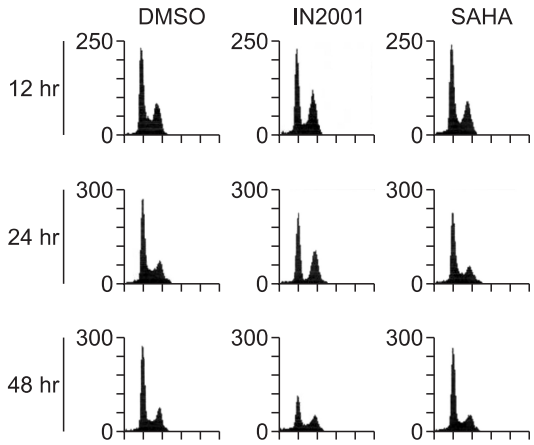

Fig. 4. Effect of IN-2001 on cell cycle distribution. Human breast cancer T47D cells were treated with vehicle (0.1% DMSO) or 1 μM IN-2001or SAHA for the indicated time periods. Cells were harvested, fixed, and stained with PI. Then 20,000 stained cells were subjected to flow cytometry analysis to determine the distribution of cells.

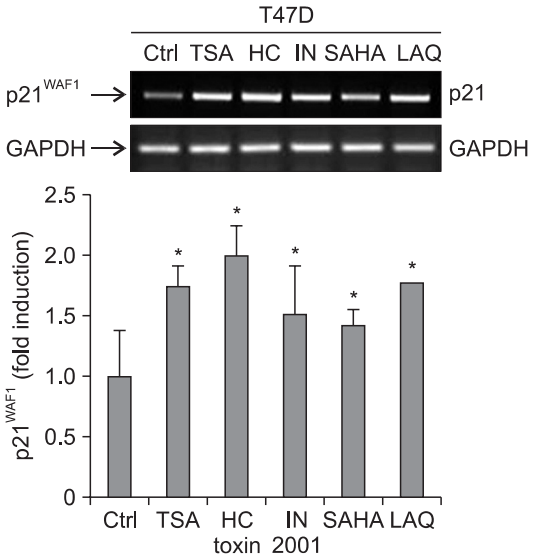

Fig. 5. Effect of HDAC inhibitor on the expression of cdk inhibitor. T47D cells were treated with vehicle (0.1% DMSO) or 1 μM HDAC inhibitors for 24 hr. Total RNA was isolated and then subjected to RT-PCR using specific primers. GAPDH served as loading control. For quantification, the band intensity of $p21^{WAF1}$ was normalized to that of GAPDH and data was expressed as fold induction compared to control group. *Significantly different from control (p<0.05).

Table 2. Cell cycle distribution by IN-2001 and SAHA in T47D cells

Times (hr)	HDAC inhibitors	Sub-G_1	Distributions of cells (%)		G_2/M
			G_0/G_1	S	
12 hr	Ctrl	3.295±0.253	65.482±1.937	7.055±0.748	27.463±1.561
	IN2001	5.181±0.156	69.086±1.370	**3.355±0.215***	27.560±1.301
	SAHA	4.567±0.496	**74.416±1.435***	4.627±0.283	20.956±1.272
24 hr	Ctrl	6.316±0.337	72.085±0.490	8.967±1.033	18.948±1.294
	IN2001	**16.140±0.447***	66.256±0.154	9.508±0.272	24.235±0.405
	SAHA	**12.780±0.364***	63.456±0.486	9.878±0.301	**26.667±0.744***
48 hr	Ctrl	7.242±1.334	61.227±4.862	19.047±4.720	19.726±1.563
	IN2001	**17.280±0.028***	60.684±0.662	10.663±0.625	28.653±1.306*
	SAHA	**13.820±1.236***	**67.965±7.265***	10.689±3.023	**21.337±5.301**

*Bold lettering indicates significant difference from control group (p<0.05).

by HDAC inhibitors.

HDAC inhibitor decreases cyclin D1 expression and increases cyclin D2 expression

As well as cdk inhibitors, one of the important cell cycle regulatoryproteins is cyclin. In this study, we examined the effect of HDAC inhibitor on the expressions of D-type cyclin (cyclin D1 and cyclin D2). T47D cells were treated with vehicle (0.1% DMSO) or 1 μM HDAC inhibitors for 24 hr and then examined for the expression of cyclin D1 and cyclin D2 by RT-PCR analysis. In T47D cells, transcription of cyclin D1 and cyclin D2 was oppositely regulated by HDAC inhibitior. HC toxin and LAQ significantly decreased cyclin D1 transcription to 58% and 17% over control level, respectively. However, cyclin D2 mRNA level was increased by 2.8-fold and 3.7-fold compared to control, respectively. TSA, IN2001, and SAHA did not change the expression of D-type cyclin in T47D cells (Fig. 7).

