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The hsp70 inhibitor VER155008 induces paraptosis requiring *de novo* protein synthesis in anaplastic thyroid carcinoma cells

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ABSTRACT

In this study, we evaluated the effect of the hsp70 inhibitor VER155008 on survival of anaplastic thyroid carcinoma (ATC) cells. In ATC cells, VER155008 increased the percentages of dead cells and vacuolated cells. VER155008 did not lead to the cleavage of caspase-3 protein regardless of pretreatment with z-VAD-fmk. VER155008 increased LC3-II protein levels but the protein levels were not changed by autophagy inhibitors. VER155008 caused the dilatation of endoplasmic reticulum (ER), and the increased mRNA levels of Bip and CHOP, suggesting paraptosis. VER155008-induced paraptosis was attenuated by pretreatment with cycloheximide. In conclusion, VER155008 induces paraptosis characterized by cytoplasmic vacuolation, independence of caspase, dilatation of ER and induction of ER stress markers in ATC cells. Moreover, VER155008-induced paraptosis requires *de novo* protein synthesis in ATC cells.

1. Introduction

Anaplastic thyroid carcinoma (ATC) is a fatal malignant tumor derived from the thyroid gland due to extrathyroidal invasion and distant metastasis [1]. In clinical fields, significant improvement of survival rate in ATC patients has not been attained despite multimodal strategies, and new therapeutic agents are under investigation [1].

Programmed cell death is divided into apoptosis, autophagy, paraptosis and mitotic catastrophe [2,3]. Paraptosis has characteristic features including extensive cytoplasmic vacuolation derived from endoplasmic reticulum (ER) and/or mitochondria swelling without detection of apoptotic hallmarks such as caspase activation [2,3]. However, whether paraptosis is involved in death of ATC cells has not been elucidated.

Heat shock protein (hsp) 70 modulates the client proteins such as Raf-1 and Akt, and maintains conformation, stability and activity of chaperone complexes [4,5]. VER155008, a hsp70 inhibitor, suppresses ATPase activity of hsp70 protein, and induces degradation of hsp70 client proteins [5]. Although VER155008 causes the repression of cell proliferation and the induction of cell death by regulating hsp70 client proteins in cancer cells [5–7], the effect of VER155008 on survival of ATC cells has not been evaluated.

In the present study, we demonstrated for the first time that the hsp70 inhibitor VER155008 induces paraptosis characterized by cell death in conjunction with cytoplasmic vacuolation, no involvement of caspase activation, dilated ERs and induction of ER stress markers in ATC cells. In addition, our results showed that VER155008-induced paraptosis is accompanied by *de novo* protein synthesis in ATC cells.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, fetal bovine serum (FBS) and streptomycin/penicillin were obtained from GIBCO (Grand Island, NY, USA). VER155008 was purchased from Sigma (St. Louis, MO, USA), while 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) was obtained from Tocris Bioscience (Bristol, UK). VER155008 and 17-AAG were dissolved in dimethylsulfoxide (DMSO, 10 mM stock solution), which was provided to the control within permissible concentrations. The final concentration of the vehicle DMSO in the control did not exceed 0.1% in all treatments. z-VAD-fmk (broad-spectrum caspase inhibitor), 3-methyladenine (3-MA, autophagy inhibitor), bafilomycin-A1 (autophagy

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inhibitor), wortmannin (inhibitor of autophagy as well as Akt) and cycloheximide (CHX, translation inhibitor) were purchased from Sigma. Primary antibodies raised against caspase-3 positive cell lysates, intact and cleaved caspase-3, microtubule-associated protein I light chain 3 (MAP1 LC3, LC3), hsp70, hsp90 and Raf-1 were obtained from Cell Signaling Biotechnology (Danvers, MA, USA), while primary antibodies raised against total and phospho-Akt (Ser473), and calnexin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody raised against β-actin was obtained from Sigma. All other reagents were purchased from Sigma unless otherwise stated.

2.2. Cell culture

For experiments, human ATC cell lines of 8505C and FRO cells were used. 8505C cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ GmbH, Braunschweig, Germany). FRO cells, authenticated as shown previously, were provided by Professor Young Joo Park (Division of Endocrinology and Metabolism, Seoul National University, Republic of Korea) [8]. 8505C cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 1% streptomycin/penicillin. FRO cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% streptomycin/penicillin. Cells received fresh medium at regular intervals. Treatments and experiments were performed using cells that were 70% confluent.

