The novel histone deacetylase inhibitor, N-hydroxy-7-(2-naphthylthio) hepatonomide, shows potent antitumor activity due to cytoplasmic free calcium mediated apoptosis in thyroid cancer both in vitro and in vivo.

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Directed by Professor Hang-Seok Chang

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Seok-Mo Kim

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Seok-Mo Kim

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ABSTRACT

The novel histone deacetylase inhibitor, N-hydroxy-7-(2-naphthylthio) hepatonomide, shows potent antitumor activity due to cytoplasmic free calcium mediated apoptosis in thyroid cancer both in vitro and in vivo.

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Epigenetic changes play a crucial role in the regulation of all DNA-based processes, such as transcription, repair, and replication. Improper histone changes can consequence in dysregulation of cell growth, leading to tumorigenic transformation and cell death. Thyroid cancer has been shown to have a higher global methylation percentage and reduced histone acetylation. Preclinical models have showed that histone gene modifiers and epigenetic changes play significant roles in

papillary (PTC) and anaplastic (ATC) thyroid cancer tumorigenesis. These days, a novel HDAC inhibitor, N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA), has been introduced therapeutic approaches to thyroid cancer as an example of a new class of anti-cancer agents. Here, we prove the ER stress (Endoplasmic Reticulum)-dependent apoptosis inducing activity of HNHA in cultured patient-derived thyroid cancer cells. Apoptosis in the HNHA-treated group was induced significantly, with marked caspase activation and Bcl-2 suppression in thyroid cells in vitro and in vivo. HNHA treatment caused cytoplasmic free calcium release from ER. HNHA also induced ER stress-dependent apoptosis, suggesting that HNHA can induce caspase-independent nuclear apoptosis in PTC and ATC. An in vivo study showed that HNHA had greater anti-tumor and pro-apoptotic effects on PTC and ATC xenografts than the established HDAC inhibitors. In conclusion, HNHA has more potent anti-tumor activity than established HDAC inhibitors and its activities are mediated by caspase-dependent and ER stress-mediated apoptosis in thyroid cancer cells. These results suggest that HNHA may offer a new therapeutic approach to thyroid cancer.

Key words: Papillary thyroid cancer (PTC), anaplastic thyroid cancer (ATC), Histone deacetylase inhibitor

The novel histone deacetylase inhibitor, N-hydroxy-7-(2-naphthylthio) hepatonomide, shows potent antitumor activity due to cytoplasmic free calcium mediated apoptosis in thyroid cancer both in vitro and in vivo.

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I. INTRODUCTION

1. Thyroid cancer

Thyroid cancer is a cancer originating from follicular or parafollicular thyroid cells. These cells give rise to both well-differentiated cancers – papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) – and anaplastic thyroid cancer (ATC), whose anaplastic cells are poorly differentiated. The second cell type, the C or parafollicular cell, produces the hormone calcitonin and is the cell of origin for medullary thyroid cancer (MTC) [1]. The most effective management of aggressive thyroid cancers is surgical removal of thyroid gland (thyroidectomy) followed by radioactive iodine ablation and TSH-suppression therapy. Chemotherapy or radiotherapy may also be used in

cases of distant metastases or advanced cancer stage. Five year survival rates are 98.1% in the United States [NIH, Cancer Statistical Summaries].

2. Thyroid cancer classification

Thyroid cancers can be classified according to their histopathological characteristics [2]. PTC (75% to 85% of cases [3]) – often in young females – excellent prognosis. May occur in women with familial adenomatous polyposis and in patients with Cowden syndrome. ATC (less than 5% of cases [4]) is not responsive to treatment and can cause pressure symptoms. ATC is one of the most aggressive human malignancies [5]. The mechanisms fundamental the progress of ATCs are incompletely understood. Presently, valid therapy for ATCs includes chemotherapy, radiotherapy, and surgery [6,7]. Nevertheless, patients with ATC still have a median survival of 5 months and less than 20% survive 1 year [8]. Furthermore early tumor dissemination results in 40% of patients having distant metastases and 90% having adjacent tissue invasion on presentation [9].

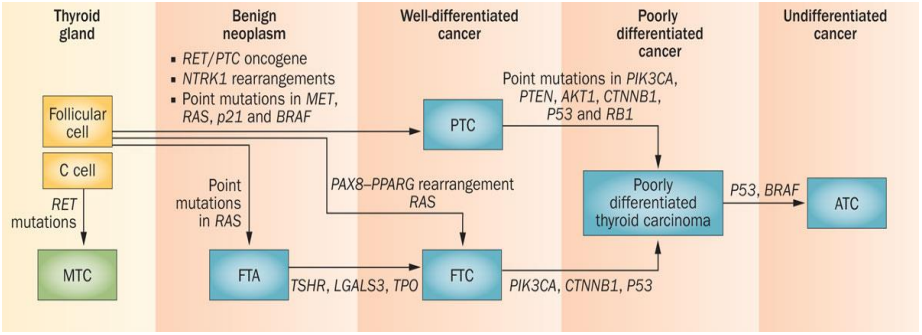


Figure 1. Classification of human thyroid carcinomas and subtype-specific genetic alterations (*Pallante, P. et al. (2013) Deregulation of microRNA expression in thyroid neoplasias Nat. Rev. Endocrinol*).

3. Histone deacetylase inhibitor

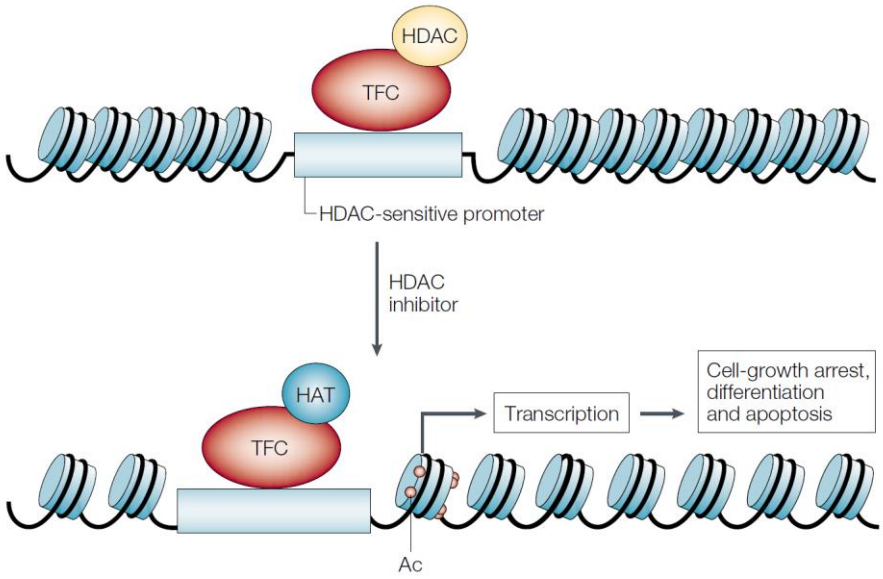


Figure 2. Proposed mechanism of action of histone deacetylase inhibitors. Ac, acetyl group; HAT, histone acetyltranferase.

