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The novel mTORC1/2 dual inhibitor INK-128 suppresses survival and proliferation of primary and transformed human pancreatic cancer cells

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ABSTRACT

Pancreatic cancer has one of worst prognosis among all human malignancies around the world, the development of novel and more efficient anti-cancer agents against this disease is urgent. In the current study, we tested the potential effect of INK-128, a novel mammalian target of rapamycin (mTOR) complex 1 and 2 (mTORC1/2) dual inhibitor, against pancreatic cancer cells *in vitro*. Our results demonstrated that INK-128 concentration- and time-dependently inhibited the survival and growth of pancreatic cancer cells (both primary cells and transformed cells). INK-128 induced pancreatic cancer cell apoptosis and necrosis simultaneously. Further, INK-128 dramatically inhibited phosphorylation of 4E-binding protein 1 (4E-BP1), ribosomal S6 kinase 1 (S6K1) and Akt at Ser 473 in pancreatic cancer cells. Meanwhile, it downregulated cyclin D1 expression and caused cell cycle arrest. Finally, we found that a low concentration of INK-128 significantly increased the sensitivity of pancreatic cancer cells to gemcitabine. Together, our *in vitro* results suggest that INK-128 might be further investigated as a novel anti-cancer agent or chemo-adjuvant for pancreatic cancer treatment.

1. Introduction

Pancreatic cancer has one of worst prognosis among all human malignancies around the world [1]. It is commonly diagnosed at an advanced stage with local infiltration or distant metastasis, when surgery is no longer able to remove the entire tumor [2–4]. The current standard therapies for pancreatic cancer include chemotherapy (gemcitabine) and/or radiation [2–4]. However, pancreatic cancer is among the most intrinsically resistant cancers to both radiation and many anti-cancer drugs [2–4]. Hence, the development of novel and more efficient agents against pancreatic cancer is extremely important and urgent [2,3,5].

The phosphoinositide 3-kinase (PI3K)/Akt pathway is frequently over-activated in human malignancies [6–9] (i.e. pancreatic cancer [10,11]) by a variety of genetic and epigenetic events. This pathway contributes to many of the hallmarks of pancreatic cancer [10,11]. As a result, a large array of agents targeting this pathway are currently undergoing clinical testing [10,11]. Mammalian target of rapamycin (mTOR) is the key player in PI3K/Akt signaling which controls cancer cell growth, proliferation, survival and apoptosis resistance [12–15].

mTOR is found in two structurally and functionally distinct multi-protein complexes termed as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [14,16–18]. These two complexes have different subunits composition, downstream substrates as well as biological effects [13–15]. mTORC1 is composed of Raptor, mLST8, PRAS40 and mTOR, while mTORC2 consists of Rictor, mSIN1, mLST8 and mTOR [13–15]. 4E-binding protein 1 (4E-BP1) and ribosomal S6 kinase 1 (S6K1) are the best-known downstream effectors of mTORC1, while mTORC2 activity is required for Akt phosphorylation at Ser 473 [13–15]. Over the past few years, a number of inhibitors of the mTOR pathway have been developed

Abbreviations: MTT, 3-[4,5-Dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; FACS, fluorescence-activated cell sorting; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; 4E-BP1, 4E-binding protein; OD, optical density; IP, immunoprecipitation; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; S6K1, S6 kinase 1; PBMNCs, peripheral blood mononuclear cells.

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in pharmaceutical companies and in academia [8,17,19,20]. The first generation of mTOR inhibitors including rapamycin and its analogs (rapalogs) only block the mTORC1 activity [8,17,19,20]. The second generation of mTOR inhibitors, or the ATP-competitive mTOR kinase inhibitors, interfere both mTORC1 and mTORC2 activities [17,19,21].

INK-128 is a novel second generation mTOR inhibitor, which inhibits mTORC1 and mTORC2 activity simultaneously with a low IC-50 [22]. A phase I clinical trial has been performed to test its efficiency in advanced solid tumors [21]. In the current study, we investigated the potential role of INK-128 against pancreatic cancer cells *in vitro*.

2. Materials and methods

2.1. Chemical and reagents

INK-128 was obtained from Selleck (Shanghai, China). Anti-Erk1/2, Akt1, S6K1, 4E-BP1, mTOR, Raptor, mSIN1, Rictor and cyclin D1, as well as rabbit and mouse horseradish peroxidase (HRP)-conjugated IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other kinase antibodies used in this study were obtained from Cell Signaling Technology (Shanghai, China). Z-VAD-fmk was purchased from Calbiochem (CA, USA). Necrostatin-1 was obtained from Sigma (Shanghai, China).

2.2. Cell culture

PANC-1 and MIA PaCa-2 human pancreatic cancer cells, purchased from Shanghai Biological Institute, were maintained in a RPMI-1640 medium (Invitrogen, Shanghai, China), supplemented with a 10% fetal bovine serum (FBS, Sigma, Shanghai, China), penicillin/streptomycin (1:100, Sigma, Shanghai, China) and 4 mM L-glutamine (Sigma), in a CO_2 incubator at 37 °C. Unless otherwise noted, experiments were conducted in 1% FBS media without antibiotics.

