Biochemical and Biophysical Research Communications 450 (2014) 973–978

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 12 June 2014
Available online 24 June 2014

Keywords:
Pancreatic cancer
INK-128
Akt/mTOR signaling
Apoptosis and gemcitabine sensitization

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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http://dx.doi.org/10.1016/j.bbrc.2014.06.081
0006-291X/

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.

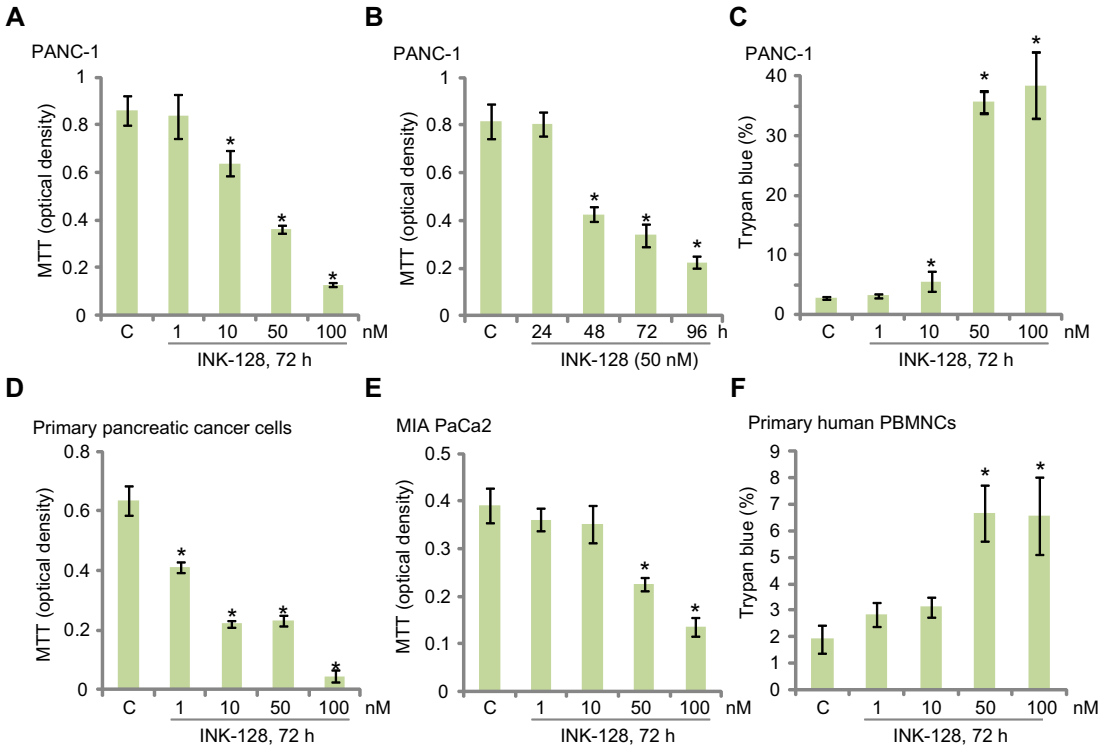

Fig. 1. INK-128 inhibits pancreatic cancer cell survival PANC-1 cell viability after indicated INK-128 treatment was tested by MTT assay (A and B), trypan blue staining was utilized to test PANC-1 cell death (C). Cell viability of primary (D) or transformed (MIA PaCa-2) (E) pancreatic cancer cells after indicated INK-128 treatment was tested by MTT assay. Trypan blue staining results of cell death after indicated INK-128 treatment in primary human PBMNCs were shown (F). Experiments in this figure were repeated three times with similar results obtained. "C" stands for untreated control group. $^{*}p < 0.05$ vs. "C" group.

2.9. Western blotting

The cells were washed with ice-cold PBS before lysed with the lysis buffer (Beyotime, Shanghai, China). The lysates (30 μg/sample) were separated by the 10% SDS-polyacrylamide gel, and were electro-transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Shanghai, China). The membranes were blocked with 10% milk in PBS plus Tween-20 (0.5%) (TBST), incubated overnight at 4 °C with indicated primary antibody, and then incubated with HRP-conjugated secondary antibody. The detection was performed by Supersignal West Pico Enhanced Chemiluminescent (ECL, Pierce, Rockville, IL). The blot intensity was quantified by Image J software. The intensity of each indicated band was normalized to that of non-phosphorylated loading controlling band. The number was expressed as fold change vs. the control band, set as "1.00".

