学 位 論 文 の 要 旨

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学位論文名	Combined Treatment With Tamoxifen and a Fusicoccin Derivative (ISIR-042) to Overcome Resistance to Therapy and to Enhance the Antitumor Activity of 5-Fluorouracil and Gemcitabine in Pancreatic Cancer Cells
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論 文 内 容 の 要 旨

INTRODUCTION

Pancreatic cancer remains a very aggressive neoplasm, and even with multimodality therapy for localized disease, patient survival is measured in months. Chemoresistance usually develops for patients who respond initially, and an effective salvage therapy is currently unavailable. Development of better therapeutic regimens for pancreatic cancer remains a high priority. Combination therapy eliminates cells that are singly resistant to either drug. Mathematical analyses reveal that triple therapy may be needed in patients with large tumor burden.

Cotylenins, fusicoccins and some diterpene glucosides are modulators of 14-3-3 proteins and have been shown to exhibit antitumor effects *in vitro* and *in vivo*. We synthesized many fusicoccin derivatives and found that ISIR-042 was the most potent at inhibiting the proliferation of tumor cells. The synergistic effects of ISIR-042 and 5FU or gemcitabine were observed at inhibiting growth of pancreatic cancer cells. Triple combination therapy would be required for an effective therapy against pancreatic cancers. Therefore, we examined the synergistic effects of various compounds and ISIR-042 on the growth of pancreatic cancer cells to identify the most potent and clinically applicable drugs. The most effective agent was tamoxifen. In the present study, we sought to clarify the synergistic effect of ISIR-042 and tamoxifen on human pancreatic cancer cells and to examine the therapeutic effects on xenografts of human pancreatic carcinoma cells in the presence or absence of 5FU or gemcitabine treatment.

MATERIALS AND METHODS

Human pancreatic cancer cell lines (Panc-1, MIAPaCa-2, BxPC-3, CFPAC-1, and Capan-2) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 80 mg/ml gentamicin at 37℃ in a humidified atmosphere of 5% CO_2 in air.

In the colony-forming assay, MIAPaCa-2 cells ($1x10^4$/dish) were plated into 1.1 ml of a semisolid methylcellulose medium with 0.8% methylcellulose and 20% fetal bovine serum in triplicates for 14 days. Colonies >0.4 mm in diameter were counted. Nucleated cells (1,000 cells/ml/dish) from a single femur bone marrow plugs of three Balb/c mice were put into a semi-solid medium containing hematopoietic growth factors and incubated for 10 days.

Human pancreatic cancer cells were seeded into 24-well multidishes, and were cultured with various concentrations of drugs for 4-6 days. The viable cells were examined by the MTT assay. Isobologram analysis was used to determine the effects of combinations of drugs on cells. The interaction of two compounds was quantified by determining the combination index (CI), in accordance with the following classic isobologram.

The cell density of the drug-treated cells was kept at 2-8 x 10^4/ml to maintain growing phase in a 24-well multidish. The viable cell number was measured by MTT assay.

The cells were homogenized with protease inhibitor cocktail. The cytoplasmic (supernatant) and mitochondrial (pellet) fractions were separated by centrifuge (10,000 x g for 30 min.) from the homogenate. Cytochrome c was determined by Western blot using monoclonal anti-cytochrome c antibody. Lipid peroxidation (LPO) was determined by measuring thiobarbituric acid-reactive substances at 535 nm.

Total RNA was extracted from cells and was converted to first-strand cDNA primed with random hexamer in a reaction volume of 20 μl using an RNA PCR kit. The quantitative RT-PCR reaction was performed using a real-time PCR system.

Xenograft was used four-week-old female athymic nude mice with a BALB/c genetic background. Mice were subcutaneously inoculated with 2×10^6 Panc-1 cells with Matrigel. Mice were given daily intraperitoneal injections of 3 mg/kg ISIR-042 in 0.2 ml PBS and/or 2 mg/kg tamoxifen in 0.1 ml corn oil with the first treatment being given 2 days after the inoculation of tumor cells. Treatment with 5 mg/kg 5FU was performed three times per week. Tumor volume was measured with vernier calipers. All experiments with animals in this study were approved by the Ethics Committee for Animal Experimentation of Shimane University and they were handled according to our institutional guidelines.

In the statistical analysis, pairs of data were compared using Student's t-test. For the *in vivo* experiment, an F-test was performed to demonstrate statistical significance. Significant differences were considered to exist for probabilities below 5% ($P<0.05$).

RESULTS AND DISCUSSION

Tamoxifen inhibited the growth of Panc-1 cells in a concentration-dependent manner. ISIR-042 produced synergistic effects with tamoxifen and the results were confirmed by isobologram analysis. This synergistic effects were found in other pancreatic cancer cell lines, although the sensitivity of pancreatic cancer cell lines to tamoxifen varied among the cell lines. It was reported that tamoxifen induces cell death by multiple non-estrogen receptor mediated mechanisms, and these mechanisms include changes in intracellular calcium, modulation of protein kinase C, changes in calmodulin activity, signaling through mitogen-activated protein kinases. The mechanism of the growth-inhibitory effect of tamoxifen on pancreatic cancer cells was examined. Estrogen and several inhibitors of protein kinases (Taurine, L-nitroarginine methyl ester, N-acetyl cysteine) had no effect. Whereas α-tocopherol, a membrane stabilizer and a lipophilic antioxidant, effectively reduced tamoxifen-induced growth inhibition of MIAPaCa-2 cells, but not ISIR-042-induced growth inhibition. Tamoxifen elevated lipid peroxidation and release of cytochrome c, and the effects of tamoxifen were reduced by α-tocopherol. These results suggest that tamoxifen is an anti-cancer drug that induces oxidative stress and apoptosis via a mitochondria-dependent pathway.

ISIR-042 preferentially inhibited stem/progenitor cells in pancreatic cancer cells. ISIR-042 effectively inhibited colony formation by MIAPaCa-2 cells in semi-solid culture. The inhibition of colony formation by tamoxifen or 5FU was similar to the inhibition of cell proliferation in liquid culture. Pancreatic cancer cells were more sensitive to combined treatment with ISIR-042 and tamoxifen than normal bone marrow cells. ISIR-042 significantly inhibited expression of stemness-related genes (*SOX2*, *NANOG*) of pancreatic cancer cells, but did not significantly affect the expression of *Oct3/4* mRNA under these conditions. These results suggest that ISIR-042 is a useful drug for cancer stem cell-targeted therapy against pancreatic carcinoma.

A triple combination (ISIR-042, tamoxifen and 5FU or gemcitabine) cooperatively inhibited the growth of MIAPaCa-2 cells *in vitro*. The combined treatment significantly inhibited the growth of Panc-1 cells as xenografts without apparent adverse effects, as judged by body weight loss. These results indicate that the combination of tamoxifen, ISIR-042 and 5FU is effective therapeutically, consistent with the *in vitro* findings.

CONCLUSION

The triple combination of tamoxifen, ISIR-042, and 5FU or gemcitabine was effective at inhibiting cell growth and the appearance of drug-resistant cells. This combined treatment significantly inhibited the growth of Panc-1 cells as xenografts without apparent adverse effects. The triple combination of tamoxifen and ISIR-042 with 5FU or gemcitabine may be highly effective against pancreatic cancer by overcoming resistance to therapy.