Hsp90 inhibitor induced autophagy and apoptosis in osteosarcoma cells.

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INTRODUCTION:
Heat shock protein (Hsp90) is an abundant chaperone which constitutes 1-2% of total protein. It interacts with a variety of intracellular client proteins in cell growth, differentiation and survival, to facilitate their folding, activity, intracellular localization and proteolytic turnover.

Hsp90 inhibitor, a benzoquinone ansamycin antibiotic, interferes with the action of the Hsp90 leading to the degradation of Hsp90 client proteins. Since Hsp90 stabilizes a number of oncogenic proteins, many of which are overexpressed in cancers, Hsp90 inhibitor inhibits the proliferation of cancer cells and shows anticancer activity in experimental animals. Some authors reported that Hsp90 inhibitor has been shown to induce apoptosis in human osteosarcoma (OS) cell lines. But recently, it was reported that autophagy is activated upon endoplasmic reticulum stress as a defensive mechanism for survival. It functions as an additional pathway that might protect OS cells against toxic misfolded proteins. So, we hypothesize that in OS cell lines Hsp90 inhibitor induce autophagy as self-protection mechanism, but the autophagy inhibitor induce apoptosis and strong cell growth suppression.

The purpose of this study is to evaluate the efficacy of geldanamycin (GA), one of Hsp90 inhibitors, against human OS cell lines. And then, we investigated autophagy and apoptosis by TUNEL (single-strand DNA fragmentation), flow cytometry (formation of sub-G1 peak), western blot (expression of autophagy-related protein: LC3-II, FBS. After 48 h, cells were treated with 1% FBS containing GA at the indicated concentration, respectively (0, 0,01, 0.1, 1, 10 µM). After 24h and 48h, the medium was removed and refreshed with fresh medium consisting of DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). The cell line was routinely maintained at 37 °C in a humidified 5% CO₂ atmosphere. In this study, we used Hsp90 inhibitor (GA, StressMarq Biosciences Inc.) and autophagy inhibitor (3-MA, Sigma Chemical Company).

The inhibitory effect of GA on cell proliferation.
The cell proliferation was assayed using the MTS assay with CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA) and instructions from the manufacture were followed. Briefly, cells were trypsinized and seeded at a density of approximately 1x10⁴ cells/well in 96-well cell culture plates in 200 µL culture medium with 10% FBS. After 24 and 48 hours (h), the medium was refreshed with fresh medium containing GA in the indicated concentrations (0, 0.01, 0.1, 1, 10 µM). After 24h and 48h, the medium was removed and refreshed with fresh medium containing MTs reagent (100 µL medium without FBS plus 20 µL MTS reagent/well). At least three independent data were performed for each study. The IC⁵₀ values for GA were calculated.

Western blotting analysis.
Cells were trypsinized and seeded at a density of approximately 6x10⁴ cells/well in 6-well cell culture plates in 2 mL culture medium with 10% FBS. After 48 h, cells were treated with 1% FBS containing GA at the indicated concentration, respectively (0, 0.01, 0.1, 1, 10 µM) at 90 min. Whole cell lysates were collected for protein content, and cell lysates were separated by SDS polyacrylamide gel electrophoresis under reducing conditions. Then gels were electrophoretically transferred to Nitrocellose membranes. The membranes were blocked for 1h and immunoblotted with anti–4E-BP1 antibody (AB1537) (R&D Systems, Minneapolis, MN, USA), anti-phospho–4E-BP1 antibody (AF1665) (R&D Systems), anti-LC3 antibody (M115-3) (MBL Co., Nagoya, Japan), and α-tubulin antibody (sc-1615) (Santa Cruz, CA, USA) overnight at 4 °C as primary antibodies. The specific HRP-conjugated secondary antibody incubations for different primary antibodies were performed for 1h at room temperature with gentle agitation. Bound antibodies were detected using the ECL plus western blotting detection system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and LAS-1000 plus image analyzer (FUJIFILM Co., Tokyo, Japan). Specific signals were quantified by densitometric analysis (NIH image software).

Detection of apoptosis and cell-cycle fraction.
In KTHOS cells, the fragmented DNA and sub-G1 fraction were used as indicators to evaluate the effect of GA. Firstly, the single-stranded DNA fragmentation was evaluated using the MEBSTAIN Apoptosis Kit Direct (MBL, Nagoya, Japan). Secondly, sub-G1 cell cycle was determined by flow cytometry.

Morphological analysis for Autophagy and Apoptosis by Fluorescence Microscopy.
Assessment of apoptosis or autophagy induced by GA were done using Annexin-V- PI staining (detection for apoptotic cells) Kit and MDC stain (detection for autophagic cells) Kit.

RESULTS:
The effect of the GA on KTHOS cells.
GA inhibited KTHOS cell proliferation in a dose- and time-dependent manner.

Detection of apoptosis and cell-cycle fraction.
Apoptotic sub-G1 was detected in cells treated with GA plus 3-MA. This tendency was also recognized by the TUNEL method in KTHOS cells.

Morphological analysis for Apoptosis and Autophagy by Fluorescence Microscopy.
Morphological analysis showed that GA treatment increased autophagic cells, and autophagic cells were reduced by adding 3-MA in MDC stain analysis (Figure.1).

DISCUSSION:
These results suggest that the inhibitory action by Hsp90 inhibitor might have antitumor effects for KTHOS cells through mTOR signaling pathway. Although, several authors say that Hsp90 inhibitor, GA, shows antitumor effects by inducing apoptosis in many malignancies, the present study indicates that GA may induce autophagy rather than apoptosis in human OS cell lines. Further studies are needed to explore the precise molecular mechanisms for the inhibition of cell growth and the induction of autophagy in KTHOS cells; our data may be one of the important results to classify the role of Hsp90 in OS cells, and support the use of Hsp90 inhibitor and autophagy inhibitor which may be an effective treatment for OS because this combination effectively induces the apoptosis pathways.

SIGNIFICANCE:
The purpose of this study is to evaluate the efficacy of Hsp90 inhibitor against human OS cell lines. And then, we investigated autophagy and apoptosis.

Figure. 1 Autophagic cells were increased by GA treatment, but reduced by GA plus 3-MA in KTHOS cells.