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# Antiproliferative Effect of Trichostatin A and HC-Toxin in T47D Human Breast Cancer Cells

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Histone deacetylase inhibitors are new class of chemotherapeutic drugs able to induce tumor cell apoptosis and/or cell cycle arrest. Trichostatin A, an antifungal antibiotic, and HC-toxin are potent and specific inhibitors of histone deacetylase activity. In this study, we have examined the antiproliferative activities of trichostatin A and HC-toxin in estrogen receptor positive human breast cancer, T47D cells. Both trichostatin A and HC-toxin showed potent antiproliferative efficacy and cell cycle arrest at $G_2$/M in T47D human breast cancer cells in a dose-dependent manner. Trichostatin A caused potent apoptosis of T47D human breast cancer cells and trichostatin A-induced apoptosis might be involved in an increase of caspase-3/7 activity. HC-toxin evoked apoptosis of T47D cells and HC-toxin induced apoptosis might not be mediated through direct increase in caspase-3/7 activity. We have identified potent activities of antiproliferation, apoptosis, and cell cycle arrest of trichostatin A and HC-toxin in estrogen receptor positive human breast cancer cell line T47D.

**Key words:** T47D cell, Trichostatin A, HC-toxin, Histone deacetylase

## INTRODUCTION

Breast cancer becomes the most common cause of cancer in women in Korea since 2001. Despite major recent advances in therapy, more effective approaches to the treatment and prevention are still necessary. Histone deacetylase inhibitors may provide an alternative therapeutic approach for the treatment of breast cancer. Histone deacetylase inhibitors have been shown to induce growth arrest, differentiation, and/or apoptosis in a variety of transformed cell lines and inhibit tumor development in rodents, including the development of *N*-methyl-*N*-nitrosourea induced mammary tumor in rats (Cohen *et al.*, 1999). Histone deacetylase inhibitors belong to a heterogenous class of compounds that include derivatives of short chain fatty acids, hydroxamic acids, cyclic tetrapeptides, and benzamides. Among the hydroxamic acids, trichostatin A and suberoylanilide hydroxamic acid are commonly used inhibitors of histone deacetylase (Lin *et al.*, 1998; Finnin *et al.*, 1999). Numerous antiproliferative effects have been reported for trichostatin A and suberoylanilide hydroxamic acid, including induction of $G_0/G_2$ cell cycle arrest, differentiations, and selective apoptosis of transformed cells (Van *et al.*, 1996; Richon *et al.*, 1996; Nusse *et al.*, 1990; Greenspan *et al.*, 1985). Suberoylanilide hydroxamic acid, in particular, shows strong antiproliferative effects but low toxicity *in vivo* and is currently under clinical trials for the treatment of solid and hematological tumor (Lin *et al.*, 1998; Finnin *et al.*, 1999). Recently there has been strong interest in histone deacetylase inhibitors as anticancer agents due to their selective toxicity against tumor cells and synergistic activity with existing therapeutic agents, including retinoic acid (Kitamura *et al.*, 2000), vitamin D analogues (Rashid *et al.*, 2001), and peroxisome proliferators-activated receptor ligands (Chang and Szabo, 2002).

Histone deacetylase inhibitors are a novel class of chemotherapeutic agents initially identified by their ability to reverse the malignant phenotype of transformed cells. They have been shown to activate differentiation programs, arrest the cell cycle, and induce apoptosis in a wide range of tumor derived cell lines, block angiogenesis and stimulate the immune system *in vivo* (Marks *et al.*, 2001; Johnstone, 2002a). Although the mechanisms through which histone deacetylase inhibitors exert these antitumor activities have not been fully understood, induction of

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histone hyperacetylation and modulation of gene transcription through chromatin remodeling are thought to be primarily responsible, leading to the selective activation of genes associated with cell growth and survival. In addition, proteins other than histones, such as p53 and GATA-1, have been identified as target for acetylation (Juan *et al.*, 2000; Boyes *et al.*, 1998), and it is possible that histone deacetylase inhibitors may exert other effects independently of their direct effects on transcription through chromatin remodeling.

In this study, we investigated the effects of histone deacetylase inhibitors, trichostatin A and HC-toxin in T47D human breast cancer cells. We found that trichostatin A and HC-toxin inhibited T47D human breast cancer cell growth by arresting cells at $G_2$/M as well as inducing apoptosis.