2.3. CCK-8 assay

Cell viability was determined by the CCK-8 Assay Kit (Dojindo laboratories, Kumamoto, Japan). Cells (5×10^3/100 μl) in each well on 96-well plates were incubated overnight, and treated with VER155008 for an additional 4 h at 37 °C. Absorbance was measured at 450 nm using a spectrophotometer (Molecular Devices, Palo Alto, CA, USA). All experiments were carried out in triplicate.

2.4. Trypan blue assay

Cells (1×10^5/500 μl) in each well on 12-well plates were incubated, and mixed with trypan blue dye at 37 °C. Stained cells were counted using a hemocytometer. All experiments were performed in triplicate.

2.5. Vacuolated cell counting

Vacuolated cells were counted using a method similar to the previous study [9]. Light micrographs were obtained from different fields and number of vacuolated cells having the total vacuoles-occupying area more than 50% of cytoplasmic area was counted in at least 300 cells for each condition.

2.6. Immunofluorescence

Cells were fixed, permeabilized with acetone for 10 min at room temperature and blocked. Cells were incubated with the calnexin antibody overnight at 4 °C and then with FITC-conjugated IgG secondary antibody for 1 h at room temperature. The secondary antibody was purchased from Vector Laboratories (Burlingame, CA, USA). Cells were mounted and identified by flexible confocal microscope LSM 700 (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany).

2.7. Transmission electron microscopy (TEM)

Cells were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde buffered with 0.1 M phosphate-buffered saline (PBS) on ice for 2 h. The cell pellets were fixed on ice for 1.5 h with 1% osmium tetroxide buffered with 0.1 M PBS, and rinsed three times with 0.1 M PBS. The fixed cell pellets were dehydrated by successive treatments at increasing concentrations of ethanol and propylene oxide for 15 min, and embedded in Epon 812 (Electron Microscopy Sciences, Hatfield, PA, USA). Ultra-thin sections (75 nm) were cut in an RMC MTXL ultramicrotome (Tucson, Arizona, USA). The sections were collected on grids, and double stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM-1011, JEOL, Japan).

2.8. Western blotting

Cells were lysed in RIPA buffer (Sigma) containing 1 × protease inhibitor cocktail and 1 × phosphatase inhibitor cocktail set V (Calbiochem, La Jolla, CA, USA). Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Equivalent amounts of protein (50 μg) were separated by 10% SDS–PAGE, and transferred to Immobilon-P Membrane (Millipore, Bedford, MA, USA). Western blotting was performed using specific primary antibodies and horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies. Bands were detected using ECL Plus Western Blotting Detection System (Pierce). β-Actin served as loading control. All reactions were carried out in triplicate.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to manufacturer's protocol. cDNA was synthesized using AmpiRevert cDNA Synthesis Platinum Master Mix (GenDEPOT, Barker, TX, USA). Amplification of the resulting cDNA was conducted. The following primers were used: Bip, 5′-ATGAGGACCTGCAAGAG-3′ and 5′-TCCTCCTCAGTCAGCC-3′; CHOP, 5′-GCACCTCCCAGAGCCCTCACTCTCC-3′ and 5′-GTCTACTC-CAAGCCTTCCCCCTGCG-3′; β-actin, 5′-CAAGAGTGGCCACGGCT GCT-3′ and 5′-TCCTTCGCATCCTGTCGGCA-3′. Reactions were successively incubated at 95 °C for 15 s, at 60 °C for 15 s and at 72 °C for 15 s using a GeneAmp PCR System 9700 thermal cycler. The number of cycle (optimized in a preliminary study the aim of which was to determine the exponential range of amplification for each gene) was 22 for Bip, CHOP and β-actin. The amplified products were analyzed by 2% agarose gel electrophoresis. The mRNA expression of Bip and CHOP was compared with the mRNA expression of β-actin.

2.10. Statistical analysis

All data are expressed as mean ± standard error (S.E.). Data were analyzed by unpaired Student's *t*-test or ANOVA as appropriate. A p value less than 0.05 was considered to be statistically significant. All analyses were performed using the SPSS version 21.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. VER155008 induces cell death and cytoplasmic vacuolation in ATC cells

To investigate the influence of VER155008 on survival of ATC cells, cells were treated with VER155008 at 10, 20, 30, 40 and 50 μM for 72 h, and at 50 μM for 24, 48 and 72 h, and cell viability and the percentage of dead cells were measured. After treatment, cell viability was reduced (Fig. 1A), while the percentage of dead cells was elevated (Fig. 1B) in a time- and dose-dependent manner.