The HDACs restrain the transcription of tumor suppressor genes and thus symbolize promising targets for cancer therapy [10,11]. HDAC inhibitors (HDACIs) are a group of small molecules that accelerate gene transcription by chromatin remodeling and have been extensively studied as potential drugs for treating cancer [11-14]. HDAC inhibitors work by affecting various cellular functions in cancer cells, including differentiation, growth arrest, senescence, autophagy and apoptosis [15-17]. They are highly selective against the cancer cells over the normal cells, owing to their up-regulation of pro-apoptotic genes, impairment of DNA repair mechanism and generation of ER (endoplasmic reticulum) stress [18,19]. Recently, N hydroxy-7-(2-naphthylthio) hepatonomide (HNHA) has been introduced. It is a novel HDAC inhibitor that potently suppresses histone hypo-acetylation and down-regulation of HDAC target genes [20,21].HNHA shows strong anti-cancer activity with pharmacological properties superior to those of the known HDAC inhibitor SAHA in human fibrosarcoma and breast cancer cells [20-22].

II. MATERIALS AND METHODS

Cell culture

SNU 80(ATC) and SNU 790(PTC), Patient-derived thyroid cancer from the Korea Cell Line Bank (Seoul National University, Seoul, Korea), they were grown in RPMI-1640 medium with 10% FBS.

Cell viability assay

Cell proliferation was measured using the MTT assay. Cells were seeded in 96-well plates at 5×10^3 per well and incubated overnight to 70% confluency. Drug was added at 0-100 μM final concentrations. After drug treatment, cells were incubated and cell viability was then measured using the MTT reagent at 490 nm according to the manufacturer's protocol. Data are presented as mean percentages of vehicle-treated cell proliferation ± SEM of triplicate experiments.

Evaluation of cell death using TUNEL assays

For the measurement of apoptotic cells, cells were fixed with 4% paraformaldehyde solution for 48 hours and stained using a Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) kit (Promega, Madison, WI, USA). The apoptotic cells (fluorescent green) and total cells were counted under a fluorescence microscope, and the data were recorded. Images were collected using a confocal microscope

(LSM Meta 700, Carl Zeiss, Oberkochen, Germany) and were analyzed using the Zeiss LSM Image Browser software program, version 4.2.0121.

Cytosolic free Ca^{2+} measurements by microspectrofluorimetry

Cells were perfused with 140 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 33 mM glucose, and 20 mM HEPES (pH 7.4, adjusted with NaOH, and 320–350 Osm with sucrose). The intracellular Ca^{2+} level of SNU 80 and SNU 790 cells were imaged using a calcium-sensitive fluorescent dye, Fura-2AM. Fluorescence intensities (ΔF) were normalized to the resting values.

Immunoblot analysis

Equal amounts of protein (20 μg) were separated in 8-10% SDS-polyacrylamide gels; the resolved proteins were then electro-transferred onto PVDF membranes (Millipore, Bedford, MA). The antibodies for acetyl-histone H3 and histone H3, acetyl-α-tubulin and α-tubulin, p53, p21 were obtained from Abcam, Cambridge, UK. Apaf-1, CDK 4, CDK 6, Cyclin D1, Bcl-2, p-NFκB, caspase-3, caspase-9, and β-actin antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. GRP78 antibody (Cell Signaling Technology, Inc., Danvers, MA, USA), ATF4 antibody (Cell Signaling Technology) and CHOP

antibody (Cell Signaling Technology) were used respectively as the primary antibodies. Antibidoies against Bax was from Novus Biologicals, Littleton, CO, USA.

Flow cytometry for cell cycle analysis

Cells were treated with HDAC inhibitor RPMI1640 medium with 10% FBS for 40 hours, harvested by trypsinization, and fixed with 70% ethanol. Cells were stained for total DNA with a solution containing 40 μg/ml propidium iodide (PI) and 100μg/ml RNase I in PBS for 30 min at 37°C. Cell cycle distribution was then analyzed with the FACS Calibur Flow Cytometer (BD Biosciences, SanJose, CA, USA). The proportions of cells in the G0/G1, S, and G2/M phases were analyzed by FACS and DNA software program (FlowJo v8, MacOSX, Tree Star, Ashland, OR, USA). This experiment was repeated three times, and the results were averaged.

Electrophoretic mobility shift assay (EMSA)

The DNA binding activity of NFκB against Bcl-2 promoter was confirmed with a ^{32}P-labeled oligonucleotide containing NFκB transcription factor binding sites found in the NFκB promoter region. The DNA binding activity of NFκB against Bcl-2 promoter was

confirmed with a ^{32}P-labeled oligonucleotide containing NFκB transcription factor binding sites found in the Bcl-2 promoter region. Double-stranded oligonucleotides containing the consensus-binding site for NFkB (GATCGAGGGGACTTTCCCTACG) were 5' end-labeled using polynucleotide kinase and ɣ-^{32}P-dATP. Nuclear extracts (5.0 μg) were incubated with 1 μl of labeled oligonucleotide (20000 c.p.m.) in 20 μl of incubation buffer (10mM Tris-HCl, 40mM NaCl, 1mM EDTA, 1mM β-mercaptoethanol, 2% glycerol, and 2 μg of poly dI-dC) for 20 min at room temperature.

Human thyroid cancer cell xenograft

Human thyroid cancer cells (2.0×10^7 cells/mouse) were cultured *in vitro* and injected subcutaneously into the upper left flank region of the mice. After 7 days, tumor-bearing mice were grouped randomly (n = 10/group) and an intraperitoneal injection of the three drugs (SAHA, TSA, and HNHA) was administered once every 2 days for a total of ten injections (25 mg/kg) when the complete tumor size reached [$4/3 \times \pi \times (0.7 \times 0.4\ \text{cm})^3 \times ½$]. Tumor size was measured every other day using calipers.

