Hsp90 Inhibitor Induced Autophagy And Apoptosis In Osteosarcoma Cells.

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Introduction: Heat shock protein 90 (Hsp90) is an ATP-dependent molecular chaperone responsible for the stability and function of a diverse range of client proteins with critical roles in cellular metabolism and trafficking, signal transduction, chromatin remodeling, growth and differentiation. Hsp90 is abundantly expressed in eukaryotes and comprises over 1% of eukaryote total cellular content. However, Hsp90 is constitutively expressed at 2-10-fold higher levels in tumor cells compared to normal cells, suggesting that it may be critically important for tumor cell growth and/or survival. Inhibition of Hsp90 activity results in rapid degradation of Hsp90 client proteins and induces apoptosis in various tumor cells. It has been reported that Hsp90 inhibitors can simultaneously activate both the death receptor pathway and the mitochondrial pathway. Hsp90 plays an important role in autophagy and Hsp90 inhibitors induce autophagy through inhibition of mTOR. However, it is still under debate whether chemotherapy-induced autophagy in tumor cells is a protective response or is invoked to promote cell death.

In this study, we provide the evidence that the Hsp90 inhibitor geldanamycin (GA) can induce apoptosis and autophagy in human osteosarcoma (OS) cells. We report that GA activates both the mitochondrial-mediated and death receptor-mediated apoptotic pathways, via the degradation of Hsp90 client proteins and that the GA can also induce autophagy in a dose-dependent manner via inhibition of Akt/mTOR/p70S6K signaling. Furthermore, we examined a combination therapy of GA and the autophagy inhibitor 3-methyladenine (3-MA) in OS cells.

Methods: Cell line and reagents.
KTHOS cell line (One of OS cell lines, established our laboratory) was used in this study. This cell line was grown in culture 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). KTHOS cells were treated with various concentrations (0, 0.01, 0.1, 1, 10 µM) of GA for 24h or 48h and cell viability was assessed using the MTS assay. The IC50 values for GA were calculated. Based on these results, we examine the cell proliferation levels of KTHOS cells treated with 5µM GA, 10mM 3-MA, 5µM GA and 10mM 3-MA.
Western blotting analysis.
We examined the protein levels of Akt, p-Akt (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mTOR, p-mTOR, p70S6K, p-p70S6K, 4E-BP1, p-4E-BP1 (Cell Signaling Technology, Beverly, MA, USA), and LC-3 (MBL, Nagoya, Japan) in KTHOS cells treated with various concentrations (0, 0.01, 0.1, 1, 10 µM) of GA for 90 min. Next, we examined the effect of GA on caspase activity to determine whether caspase activation occurs during GA-induced apoptosis. KTHOS cells were treated with various concentrations (0,
0.01, 0.1, 1, 10 µM) of GA for 24h, and total pro-caspase expression, caspase (Cell Signaling Technology), and PARP (BD Biosciences, San Jose, CA, USA) cleavage were analyzed. Next, we investigated whether the mitochondrial pathway contributes to GA-induced apoptosis. KTHOS cells were treated with various concentrations (0, 0.01, 0.1, 1, 10 µM) of GA for 24h, and cytosolic and mitochondrial cytochrome c (Santa Cruz Biotechnology) levels were analyzed. 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 (FUJI FILM Co., Tokyo, Japan). Specific signals were quantified by densitometric analysis (NIH image software).

Detection of apoptosis.

In KTHOS cells, the fragmented DNA was used as indicators to evaluate the effect of GA. The single-stranded DNA fragmentation was evaluated using the MEBSTAIN Apoptosis Kit Direct (MBL).

Morphological analysis for Autophagy and Apoptosis by Fluorescence Microscopy.

Assessment of apoptosis or autophagy induced by GA were done using AnnexinV- 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. In GA and 3-MA group, the cell proliferation levels of KTHOS cells were strongly decreased than in GA group.

Detection of apoptosis.

Apoptosis levels were increased in GA and 3-MA treated group by TUNEL method.

Western blotting analysis.

The protein levels of p-mTOR, p-p70S6K, p-4E-BP1 decreased in dose-dependent manner. In addition, LC3-II expression levels were increased by GA in dose-dependent manner in KTHOS cells. GA induced cleavage of PARP, as well as dose-dependent activation of caspase-3, caspase-7, caspase-8 and caspase-9 and a dose-dependent release of mitochondrial cytochrome c into the cytosol of 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. In Annexin and PI stain analysis, apoptosis was detected in GA treated group, and apoptosis level was increased in GA plus 3-MA treated group.

**Discussion:** This study provides the evidence that GA simultaneously induces apoptosis and autophagy in KTHOS cells. GA induces degradation of Hsp90 client proteins, activates both the mitochondrial and death receptor-mediated apoptotic pathways and leads to Bcl-2 and Bcl-xL downregulation, Bid upregulation, cleavage of caspase-9, caspase-7, caspase-3 and PARP, and activation of caspase-8. Additionally, GA induces autophagy via inhibition of Akt/mTOR/p70S6K signaling. 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 and in combination with autophagy inhibitor against human OS cell lines. And then, we investigated autophagy and apoptosis.