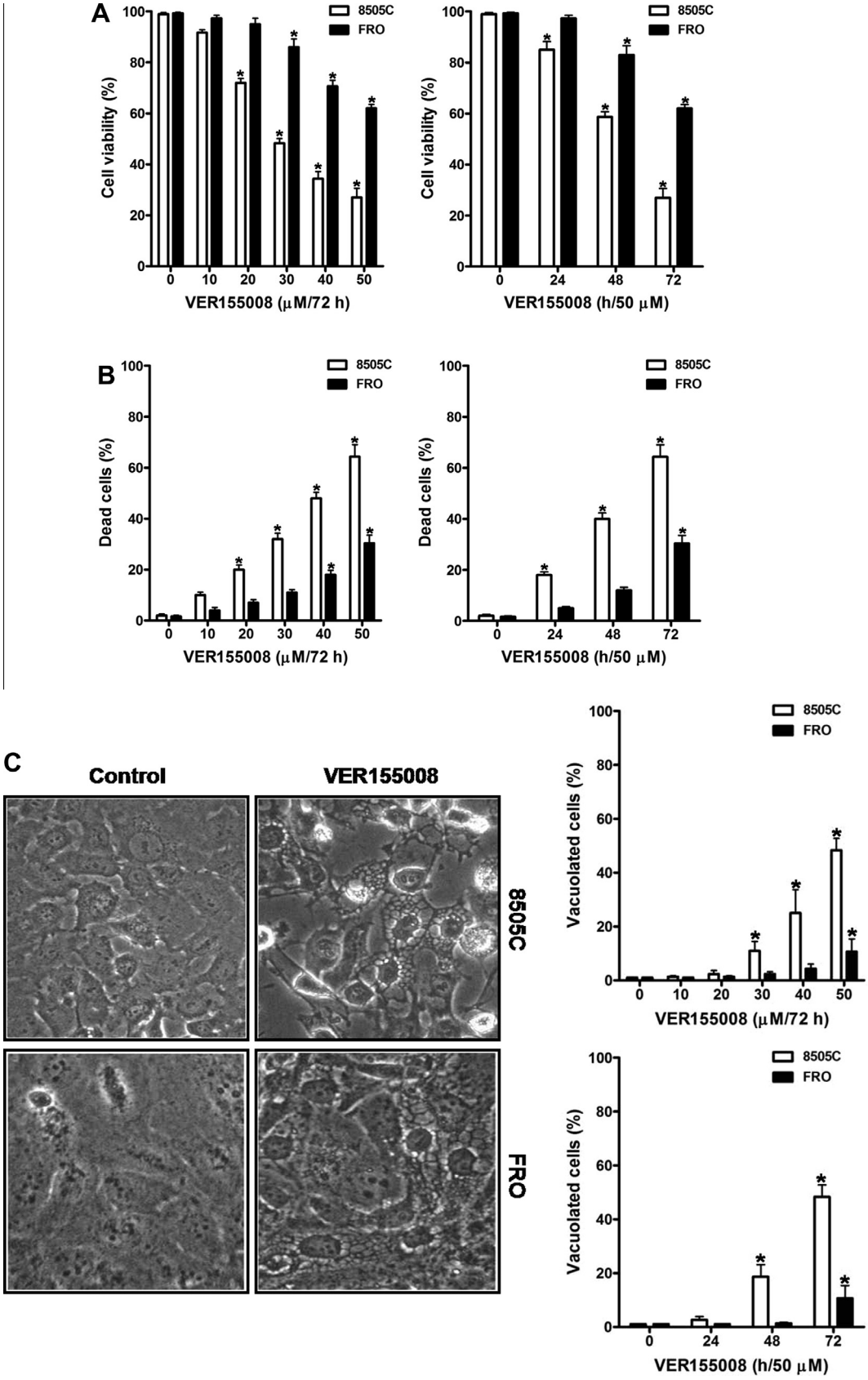

Fig. 1. The influence of VER155008 on survival and morphology of ATC cells. (A and B) Cells were treated with VER155008 at 10, 20, 30, 40 and 50 μM for 72 h, and at 50 μM for 24, 48 and 72 h. Cell viability (A) and the percentage of dead cells (B) were measured using CCK-8 assay and trypan blue assay, respectively. (C) Left panel: cells were treated with VER155008 at 50 μM for 72 h, and morphology was examined using light microscope. Right panel: cells were treated with VER155008 at 10, 20, 30, 40 and 50 μM for 72 h, and at 50 μM for 24, 48 and 72 h, and the percentage of vacuolated cells was measured using light microscope. Data are expressed as mean ± S.E. $^{*}p < 0.05$ vs. each matched control.