In vivo toxicity study

In vivo toxicity assays were performed using BALB/c nude mice.

Six-week-old mice were caged for 1 week for acclimatization. Each group of 10 mice was injected intraperitoneally with SAHA, TSA, or HNHA at a dose of 25 mg/kg per mouse. The animals were monitored regularly for external signs of toxicity or lethality.

Immunohistochemistry

All tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin wax using standard protocols. Tissue sections (5 μm) were dewaxed and antigen retrieval was performed in citrate buffer (pH 6), using an electric pressure cooker set at 120°C for 5 min. Sections were incubated for 5 min in 3% hydrogen peroxide to quench endogenous tissue peroxidase. All tissue sections were counterstained with hematoxylin, dehydrated, and mounted.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Immunohistochemistry results were subjected to one-way ANOVA followed by a Bonferroni *post hoc* test. Values are expressed as means ± SEM. P values < 0.05 were considered to indicate statistical significance.

III. RESULTS

3.1. HNHA inhibits the proliferation of ATC and PTC

Table 1. IC50 (half maximal inhibitory concentration) determination using a cell proliferation assay. each data point represents the mean of three independent IC50 experiments performed in triplicate by MTT assay. SD, standard deviation.

Cell Line	Histopathology	Animal	Cell proliferation IC_{50}*)(μM)		
			HNHA	TSA	SAHA
SNU-80	Thyroid: anaplastic	Human	2.74 (± 0.9)*	4.02 (± 1.0)	6.74 (± 1.1)
SNU-790	Thyroid : papillary	Human	2.32 (± 1.0)*	3.91 (± 1.2)	5.31 (± 1.4)

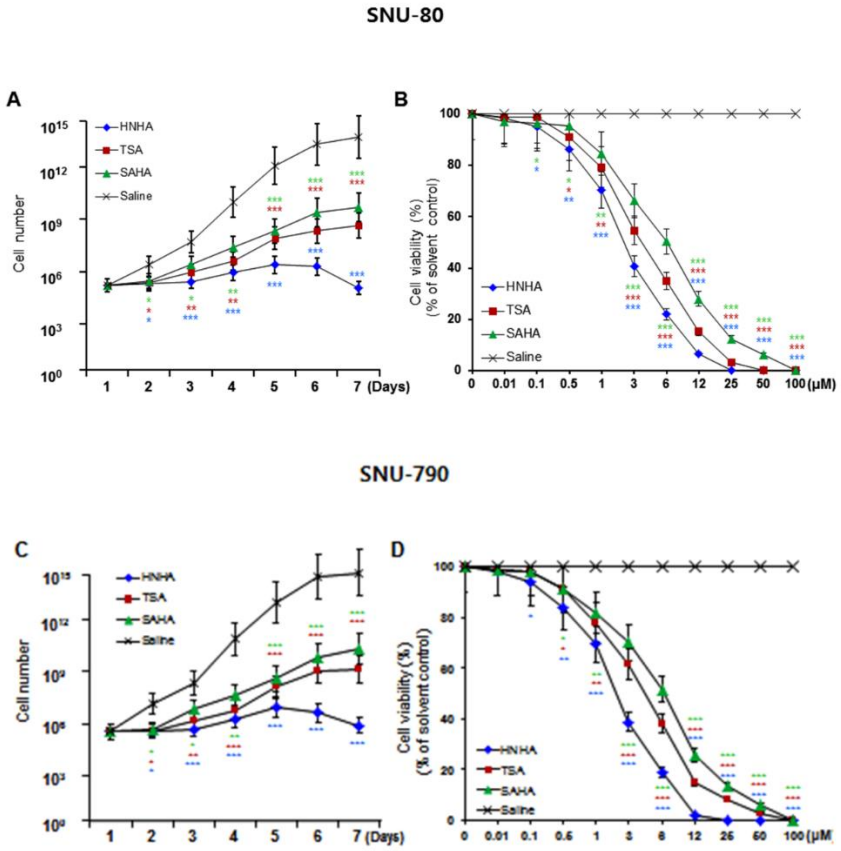


Figure 3. HNHA suppressed ATC and PTC cells proliferation. Cell viability and proliferation assays showed that HNHA induced the greatest inhibition of thyroid cancer cell proliferation in SNU 80 (A and B) and SNU 790 cells (C and D). Points indicate mean % of the solvent-treated control. All experiments were repeated at least three times with similar results.

To study the anti-cancer effects of HNHA in distinction with previously well-established HDAC inhibitors on ATC and PTC, a cell proliferation assay using SAHA, TSA, and HNHA were performed.

These results were compatible with the concentration-dependent cytotoxicity of HNHA, which induced greater loss of viability at low concentrations than the other HDAC inhibitors used (Table 1). Compared with SAHA and TSA, HNHA had a lower IC50 in ATC and PTC tested. To further characterize the results of HNHA on ATC and PTC cells viability, we evaluated SNU-80 and SNU-790 cells. All HDAC inhibitors reduced the viability of ATC and PTC cells compared to controls. However, HNHA showed the most significant suppression of cell proliferation among the HDAC inhibitors (Figure 3 A and C); moreover, the suppression of proliferation was dose-dependent (Figure 3 B and D).