## MATERIALS AND METHODS

### Cell culture

The human breast cancer cell line, T47D cells were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI1640 supplemented with 5% fetal bovine serum and penicillin-streptomycin at 37 °C and in 5% $CO_2$.

### Cell proliferation

T47D cells were plated in 96 well plates at a density of 3,000 cells per well. The following day, the cells were treated with various concentrations of histone deacetylase inhibitors for 72 h. The number of cells was measured based on the modified SRB assay (Soto *et al.*, 1995).

### DAPI staining

T47D cells were plated in 6 well plates and treated with various concentrations of the histone deacetylase inhibitors for 72 h. Cells were washed with 1x PBS. After hypotonic swelling with 75 mM KCl for 1 min at room temperature, cells were fixed in ice-cold methanol : acetic acid (3:1). Cells were air-dried and incubated with the DNA specific fluorochrome DAPI for 20 min. The excess of DAPI was removed and stained nuclei were visualized under a fluorescence microscopy.

### Flow cytometric anlaysis

For flow cytometric analysis, cells were plated in 35 mm dishes and treated with vehicle solvent or histone deacetylase inhibitors. After 12, 24, or 72 h, the medium was removed and cells were detached using trypsin-EDTA, washed in PBS and fixed using 70% ethanol. After centrifugation, cell pellet was resuspended and treated with RNase A for 20 min at 37 °C. The DNA content per cell was evaluated in a FACScalibur (Becton-Dickinson, Mountain View, Cal., USA) after staining the cells with propidium iodide for 30 min at 37 °C in the dark.

### Caspase assay

Caspase 3/7 activity was assayed by fluorometric methods. Cells were treated with histone deacetylase inhibitors for 24 h, mixed with Apo-One homogenous cell lysis/activity buffer containing profluorescent substrate, z-DEVD-rhodamine 110 in 96 well plates, and incubated for 30 min at room temperature. Upon sequential cleavage and removal of the DEVD peptide by caspase-3/7 activity and excitation, the rhodamine 110 leaving group becomes intensively fluorescent. The release of rhodamine 110 was monitored at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

### Statistical analysis

Statistical analysis of data was carried out by Student's *t*-test.

## RESULTS

### Trichostatin A and HC-toxin inhibit cell proliferation in T47D human breast cancer cells

The estrogen receptor positive human breast cancer cell line T47D was cultured with increasing concentrations of histone deacetylase inhibitor suberoylanilide hydroxamic acid (1 nM~10 μM). As shown in Fig. 1, concentrations of suberoylanilide hydroxamic acid of >1 μM caused a profound cell growth inhibition. The $IC_{50}$ of 0.33 μM was

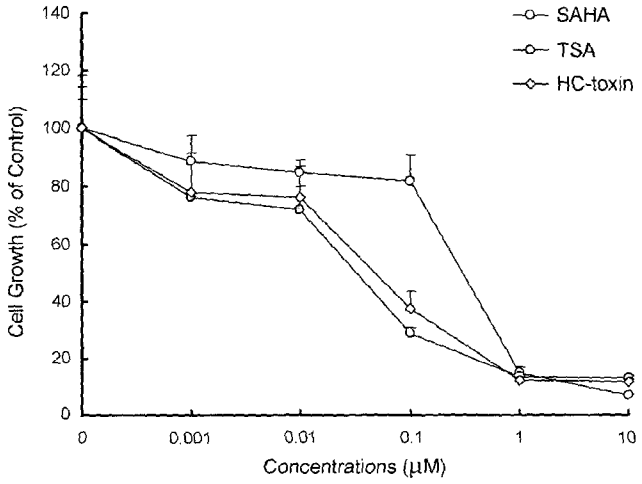


**Fig. 1.** Effect of HDAC inhibitors on the T47D human breast cancer cell proliferation. T47D cells were plated in 96 well plates at a density of 3,000 cells per well. After 24 h incubation, cells were treated with increasing concentrations of the HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA), Trichostatin A (TSA), or HC-toxin for 72 h. Cell growth was determined by measuring the absorbance at 570 nm as described in Materials and Methods. Data represent the mean ± S.D. of three independent experiments. ○, SAHA; ●,TSA; □, HC-toxin.