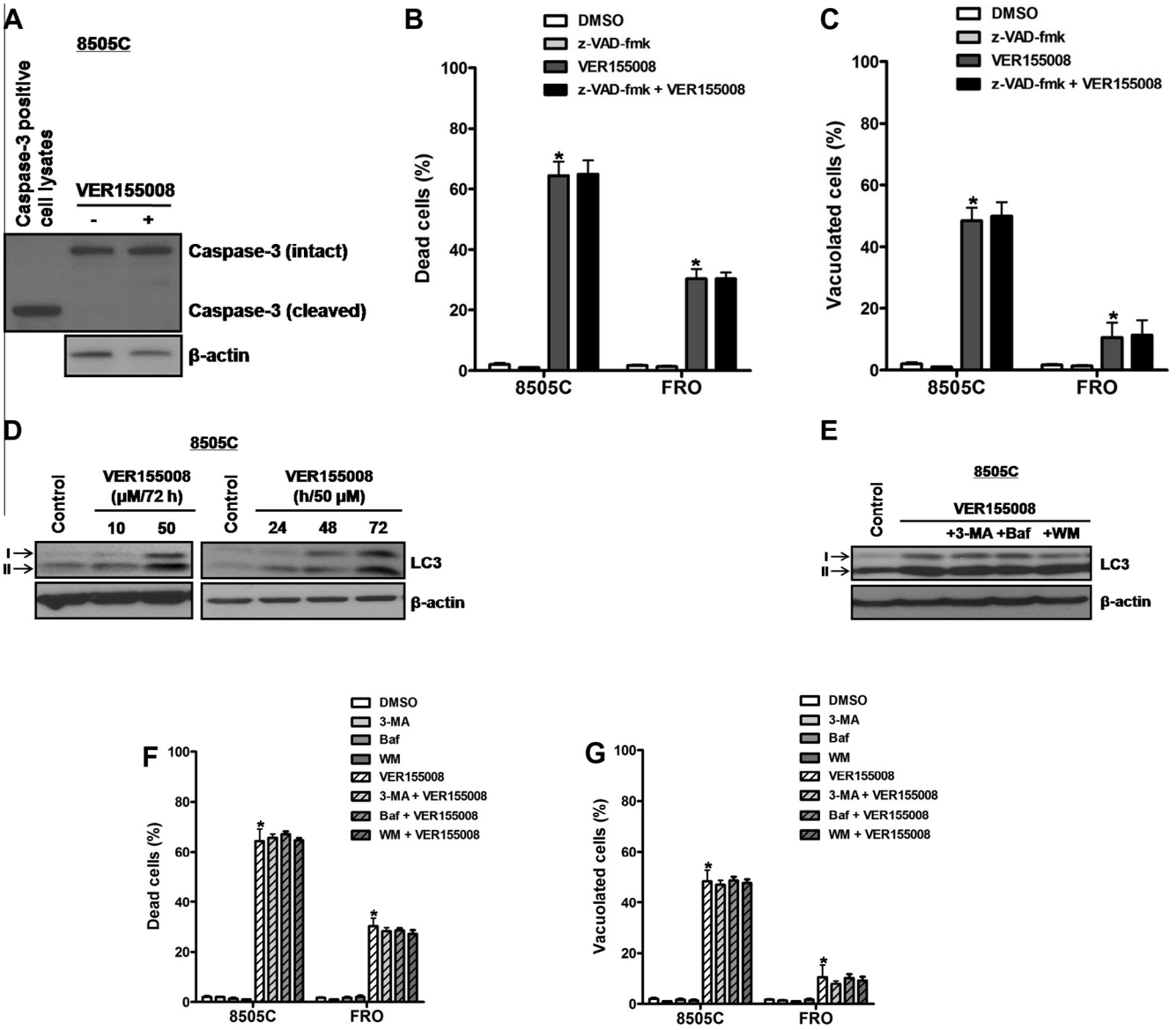

Fig. 2. The effect of VER155008 on caspase activation and autophagic flux in ATC cells. (A) Cells were treated with VER155008 at 50 μM for 72 h, and caspase-3 protein levels were measured. The caspase-3 positive cell lysates were used as positive control. (B and C) Cells were pretreated with z-VAD-fmk at 50 μM for 1 h before treatment of VER155008 at 50 μM for 72 h, and the percentages of dead cells (B) and vacuolated cells (C) were measured. (D) Cells were treated with VER155008 at 10 and 50 μM for 72 h, and at 50 μM for 24, 48 and 72 h. LC3-II protein levels were measured. (E–G) Cells were pretreated with 3-MA (10 mM, 1 h), bafilomycin-A1 (20 nM, 1 h) and wortmannin (10 μM, 1 h) prior to treatment of VER155008 at 50 μM for 72 h. LC3-II protein levels (E), and the percentages of dead cells (F) and vacuolated cells (G) were measured. Data are expressed as mean ± S.E. $^{*}p < 0.05$ vs. control. Baf, bafilomycin-A1. WM, wortmannin.

When cells were treated with VER155008 by doses and by times, cytoplasmic vacuoles were observed (Fig. 1C, left panel), and the percentage of vacuolated cells was elevated in a time- and dose-dependent manner (Fig. 1C, right panel). In consideration of the dose and the time leading to cell death and cytoplasmic vacuolation, we performed further experiments under treatment of VER155008 at 50 μM for 72 h.