3.2. ER stress is increase cytoplasmic free calcium release from ER

stress by HNHA

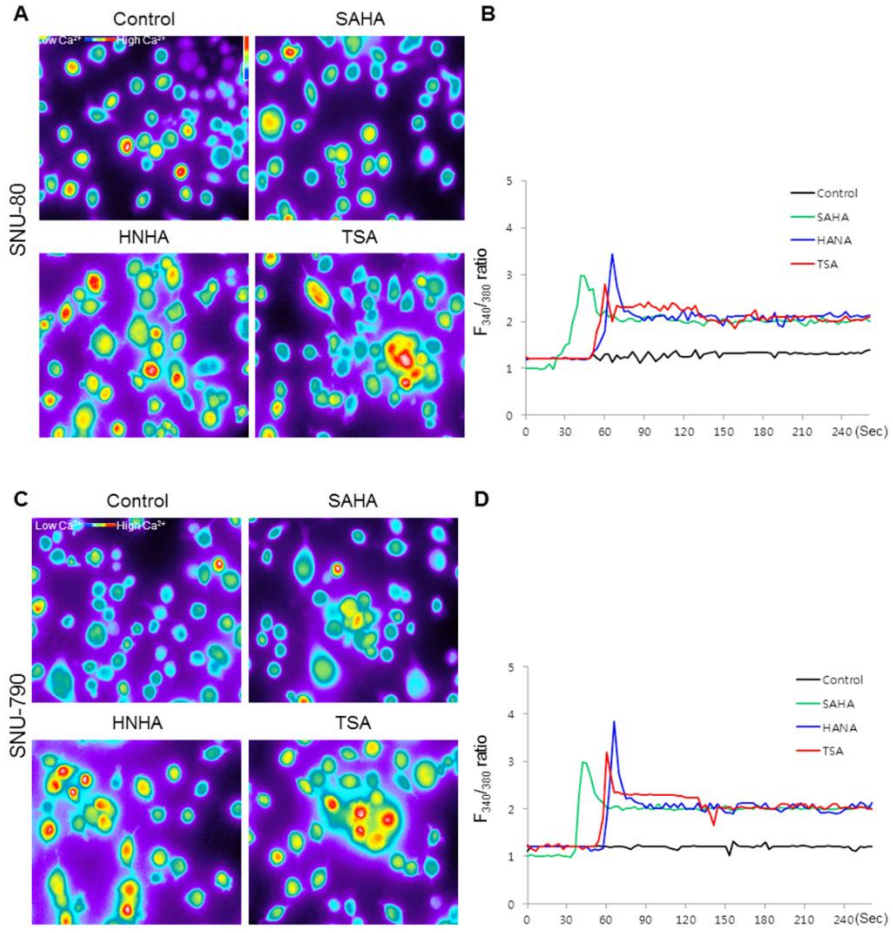


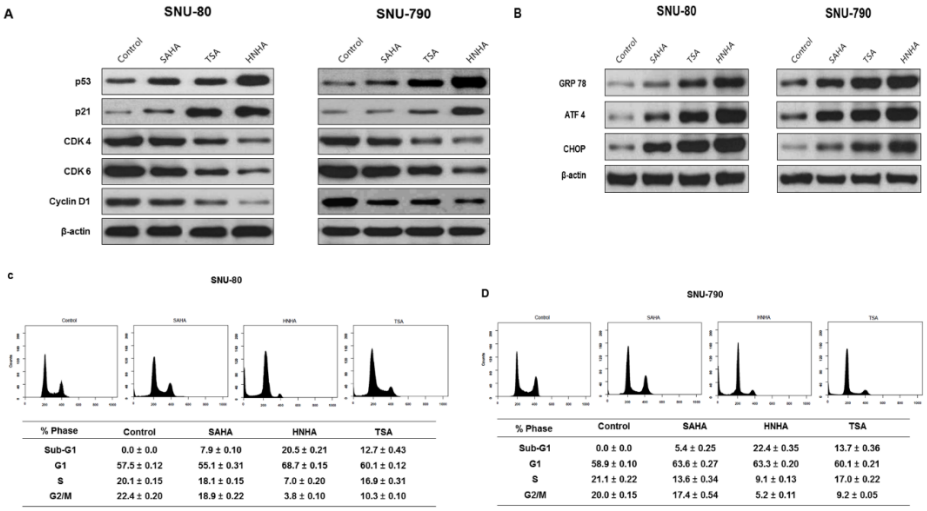
Figure 4. Cytosolic free Ca2+ measurements by microspectrofluorimetry on ATC and PTC cells that were HDAC inhibitor treated. Calcium response from Fura 2AM loaded ATC (A and B) and PTC (C and D) cells.

We measured the change of intracellular Ca2+ levels using

microspectrofluorimetry. There were significant differences in the levels of cytoplasmic Ca2+ between control and experimental group, the levels of cytoplasmic Ca2+ were different after the spike of intracellular Ca2+. As shown in Figure 2, the basal level of intracellular Ca2+ increased in HDAC inhibitor-treated cells, whereas non-treated cells level of intracellular Ca2+ was no significant difference (Figure 4A and C). The cytoplasmic Ca2+ level of experimental group failed to return to the basal levels while that control group returned to the basal levels after the spike of intracellular Ca2+ (Figure 4B and D). These differences in cytoplasmic Ca2+ levels between control and experimental group were correlated with ER stress levels.

3.3. HNHA induce ER stress dependent cell cycle arrest to ATC and

PTC



% Phase	Control	SAHA	HNHA	TSA
Sub-G1	0.0 ± 0.0	7.9 ± 0.10	20.5 ± 0.21	12.7 ± 0.43
G1	57.5 ± 0.12	55.1 ± 0.31	68.7 ± 0.15	60.1 ± 0.12
S	20.1 ± 0.15	18.1 ± 0.15	7.0 ± 0.20	16.9 ± 0.31
G2/M	22.4 ± 0.20	18.9 ± 0.22	3.8 ± 0.10	10.3 ± 0.10

% Phase	Control	SAHA	HNHA	TSA
Sub-G1	0.0 ± 0.0	5.4 ± 0.25	22.4 ± 0.35	13.7 ± 0.36
G1	58.9 ± 0.10	63.6 ± 0.27	63.3 ± 0.20	60.1 ± 0.21
S	21.1 ± 0.22	13.6 ± 0.34	9.1 ± 0.13	17.0 ± 0.22
G2/M	20.0 ± 0.15	17.4 ± 0.54	5.2 ± 0.11	9.2 ± 0.05

Figure 5. HNHA induced cell cycle arrest and ER stress in ATC and PTC cells. Immunoblot analysis for that HNHA induced the cell cycle arrest proteins in ATC and PTC cells (A). Immunoblot analysis showed that HNHA potently induced the expression of cell cycle arrest proteins and reduced positive regulator (A). HDAC inhibitor treated SNU 80 and SNU 790 for 24 hours and analyzed the expression of GRP 78, ATF 4, and CHOP, an ER stress marker, in the cells by immunoblot analysis (B). Flow cytometry analysis in HDAC inhibitor, cells were harvested and stained with PI (Propidium iodide), and analyzed by FACS and a DNA software program (FlowJo v8, MacOSX) (C and D).

