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, 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; Bolden et al., 2006; 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 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; Marladason et al., 2000; Suzuki et al., 2002), are those encoding known tumor-associated proteins that medi- rate proliferation and cell cycle progression, survival factors, growth factor receptors, kinase and signaling transduction intermediates, DNA synthesis/repair enzymes, shuffling pro- teins, transcription factors, and proteases. p21WAF1 mediates growth arrest in the Go 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 (Na- kano 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 p21WAF1 gene by HDAC inhibitors is associated with an increased level of histone acetylation at the p21WAF1 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 dis-tribution, 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). So- dium pyruvate, penicillin-streptomycin, fetal bovine serum (FBS), trypsin-EDTA, minimum essential medium (MEM), and RPMI were acquired from GibcoBRL (Rockville, MD, USA). T47D cells were maintained in RPMI containing 1% acetic acid. Cells were washed with tap water and centrifuged at 14,000 g for 5 min at 4°C and in 5% CO2.

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% CO2.

Cell proliferation assay
Cells were plated in 96 well plates at a density of 104 cells per well. The following day, the cells were treated with chemica- lants. The number of cells was measured based on the modi- fied SRB assay. Cells were treated with cold 10% trichloroace- tic 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 4% (w/v) sulfurphodamineB (SRB) dis- solved 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 mm2 dishes and exposed to chemi- cals. 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 mm2 dishes and exposed to chemi- cals 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 μl RNasin (40 U/μl), and H2O. 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 prelimi- nary experiments; and extended at 72°C for 1 min. The PCR reaction ended with 5 min incubation at 72°C. Special primers (GAPDH: 5’ACATCGTCAGACACCATgg3’; 5’ gTAgTtgAg-gTCAATgAaggg3’; p21: 5’gAACTTCgACCTTCTgACgAg3’; 5’ CgTTTTCCgACCTTCTgAgTCTG3’; Cyclin D1: 5’AgCCATggAA CACCagCTC3’; 5’ gCACCTCCTagCATCCAgT3’; Cyclin D2: 5’ TACCTTCAAgTgCgAgAgAC3’; 5’ TCCCACTTCTCCAg TTgCatCAT3’) for PCR amplification and PCR products were analyzed on 2% agarose gels.

Western blot analysis
Cells were plated in 100 mm2 dishes and then incubated with chemicals for 24 hr. Cells were collected and homogenized in a lysis buffer (Pro-prep protein extraction solution, INFRON; 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 mMthiourelitol (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-acylamide 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.