3.2. VER155008-induced cytoplasmic vacuolation cell death does not depend on caspase activation and autophagic flux in ATC cells

In this study, VER155008 resulted in cell death in conjunction with cytoplasmic vacuolation, and thus we identified the mode of cell death caused by VER155008. First, to examine the involvement of caspase activation, cells were treated with VER155008, and caspase-3 protein levels were measured. The caspase-3 positive cell lysates were used as positive control. As a result of treatment, intact caspase-3 protein levels were not changed, and cleaved caspase-3 protein was not detected (Fig. 2A). When cells were pretreated with the broad spectrum caspase inhibitor z-VAD-fmk before treatment of VER155008, the percentages of dead cells (Fig. 2B) and vacuolated cells (Fig. 2C) were not altered regardless of pretreatment with z-VAD-fmk.

Next, to explore the involvement of autophagic flux, cells were treated with VER155008, and LC3-II protein levels were measured in situations with or without the autophagy inhibitors 3-MA, bafilomycin-A1 and wortmannin. VER155008 increased LC3-II protein levels (Fig. 2D) but the increment was not augmented by pretreatment with the autophagy inhibitors (Fig. 2E), suggesting that increased LC3-II protein levels by VER155008 are due to not autophagic flux but suppression of lysosomal degradation. In addition, pretreatment with 3-MA, bafilomycin-A1 and wortmannin did not