In order to research HNHA role of changes to cell cycle arrest response to ER stress, we treated three established HDAC inhibitor to ATC and PTC. ER stress-induced cell cycle arrest was shown to be activated by HDAC inhibitor. HNHA induced p53 and p21, a well-

known arrestor of cell cycle, but reduced cyclin D1, CDK 4 and 6 a positive regulator of the cell cycle, compared with other drugs (Figure 5A). HNHA resulted in most significantly increased levels of the cell-cycle arrest (p53 and p21) and reduced positive regulator (cyclin D1, CDK 4 and 6) proteins in ATC and PTC. We tested whether induction of ER stress could be related to PTC and ATC by HDAC inhibitor. We treated SNU 80 and SNU 790 with SAHA, TSA and HNHA for 24 hours and analyzed the expression of GRP 78, ATF 4, and CHOP, an ER stress marker, in the cells by immunoblot analysis (Figure 5B). To investigate the effects of HNHA on cell cycle progression, propidium iodide staining and flow cytometry were performed after treatment with several HDAC inhibitors, including HNHA. The HDAC inhibitors induced G0/G1 phase arrest and increased the sub G0 population ($p < 0.05$), suggesting induction of cell cycle arrest and apoptosis in ATC and PTC (Figure 5C and D). HNHA treated group was strongest increase an ER stress marker.These data suggest that HNHA is the most potent ER stress inducer of the compounds tested and exerts this effect via ER stress dependent apoptosis and cell cycle arrest activation, which is comparable to the highest inhibition of viability in ATC and PTC.

3.4. HNHA-induced apoptosis of ATC and PTC were caspase-

dependent

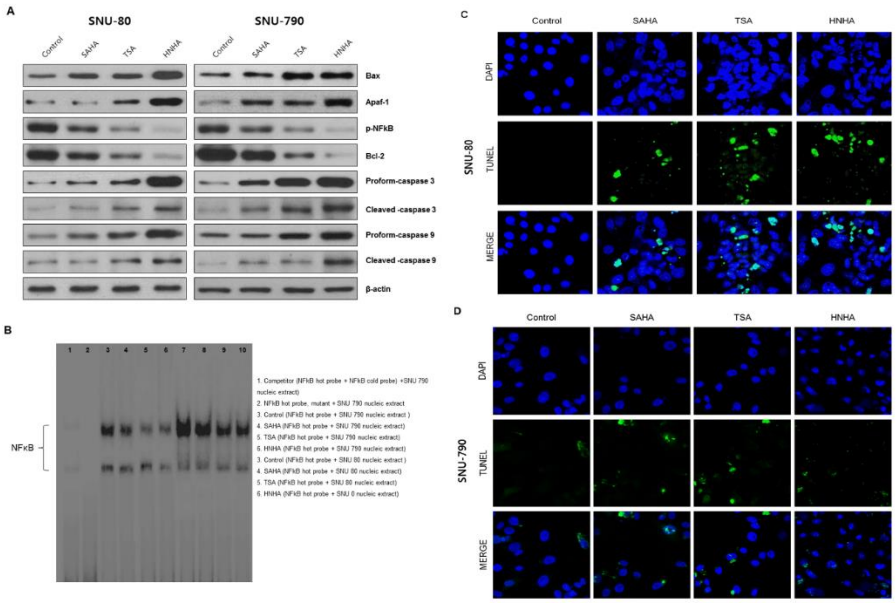


Figure 6. HNHA induced apoptotic death in ATC and PTC cells. Immunoblot analysis suggested that HNHA induced the apoptotic and reduced anti-apoptotic proteins in ATC and PTC cells (A). An electrophoretic mobility shift assay was carried out with a 32P-labeled oligonucleotide containing NF-κB binding sites found in the Bcl-2 promoter (B). TUNEL assay of apoptotic cells in ATC and PTC cells; TUNEL-positive (apoptotic) cells are indicated (×400) (C, D).

To investigate pro-apoptotic signaling pathways activated following the incubation of these drugs, the expression of pro- (Bax and Apaf-1) and anti-apoptotic (phosphorylation NF-κB p65 and Bcl-2) members of Bcl-2 family and the activation of caspase-3 and caspase 9,

major executioners in apoptosis, in PTC and ATC were investigated by immunoblot analysis (Figure 6A).

These results imply that HNHA enriched the 'pro' form of caspase-3 and induced the cleavage of pro-caspase-3 and pro-caspase-9 more potently than did TSA or SAHA (Figure 6A).

As NF-κB is known to work as a transcriptional activator, we investigated potential p-NF-κB binding sites in the Bcl-2 promoter region and identified a few p-NF-κB binding sites. An electrophoretic mobility shift assay (EMSA) was carried out with a 32P-labeled oligonucleotide containing NF-κB binding sites found in the Bcl-2 promoter (Figure 6B). Labeled NF-κB probe-nuclear extract (SNU 80 and SNU 790) complexes produced two bands, corresponding to the NF-κB transcription factor-labeled NF-κB probe (Figure 6B, lanes 3~6; SNU 790, lanes 7~10; SNU 80). The specificity of the EMSA result was confirmed by complete inhibition of NF-κB DNA binding by excess unlabeled NF-κB (non-labeled probe, Figure 6B, lane 1). In addition, a similar amount of mutated NF-κB probe also failed to bind the NF-κB transcription complex (Figure 6B, lane 2). HNHA treated group was strongest decrease an NF-κB transcription factor. Together, these results demonstrated that HNHA, transcriptionally inhibitor Bcl-2 expression.

The TUNEL assay confirmed that HNHA induced apoptosis in ATC and PTC more potently than did TSA or SAHA (Figure 6C and D). These data suggest that HNHA is the most potent apoptosis inducer of the compounds tested and exerts this effect via caspase activation and inhibition of the Bcl-2 pathway in ATC and PTC.

