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.

論文審査及び最終試験又は学力確認の結果の要旨

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	副査	田島義証 印
	副査	松崎有未 印

論文審査の結果の要旨

膵癌は代表的な難治性がんである。現在、FORFIRINOX と呼ばれる 3 剤併用療法が開発されているが、副作用の問題があるためさらなる改善が必要とされている。申請者らは、同グループが開発した抗腫瘍物質である fusicoccin 誘導体(ISIR-042)を軸とする多剤併用療法の確立を目的に、膵癌培養細胞を用いて、この物質との併用で相乗効果の得られる薬剤を比較的毒性の少ない薬剤の中から探索した。その結果、tamoxifen が最も効果的であることが明らかになったため、その作用機序について検討を行った。Tamoxifen の効果が膜安定化剤α-tocopherol で阻止されることから膜に作用点があると考え、培養細胞への Tamoxifen 添加により、ミトコンドリア膜の lipid peroxidation の増加と cytochrome C の遊離が惹起されることから、ミトコンドリア膜障害がアポトーシスを誘導していることを明らかにした。一方、ISIR-042 が膵癌細胞のコロニー形成を効果的に抑制し、癌幹細胞の特徴となる遺伝子の発現を抑制することから、ISIR-042 が癌幹細胞の抑制に有効であることを示した。また、3 剤併用療法の可能性を検討するため、膵癌治療に頻用される 5-fluorouracil (5FU)や gemcitabine (GEM)との併用について検討したところ、ISIR-042、tamoxifen と 5FU もしくは GEM の併用で薬剤耐性細胞の出現を効果的に抑制できることを明らかにした。申請者は更に、5FU/tamoxifen/ISIR-042 の 3 剤併用療法の有効性を、ヌードマウスに移植した膵癌組織に対する *in vivo* での治療実験においても確認した。これらの結果は膵癌に対する有効な多剤併用療法の確立につながる臨床的に重要な成果である。

最終試験又は学力の確認の結果の要旨

申請者は、同グループの開発した新規抗腫瘍物質 ISIR-042 を含む新たな多剤併用療法が *in vitro, in vivo* で膵癌治療に有効であることを示し、またその作用機序に関する解析を行った。これは、膵癌治療改良につながる臨床的意義のある成果である。審査時の質疑応答も適切で背景の知識も充分であることから、学位授与に値すると判断した。（主査　並河 徹）

申請者は，難治性疾患の膵癌に対する tamoxifen+fusicoccin 誘導体(ISIR-042)+5FU (GEM)の3剤併用化学療法を考案し，ヒト膵癌細胞株を用いた *in vitro, in vivo* 実験の何れにおいても優れた抗腫瘍効果を有することを示した。臨床応用に向けて有益な結果であり，学位審査における質疑応答も的確で，学位授与に値すると判断した。（副査：田島義証）

申請者は低分子化合物 ISIR-042 と tamoxifen による 2 剤併用、あるいは GEM/5FU を加えた 3 剤併用療法が、膵がん細胞株に対しアポトーシスを誘導し、増殖抑制的に作用することを明らかにした。また動物実験にて大きな副作用なしに著効を確認しており、膵がん治療の新たな可能性を示唆する重要な研究と認められ、学位授与に値すると判断した。（副査：松崎有未）

（備考）要旨は、それぞれ 400 字程度とする。