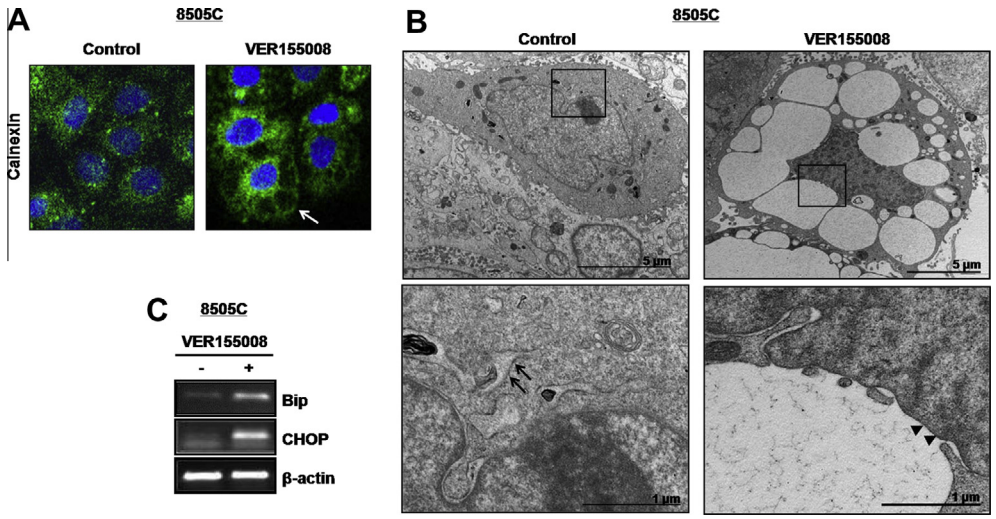

Fig. 3. The impact of VER155008 on morphology of ER and expression of ER stress markers in ATC cells. (A–C) Cells were treated with VER155008 at 50 μM for 72 h. (A) ER was immunostained with calnexin, and DAPI was used to label nucleus. Arrow indicates cytoplasmic vacuole. (B) The ultrastructure of cells showing cytoplasmic vacuolation was observed by TEM. Arrows indicate ribosomes aligned with ER membrane in control cells, and arrowheads indicate swollen ER membrane without ribosome in VER155008-treated cells. (C) The mRNA levels of Bip and CHOP were measured.

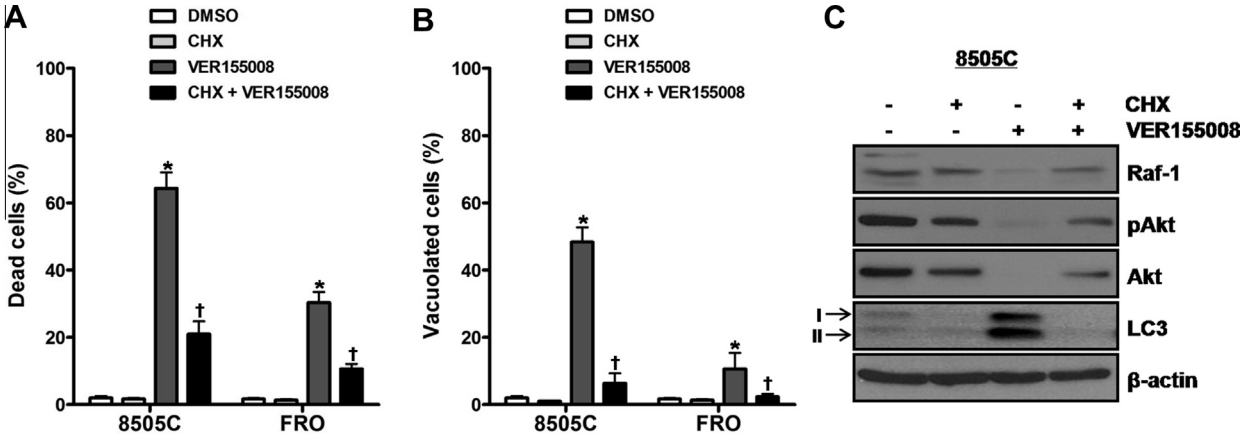

Fig. 4. The involvement of *de novo* protein synthesis in VER155008-induced paraptosis in ATC cells. (A–C) Cells were pretreated with CHX (100 μM, 1 h), after which treated with VER155008 at 50 μM for 72 h. The percentages of dead cells (A) and vacuolated cells (B), and the protein levels of Raf-1, total and phospho-Akt, and LC3-II (C) were measured. Data are expressed as mean ± S.E. $^{*}p < 0.05$ vs. control. $^{\dagger}p < 0.05$ vs. cells treated with VER155008 alone.

affect the percentages of dead cells (Fig. 2F) and vacuolated cells (Fig. 2G) in cells treated with VER155008.

3.3. VER155008 induces paraptosis in ATC cells

In this study, VER155008 led to cytoplasmic vacuolation cell death without caspase activation and autophagic flux. Since non-apoptotic and non-autophagic cytoplasmic vacuolation cell death has been shown to be paraptosis characteristic of dilatation of ER and induction of ER stress markers [9–11], we evaluated morphology of ER and expression of ER stress markers under exposure of VER155008. In VER155008-treated cells showing cytoplasmic vacuolation, ER was immunostained with the ER-specific marker calnexin, and cytoplasmic vacuoles were seen through dilated ERs (Fig. 3A). The inflated cells with cytoplasmic vacuoles and dilated ERs were detected by TEM, and cytoplasmic vacuoles appeared to be fused and swollen ER cisternae (Fig. 3B), indicating that cytoplasmic vacuoles induced by VER155008 are derived from dilated ER cisternae in cells. When cells were treated with VER155008, the mRNA levels of Bip and CHOP were elevated (Fig. 3C). Taken together, our results suggest that VER155008-induced cell death is paraptosis characterized by cytoplasmic vacuolation, independence of caspase, dilated ERs and activation of ER stress markers in ATC cells.