3.5. HNHA induced histone H3 acetylation in thyroid cancer cells

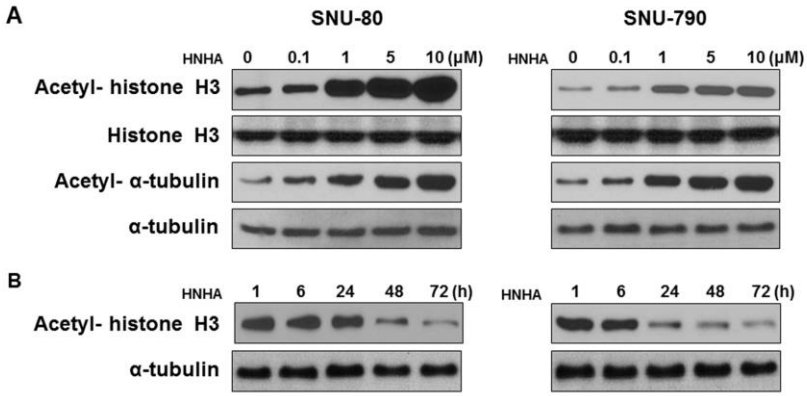


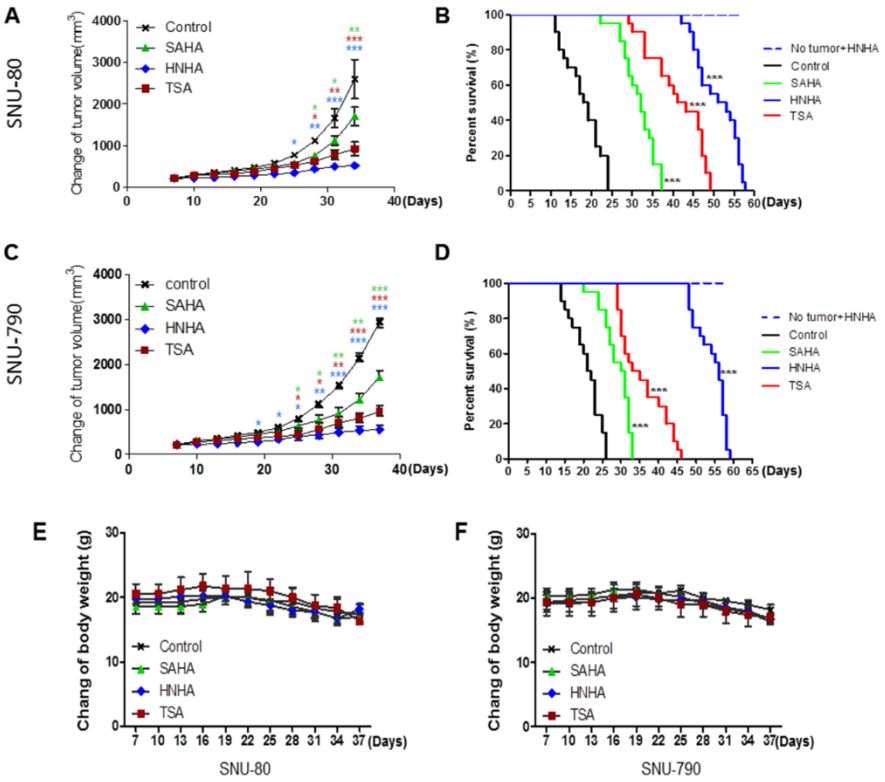
Figure 7. HNHA induced acetylation of histone H3 on ATC and PTC cells. SNU 80 and SNU 790 cells were treated for 24 h with 0.1, 1, 10, or 20 μM HNHA (A). SNU 80 and SNU 790 were treated with HNHA (15 μM) for 1, 6, 24, 48, or 72 h (B). Total proteins were isolated and histone H3 and α-tubulin acetylation was evaluated by immunoblot analysis.

To investigate the effects of HNHA on histone acetylation in

ATC and PTC, we exposed SNU-80 and SNU-790 cells to HNHA at various doses and then evaluated histone H3 acetylation by immunoblot analysis. Acetylation of histone H3 was increased by HNHA in a dose-dependent manner (Figure 7A). We also assessed the effects of HNHA on the acetylation of non-histone proteins using α-tubulin; α-tubulin acetylation was also increased by HNHA in a dose-dependent manner (Figure 7A). To determine the duration of maintenance of histone H3 acetylation by HNHA, protein levels were evaluated by Immunoblot analysis at 1, 6, 24, 48, and 72 h after HNHA treatment. Histone H3 acetylation peaked at 1 h after administration of HNHA and remained stable until 48 h (Figure 7B). These data show that HNHA can induce stable acetylation of histone H3, and also non-histone proteins, in ATC and PTC.

3.6. HNHA reduced the tumor burden and improved survival in

thyroid cancer cells xenografts



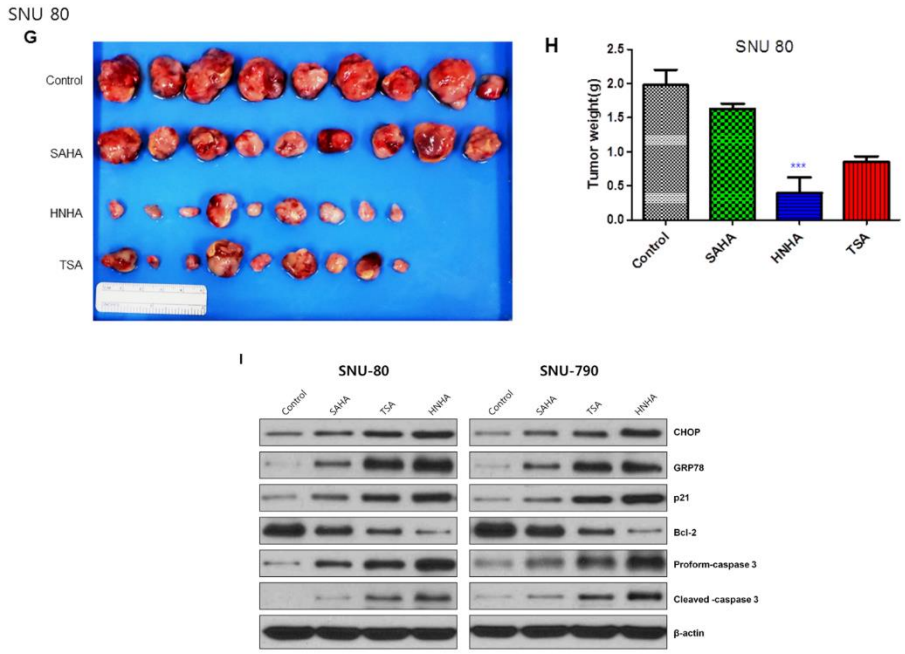


Figure 8. HNHA inhibited the most potent anti-tumor effects in thyroid cancer cell xenografts suggesting induction of cell cycle arrest and apoptosis by ER stress.