**Table I.** Inhibition of cell proliferation ($IC_{50}$) of T47D human breast cancer cell by HDAC inhibitors

| Compounds | $IC_{50}$ (μM) |
|---|---|
| SAHA | 0.330 |
| TSA | 0.023 |
| HC-toxin | 0.037 |

The $IC_{50}$ values indicate the concentrations of HDAC inhibitors (SAHA, TSA, or HC-toxin) that resulted in 50% reduction of cell number. The $IC_{50}$ was calculated by plotting the inhibition of cell growth. Data were expressed as the average of triplicate values from different experiment.

calculated as 50% reduction in cell number after 72 h of continuous exposure to suberoylanilide hydroxamic acid (Table I). Various concentrations of trichostatin A (1 nM~10 μM) were treated to T47D human breast cancer cells for 72 h. Fig. 1 showed strong inhibition of T47D human breast cancer cell proliferation by trichostatin A treatment in a dose-dependent manner. The $IC_{50}$ of 0.023 μM and 0.037 μM were calculated by trichostatin A and HC-toxin treatment for 72 h, respectively (Table I).

### Trichostatin A and HC-toxin arrest cell cycle at $G_2$/M

We found that treatment of T47D cells with trichostatin A or HC-toxin for 12, 24, or 48 h induced cell cycle arrest (Fig. 2A). Treatment with trichostatin A for 12 h or 24 h to T47D human breast cancer cells resulted in dose-dependent