In MDA-MB-231 cells, TSA, HC toxin, and LAQ significantly down-regulated cyclin D1 mRNA level but did not change cyclin D2 mRNA level. Cyclin D2 mRNA level was up-regulated by IN2001 and SAHA to 1.6-fold and 1.8-fold, respectively (Fig. 7).

HDAC inhibitor decreasesthymidylate synthase expression

Thymidylate synthase (TS) is an essential enzyme for DNA replication and repair because it provides the sole intracellular source of dTMP. Thus, it has been a major target of chemotherapeutic agents, such as fluoropyrimidines (i.e. 5-FU) and antifolates (i.e. TDX, ZD931, and MTA). Therefore, we examined the effect of HDAC inhibitor on the TS gene expression. T47D cells were treated with vehicle (0.1% DMSO) or 1 μM HDAC inhibitors for 24 hr and then TS mRNA level

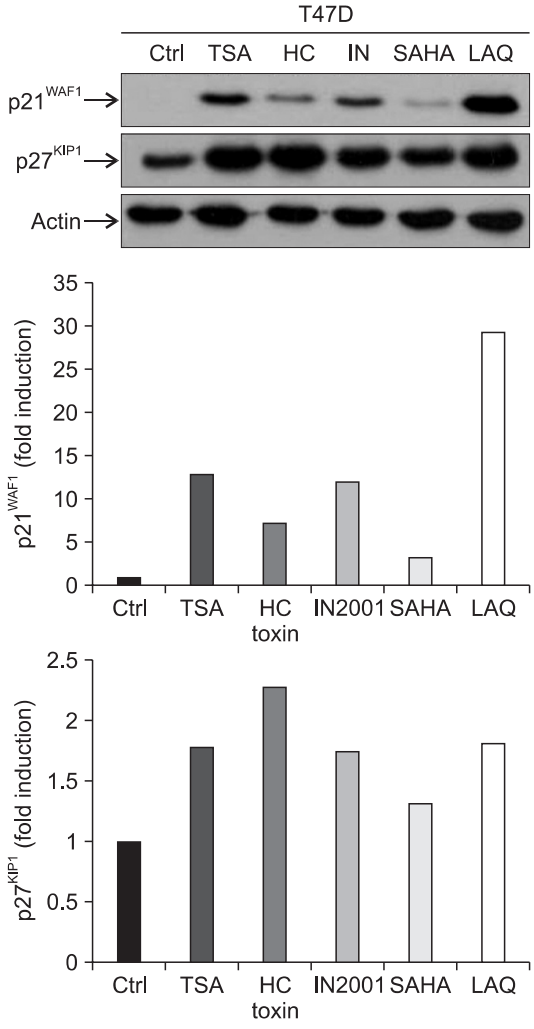

Fig. 6. Effect of HDAC inhibitor on the expression of cdk inhibitor. T47D cells were treated with vehicle (0.1% DMSO) or 1 μM HDAC inhibitors for 24 hr. Protein extracts were prepared and 50 μg of protein extracts were separated by 12% SDS-PAGE. Blots were probed with the corresponding antibodies. actin served as the loading control. For quantification, the band intensity of $p21^{WAF1}$ and $p27^{KIP1}$ was normalized to that of actin and data was expressed as fold induction compared to control group.

