Hsp70 Inhibition Potentiates Radicicol-induced Cell Death in Anaplastic Thyroid Carcinoma Cells

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Abstract. Background/Aim: The aim of the present study was to evaluate the effect of radicicol, an inhibitor of heat shock protein (hsp) 90, alone or in combination with hsp70 inhibition on survival of anaplastic thyroid carcinoma (ATC) cells. Materials and Methods: Antitumor activity of radicicol-alone or in combination with the hsp70 inhibitor VER155008 was investigated in 8505C and CAL62 cells. Results: Radicicol decreased cell viability and Akt protein levels, and increased the percentage of dead cells and hsp70 protein levels. In PIK3CA plasmid-transfected cells, compared to cells treated with radicicol-alone, cell viability increased and cellular death decreased. In cells treated with both radicicol and VER155008, compared to cells treated with radicicol-alone, cell viability further decreased and the percentage of dead cells further increased, with a parallel decrease of the protein levels of heat shock cognate 70, Akt and survivin. Conclusion: Our results suggest that radicicol induces cell death mediated through PI3K/Akt signaling with modulation of hsp90 client proteins and hsp70 inhibition enhances radicicol-induced cell death with suppression of survivin in ATC cells.

Anaplastic thyroid carcinoma (ATC) is the most lethal thyroid malignancy and resistant to all types of therapy despite available multimodal approaches (1, 2). Various molecular derangements of ATC render it the most difficult thyroid malignancy to treat and new therapeutic agents are urgently needed (1, 2).

Radicicol, a macrocyclic lactone produced by fungus, is a heat shock protein (hsp) 90 inhibitor that exhibits anti-tumor activity by binding the N-terminal domain of hsp90 and destabilizing hsp90 client proteins, including Akt, mutant p53 and HER2/ERbB2, which are involved in cancer progression (3, 4). Radicicol represses the transformation caused by ras and mos oncogenes and reverses the malignant phenotype of v-src-transformed fibroblasts and exerts a cytotoxic effect on breast cancer cells by inhibiting angiogenesis and estrogen receptor signaling (5-8). However, the effect of radicicol on cell survival and hsp90 client proteins in thyroid cancer, including ATC, has not been evaluated.

The hsp70 family, including hsp72 and heat shock cognate (hsc) 70 (also known as hsp73), plays crucial roles in the regulation of client proteins and transcription factors involving structural conformation, stabilization and activation in the chaperoning program, while it is considered as a family of cytoprotective proteins capable of blocking cell death (9-12). In this regard, it was reported that the hsp70 inhibitor VER155008 enhances the effect of hsp90 inhibitors (10, 13, 14). However, whether hsp70 inhibition in combination with hsp90 inhibitors affects cell survival in thyroid cancer, including ATC, has not been investigated.

In the present study, we evaluated the effect of radicicol-alone and in combination with hsp70 inhibition on survival of ATC cells. Our results, for the first time, demonstrate that radicicol induces cell death mediated through PI3K/Akt signaling with modulation of hsp90 client proteins in ATC cells. Moreover, our results indicate that hsp70 inhibition augments radicicol-induced cell death with suppression of hsp70 and survivin in ATC cells. These findings provide clinical implications of radicicol alone and in combination with hsp70 inhibition, for the treatment of human ATC, which is refractory to conventional therapies.

Key Words: Anaplastic thyroid carcinoma, radicicol, hsp90, hsp70, Akt.