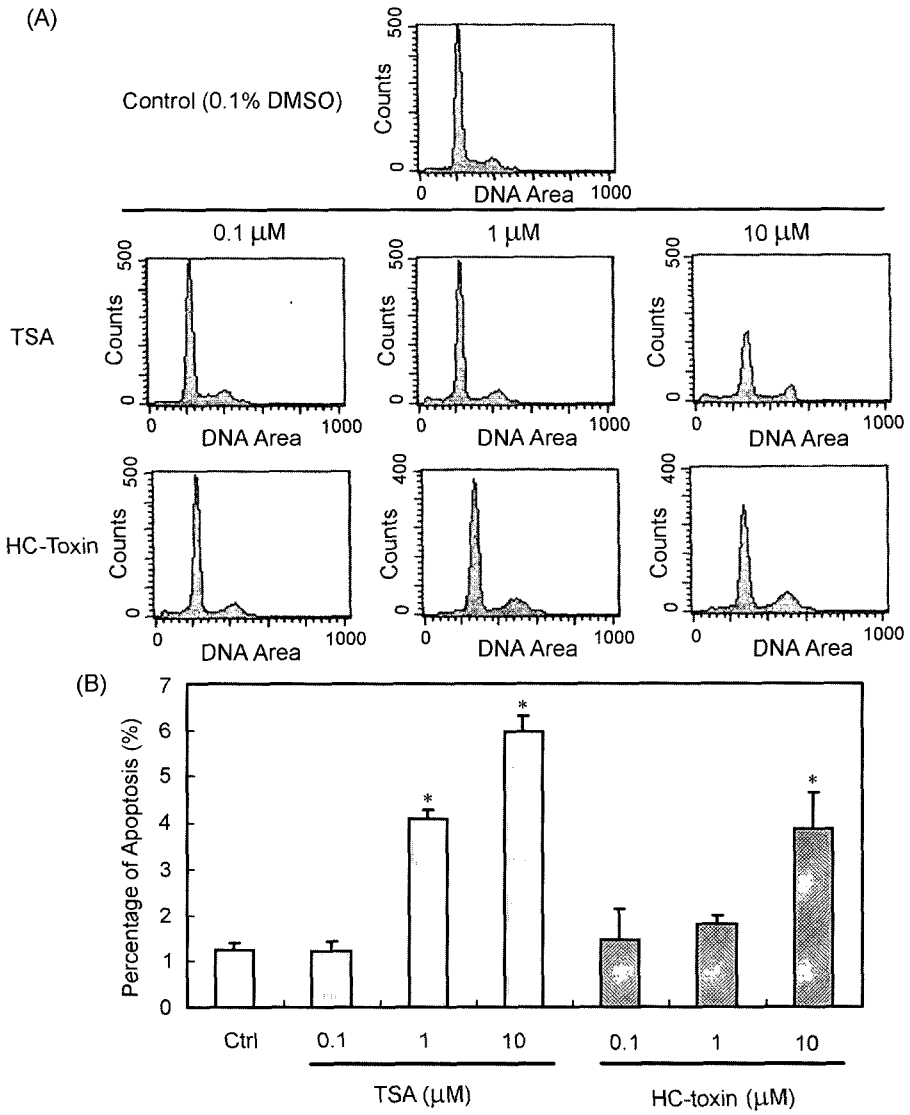


**Fig. 2.** Effect of HDAC inhibitors on the cell cycle distribution. (A) Representative profiles of cell cycle status. T47D cells were treated with vehicle solvent (0.1% DMSO) or HDAC inhibitors (trichostatin A or HC-toxin) at the indicated concentration for 12, 24, or 48 h, respectively. Cells were harvested, fixed, and stained with propidium iodide. Then 20,000 stained cells were subjected to flow cytometry analysis to determine the distribution of cells. (B) Quantitative analysis of apoptosis. Quantitative analysis of apoptosis was done using profiles as those shown in panel A. T47D cells were treated with vehicle solvent (0.1% DMSO) or HDAC inhibitors (trichostatin A or HC-toxin) at the indicated concentration for 12 h. *Significant different from control at $p < 0.05$. □, control (0.1% DMSO); , TSA; □, HC-toxin.

**Table II.** Kinetic analysis of cell cycle distribution by HDAC inhibitors in T47D cells

| Treatment (μM) | | Distribution (%) | | | |
|---|---|---|---|---|---|
| | | Sub-G1 | G0/G1 | S | G2/M |
| 12 hr | | | | | |
| Control | | 1.24 | 64.94 | 22.08 | 12.98 |
| TSA | 0.1 | 1.23 | 63.38 | 24.85 | 11.80 |
| | 1 | 4.10 | 69.11 | 13.92 | 16.97 |
| | 10 | 5.95 | 66.41 | 20.15 | 20.15 |
| HC-toxin | 0.1 | 1.46 | 66.32 | 13.42 | 20.25 |
| | 1 | 1.82 | 62.14 | 17.35 | 20.51 |
| | 10 | 3.90 | 67.39 | 19.83 | 12.77 |
| 24 hr | | | | | |
| Control | | 3.57 | 69.00 | 24.82 | 6.13 |
| TSA | 0.1 | 2.40 | 71.00 | 21.56 | 7.44 |
| | 1 | 3.99 | 78.50 | 9.99 | 11.51 |
| | 10 | 17.45 | 64.41 | 12.65 | 25.02 |
| HC-toxin | 0.1 | 5.57 | 66.68 | 24.14 | 9.17 |
| | 1 | 3.50 | 74.94 | 9.53 | 15.53 |
| | 10 | 4.28 | 68.49 | 11.27 | 20.57 |
| 48 hr | | | | | |
| Control | | 2.95 | 69.60 | 21.16 | 10.24 |
| TSA | 0.1 | 2.62 | 65.61 | 21.99 | 12.40 |
| | 1 | 8.06 | 77.23 | 10.91 | 11.87 |
| | 10 | N.D. | N.D. | N.D. | N.D. |
| HC-toxin | 0.1 | 4.16 | 82.49 | 8.35 | 9.15 |
| | 1 | 3.68 | 69.08 | 16.59 | 14.33 |
| | 10 | 18.21 | 66.78 | 4.98 | 28.24 |

T47D human breast cancer cells were treated with vehicle solvent (0.1% DMSO) or HDAC inhibitors (Trichostatin A or HC-toxin) for 12, 24, or 48 h at the indicated concentrations. Cell cycle distribution was measured by flow cytometry.

cell cycle arrest at $G_2$/M (Fig. 2A, Table II). Treatment of T47D cells for 12, 24, or 48 h with HC-toxin also induced $G_2$/M arrest in a dose-dependent manner (Fig. 2A, Table II). However, the cells seemed to be killed by treating with trichostatin A for 48 h.

### Trichostatin A and HC-toxin induced apoptosis of T47D human breast cancer cells

Treatment of T47D human breast cancer cells with trichostatin A or HC-toxin for 12 h induced apoptosis of cells in a dose-dependent manner (Fig. 2B, Table II). Cell death induced by trichostatin A (0.1, 1.0, or 10 μM) was compared with that by HC-toxin (0.1, 1.0, or 10 μM) in T47D human breast cancer cells. Trichostatin A appeared to be 10-fold more potent than HC-toxin (Fig. 2B, Table II). In T47D human breast cancer cells, a significant cell death was observed after treatment with trichostatin A or HC-toxin for 12 h and steadily increased over 48 h time period (Table II). Similar results were obtained using PI staining and DAPI staining (Fig. 3).