2.10. Immunoprecipitation (IP)

After treatment, PANC-1 cell lysates (1000 μg) in 1 mL IP lysis buffer (Beyotime, Shanghai, China) were pre-cleared with 20 μl of protein A/G-agarose (Sigma) for 30 min. After centrifugation for 10 min at 4 °C in a micro-centrifuge, the supernatants were rotated overnight with 2 μg of indicated primary antibody (mTOR, Santa Cruz). Samples were then centrifuged for 5 min at 4 °C in a micro-centrifuge to remove nonspecific aggregates that formed overnight. Protein A/G-agarose (35 μl/sample) were then added to the supernatants for 3 h at 4 °C. Pellets were washed six times with PBS, resuspended in the lysis buffer, and then assayed in Western blotting to test targeted protein–protein binding.

2.11. Statistical analyses

For all experiments, the time point was chosen based on pre-experiment results where the most significant effect was detected. The data were expressed as means ± S.D. Statistical differences were analyzed by one-way *ANOVA* followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 16). Values of $p < 0.05$ were considered statistically significant. The significance of the difference between two specific groups was tested using paired-samples t test when needed.

3. Results

3.1. INK-128 inhibits pancreatic cancer cell survival

We examined the potential role of INK-128 against pancreatic cancer cells. MTT cell viability assay results in Fig. 1A demonstrated that INK-128 dose-dependently inhibited PANC-1 (human pancreatic cancer cell line) cell survival. INK-128 at concentration of 10–100 nM significantly reduced PANC-1 cell viability (Fig. 1A). Meanwhile, we detected a time-dependent inhibitory effect of INK-128 on PANC-1 cell survival (Fig. 1B), and no significant viability decrease was detected until 48 h after INK-128 treatment (Fig. 1B). Further, INK-128 induced dramatic PANC-1 cell death, as the percentage of trypan blue positive ("dead") PANC-1 cells increased after INK-128 (10–100 nM) treatment (Fig. 1C). INK-128 also dose-dependently inhibited the survival of primary (Fig. 1D) and another transformed (MIA PaCa-2) (Fig. 1E) pancreatic cancer cells. We also tested the effect of INK-128 on primary PBMNCs of same cancer patients. Trypan blue staining results showed that same concentrations of INK-128 (1–100 nM) only induced minor PBMNC death (less than 6–7%), indicating the specific inhibitory effect of INK-128 in cancer cells (Fig. 1F). Together, these results confirm the cytotoxic effect of INK-128 against pancreatic cancer cells.

