INTRODUCTION

Histone acetylation is the most thoroughly studied and appreciated post-translational modification mechanism (Grunstein, 1997). Generally, transcriptionally active euchromatin domains tend to be relatively hyperacetylated whereas transcriptionally repressed heterochromatin domains are hypoacetylated (Göttlicher et al., 2001; Gui et al., 2004). The acetylation status of histones is regulated by the opposing action of two classes of enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs) (Struhl, 1998; Kouzarides, 2000). Acetylation of the histone tails enhance the accessibility of transcription factors, transcriptional regulatory complexes, and RNA polymerases to promoter regions of DNA (Reynisdóttir et al., 1995; Richon et al., 2000; Roth et al., 2001; Rosato et al., 2001). This explains the role of HATs as transcriptional coactivators, and DNA binding proteins including PCAF (p300/cyclic AMP-response-element binding protein-associated factor) and members of the p300/ CBP family of transcriptional coactivators can recruit them to their site of action (Qui et al., 1999; Roth et al., 2001). However, the acetyltransferase activity of HATs extends beyond histones; various nuclear proteins, in particular transcription factors such as p53, GATA-1, E2F, estrogen receptor, and various cell cycle regulatory proteins with variable functional consequences (Kouzarides, 2000; Roth et al., 2001; Marks et al., 2001). HDACs counteract the activity of HATs and catalyse the removal of acetyl groups from lysine residues in histone N-terminus, leading to chromatin condensation and transcriptional repression (David et al., 1998; Davis et al., 2000; Gray and Ekström, 2001). This condensed chromatin structure inhibits transcription, presumably, because transcription factors, transcriptional regulatory complexes, and RNA polymerase do not have access to the DNA. In addition, HDACs are part of multiprotein transcriptional repressor complex or interact with DNA binding proteins. In addition to regulating the acetylation state of histones, histone deacetylase (HDAC) can bind to, deacetylate and regulate the activity of a number of other proteins, including transcription factors such as p53, E2F transcription factor 1 (E2F1), STAT1, STAT3, and nuclear factor-

Anti-Cancer Effect of IN-2001 in MDA-MB-231 Human Breast Cancer

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Abstract
In recent years, inhibition of HDACs has emerged as a potential strategy to reverse aberrant epigenetic changes associated with cancer, and several classes of HDAC inhibitors have been found to have potent and specific anticancer activities in preclinical studies. But their precise mechanism of action has not been elucidated. In this study, a novel synthetic inhibitor of HDAC, 3-(4-di-methylamino phenyl)-N-hydroxy-2-propenamide [IN-2001] was examined for its antitumor activity and the underlying molecular mechanisms of any such activity on human breast cancer cell lines. IN-2001 effectively inhibited cellular HDAC activity (IC50 = 0.585 nM) in MDA-MB-231 human breast cancer cells. IN-2001 caused a significant dose-dependent inhibition of cell proliferation in estrogen receptor (ER) negative MDA-MB-231 human breast cancer cells. Cell cycle analysis revealed that the growth inhibitory effects of IN-2001 might be attributed to cell cycle arrest at G0/G1, and/or G2/M phase and subsequent apoptosis in human breast cancer cells. These events are accompanied by modulating several cell cycle and apoptosis regulatory genes such as CDK inhibitors p21WAF1 and p27KIP1 cyclin D1, and other tumor suppressor genes such as cyclin D2. Collectively, IN-2001 inhibited cell proliferation and induced apoptosis in human breast cancer cells and these findings may provide new therapeutic approaches, combination of antiestrogen together with a HDAC inhibitor, in the hormonal therapy-resistant ER-negative breast cancers. In summary, our data suggest that this histone deacetylase inhibitor, IN-2001, is a novel promising therapeutic agent with potent antitumor effects against human breast cancers.

