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Original Articles

The molecular mechanisms of a novel multi-kinase inhibitor ZLJ33 in suppressing pancreatic cancer growth

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ABSTRACT

ZLJ33, an oral active multi-kinase inhibitor, was evaluated both in vitro and in vivo against human pancreatic cancer. It could effectively inhibit cell proliferation, induce apoptosis, and cause inhibition of invasion in pancreatic cancer cells. At a dose of 15.0 mg/kg, ZLJ33 induced tumor shrink in Mia-PaCa2, Capan2, and AsPC-1 xenografts models by 60.59%, 74.19%, and 71.54% according to the tumor weight, respectively. The effect of ZLJ33 on pancreatic cancer was mainly mediated by inactivation of p-PDGFRβ, p-c-Raf, and p-RET. Treatment with ZLJ33 did not cause side effect of hematology indexes in the pancreatic cancer xenograft model. ZLJ33 could be a potential therapeutic agent against pancreatic cancer.

Introduction

The incidence of pancreatic cancer has risen steadily in recent years. Moreover, pancreatic cancer is an aggressive human cancer with a median survival time of 4–6 months and a 5-year survival rate of <5% [1], and it is the fourth leading cause of death from cancer in the USA [2]. Yet pancreatic cancer shows limited susceptibility or high resistance to conventional classes of cytotoxic drugs and radiation treatment [3]. Therefore, there is an urgent need for new therapies based on the molecular biology of malignant progression in pancreatic cancer to improve overall survivals of patients.

Pancreatic cancer displays several molecular genetic mutations. Activating mutation in KRAS occurs in 90% of tumors, leading to constitutive activation of downstream signal transduction pathway such as the mitogen-activated protein kinase (MAPK) [4,5] and PI3K/AKT-mammalian target of rapamycin (mTOR) signaling cascade [6–9], which play an important role in regulation of cell proliferation, survival, and angiogenesis. Overexpression of PDGFRβ signaling pathway is associated with progressive growth of human pancreatic cancer, and PDGFRβ activation leads to activation of downstream signaling pathway, such as PI3K/AKT and Ras/MAPK [10–12]. The Rearranged During Transfection (RET) gene is identified as a novel proto-oncogene which was activated by DNA rearrangement [13], and RET is expressed highly in pancreatic cancers. Formation of RET complexes with ligand/co-receptor results in RET dimerization is also shown to cause activation of several downstream pathway, including Ras/Raf/ERK, PI3K/AKT and STAT transcription [14,15]. These results suggest that those pathways can become important targets for pancreatic cancer therapy. Inhibitors targeting these pathways simultaneously might be effective in pancreatic cancer therapy [16].

Sorafenib (Nexavar) is one such inhibitor targeting a number of kinase kinases [17]Preclinical studies showed that sorafenib exerted its broad inhibiting activity on tumor angiogenesis and cell proliferation through inhibiting signaling kinases including c-Raf, VEGFR, PDGFRβ, Flt-3, and c-kit in several tumor types such as renal cell carcinoma, pancreatic cancer, colon cancer, breast cancer, and melanoma [18,19].

In this paper, we studied another novel multi-kinase inhibitor identified in biochemical assay, named ZLJ33, which is a picolinohydrazide derivative with a 1,3-diphenylurea group similar

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to that of sorafenib. In particular, we evaluated the anti-tumor activity of this compound and its molecular mechanism as a multi-kinase inhibitor in pancreatic cancers. The results showed that ZLJ33, like sorafenib, strongly inhibited cell proliferation and invasion, induced cell apoptosis in Mia-PaCa2 and Capan2 pancreatic cancer cells. Moreover, ZLJ33 at a dose of 15.0 mg/kg significantly reduced the tumor growth of pancreatic cancer in Mia-PaCa2, Capan2, and AsPC-1 xenograft models. The anti-tumor activities of ZLJ33 on pancreatic cells were associated with suppression of p-PDGFRβ, p-RET, and p-c-Raf which might account for the blockade of the regulation pathways of apoptosis, proliferation, and invasion, such as Raf/MEK/ERK, AKT/mTOR, and β-catenin. Unlike sorafenib, ZLJ33 had little side effect on the hematology indexes and body weight under the dose of 15.0 mg/kg in the Mia-PaCa2 xenograft model.

In summary, we demonstrated that the action of ZLJ33 on the targets involved in c-Raf, PDGFRβ, and RET might be associated with its anti-tumor effect in pancreatic cancer in vitro and in vivo, suggesting that ZLJ33 could be developed into a more potent agent for the treatment of human pancreatic cancer.

Materials and methods

Cell culture

Pancreatic cancer cell lines SW1990, PANC1, JF305, AsPC-1, normal human fibroblasts (HELF), and human kidney proximal tubular cell (HK2) were obtained from the cell center of CAMS & PUMC. Capan2, Mia-PaCa2, and PL45 were obtained from ShangHai Xiangf Bio Company. All the cells were maintained in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator containing 5% CO_2 at 37 °C.

Drugs and compound

Sorafenib and ZLJ33 were synthesized by the Department of Pharmacochemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (purity > 98% (HPLC)). For in vitro experiments, sorafenib and ZLJ33 were dissolved in DMSO (dimethyl sulfoxide) and stored at 4 °C till use for experiments. For in vivo experiments, sorafenib and ZLJ33 were dissolved in the solution with Cremophor EL/ethanol/water (12.5:12.5:75) [19].

Kinases assay

Inhibition of kinase activity against target kinases was measured using Caliper assay and Glo-ATP assay. The biochemical assay was performed by HD Biosciences (China) Co., Ltd.