### Trichostatin A induced caspase activity in T47D human breast cancer cells

In order to examine if caspases are involved in the apoptotic activity of trichostatin A and HC-toxin, we measured caspase-3/7 activity using z-DEVD-rhodamine 110 as a substrate. As shown in Fig. 4, trichostatin A treatment of T47D human breast cancer cells for 24 h showed a dose-dependent increase in caspase-3/7 activity. Treatment of T47D cells with HC-toxin for 24 h showed minimal increase of caspase-3/7 activity (Fig. 4). These data indicated that trichostatin A and HC-toxin mediated apoptosis through different mechanisms.

## DISCUSSION

The antifungal antibiotic trichostatin A is a noncompetitive reversible inhibitor of histone deacetylase activity in cultured mammalian cells and in fractionated cells nuclear extracts at low nanomolar concentrations. Trichostatin A has been shown to arrest at $G_1$ and $G_2$ phases of the cell cycle, induce differentiation, and reverse the transformed morphology of cells in culture (Yoshida *et al.*, 1995). Trichostatin A has been reported to be a potent inhibitor of proliferation and histone deacetylase activity in human breast cancer cell line and antitumor agent without measurable toxicity in the *N*-methyl-*N*-nitrosourea induced mammary carcinoma model (Vigushin *et al.*, 2001). In this study, we have examined the effect of trichostatin A and HC-toxin on estrogen receptor positive human breast cancer cell line T47D. As shown in Fig. 1, trichostain A has potent antiproliferative activity against T47D human breast cancer cells, strongly supporting histone deacetylase as a molecular target for anticancer therapy in breast cancer. Chemotherapeutic drugs can induce cell death by activating either one of two major pathways (Johnstone *et al.*, 2002b). The death receptor pathway involves ligation of receptors such as Fas, tumor necrosis factor receptor, and TRAILR by their ligands, resulting in binding of adaptor proteins such as FADD and TRADD and recruitment and activation of membrane proximal caspases such as caspase-8 and caspase-10. These enzymes subsequently activate other caspases (i.e. caspase-3), resulting in the cleavage of a number of nuclear and cytoplasmic substrates to induce the morphological changes, characteristic of apoptosis. The second pathway, known as the intrinsic pathway, is primarily activated by stress stimuli such as chemotherapeutic drugs, ionizing radiation, and growth factor withdrawal (Cory and Adams, 2002). These stimuli induce the release of mitochondrial proteins such as cytochrome c, together