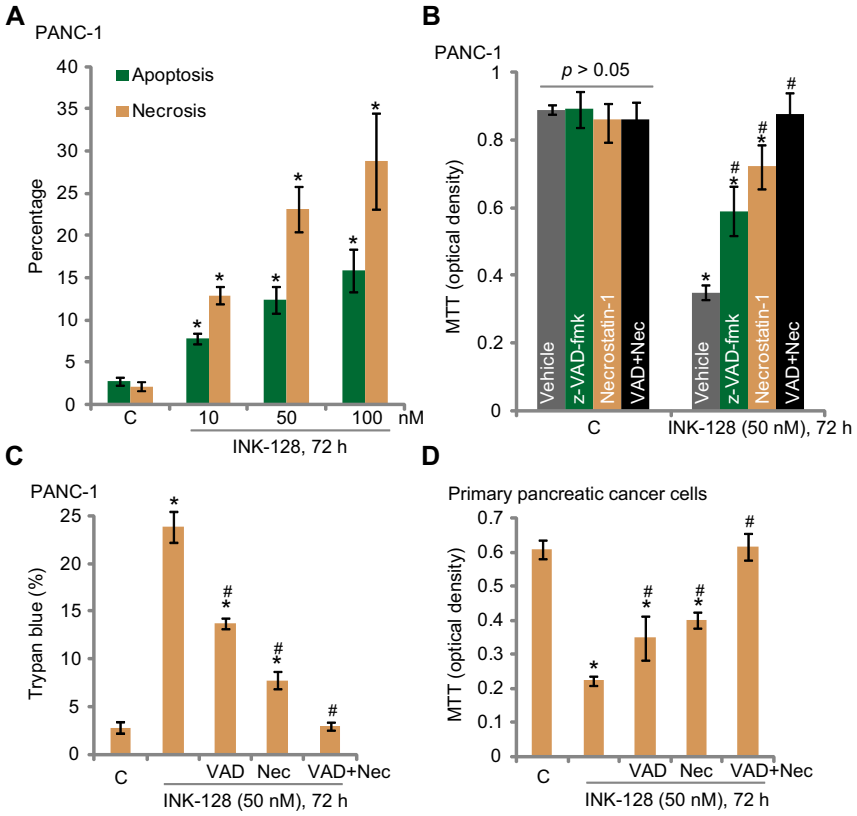

Fig. 2. Both apoptosis and necrosis attribute to INK-128-induced cytotoxicity in pancreatic cancer cells PANC-1 cells treated with indicated INK-128 were analyzed by Annexin V/PI FACS assay (A). Primary or PANC-1 pancreatic cancer cells were pre-treated with z-VAD-fmk (VAD, 50 μM), necrostatin-1(Nec, 10 μM), or both (VAD + Nec) for 1 h, followed by indicated INK-128 stimulation, cell viability (B and D) and cell death (C) were tested. Experiments in this figure were repeated three times with similar results obtained. "C" stands for untreated control group. Vehicle stands for 0.1% DMSO (B). $^{*}p < 0.05$ vs. "C" group. $^{\#}p < 0.05$ vs. INK-128 only group.

3.2. Both apoptosis and necrosis attribute to INK-128-induced cytotoxicity in pancreatic cancer cells

The results in Fig. 1 have confirmed the cytotoxic effect of INK-128 in primary and transformed pancreatic cancer cells. Next, we tested the involvement of apoptosis and necrosis in the process. Annexin V/PI staining was applied. Results in Fig. 2A showed that INK-128 dose-dependently induced apoptosis (Annexin $V^{+/+}$) and necrosis (Annexin $V^{-/-}/PI^{+/+}$) simultaneously in PANC-1 cells. The apoptosis inhibitor z-VAD-fmk as well as the necrosis inhibitor necrostatin-1 alleviated INK-128-induced PANC-1 cell viability decrease (Fig. 2B), while combination of z-VAD-fmk and necrostatin-1 almost blocked INK-128's effect (Fig. 2B). Meanwhile, both necrostatin-1 and z-VAD-fmk attenuated INK-128-induced PANC-1 cell death, and combination of the two almost blocked INK-128-induced cytotoxicity (Fig. 2C). Results in Fig. 2D demonstrated that necrostatin-1 and z-VAD-fmk inhibited INK-128-induced viability decrease in primary pancreatic cancer cells, and combination of the two again showed the additive effect. These results indicate that INK-128 induces both apoptotic and necrotic cell death in pancreatic cancer cells.

3.3. INK-128 inhibits Akt-mTORC1/2 activation in pancreatic cancer cells

As discussed, over-activation of Akt/mTOR signaling is one of the most frequent occurrences in pancreatic cancer, and this pathway is an important drug target for interfere of this malignancy [10,11]. INK-128 is a novel mTORC1 and 2 dual inhibitor [22], thus we examined the activation of Akt/mTOR signaling in INK-128-treated pancreatic cancer cells. Western blotting results in Fig. 3A showed that INK-128 dramatically inhibited phosphorylation of 4E-BP1-S6K1 (mTORC1 activation indicators) and Akt at Ser 473 (the mTORC2 activation indicator) [26] in both PANC-1 and primary pancreatic cancer cells. Phosphorylation of Erk1/2, an indicator of mitogen-activated protein kinase (MAPK) activation, was not affected by INK-128. Meanwhile, IP results in PANC-1 cells showed that assembly of mTORC1 (mTOR-raptor) and mTORC2 (mTOR-mSIN1-Rictor) was also disrupted by INK-128 (Fig. 3B). Note that the expression of above mTORC1/2 components was not affected by INK-128 (Fig. 3B, Input). Activation of mTOR induces the mRNA transcription of a number of key oncogenic proteins, i.e. cyclin D1 [27,28], the latter is a transcription factor that plays a central role in cell cycle progression. In our study, we found that INK-128 caused cyclin D1 downregulation in both PANC-1 cells and primary cells (Fig. 3A and B). As a result, PANC-1 cell cycle progression (Fig. 3C) and cell proliferation (Fig. 3D) were also disrupted. Results in Fig. 3E showed that INK-128 inhibited proliferation of primary pancreatic cancer cells.

3.4. INK-128 increases gemcitabine sensitivity in cultured pancreatic cancer cells

Akt/mTOR activation is an important gemcitabine resistance factor in pancreatic cancer [29,30], thus we tested whether INK-128 could affect the sensitivity of gemcitabine in cultured pancreatic cancer cells. The MTT viability results in Fig. 4A showed that gemcitabine at concentrations of 0.25–1 μM only slightly inhibited PANC-1 cell survival. However, co-treatment with INK-128 significantly increased gemcitabine's sensitivity, as more than 85% of cells were non-viable after INK-128/gemcitabine co-administration (Fig. 4A). Note that we utilized a relative low concentration of INK-128 (10 nM) to achieve the significant sensitization effect.