Key Words: IN-2001, MDA-MB-231, HDAC
H4, H3, H2A, H2B (Marks 2003). The treatment of normal and tumor cells with HDAC inhibitors might also be attributed to transcription-independent mechanisms by modulating the acetylation status of a series of non-histone targets. HDAC inhibitors affect tumor cell growth and survival through multiple biological effects. HDAC inhibitors induce cell cycle arrest and apoptosis, and have anti-angiogenic and immunomodulatory effects by modulating the acetylation of a series of non-histone proteins. HDAC inhibitors have been found to induce cell growth arrest, differentiation, and/or apoptosis, and exhibit potent anti-metastatic, antiangiogenic, and immuno-modulatory properties in a variety of transformed cells in vitro and in vivo that can contribute to the inhibition of tumor development and progression (Marks et al., 2000; Johnstone, 2002; Johnstone and Licht, 2003). The treatment of normal and tumor cells with HDAC inhibitors causes a similar accumulation of acetylated histones H4, H3, H2A, H2B (Marks et al., 2001; Vugushin et al., 2001; Vigushin and Coombes, 2002). Nevertheless, tumor cells appear to be much more sensitive to growth arrest, differentiation, and apoptotic effects of these agents than normal cells (Qui et al., 1999; Butler et al., 2000a, 2000b; Krammer et al., 2001; Johnstone, 2002).

In this study, we tried to evaluate the anti-tumor effects of various HDAC inhibitors on MDA-MB-231 human breast cancer. Moreover, the underlying chemotherapeutic mechanisms of them were explored also. To examine the anti-tumor effect of HDAC inhibitors, we examined the effect of HDAC inhibitors on the cell proliferation, cell cycle distribution, and apoptosis in MDA-MB-231 human breast cancer cells. To find out the mechanism of anti-tumor activity of HDAC inhibitors, we examined the effect of IN-2001 on the expression of cell cycle regulatory protein and apoptosis-related proteins.

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 pyruvate, 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 Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell lines and cell culture conditions
MDA-MB-231 cells were obtained from Korean Cell Line Bank (KCLB, Seoul, South Korea). MDA-MB-231 cells were maintained in RPMI1640 medium, supplemented with fetal bovine serum and penicillin-streptomycin. Cells were routinely maintained at 37°C and in 5% CO2.

Cell proliferation assay
Cells were plated in 96 well plates and were treated with chemicals. Cells were treated with cold 10% trichloroacetic acid (TCA) and TCA-fixed cells were stained for 30 min with 0.4% (w/v) sulforhodamine B (SRB) dissolved in 1% acetic acid. Bound dye was solubilized with 10 mMTris base (pH 10.5) and optical density was read using ELISA reader (Bio-Rad, Hercules, CA, USA) at 570 nm.

Flow Activated Cell Sorter (FACS) Analysis
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) and stained with propidium iodide (2 μg/ml). The DNA content per cell was evaluated in a FACS caliber (Becton Dickinson, San Diego, CA, USA).

RT-PCR analysis
Total RNA was extracted using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) from cells were exposed to chemicals for 24 hr. Reverse transcription was carried out on total RNA in a mixture containing random prime, dNTPs, DTT, RT buffer (5X), M-MLV reverse transcriptase, RNase in at 37°C for 1 hr. cDNA was stored at -20°C or cDNA was subjected to PCR amplification with special primer (GAPDH; 5’ACATCTcGCTCAAGACCATggg3’; 5’gTAgTTgAggTCAATgAAg3’; p21; 5’gAACCTCGACTTggTCAACgAg3’; 5’gTgTTTTCgACCCCTgAgAgtgC3’; Cyclin D1; 5’gAACTTTCgACTTTgTCACCCAgAg3’; 5’gTTTCTCGACCCCTgAgAgtgC3’; Cyclin D2; 5’TACTTCAgTgCgAggC3’; 5’TCCCCACCTTCC-AgTTgCgATCAT3’ in reaction containing dNTP and Taqpolymerase. DNA was denatured at 95°C extended at 72°C and PCR products were analyzed on 2% agarose gels.

Western blot analysis
After the incubation with chemicals for 24 hr, cells were homogenized in a lysis buffer (Pro-prep protein extraction solution, INIRON; 20 mMTris, 160 mMNaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM NaF, 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, pepstatin, leupeptin, and aprotonin) on ice and cell lysates were centrifuged at 14,000 g for 5 min at 4°C, divided into aliquots and stored at -80°C. Protein was separated by electrophoresis on 10-15% SDS-acrylamide gels and then electrophoretically transferred to polyvinylidenedifluoride (PVDF) transfer membrane (Hybond-P; Amer sham). Membranes were blocked with 5% dry milk in PBS.
(PBS with 0.1% Tween) over night at 4°C and incubated with specific first antibodies and second antibodies conjugated to horse radish peroxidase. Membranes were washed and air dried for ECL detection (ECL Plus; Amersham).