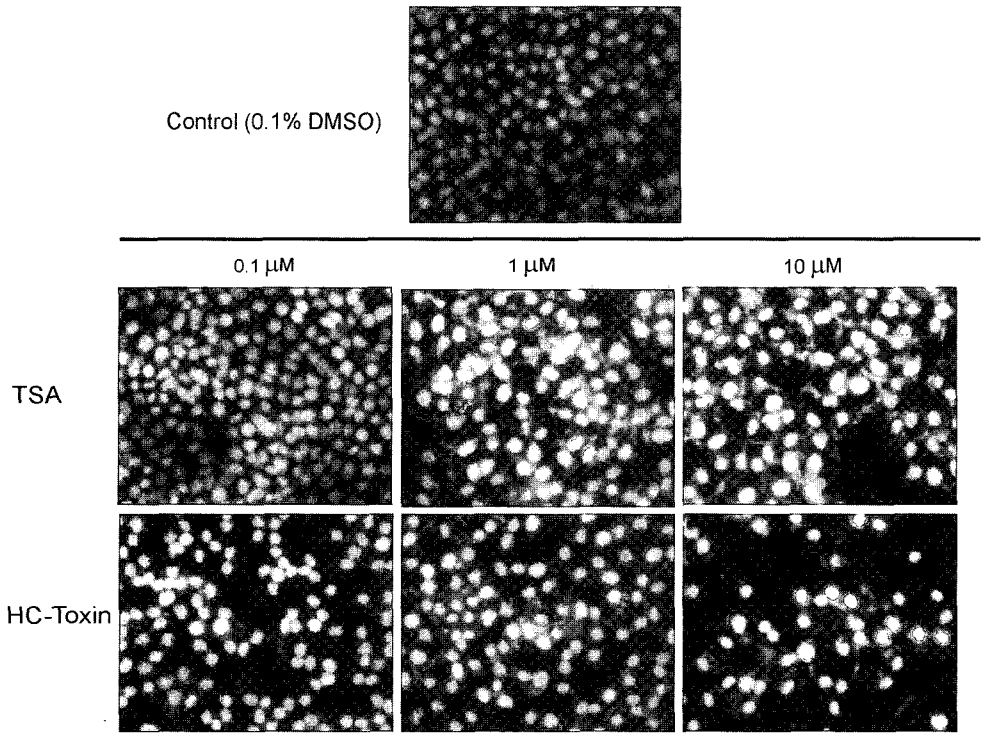


**Fig. 3.** Induction of apoptosis by HDAC inhibitors, shown by DAPI staining. Apoptosis induction by trichostatin A or HC-toxin in T47D human breast cancer cell line was analyzed by immunofluorescence. T47D cells were treated with vehicle solvent (0.1% DMSO) or HDAC inhibitors (trichostatin A or HC-toxin) for 24 h and then stained with DAPI. Stained nuclei were visualized under a fluorescence microscopy (x 400).

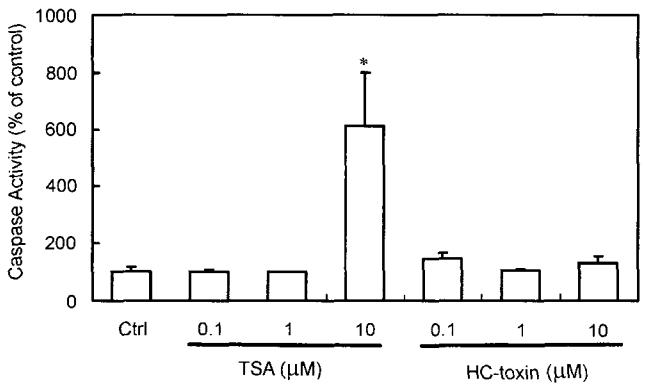


**Fig. 4.** Activation of caspase-3/7 by HDAC inhibitor. T47D human breast cancer cells were treated with vehicle solvent (0.1% DMSO) or HDAC inhibitors (trichostatin A or HC-toxin) for 24 h at 37 °C. The enzyme activity of caspase-3/7 was determined by incubation with profluorescent substrate, z-DEVD-rhodamine 110 for 30 min. Upon cleavage on the C-terminal side of the aspartate residue in the DEVD peptide substrate sequence by caspase-3/7 enzymes and excitation, the rhodamine 110 leaving group becomes intensely fluorescent. The release of rhodamine 110 was monitored at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Caspase activity indicated relative percent activity of HDAC inhibitors versus vehicle control. *Significant different from control at $p < 0.05$. □, control (0.1% DMSO); , TSA; □, HC-toxin.

with Apaf-1, induces caspase-9 activation, and HtrA2 and Smac/DIABLO, which neutralize the caspase inhibitory actions of inhibitors of apoptosis protein (IAP). Caspase-9 can then activate downstream effector caspases (i.e. caspase-3). The intrinsic pathway is primarily regulated by the Bcl-2 family of proteins, which include the multidomain antiapoptotic (i.e. Bcl-2 and Bcl-XL) and pro-apoptotic (i.e. Bak and Bax) members, and BH3 domain only proapoptotic (i.e. Bid, Bad, NOXA, PUMA, and HrK). Interestingly, death receptors can also activate the intrinsic pathway through caspase-8-mediated activation of Bid. In this way, Bid serves as a molecular link between the two pathways and can amplify the death receptor stimulated response. There was a report that histone deacetylase inhibitor, suberoylanilide hydroxamic acid mediated apoptosis *via* a mechanism involving activation of the intrinsic death pathway by inducing the caspase independent cleavage and activation of Bid (Ruefli *et al.*, 2001). In this study, both histone deacetylase inhibitors trichostatin A and HC-toxin induced apoptosis (Fig. 2A, 2B, Table II), however, mechanisms of actions of these two compounds appeared to be different. Apoptotic activity of trichostatin A might be involved in the induction of caspase-3/7, but apoptotic activity of HC-toxin might be mediated through other pathway other than activation of caspase-3/7 (Fig. 4). Taken together, these data suggested that trichostatin A might be a candidate for the anticancer agent against estrogen receptor positive human breast cancer.

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