3.4. VER155008-induced paraptosis requires new protein synthesis in ATC cells

Paraptosis has been reported to need *de novo* protein synthesis [9,10,12], and thus we documented whether new protein synthesis is necessary for VER155008-induced paraptosis. When cells were pretreated with the translation inhibitor CHX prior to treatment of VER155008, the percentages of dead cells (Fig. 4A) and vacuolated cells (Fig. 4B) decreased in cells treated with both VER155008 and CHX, compared with cells treated with VER155008 alone. After treatment of the hsp90 inhibitor 17-AAG at 2 μM for 72 h and VER155008 at 50 μM for 24, 48 and 72 h, 17-AAG increased the protein levels of hsp70 and hsp90, whereas VER155008 did not change those protein levels (Fig. S1). In contrast, both 17-AAG and VER155008 decreased the protein levels of Raf-1, and total and phospho-Akt. Pretreatment with CHX

attenuated the alterations in the protein levels of Raf-1, total and phospho-Akt, and LC3-II resulted from VER155008 (Fig. 4C).

4. Discussion

Hsp70 regulates oncogenic client proteins and transcription factors, and contributes to maintenance of conformation, stability and activity of chaperone machinery [4,5]. Even though the hsp70 inhibitor VER155008 has been shown to induce cytostatic and cytotoxic effects in cancer cells [5–7], the influence of VER155008 on survival of ATC cells has not been investigated. In our study, VER155008 led to cell death with concomitant changes in the levels of hsp70 client proteins in ATC cells. Our data are the first to demonstrate that VER155008 induces cell death with modulation of hsp70 client proteins in ATC cells. Meanwhile, when the involvement of caspase activation was examined in VER155008-induced cell death, cleaved (activated) caspase-3 protein was not detected regardless of pretreatment with broad spectrum caspase inhibitor. The pretreatment with broad spectrum caspase inhibitor did not alter the percentages of dead cells and vacuolated cells, indicating no involvement of caspase activation. Because VER155008 resulted in cytoplasmic vacuolation cell death, the involvement of autophagy, characterized by the appearance of acidic vesicular organelles and the localization of LC3-II on autophagosomes, was explored [2,13,14]. VER155008 elevated LC3-II protein levels but the elevation was not potentiated by pretreatment with autophagy inhibitors. The pretreatment with autophagy inhibitors did not affect the percentages of dead cells and vacuolated cells, indicating no involvement of autophagic flux. Taken together, these results suggest that VER155008-induced cytoplasmic vacuolation cell death is not related to caspase activation and autophagic flux in ATC cells.

The non-apoptotic and non-autophagic cytoplasmic vacuolation cell death has been reported to be paraptosis characteristic of dilatation of ER and induction of ER stress markers [9–11]. In this regard, it was shown that epidermal growth factor, glucocorticoids and calcium influx induce paraptosis [10,15–18]. In our study, when vacuole-containing cells were observed by immunofluorescence and TEM after treatment of VER155008, the cytoplasmic vacuoles were identified as dilated ERs. Furthermore, VER155008 enhanced the expression of ER stress markers. Given that LC3-II is overexpressed in paraptosis as well [9,10], our results suggest that VER155008 induces paraptosis characteristic of cytoplasmic vacuolation, independence of caspase, dilated ERs and activation of ER stress markers in ATC cells. In viewpoint of clinical implication, VER155008-induced paraptosis may provide the basis of development of ‘new concept’ therapeutic agents in human ATC, which is resistant to chemotherapeutic drugs.

The *de novo* protein synthesis is required in paraptotic process [9,10,12], and thus we determined whether new protein synthesis is needed for VER155008-induced paraptosis. When cells were pretreated with translation inhibitor before treatment of VER155008, the percentages of dead cells and vacuolated cells were rescued, and the changes in the levels of hsp70 client proteins and LC3-II protein were attenuated by pretreatment with translation inhibitor. These results suggest that VER155008-induced paraptosis requires *de novo* protein synthesis in ATC cells.

In conclusion, VER155008 induces paraptosis characterized by cytoplasmic vacuolation, independence of caspase, dilatation of ER and induction of ER stress markers in ATC cells. Moreover, VER155008-induced paraptosis requires *de novo* protein synthesis in ATC cells. The present study will provide the clinical implications of hsp70 inhibitors in the treatment of human ATC, is refractory to conventional chemotherapeutic agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.060.

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