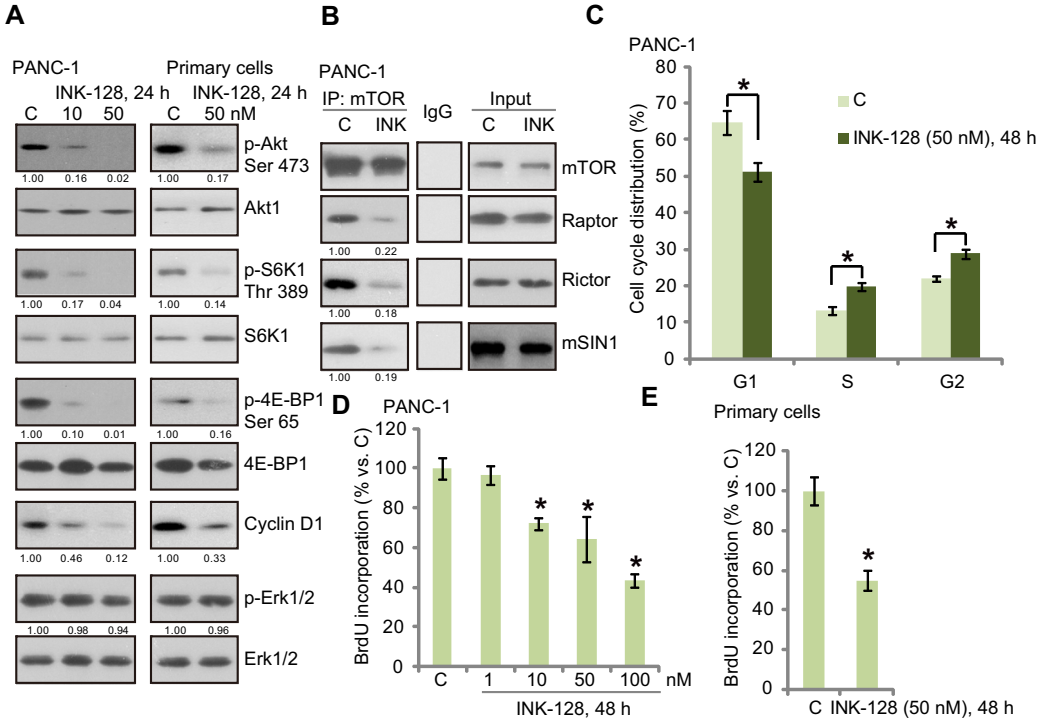

Fig. 3. INK-128 inhibits Akt-mTORC1/2 activation in pancreatic cancer cells PANC-1 and primary cultured pancreatic cancer cells were treated indicated INK-128, expression of indicated proteins was tested by Western blotting (A) p-Akt/S6K1/4E-BP1 and total cyclin D1 expression were quantified (A). mTOR-mSIN1-Raptor-Rictor association in PANC-1 cells was tested by IP (B), and input loadings were tested by Western blotting (B, input), mTOR bound Raptor, Rictor and mSIN1 was quantified (C). PANC-1 cell cycle distribution after indicated INK-128 treatment was shown (C). PANC-1 (D) and primary pancreatic cancer cells (E) were treated with indicated INK-128 for 48 h, cell proliferation was analyzed by BrdU incorporation assay. Experiments in this figure were repeated three times with similar results obtained. "C" stands for untreated control group. $^{*}p < 0.05$ vs. "C" group.