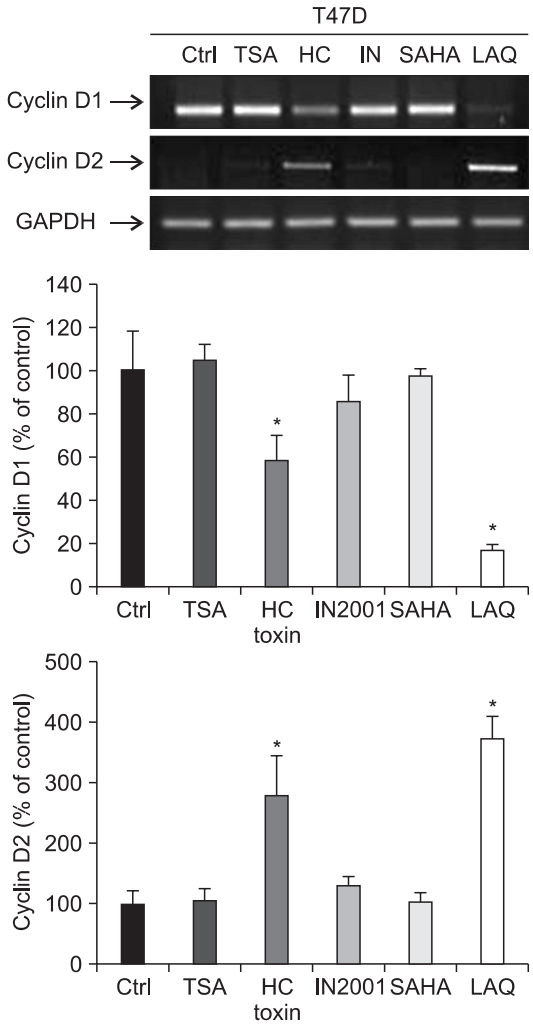

Fig. 7. Effect of HDAC inhibitor on the cyclin D expression. T47D cells were treated with vehicle (0.1% DMSO) or 1 μM HDAC inhibitors for 24 hr. Total RNA was isolated and then subjected to RT-PCR using specific primers. GAPDH served as the loading control. For quantification, the band intensity of cyclin D was normalized to that of GAPDH and data was expressed as fold induction compared to control group. *Significantly different from control (p<0.05).

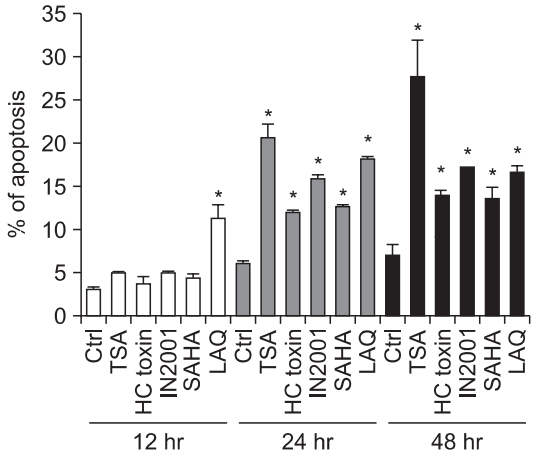

Fig. 8. Effect of HDAC inhibitor on the thymidylate synthase expression. T47D cells were treated with vehicle (0.1% DMSO) or 1 μM HDAC inhibitors for 24 hr. Total RNA was isolated and then subjected to RT-PCR using specific primers for thymidylate synthase. GAPDH served as the loading control. For quantification, the band intensity of TS was normalized to that of GAPDH and data was expressed as fold induction compared to control group. *Significantly different from control (p<0.05).

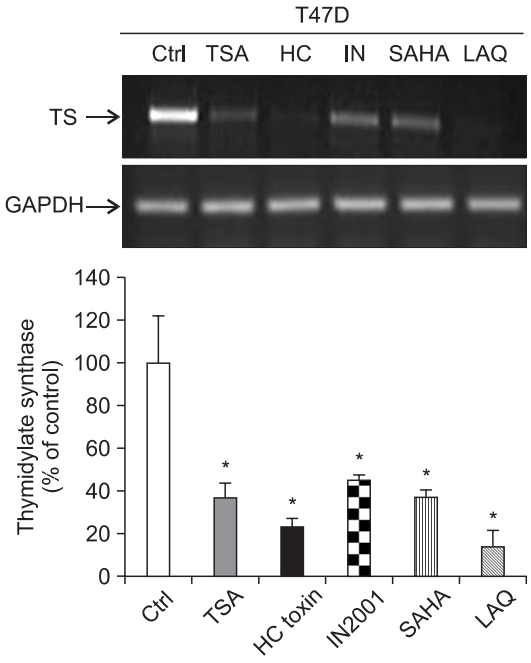

Fig. 9. Quantitative analysis of HDAC inhibitor-induced apoptosis. T47D cells were treated with vehicle (0.1% DMSO) or 1 μM HDAC inhibitors for 12, 24 or 48 hr. Cells were harvested, fixed, and stained with PI. Then 20,000 stained cells were subjected to flow cytometry analysis. Quantitative analysis of apoptosis was done using ModiFit program. Data present mean ± S.D. (N=4). *Significantly different from control at each time point (p<0.05).

was determined using RT-PCR technique. As shown in Fig. 8, T47D cells treated with TSA, HC toxin, IN2001, SAHA, and LAQ showed down-regulation of TS mRNA level to 37%, 24%, 45%, 37%, and 15% over control level, respectively.