2.3. Primary human pancreatic adenocarcinoma cells isolation and culture

Similar to previously reported [23], pancreatic adenocarcinoma tissues from three early-stage patients (male, 35/41/44, at their early stages) hospitalized at Department of Hepatopancreatobiliary Surgery, First People's Hospital of Hangzhou were obtained at the time of surgery. The fresh pancreatic adenocarcinoma tissues were thoroughly washed in phosphate buffer solution (PBS) containing 200 units/ml penicillin–streptomycin and 1 mM Dithiothreitol (DTT) (Sigma) to remove debris, and then minced by scalpel into small pieces in high glucose DMEM containing 200 units/ml penicillin–streptomycin. Pancreatic cancer cell pellets were thoroughly washed, then re-pelleted at 500 g for 5 min. Single-cell suspensions were achieved by re-suspending cells in 0.05% (w/v) collagenase-I dissolved in DMEM and incubating the suspension at 37 °C and 5% CO_2. After 1 h, individual cells were pelleted and rinsed twice with DMEM before re-suspending the cell pellets in cell culture medium (DMEM, 20% FBS, 2 mM glutamine, 1 mM pyruvate, 10 mM HEPES, 100 units/ml penicillin/streptomycin, 0.1 mg/ml gentamicin, and 2 g/liter fungizone). Primary cells were cultured in culture medium for 6–7 passages. Fresh peripheral blood mononuclear cells (PBMNCs) from same patients were collected and separated by Ficoll–Hypaque density sedimentation as previously reported [24], the cells were then cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G and 100 μg/mL streptomycin. The study was approved by the institutional review board of all authors' institutions, the written informed consent was obtained from each patient enrolled. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

2.4. Cell survival assay

Pancreatic cancer cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay [25]. Briefly, after treatment, twenty micro-liter (20 μl) of MTT tetrazolium salt (Sigma, St. Louis, MO) dissolved in PBS at a concentration of 5 mg/ml was added to cancer cells, and incubated in CO_2 incubator for 3 h. The medium was then aspirated very carefully, and 150 μl of dimethyl sulfoxide (DMSO)/well was then added to dissolve formazan crystals, the absorbance of each well was obtained using plate reader at a test wavelength of 490 nm with a reference wavelength of 630 nm. Optical density (OD) was utilized as the indicator of cell survival.

2.5. Trypan blue staining assay

Dead pancreatic cancer cells after treatment were stained by trypan blue, and were counted based on the protocol from Lonza (Shanghai, China). Non-viable cells will be blue, viable cells will be unstained. The percentage (%) of dead cells was calculated by the number of the trypan blue stained cells divided by the total cell number.

2.6. Annexin V/propidium iodide (PI) fluorescence-activated cell sorting (FACS)

After treatment, cells were collected by trypsinization, and the concentration of cells was adjusted to $\sim 1 \times 10^6$ cells/ml. Cells were washed with ice-cold DMEM and were centrifuged to collect the cell pellet, which was resuspended in ice-cold binding buffer. Afterward, Annexin V-FITC (10 μl/ml, Beyotime, Shanghai, China) and propidium iodide (PI) (10 μl/ml, Beyotime) were added to the cell suspension and mixed gently. The tube was then incubated for 15 min in the dark before being analyzed by fluorescence-activated cell sorting (FACS) (BD, Shanghai, China). The Annexin $V^{+/+}$/$PI^{-/-}$ cells plus Annexin $V^{+/+}$/$PI^{+/+}$ cells were detected as apoptotic cells. Annexin $V^{-/-}$/$PI^{+/+}$ cells were labeled as necrotic cells.

2.7. FACS analysis of cell cycle distribution

The cell suspension was prepared by trypsinization, and $\sim 1 \times 10^6$ cells/ml were washed twice with PBS. The cells were resuspended with 10 ml of 70% ethanol (−20 °C), incubated at 4 °C for 4 h, washed twice in cold PBS, incubated with RNase (Sigma) at a concentration of 0.25 mg/ml at 37 °C for 15 min, followed by treatment with PI (10 μl/ml), and incubated for 15 min at 4 °C in the dark. DNA histograms were analyzed using same FACS machine to evaluate the cell cycle distribution.

2.8. BrdU incorporation assay

Pancreatic cancer cells were seeded at a density of 2×10^5 cells/well in 1 ml RPMI containing 10% FBS into the 12-well tissue culture plates, cells were then exposed to indicated concentration of INK-128 for 48 h. Cell proliferation was assessed by BrdU incorporation though BrdU enzyme linked immunosorbent assay (ELISA) colorimetric assay (Roche, Indianapolis, IN) according to the manufacturer's recommendations. The ELISA OD value of treatment group (indicator of cell proliferation ability) was normalized to that of the control group.