Cell viability assay

Cell lines Mia-PaCa2, Capan2, AsPC-1, SW1990, PANC1, PL45, JF305, HELF and HK2 were seeded in a 96-well plate. After 24 h incubation, cells were treated with varied concentrations of both sorafenib and ZLJ33. After 120 h incubation, the CCK-8 assay was performed to evaluate cell viability. IC_{50}, defined as the compound concentration at which cell growth was inhibited by 50% was assessed from the dose–response curve. Each assay was performed in triplicate.

Colony formation assay

Mia-PaCa2, Capan2, SW1990, and PANC1 cells were trypsinized and plated (400 cells/well) in 6 well tissue culture plates. After 24 h, the cells were treated with either ZLJ33, sorafenib, or 0.1% DMSO, or were left untreated. Cells were incubated in 5% CO_2 at 37 °C for 14 days, and colonies were washed, fixed and stained with 0.005% crystal violet in methanol. Numbers of colonies were manually counted. Experiments were performed in triplicate and repeated thrice.

Apoptosis analysis

Cells were treated with either ZLJ33, sorafenib, or 0.1% DMSO, or were left untreated for 96 h, washed in PBS, and fixed with ice-cold 70% ethanol overnight. Cells were then suspended in PBS containing RNase A (100 μg/mL), propidium iodide (50 μg/mL) and 0.1% Triton X-100, and incubated in the dark for at least 1 h [20]. Fluorescence-activated cell sorting (FACS) was performed to quantitate the apoptotic population based on DNA levels.

2.7 In vitro invasion assay

Invasion was determined using a variation of the Boyden chamber assay, as described in [21]. Briefly, 1×10^6 cells (Mia-PaCa2 and Capan2 cells treated with DMSO or ZLJ33, respectively) were seeded into the upper compartment (Costar). Each polycarbonate filter had been coated with 10 μL of 0.5% Matrigel before the addition of cells. After 18 h of incubation at 37 °C in 5% CO_2, the cells on the underside of the chamber were fixed, stained (HE stain), and photographed. The five visual fields were photographed in every membrane, with manual counting of nuclear-stained cells. All samples were run in triplicate.

Tumor implantation and growth in Mia-PaCa2, Capan2 and AsPC-1 xenografts

All animal studies were in compliance with policies of the Institute of Materia Medica Animal Care and Use Committee. Six-week-old female BALB/c/nu nude mice (Vital River Laboratories (VRL)) were used. Ten million Capan2, Mia-PaCa2 and AsPC-1 cells were subcutaneously implanted in the left flank of each mouse. When tumors grew up to an average volume of 100 ~ 250 mm^3, tumor-bearing mice were randomly separated into four groups of 5 animals (Mia-PaCa2 xenograft model) or three groups of 5 animals (Capan2 xenograft model) or five groups of 5 animals (AsPC-1 xenograft model). One group orally received 1 × solution with cremophor EL/ethanol/water (12.5:12.5:75) as a vehicle control; other groups received oral dose of 30 mg/kg of sorafenib, 7.5 mg/kg, and 15.0 mg/kg of ZLJ33 for six days/week respectively (Mia-PaCa2 xenograft model) or 15.0 mg/kg of ZLJ33 for six days/week (Capan2 xenograft model) or 3.75 mg/kg, 7.5 mg/kg and 15.0 mg/kg of ZLJ33 six days/week (AsPC-1 xenograft model); mice were euthanized at the end of the treatment period. Tumor size was measured with vernier calipers, and tumor volume (length × $width^2/2$) was calculated twice a week. Tumors were excised, weighed, and photographed. A portion of each tumor was kept in 4% paraffin for IHC analysis, and the remaining tissues were stored at −80 °C for Western blot analysis and future other analyses.

Western blot analysis

Lysates were prepared by washing cells with PBS then following the methods described in Ref [22]. Lysates of nuclear were prepared according to the protocol of Nuclear and Cytoplasmic Extraction Kit (Beijing ComWin Biotech Co., Ltd.). For Western blot, samples transferred to a nitrocellulose membrane by semi-wet electrophoresis were incubated with dedicated primary antibody (CST) overnight at 4 °C. The samples were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz), and developed using an ECL Western blot detection and analysis system (Applygen Technologies Inc.). Membranes were tested for equal loading by probing for β-Actin.

Immunohistochemistry

Immunohistochemical staining was done using 4-μm formalin-fixed, paraffin-embedded tissue sections. Antigen retrieval was carried out in citrate buffer (10 mmol/L, pH 6.0) for 15 mins at 100 °C in a microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for 10 mins. The sections were then washed in water and preblocked with normal horse serum for 30 mins. The slides were incubated with a primary rabbit anti-PCNA, rabbit anti-p-ERK, and rabbit anti-β-catenin (CST) overnight at 4 °C. Sections were then incubated with biotinylated secondary anti-rabbit IgG (Santa Cruz) for 1 h. After washing with 1 × TTBS, sections were incubated with Vectastain ABC reagent (Santa Cruz). The immune complex was visualized using DAB substrate solution (Santa Cruz). Each section was examined under a magnification of 100× and 200×, and analyzed by a computer image analysis system. The quantitation of PCNA, p-ERK, and β-catenin saw the description in Huang et al. [23].

Statistical analyses

Data were expressed as mean ± standard deviation. Statistical analysis to test the differences was performed using Student's t-test. P-values were considered as significant as it was less than or equal to 0.05.

Results

Inhibition of ZLJ33 on kinases

The inhibitory activities of ZLJ33 were analyzed by performing Caliper and ADP-Glo assays on a panel of kinases. The results showed that kinases' activities related with oncogenes could be blocked