Materials and Methods

Materials. Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS) and streptomycin/penicillin were purchased from GIBCO (Grand Island, NY, USA). Radicicol and the hsp70 inhibitor VER155008 were purchased from Calbiochem (San Diego, CA, USA). The Pseudomonas aeruginosa gene for PIK3CA was purchased from Addgene (Cambridge, MA, USA). The hsp70 inhibitor VER155008 was purchased from Calbiochem (San Diego, CA, USA). The Pseudomonas aeruginosa gene for PIK3CA was purchased from Addgene (Cambridge, MA, USA). The hsp70 inhibitor VER155008 was purchased from Calbiochem (San Diego, CA, USA).
inhibitor VER155008 were obtained from Sigma (St. Louis, MO, USA). Radicicol was dissolved in dimethyl sulfoxide (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. Primary antibodies raised against total and cleaved poly (ADP-ribose) polymerase (PARP), hsp90, hsp70, hsc70, Raf-1, total and phospho-GSK3β (Ser9), and survivin were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). The primary antibodies raised against total and phospho-Akt (Ser473) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while the primary antibody raised against β-actin was from Sigma (St. Louis, MO, USA). All other reagents were obtained from Sigma unless otherwise stated.

Cell culture. For experiments, human ATC cell lines of 8505C and CAL62 cells were used. 8505C and CAL62 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ GmbH, Braunschweig, Germany) and grown in DMEM 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 50% confluent.

CCK-8 assay. As previously described, cell viability was determined by the CCK-8 Assay Kit (Dojindo laboratories, Kumamoto, Japan) (15). Cells (5×10^3/100 μl) in each well on 96-well plates were incubated overnight and treated with radicicol and/or VER155008 for an additional 4 h at 37°C. Absorbance was measured at 450 nm using a spectrophotometer (Molecular Devices, Palo Alto, CA, USA).

Trypan blue assay. Cells (5×10^4/500 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 carried-out in triplicate.

Western blotting. The total protein was extracted by RIPA buffer (Sigma) containing 1x protease inhibitor cocktail and 1x phosphatase inhibitor cocktail set V (Calbiochem, La Jolla, CA, USA). Western blotting was performed using primary antibodies and horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies. Bands were detected using an ECL Plus Western Blotting Detection System (Pierce, Rockford, IL, USA). β-actin was used as positive control. The protein levels were quantified by densitometry using the ImageJ software (National Institutes of Health) and normalized to β-actin levels. The relative levels of protein to β-actin were calculated. All reactions were carried out in triplicate.

Transfection of plasmid. A PIK3CA plasmid was purchased from Addgene (Cambridge, MA, USA). Cells were transfected with plasmid using the Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol. Transfection efficiency was tested by Western blotting.

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).

**Results**

Radicicol induces cell death with modulation of hsp90 client proteins in ATC cells. In 8505C and CAL62 cells, to evaluate the effect of radicicol on cell survival, cells were treated with radicicol at 1, 2, 5 and 10 μM for 24, 48 and 72 h and cell viability was measured (Figure 1A). After treatment, cell viability decreased in a time- and concentration-dependent manner. When cells were treated with radicicol at 1, 2, 5 and 10 μM for 72 h, the percentage of dead cells increased in a concentration-dependent manner (Figure 1B).

To investigate the influence of radicicol on hsp90 client proteins, cells were treated with radicicol at 10 μM for 72 h and the protein levels of hsp90, hsp70, HER2/ERbB2, Raf-1, GSK3β and Akt were measured (Figure 1C). After treatment, the protein levels of hsp90 and hsp70 increased and those of HER2/ERbB2, Raf-1, phospho-GSK3β and total and phospho-Akt decreased without change in total GSK3β protein levels.

To identify the impact of radicicol on the activation of PARP, cells were treated with radicicol at 10 μM for 72 h and total and cleaved PARP protein levels were measured (Figure 1D). After treatment, cleaved PARP protein levels increased without alteration in total PARP protein levels.

Radicicol-induced cell death is mediated through PI3K/Akt signaling in ATC cells. In the present study, radicicol caused cell death with a concomitant decrease of Akt, and thus the role of PI3K/Akt signaling in radicicol-induced cell death was examined. Cells were transfected with the PI3KCA plasmid before radicicol treatment at 10 μM for 72 h and cell viability, the percentage of dead cells and cleaved PARP protein levels were measured. In PI3KCA plasmid-transfected cells, compared with cells treated with radicicol alone, cell viability was elevated (Figure 2A) and the percentage of dead cells (Figure 2B) as well as cleaved PARP protein levels (Figure 2C) were reduced.