Effect of HDAC inhibitor on the apoptosis

To determine whether anti-proliferative effect of HDAC inhibitor is related with induction of apoptosis, we examined the effect of HDAC inhibitor on the apoptosis. Moreover, we tried to elucidate the underlying mechanism of apoptosis induced by HDAC inhibitors. T47D cells treated with vehicle (0.1% DMSO) or 1 μM HDAC inhibitors for various time period (12 hr, 24 hr, or 48 hr). And then cell were stained with fluorescent PI dye and then subjected to FACS analysis to measure the sub-G_1 populations, which represent apoptotic cells with less than 2N DNA content. As shown in Fig. 9, T47D cells showed significant inductin of apoptosis when cells were treated with HDAC inhibitors for more than 24 hr. However, exceptionally, LAQ significantly increased apoptotic sub-G_1 populations with 12 hr treatment (11.44%, compared to 3.30% in control). 24 hr treatment of HDAC inhibitors increased in apoptosis ranging from 12.3% to 20.86% compared to the control value of 6.32%. With 48 hr treatment, more apoptotic cells were detected by HDAC inhibitors. When cells were treated with HDAC inhibitors for 48 hr, 13.8-27.8% of apoptotic cells were observed, whereas untreated control cells showed 7.2% of sub-G_1 peak.

DISCUSSION

HDAC inhibitors have been found to induce cell growth arrest, differentiation, and/or apoptosis, and exhibit potent antimetastatic, antiangiogenic, and immuno-modulatory properties in a variety of transformed cells *in vitro* and *in vivo* that can contribute to the inhibition of tumour development and progression (Zhou *et al.*, 2000; Marks *et al.*, 2001; Wittich *et al.*, 2002; Johnstone and Licht, 2003; Yang, 2004).

HDAC inhibitors may achieve their antitumor effects through reactivation of dormant tumor suppressor genes (Vrana *et al.*, 1999; Wade, 2001; Villar-Garea and Esteller, 2004). In cancer, some genes are transcriptionally silenced by the inappropriated recruitment of HDACs, e.g., tumor suppressor genes (Glaser *et al.*, 2003). Known repressors are multiproteins that contain DNA binding proteins (e.g., NcoR, SMRT, MEF, MeCP2, and sin3A) that commonly use HDACs to repress transcription and block the function of the tumor suppressor gene. The arche-typical gene silenced in this manner in human cancer is the cyclin-dependent kinase inhibitor $p21^{WAF1}$. Epigenetic reactivation of $p21^{WAF1}$ by HDAC inhibitors has been reported in cancer cell lines (Archer *et al.*, 1998), and the restoration of $p21^{WAF1}$ gene expression by HDAC inhibitors is associated with enrichment of hyperacetylated histones at the $p21^{WAF1}$ promoter (Bereshchenko *et al.*, 2002; Gui *et al.*, 2004). Demethylatingagents such as 5-aza-2'-deoxycytidine are particularly interesting owing to the interaction of DNA methylation with histone deacetylation in gene silencing of tumor suppressor genes. Combinations of 5-aza-2'-deoxycytidine with HDAC inhibitors, TSA or depsipeptide, were shown to reactivate silenced tumor suppressor genes including MLH1, TIMP3, CDKN2B, CDKN2A, ARHI, gelsolin, and maspin, synergistically increasing the level of tumor cell apoptosis (Drummond *et al.*, 2005). In addition to stand alone-therapeutics for chemotherapy, HDAC inhibitors seem to be suitable for combination therapy as "sensitizer drugs", enhancing the antitumor effects of specific chemotherapeutics. In fact, a proportion of the clinical trials using

HDAC inhibitors involve a combination of an established anti-tumor compound together with a HDAC inhibitor (Villar-Garea and Esteller, *et al.*,2004). The drug combinations may have 2 advantages: first, the dose of each substance necessary for cell growth inhibition or apoptosis is usually much lower than if used separately, reducing side effects and toxicity, and second, resistance to certain chemicals can be overcome in some cases by combining drugs. For instance, cell death after treatment with etoposide, comptothecin, and other substances that cross-link DNA and Topo II enzymes increases if the cell lines are pretreated with either TSA and SAHA, probably because the chromatin changes caused by the hydroxamic acids facilitate cross-linker access to the target (Kim *et al.*,2003). Combinations of nuclear receptor ligands, such as all trans retinoic acid (ATRA), or vitamin D analaogs, such as 1,25-dihydroxyvitamin D, with HDAC inhibitors have been shown to increase differentiation and apoptosis in cancer cells and also inhibit tumor growth in vivo (Banwell *et al.*,2003; Drummond *et al.*, 2005; Joung *et al.*, 2006).

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