Tumors were established in athymic nude mice and treated with HDAC inhibitor. Tumor size was measured with a caliper every other day. Data were presented as the mean tumor volumes of mice in both treatment and vehicle groups. HNHA induced more potent inhibition of tumor progression than the established HDAC inhibitors and resulted in the greatest prolongation of survival in ATC (A, B) and PTC (C, D) xenografts (Each group, n = 10). 'No tumor + HNHA' group indicates HNHA treated mice without a xenograft for no evidence of systemic toxicity or treatment-related death was found in HNHA treated group (B and D). Each group = 10 mice, survival curves for 55 ~60

days. There was no significant effect on the body weight of mice treated with HDAC inhibitor compared to the control group (E and F). Photomicrograph of the dissected tumors treated and control group (G). Tumor weight of the dissected tumors as indicated (H). Immunoblot analysis, total proteins were isolated to tumor (I). * $P < 0.05$ vs. Control, ** $P < 0.01$ vs. Control, *** $P < 0.01$ vs. Control.

To investigate the antitumor effect of HNHA in vivo, we developed subcutaneous thyroid cancer cells xenograft mouse models using SNU-80 and SNU-790 cells. SAHA and TSA showed significant suppression of tumor growth in thyroid cancer cells xenografts; however, HNHA exhibited greater suppression of the growth of thyroid cancer cells xenografts (Figure 8A and C). Also, survival was prolonged significantly by all HDAC inhibitors, but to a greater extent by HNHA than SAHA or TSA (Figure 8B and D). No evidence of systemic toxicity or treatment-related death was found in any group. There was no significant effect on the body weight of mice treated with SAHA, TSA and HNHA compared to the control group (Figure 8E and F). HNHA treatment group showed significantly smaller tumor volumes compared to SAHA and TSA treated group (Figure 8G and H). The HDAC inhibitors induced p21-cell cycle arrest protein- and increased the GRP78-ER stress protein- and cleaved caspase, suggesting induction of

cell cycle arrest and apoptosis by ER stress in ATC and PTC mouse xenografts (Figure 8I). In summary, these data suggest that HNHA induced more potent subcutaneous thyroid cancer suppression in an animal model.

3.7. HNHA inhibits the proliferation of ATC and PTC in thyroid cancer cells xenografts

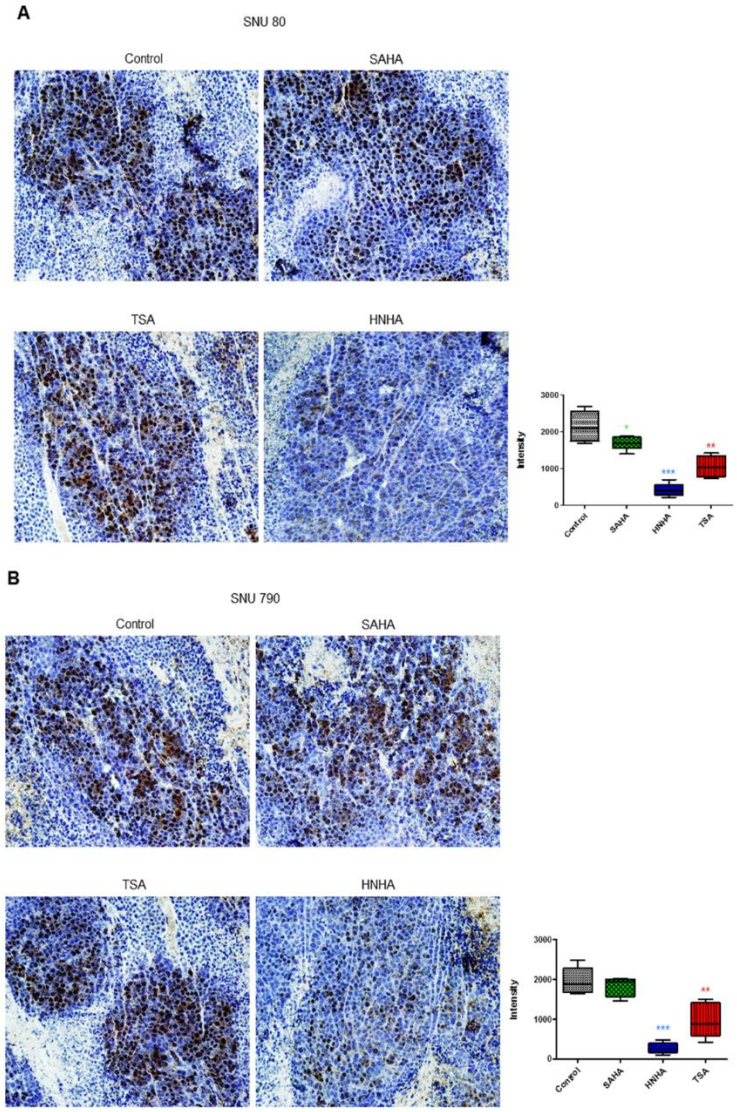


Figure 9. HNHA reduced cell proliferative activity Ki -67 expressions in tumor tissues by immunohistochemical analysis. Immunohistochemistry analysis of the Ki-67 protein levels in paraffin embedded xenograft tumor tissues with ATC (SNU 80, A) and PTC (SNU 790, B). The MetaMorph 4.6 image-analysis software was used to quantify the immunostained target protein. * $P < 0.05$ vs. Control, ** $P < 0.01$ vs. Control, *** $P < 0.01$ vs. Control.

Cell proliferative activity is one of the important factors for assessing the biological behavior of carcinoma. At present, the most useful marker to evaluate cell proliferative activity is Ki-67, which is expressed in all cells except those in the G0 phase. Consequently, we detected this markers expression by using immunohistochemistry. HNHA treated group was strongest decrease a Ki-67 expression (Figure 9A and B). These data show that HNHA is most potent anti-cancer agent to thyroid cancer.