Hsp70 inhibition enhances radicicol-induced cell death in ATC cells. In the present study, radicicol caused cell death accompanied by an increase of hsp70; thus, the impact of hsp70 inhibition-alone or in combination with radicicol was explored.

To document the effect of hsp70 inhibition-alone on cell survival, cells were treated with the hsp70 inhibitor VER155008 at 10, 20, 30 and 40 μM for 24, 48 and 72 h. After treatment at 30 μM for 72 h, and at 40 μM for 24, 48 and 72 h, cell viability was diminished (Figure 3A) and the percentage of dead cells was enhanced (Figure 3B).

To determine the influence of hsp70 inhibition in combination with radicicol on cell survival, cells were co-treated with radicicol at 1, 2, 5 and 10 μM and VER155008 at 10 and 20 μM for 72 h. In addition, cells were co-treated...
Figure 1. The effect of radicicol on survival of ATC cells. A: Cells were treated with radicicol at 1, 2, 5 and 10 μM for 24, 48 and 72 h and cell viability was measured using the CCK-8 assay. B: Cells were treated with radicicol at 1, 2, 5 and 10 μM for 72 h and the percentage of dead cells was measured by trypan blue assay. C and D: Cells were treated with radicicol at 10 μM for 72 h and the protein levels of hsp90, hsp70, HER2/ERbB2, Raf-1, GSK3β and Akt (C) as well as those of total and cleaved PARP (D) were measured. All experiments were performed in triplicate. The blots (C and D) are representative of independent experiments. Data are expressed as mean±S.E. *p<0.05 vs. each matched control.
with radicicol at 10 μM and VER155008 at 20 μM for 24, 48 and 72 h. In cells treated with both radicicol and VER155008, compared to cells treated with radicicol-alone, cell viability further decreased (Figure 4A and C) and the percentage of dead cells further increased (Figure 4B and D) in a time- and concentration-dependent manner.

When cells were co-treated with radicicol at 10 μM and VER155008 at 20 μM for 72 h, the protein levels of hsc70, Akt and survivin decreased, while cleaved PARP protein levels increased without a change in the protein levels of hsp90 and hsp70 in cells treated with both radicicol and VER155008, compared with cells treated with radicicol alone (Figure 4E and F).

Discussion

Radicicol exerts anti-tumor activity and modulates hsp90 client proteins in human malignancies (3-8). However, the effect of radicicol on cell survival and the expression of hsp90 client proteins in ATC cells has not been elucidated. In the present study, radicicol caused death of ATC cells in a time- and concentration-dependent manner. Moreover, radicicol modified the protein levels of hsp90 client proteins including Akt in ATC cells. Our data are the first to demonstrate that radicicol induces cell death with regulation of hsp90 client proteins in ATC cells. These results suggest that radicicol may have clinical implications for the treatment of human ATC, which is refractory to conventional therapies.

The hsp70 family plays pivotal roles in the hsp90 chaperone machinery, which is operated with a variety of oncogenic client proteins and transcription factors to maintain cellular conformation, stability and activity (9). Hsp70 is induced following treatment with hsp90 inhibitors as a potential mechanism of resistance to hsp90 inhibitors (10-12). In this regard, it was reported that inhibition of hsp70 or its isoforms, such as hsc70, augments pro-apoptotic efficacy of the hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin, while the hsp70 inhibitor VER155008 potentiates a cytotoxic effect induced by hsp90 inhibitors (10, 13, 14, 16). However, the combination of hsp70 inhibition with hsp90 inhibitors in ATC cells has not been challenged. In the present study, we found that radicicol induced cell death with a concomitant increase of hsp70 protein levels in ATC cells, and thus we hypothesized that hsp70 inhibition would enhance radicicol-induced cytotoxicity in ATC cells. For combination experiments, the concentration and incubation time of VER155008 were chosen to be 20 μM and 72 h, respectively, which, although maximal, were found not to affect cell survival. Our data indicate that hsp70 inhibition augments radicicol-induced cytotoxicity in ATC cells, implying that combination of hsp70 inhibition with radicicol may be a beneficial option to overcome resistance to radicicol in the treatment of ATC.

