The antitumor effects of chloroquine involve the suppression of autophagy and inhibition of the MAPK pathway in osteosarcoma cells

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INTRODUCTION: Autophagy promotes tumor growth by mitigating metabolic stress and inducing resistance to chemotherapy and/or irradiation in various types of human cancer. Many studies have shown that autophagy is a self-defense mechanism of cancer cells that are subjected to anti-tumor agents, and blocking autophagy can trigger apoptosis. Chloroquine (CQ), which was originally developed as an anti-malarial agent, has been evaluated as an autophagy inhibitor for cancer treatment. In this study, we investigated the effects of CQ on the inhibition of autophagy in osteosarcoma cells. In addition, the efficacy of rapamycin, which is an mTOR inhibitor and potential targeted therapy for osteosarcoma, was analyzed alone or in combination with CQ.

METHODS: Drug: CQ was purchased from Sigma Chemical Company (St. Louis, MO), dissolved in distilled water to a concentration of 1 mM, and stored at room temperature. Rapamycin was purchased from Merck KGaA (Darmstadt, Germany), dissolved in DMSO, and stored at -20°C. Cell culture: MG63 osteogenic sarcoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St.Louis, MO) with 10% FBS and were routinely maintained at 37°C in a humidified 5% CO2 atmosphere. Cells were seeded onto culture dishes and cultured in growth medium for 24 or 48 h. The growth medium was then replaced with fresh medium with or without inhibitors.

In vitro proliferation assay: Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyoxothiophenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Briefly, cells were trypsinized and seeded at a density of approximately 1 × 10^4 cells/well in 96-well cell culture plates containing 200 µl of culture medium with 10% fetal bovine serum (FBS) in each well. After 24 h, the medium was changed to fresh medium with 1% FBS in the presence or absence of rapamycin and/or CQ. After 24 or 48 h, the medium was removed and replaced with fresh medium containing FBS reagent. The percent viability of the cells in each well was calculated. At least three independent experiments were performed.

Western blot analyses: Cells were trypsinized and seeded at a density of approximately 1 × 10^6 cells/well in 6-well cell culture plates containing 2 ml of culture medium with 10% FBS and 5% FBS. They were then treated with rapamycin at various concentrations. Cells were lysed and the cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The resolved proteins were then electrochemically transferred onto nitrocellulose membranes, which were subsequently incubated with primary and secondary antibodies in iBind Western System, Life Technologies, Carlsbad, CA for 2.5 h. Bound antibodies were detected using the Novex® AP Chemiluminescent Detection Kit (Life Technologies) and an LAS-1000 Plus Image Analyzer (Fuji Film, Tokyo, Japan). Specific signals were quantified by densitometric analysis using Image J software (NIH, Bethesda, MD).

Analysis of cell cycle progression and apoptosis by flow cytometry: Cells were seeded at a density of 6 × 10^4 cells/well in 6-well cell culture plates and cultured for 48 h. They were then treated with CQ for 24 h, washed in phosphate-buffered saline, fixed with 70% ethanol and stored at -30°C. The next day, the samples were washed and incubated with 10 µM DNase-free RNase (Roche Applied Science, Penzberg, Germany) and 50 µM propidium iodide (PI) for 20 min at 37°C. Cell cycle analysis was performed using a flow cytometer (FC-500, Beckman Coulter, Inc., Brea, CA) for the evaluation of the sub-G1 fraction.

Fluorescence microscopy: Cells were trypsinized and seeded at a density of approximately 1 × 10^5 cells/well on 25-mm circular coverslips. For the detection of autophagy, cells were stained immunocytochemically with anti-LC3 antibody (Code No. PM036, MBL, Nagoya, Japan) and transfected with a GFP-LC3 expression vector (P36235, Invitrogen). Assessments of apoptosis were performed using an Annexin V/PI and Hoechst 33342 triple-staining assay. The stained cells were detected under an epifluorescence microscope (FSX100, Olympus Optical Co., Tokyo, Japan).

RESULTS: In vitro cell proliferation and cytotoxicity: Rapamycin and CQ decreased the proliferation of MG63 cells in a dose-dependent manner with IC_{50} of 20 µM and IC_{50} of 30 µM, respectively. Furthermore, cell proliferation was significantly inhibited by rapamycin and CQ combination treatment.

Western blot analyses: The expression level of the autophagy marker, LC3-II, was increased by rapamycin alone, and further increased by the combination treatment. In contrast, the expression level of p62/SQSTM1 was decreased by rapamycin alone, but significantly increased by the combination treatment. These results suggest autophagy was induced by rapamycin, and inhibited by CQ. In addition, the expression levels of apoptosis-related proteins, caspase-3, caspase-9, and PARP were increased by rapamycin and CQ treatment, and further increased by combination treatment. The levels of p44/42 MAPK phosphorylation were increased by rapamycin alone, but decreased by CQ and combination treatment. These results suggest that CQ induced apoptosis by inhibiting autophagy and the MAPK pathway in MG63 cells.

Fluorescence microscopy: LC3 expression was increased by rapamycin treatment, and was further enhanced by rapamycin and CQ combination treatment. In the Annexin V/PI and Hoechst 33342 triple-staining assay used to detect apoptotic cells, we observed several Annexin V-FITC-positive cells (early stage of apoptosis) and a high number of Annexin V-FITC plus PI-positive cells (late stage of apoptosis) among the cells treated with the rapamycin and CQ combination.

DISCUSSION: Autophagy is a lysosome-dependent degradative process that protects cancer cells from multiple stresses, including chemotheraphy, and is regulated by several signal transduction pathways, including the mTOR and MAPK cascades. Our previous studies have shown that rapamycin can induce cytoprotective autophagy by activating the MAPK signaling pathway, and combination therapy with mTOR and autophagy inhibitors may provide an effective treatment for soft tissue sarcoma by effectively inducing apoptotic pathways.

Furthermore, the MAPK pathway activates cell proliferation, and inhibition of this pathway is effective for tumor suppression. This study demonstrated that CQ mediated inhibition of autophagy increases MG63 cell sensitivity to rapamycin treatment by inhibiting the PI3K pathway and activating apoptosis pathways. These results suggest that CQ combination therapy may improve patient responses to cell signaling-targeted therapies currently being considered for the treatment of osteosarcoma.

SIGNIFICANCE: This study highlights the potential of CQ as a new targeted therapy for osteosarcoma.

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