学 位 論 文 の 要 旨

氏名　Mohammad Mahbubul Hoque

学 位 論 文 名　Hydroxychloroquine Promotes Bcl-xL Inhibition-induced Apoptosis in BxPC-3 Human Pancreatic Cancer Cells

発 表 雑 誌 名（巻，初頁～終頁，年）　Anticancer Research (**42**, 3495-3506, 2022)

著 者 名　Mohammad Mahbubul Hoque, Yuichi Iida, Hitoshi Kotani, Irna Diyana Kartika, Mamoru Harada

論 文 内 容 の 要 旨

INTRODUCTION

The goal of anticancer therapy is to induce cancer cell death without damage to normal cells and tissues. However, cancer cells evade therapies via various mechanisms. One major mechanism is resistance to apoptosis and Bcl-2 family proteins play crucial roles in resistance to apoptosis by cancer cells. Bcl-2 family proteins consist of anti-apoptotic proteins, including Bcl-2, Bcl-xL, Bcl-w, and Mcl-1, and pro-apoptotic proteins, including Bax and Bak. ABT-263 is an orally bioavailable Bcl-2 family inhibitor against Bcl-2, Bcl-xL, and Bcl-w. We previously reported that ABT-263, and its analogue ABT-737 with the same specificity, can enhance drug-induced antitumor effects against human prostate and pancreatic cancer cells.

Hydroxychloroquine (HCQ) has been used for the treatment of malaria and autoimmune diseases. Chloroquine (CQ) and HCQ belong to the quinolone family and show similar pharmacological effects. Both CQ and HCQ have the potential to exert antitumor activity. However, because HCQ is less toxic than CQ, HCQ was expected to be a better anticancer drug.

Pancreatic cancer is an aggressive malignancy with a high metastatic risk and low survival rate compared to other cancers. Its diagnosis and treatment are challenging, and new treatment modalities are required to improve its prognosis. In the present study, we investigated the combination effect of HCQ with Bcl-2 family inhibitors, including ABT-263 and ABT-199, using three human pancreatic cancer cell lines.

MATERIALS AND METHODS

Three human pancreatic cancer cell lines (PANC-1, MiaPaCa-2, and BxPC-3) were used. HCQ was purchased from InvivoGen and diluted in saline. ABT-263 and ABT-737 were purchased from Active Biochemicals Co., Ltd.. ABT-199 was purchased from ChemieTek. A1331852 was purchased from Selleck. Pan-caspase inhibitor (Z-VAD-FMK) was purchased from Enzo Life Sciences. Both caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) were purchased from R&D Systems. Cell viability was measured using the WST-8 assay with 96-well flat-bottom plates. For colony-forming assay, cells were seeded into 6-well flat-bottom plates and cultured with the indicated doses of HCQ for 12–16 days. Next, to visualize colonies, the cells were fixed in methanol, stained with 0.05% (w/v) crystal violet, and counted. For immunoblotting, cells were lysed using a mammalian protein extraction reagent containing a protease inhibitor cocktail. Equal amounts of protein were loaded and transferred to polyvinylidene fluoride membranes. The membranes were blocked and then incubated with the following primary antibodies: anti-Bcl-2, anti-Bcl-xL, or anti-β-actin. The membranes were incubated at room temperature for 30 min with either goat anti-rabbit or goat anti-mouse alkaline phosphatase-conjugated secondary antibodies. siRNA transfection was performed using Lipofectamine RNAiMAX. Flow cytometric analysis. Cancer cells (4×10^4/well) were seeded in a volume of 1 mL in 24-well plates with HCQ and/or ABT-263. Two days later, the cultured cells were harvested and stained with FITC-conjugated annexin V (AV) and propidium iodide (PI). Then, the cells were analyzed using CytoFLEX or FACSCalibur. *In vivo* xenograft model. Female BALB nu/nu mice were inoculated in the right flank with 2×10^6 BxPC-3 cells and Matrigel at a 1:1 volume ratio in a total volume of 100 μL. The mice were pooled and divided into four groups. HCQ (60 mg/kg) was intraperitoneally administered on days 0, 1, 3, 4, 6, 7, 9, 10, 12, and 13 after grouping. ABT-737 (50 mg/kg) was administered intraperitoneally on days 2, 5, 8, 11, and 14 after grouping. The tumor volume was calculated as follows: tumor volume (mm^3) = (length × width^2) ÷ 2. All experiments with animals in this study were approved by the Animal Care and Use Committee of Shimane University (IZ3-94). Data were analyzed using the unpaired two-tailed Student's t-test (between two groups) or an analysis of variance (ANOVA) with Tukey–Kramer test (for more than two groups). *P*-values < 0.05 were considered indicative of statistical significance.