IV. DISCUSSION

In this research, we proved that HNHA is a potent cytotoxic drug on PTC and ATC both in vitro and in vivo. HNHA is much more powerful apoptosis inducer than the other HDAC inhibitor we tested in the thyroid cancer cell lines, and other HDAC inhibitors previously used against thyroid cancer cell [9,23], which are effective only in the high dose compared than HNHA . The mechanisms fundamental the cytotoxic effect of HNHA on ATC and PTC cell lines included both apoptosis-induction and cell cycle arrest. Apoptosis was showed by the increased percentage of cells in sub G_1, by the formation of cytoplasmatic nucleosomes and by the activation of caspase3. HNHA effect on cell

cycle progression showed a characteristic marks: actually, G_1 arrest was already evident when cells were treated with HNHA at lower doses compare than SAHA and TSA. This behavior is in concurrence with previous study showing that HDAC inhibitors usually affect cytotoxicity and induce G_1 arrest at lower doses [20,21]. The major molecular mechanism of HDAC inhibitor action is to change the acetylation status of the core histone proteins, consequently facilitating chromatin remodeling with consequent alteration in gene expression, and cell differentiation [24-26]. In concurrence with this, we investigated that HNHA acetylates histones of papillary and anaplastic thyroid cancer cells, finally principal to the upregulation of p21 and the downregulation of cyclin D1. Nevertheless, histones were regarded the canonical substrate of acetylation, some research have challenged this minimalist paradigm and involved protein acetylation in amazingly various array of cellular step comprising protein transport, apoptosis and cell motility [27]. Here, we proved that low doses of HNHA induce to apoptosis and cell cycle arrest as evidenced by increased apoptotic and cell cycle arrest protein. Consequently, HNHA treatment resulted in a block during mitosis as proved by the presence of multiple nuclei. Histone modification is now a well-known epigenetic modification [28]. Among some types of histone

modification, histone deacetylation is deregulated in many cancers. A recent study revealed that HDAC1, HDAC2, and HDAC3 are highly expressed in RCC [29]. Some studies focus to overexpression of class I HDACs, in particular HDAC1, as a cancer marker connected with a poor prognosis [30]. HDAC inhibitors have been progressed to reverse gene silencing by inhibiting HDAC activity and increasing histone acetylation. These inhibitors perform by binding to the catalytic site of the enzyme. There are four distinct classes of HDAC inhibitor: short chain fatty acids (valproic acid and butyrates), hydroxamic acids (trichostatin A, TSA, and suberoylanilide hydroxamic acid), cyclic tetrapeptides (trapoxin and depsipeptide), and benzamides [31,32].

Preclinical studies have shown the potential of HDAC inhibitors in the treatment of ATC. LBH589 altered cell-cycle-regulating proteins, particularly cyclin D1 and p21, significantly inhibited the growth of ATC in subcutaneous xenografts, accompanied by strong decrease of ki67 in tissue specimens of LBH589-treated animals [33].

The novel HDAC inhibitor HNHA was introduced recently and showed strong anti-tumor activity in breast cancer *in vitro* and *in vivo* [22,34]. Here, we showed that HNHA had more potent anti-tumor activity than the established HDAC inhibitors SAHA and TSA with induction of

apoptosis through expression of Bcl-2 and engagement of the Cell Cycle G1/S Checkpoint signaling pathway in PTC and ATC cells *in vitro* and *in vivo*. HNHA activated the caspase-dependent apoptotic pathways by inducing release of cytoplasmic free calcium from ER (endoplasmic reticlum) and its translocation into the mitochondria in ATC and PTC cells. In our study, GRP78 was found to be upregulated noticeably by all HDAC inhibitors tested—albeit to a greater degree by HNHA than SAHA or TSA—in ATC and PTC cells. HNHA treatment resulted in the greatest level of cytoplasmic free calcium release from ER among the HDAC inhibitors tested.

All things considered, although the precise mechanism(s) of the anti-cancer effects of HDAC inhibition should be investigated further, our results suggest that HNHA may be a potent therapeutic option. The role of HDAC inhibition as an anti-cancer strategy in ATC and PTC should be estimated using agents more potent than those tested previously. HNHA has more potent anti-tumor activity than established HDAC inhibitors. Its effects are mediated by caspase-dependent apoptosis in ATC and PTC cells. These results suggest that HNHA may offer a new therapeutic approach to ATC and PTC.

V. CONCLUSION

The anticancer activity of HNHA opens up therapeutic approach for the ATC and PTC, which do not respond to conventional therapy. Translational and clinical researchs will ultimately determine the clinical benefit and safety of HNHA, used alone or in combination with chemotherapics, as an option for the treatment of this kind of tumor.

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ASTRACT (IN KOREAN)

새로운 histone deacetylase inhibitor인 N-hydroxy-7-(2-naphthylthio) hepatonomide의 cytoplasmic free calcium mediated apoptosis를 통한 갑상선암의 항암효과 분석

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갑상선암은 전 세계적으로 methylation 비율이 높고 histone acetylation이 감소하는 것으로 나타난다. 전임상 모델은 히스톤 유전자 수정 자 및 후 성적 변화가 갑상선 유두암 및 역형성 갑상선암의 종양 형성에 중요한 역할을 한다는 것을 보여 준다. 새로운 HDAC 억제제 인 HNHA가 새로운 종류의 항암제의 한 예로 갑상선암에 대한 치료법을 도입되었다. 본 연구에서 갑상선암 세포에서 HNHA의 ER(Endoplasmic Reticulum) 스트레스 의존성 세포 사멸 유도 활성을 입증하고자 하였다. HNHA로 처리 한 그룹에서 세포 사멸은 생체 외부

및 생체 내에서 갑상선암 세포에서 현저한 caspase 활성화 및 Bcl-2 억제와 함께 유의하게 유도되었다. HNHA 처리로 ER에서 세포질 자유 칼슘 방출이 일어났으며, HNHA는 또한 ER 응력 - 의존적 세포 사멸을 유도하여 HNHA가 PTC 및 ATC에서 독립적인 핵 세포 사멸을 유도 할 수 있음을 시사한다. 생체 내 연구에서 HNHA는 확립 된 HDAC 억제제보다 PTC 및 ATC에서 보다 효과적인 항암 효과 및 친 사멸 효과를 나타냈다. 결론적으로, HNHA는 확립된 HDAC 억제제보다 더 강력한 항 종양 활성을 가지며 그 활성은 갑상선암 세포에서 Caspase 의존성 및 ER 스트레스 매개 세포 사멸에 의해 매개된다. 이러한 결과는 HNHA가 갑상선암에 대한 새로운 치료법을 제시 할 수 있음을 시사한다.

중심어: Papillary thyroid cancer (PTC), anaplastic thyroid cancer (ATC), Histone deacetylase inhibitor