**RESULTS**

**IN-2001 causes dose-dependent growth inhibition**

In recent years, an increasing number of structurally diverse HDAC inhibitors have been identified as an exciting new class of potential anti-cancer agents. In this study, we evaluated the anti-tumor effects of various kinds of HDAC inhibitors (Fig. 1) in the human breast cancer cells in an attempt to find out better therapeutic agents for breast cancer treatment. To determine the antiproliferative effect of IN-2001 on the human breast cancer MDA-MB-231 cells were treated with vehicle (0.1% DMSO) or various concentrations (0.001-10 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 anti-proliferative effect in a dose-dependent manner. The IC\(_{50}\) values of HDAC inhibitors in each cell lines were shown in Table 1. These data indicated that the anti-proliferative effects of HDAC inhibitors were cell type specific and ER positive breast cancer cells seemed to be more susceptible to HDAC inhibitors than ER negative breast cancer cells.

**IN-2001 time-dependent growth inhibition**

In the next experiment, we carried out time-course experiment with 1 μM IN-2001. As shown in Fig. 3, IN-2001 decreased the proliferation of MDA-MB-231 human breast cancer cells in a time-dependent manner. MDA-MB-231 cells showed significant growth inhibition when cells were exposed to for more than 24 hr. In MDA-MB-231 cells, cell growth was decreased by 10-15% over control with IN-2001 treatment for 24.

**IN-2001 induces cell cycle arrest**

To investigate whether the growth inhibitory effect of IN-2001 is related to cell cycle alteration, we analyzed the cell cycle distribution of IN-2001-treated breast cancer cells. ER negative MDA-MB-231 cells were treated with vehicle (0.1% DMSO) or 1 μM IN-2001 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-2001 showed G2/M arrest with decrease of G0/G1 phase or S phase in MDA-MB-231 cells. When cells were treated with IN-2001 for 12 hr, MDA-MB-231 cells yielded 42.7% of cells in G2/M phase,  

**Table 1. 50% inhibitory concentration (IC\(_{50}\)) of HDAC inhibitor**

|        | IC\(_{50}\) (μM) |
|--------|-----------------|
| IN2001 | 0.585           |
| SAHA   | 0.923           |

**Fig. 2. Dose-dependent growth inhibition by IN-2001.** MDA-MB-231 human breast cancer cells were treated with vehicle (0.1% DMSO) or indicated concentrations (0.001-10 μM) of IN-2001 for 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).

**Fig. 3. Time-dependent growth inhibition by IN-2001.** Human breast cancer MDA-MB-231 cells were treated with vehicle (0.1% DMSO) or 1 μM IN-2001 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).

**Fig. 4. Effect of IN-2001 on cell cycle distribution.** MDA-MB-231. Human breast cancer cells were treated with vehicle (0.1% DMSO) or 1 μM IN-2001 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.
whereas untreated control cells showed 34.3% of cells in G2/M phase. With 24 hr treatment IN-2001 accumulated 42.4% of cells in G2/M phase, whereas untreated control cells showed 26.9% of cells in G2/M phase. When cells were treated with IN-2001 for 48 hr showed 39.5% of cells in G2/M phase, whereas untreated control cells showed 27.3% of cells in G2/M phase. SAHA did not affect cell cycle distribution of MDA-MB-231 cells.

**IN-2001 increases p21WAF1 and p27KIP1 expression**

In the previous study, we found that HDAC inhibitors induced cell cycle arrest. In relation to cell cycle arrest, we examined the effects of HDAC inhibitor on the cell cycle regulatory proteins, such as cyclins and cyclin dependent kinase (cdk) inhibitors. MDA-MB-231 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 p21WAF1 and p27KIP1 was examined by RT-PCR and western blot analysis. As shown in Fig. 5 in MDA-MB-231 cells, IN2001, and SAHA slightly increased p21WAF1 mRNA level. In contrast, p21WAF1 protein level was significantly up-regulated by all kinds of IN-2001 (Fig. 6). IN2001, and SAHA treatment showed 1.9-fold, and 1.4-fold increase in p21WAF1 protein level, respectively. In addition, p27KIP1 protein level was also increased to 2.6-fold, and 1.5-fold with IN2001, and SAHA, respectively.