Besides hsp70, survivin is known to be a resistance factor to chemotherapeutic agents and detected during chemotherapy (17-19). In this regard, it was reported that the expression of survivin is higher in ATC than well-differentiated or non-well differentiated thyroid cancers (20). In the present study, hsp70
inhibition attenuated the increment of survivin protein levels in radicicol-treated ATC cells. Considering that hsp70 and survivin are associated with cytoprotection, it may be inferred that hsp70 and/or survivin are involved in the mechanism of the combined effect of radicicol and VER155008 in ATC cells (10-12, 17-19). The precise mechanism of such combined effect should be further investigated.

PI3K/Akt signaling modulates various cellular processes, including survival, growth, proliferation and differentiation (21). PI3K/Akt signaling is de-regulated in ATC cells and Akt

Figure 3. The effect of VER155008 on survival of ATC cells. A and B: Cells were treated with VER155008 at 10, 20, 30, and 40 μM for 24, 48 and 72 h. Cell viability at 24, 48 and 72 h (A) as well as the percentage of dead cells at 72 h (B) were measured. All experiments were performed in triplicate. Data are expressed as mean±S.E. *p<0.05 vs. each matched control.
is activated in most ATC patients (21, 22). As for cell survival, we recently showed that PI3K/Akt signaling is involved in survival of ATC cells exposed to cytotoxic agents (23-25). In the present study, radicicol led to cell death in conjunction with decreased Akt protein levels in ATC cells, and thus the role of PI3K/Akt signaling in radicicol-induced death of ATC cells was examined. Our data manifest that activation of PI3K/Akt signaling by PI3KCA plasmid transfection mitigates radicicol-induced cytotoxicity in ATC cells, connoting that radicicol-induced cytotoxicity is mediated through PI3K/Akt signaling in the ATC cellular system.

In conclusion, our results suggest that radicicol induces cell death mediated through PI3K/Akt signaling with modulation of hsp90 client proteins and hsp70 inhibition enhances radicicol-induced cell death with suppression of survivin in ATC cells. The present study will provide clinical implications of radicicol alone and in combination with hsp70 inhibition for the treatment of human ATC, in which conventional therapies are not effective.
Figure 4. continued
Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2012R1A1A2008786) to S.J. Lee, Republic of Korea, and also was supported by Hallym University Research Fund 2013 (HURF-2013-16) to S.J. Lee, Republic of Korea.

References


Figure 4. The influence of VER155008 in combination with radicicol on survival of ATC cells. A and B: Cells were co-treated with radicicol at 1, 2, 5 and 10 μM and VER155008 at 10 and 20 μM for 72 h. Cell viability (A) and the percentage of dead cells (B) were measured. C and D: Cells were co-treated with radicicol at 10 μM and VER155008 at 20 μM for 24, 48 and 72 h. Cell viability (C) and the percentage of dead cells (D) were measured. E and F: Cells were co-treated with radicicol at 10 μM and VER155008 at 20 μM for 72 h. The protein levels of hsp90, hsp70, hsc70, Akt, survivin and cleaved PARP were measured (E). The protein levels were quantified by densitometry and normalized to β-actin levels. The relative levels of protein to β-actin were calculated (F). All experiments were performed in triplicate. The blots (E) are representative of independent experiments. Data are expressed as mean±SEM. *p<0.05 vs. control. **p<0.05 vs. cells treated with radicicol alone.