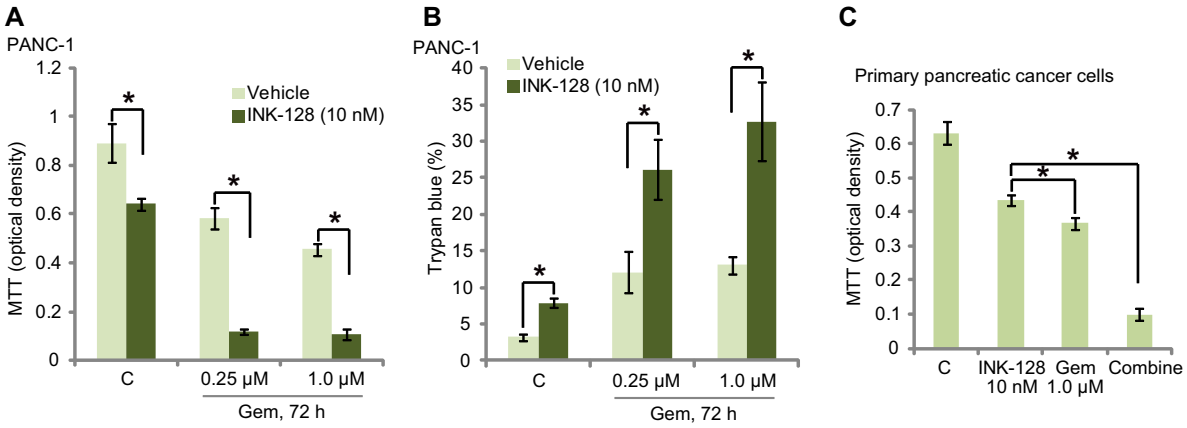

Fig. 4. INK-128 increases gemcitabine sensitivity in cultured pancreatic cancer cells PANC-1 and primary cultured pancreatic cancer cells were treated with indicated gemcitabine (Gem, 0.25/1.0 μM), in the presence or absence of INK-128 (10 nM) for 72 h, cell viability and cell death were tested by MTT assay (A and C) and trypan blue staining assay (B), respectively. "C" stands for untreated control group. Vehicle stands for 0.1% DMSO (A–B). $^{*}p < 0.05$ (*ANOVA*).

The trypan blue staining assay further confirmed the gemcitabine-sensitivity effect by INK-128 in PANC-1 cells (Fig. 4B). In primary pancreatic cancer cells, INK-128 and gemcitabine co-administration again demonstrated a synergistic efficiency in inhibiting cancer cell survival (Fig. 4C). Thus, INK-128 increases gemcitabine sensitivity in pancreatic cancer cells.

4. Discussions

Pancreatic cancer is one of the leading causes of cancer-related mortality in the US and around the world [1]. Five-year survival is less than 5%, mainly because it has a propensity to invade surrounding tissue and organs, which prevents surgical resection in majority pancreatic cancer patients [2,3,11]. Most pancreatic cancers are resistant to conventional chemotherapies (i.e. gemcitabine) due to constitutively active factors that stimulate growth and inhibit apoptosis. mTOR pathway is one of the major pathways modulating cell growth, proliferation, survival, and apoptosis [14,18,31,32]. Hyper-activation or over-expression of this pathway is one of the most frequent occurrences in pancreatic cancer [10,11]. In the current study, we found that INK-128 inhibited Akt-mTOR activation, and induced apoptosis as well as necrosis in primary and transformed human pancreatic cancers. Further, INK-128 inhibited cyclin D1 degradation, disrupted pancreatic cancer cell cycle progression and cell proliferation.

Meanwhile, a low concentration of INK-128 significantly increased the sensitivity of pancreatic cancer cells to gemcitabine.

mTORC1 and mTORC2 complexes are formed and regulated by different proteins and are also driven by multiple different compensatory feedback loops [33]. The cytotoxic activity and anti-proliferative property of first generation of mTOR Inhibitors (rapalogs) were limited [33]. Further, mTORC1 inhibition by rapalogs will result in a feedback activation of the PI3K-Akt pathway [34,35], and simultaneously increase of MAPK activity [36]. Whereas rapalogs exert their actions almost exclusively through mTORC1 inhibition, the second generation of inhibitors target the ATP site of mTOR kinase domain [17,21,33,37]. These compounds (i.e. AZD-8055, AZD-2014, INK-128, and OSI-027) are able to block simultaneously both complexes due to their catalytic activity over mTOR [17,21,33,37]. Their most important advantage of these inhibitors, besides exerting a more efficient mTORC1 inhibition, would be the mTORC2 blockade leading to significant decrease of Akt phosphorylation at Ser 473 [17,21,33,37]. Some of these inhibitors (including INK-128) have entered into phase I clinical trials [17,21,33,37]. Preclinical data with INK-128 have shown the important anti-proliferative activity against mice xenograft *in vivo*. In consistent with these studies, our results found that INK-128 blocked the phosphorylation of S6K1, 4E-BP1, and Akt at Ser 473 in primary and transformed human pancreatic cancer cells. While phosphorylation of Erk1/2, an indicator of MAPK activation, was not affected by INK-128.

Our *in vitro* results demonstrated that INK-128 significantly augmented the cytotoxicity of gemcitabine in both transformed and primary pancreatic cancer cell. Given the facts that gemcitabine is the only clinically approved anti-pancreatic cancer agent, and INK-128 shows low toxicity to normal tissues [22,38] (currently undergoing phase I clinical trial [17,21,33,37]), we propose that INK-128 could also synergize with gemcitabine against human pancreatic cancer.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgments

This research was supported by grants from the Program for Innovative Research Team in Zhejiang Province 2012R10046, Administration of Chinese Traditional Medicine of Zhejiang Province 2011ZB080, and Natural Science Foundation of Zhejiang Province LY12H16022.

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