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-2001 decreases cyclin D1 expression and increases cyclin D2 expression**

As well as cdk inhibitors, one of the important cell cycle regulatory proteins is cyclin. In this study, we examined the effect of IN-2001 on the expressions of D-type cyclin (cyclin D1 and cyclin D2). MDA-MB-231 cells were treated with vehicle (0.1% DMSO) or 1 μM IN-2001 for 24 hr and then examined for the expression of cyclin D1 and cyclin D2 by RT-PCR analysis. 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 decreases thymidylate 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. MDA-MB-231 cells were treated with vehicle (0.1% DMSO) or 1 μM HDAC inhibitors for 24 hr and then TS mRNA level was determined using RT-PCR technique. As shown in Fig. 8, TSA, HC toxin, IN2001, and LAQ treatment decreased TS mRNA level to 24%, 22%, 80%, and 33% of control level, respectively in MDA-MB-231 cells. But SAHA did not show sig-

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**Table 2. Cell cycle distribution by IN-2001**

| Times (hr) | HDAC inhibitors | Distributions of cells (%) |
|-----------|----------------|--------------------------|
|           | Sub-G1 | G0/G1 | S  | G2/M |
| 12 Ctrl | 1.706 ± 0.212 | 53.832 ± 0.655 | 11.487 ± 0.291 | 34.314 ± 0.731 |
| IN2001  | 2.376 ± 0.281 | 49.477 ± 0.883 | 7.878 ± 0.267 | 42.667 ± 1.098* |
| SAHA    | 2.678 ± 0.352 | 54.997 ± 1.681 | 10.299 ± 0.436 | 33.762 ± 1.369 |
| 24 Ctrl | 2.067 ± 0.151 | 59.915 ± 0.916 | 12.870 ± 0.211 | 26.936 ± 0.714 |
| IN2001  | 2.845 ± 0.237 | 52.017 ± 1.137 | 5.565 ± 0.284 | 42.418 ± 0.906* |
| SAHA    | 3.078 ± 0.027 | 60.993 ± 0.481 | 12.502 ± 0.702 | 26.497 ± 0.555 |
| 48 Ctrl | 2.583 ± 0.155 | 61.213 ± 1.107 | 10.910 ± 0.112 | 27.275 ± 1.182 |
| IN2001  | 7.595 ± 0.802 | 49.338 ± 0.645 | 11.542 ± 0.480 | 39.458 ± 0.796* |
| SAHA    | 3.474 ± 0.712 | 62.667 ± 1.875 | 10.879 ± 0.590 | 26.103 ± 1.341 |

*Bold lettering indicates significant difference from control group (p<0.05).
significant changes in TS expression, instead slightly increased TS expression.

**HDAC inhibitor induces dose-dependent 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. MDA-MB-231 cells treated with vehicle (0.1% DMSO) or 1 μM IN-2001 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\textsuperscript{WAF1} and p27\textsuperscript{KIP1} was normalized to that of Actin and data was expressed as fold induction compared to control group.

**DISCUSSION**

In cancer, some genes are transcriptionally silenced by the inappropriate recruitment of HDACs, e.g., tumor suppressor genes (Glaser et al., 2003). Known repressors are multipeptides 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 archetypical gene silenced in this manner in human cancer is the cyclin-dependent kinase inhibitor p21\textsuperscript{WAF1}. Epigenetic reactivation of p21\textsuperscript{WAF1} by HDAC inhibitors has been reported in cancer cell lines (Archer et al., 1998), and the restoration of p21\textsuperscript{WAF1} gene expression by HDAC inhibitors sub-G\textsubscript{1} peak. When cells were exposed to HDAC inhibitors for 48 hr, HDAC inhibitors except SAHA increased apoptotic peak ranging from 7.6% to 12.9% compared to the control value of 2.6%. However, 1 μM SAHA did not show apparent apoptotic sub-G\textsubscript{1} peak (Fig. 9).
Effect of HDAC inhibitor on the thymidylate synthase
Quantitative analysis of HDAC inhibitor-induced apoptosis. MDA-MB-231 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. MDA-MB-231 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 ModFit program. Data present mean ± S.D. (N=4). *Significantly different from control at each time point (p<0.05).

Fig. 8. Effect of HDAC inhibitor on the thymidylate synthase expression. MDA-MB-231 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).

ACKNOWLEDGMENTS

This work was supported by grant 2006-KRF-531-E00112 from KOSEF.

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