RESULTS AND DISCUSSION

HCQ with ABT-263, but not ABT-199, effectively decreased BxPC-3 cell viability. Initially, the effects of HCQ on the *in vitro* growth of three human pancreatic cancer cell lines, PANC-1, MiaPaCa-2, and BxPC-3, were examined. HCQ significantly reduced the viability of three cell lines in a dose-dependent manner. In addition, HCQ similarly inhibited the colony number in the

three cell lines. In the colony-forming assay, BxPC-3 cells were more sensitive to HCQ than the other two cell lines. When BxPC-3 cells were cultured with HCQ (10 and 20 μM) and ABT-263 (2.5, 5, and 10 μM), their viability decreased more profoundly compared to the combination of HCQ and ABT-199. In contrast, ABT-199 decreased MiaPaCa-2 cell viability with or without 10 μM HCQ. The combination of ABT-263 (5 μM) and HCQ (10 μM) effectively decreased cell viability compared with either treatment alone. On the other hand, ABT-199 could not enhance the antitumor effect of HCQ on BxPC-3 cells.

HCQ plus ABT-263 promoted caspase-dependent apoptosis in BxPC-3 cells. Next, whether the cell death was due to apoptosis was investigated. An apoptosis assay using AV and PI showed that combination of HCQ and ABT-263 significantly increased the percentages of AV^+ PI^- early apoptotic cells compared with monotherapy. A statistical difference was not observed regarding AV^- PI^+ late apoptosis between ABT-263 alone and ABT-263 combined with HCQ. The addition of the pan-caspase inhibitor Z-VAD significantly decreased the percentage of AV^+ PI^- early apoptotic BxPC-3 cells.

Inhibition of Bcl-xL, but not Bcl-2, promoted apoptosis in HCQ-treated BxPC-3 cells. The effects of Bcl-xL inhibition on apoptosis of HCQ-treated BxPC-3 cells were examined. The combination of HCQ with A1331852, a Bcl-xl-specific inhibitor, or ABT-263 significantly increased the percentage of early apoptotic BxPC-3 cells more profoundly compared with HCQ and ABT-199. In addition, experiments with transfection with Bcl-2 siRNA and Bcl-xL siRNA were performed. Although siRNA-mediated knockdown of Bcl-2 increased the percentage of late apoptotic BxPC-3 cells, higher levels of apoptotic cells were observed in Bcl-xL-siRNA transfected cells.

In vivo *antitumor effect of HCQ and ABT-737 combination on BxPC-3 cells.* In the xenograft model, either HCQ or ABT-737 alone showed no significant antitumor effect, but combination of them slightly but significantly suppressed the growth of BxPC3 on days 18 and 21 after starting the treatment compared with the untreated group. However, this significant effect disappeared on day 25 because variations of tumor size increased.

CONCLUSION

We showed that HCQ effectively promoted ABT-263-induced apoptosis in BxPC-3 human pancreatic cancer cells, and that ABT-263 in a combination treatment was suggested to be due to Bcl-xL inhibition. These findings indicate that HCQ is a promising modality to augment the therapeutic efficacy of ABT-263 against pancreatic cancer cells.