Synergistic antitumor effects of CDK inhibitor SNS-032 and an oncolytic adenovirus co-expressing TRAIL and Smac in pancreatic cancer

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Abstract. Gene therapy using oncolytic adenoviruses is a novel approach for human cancer therapeutics. The current study aimed to investigate whether the combined use of an adenovirus expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and second mitochondria-derived activator of caspase (Smac) upon caspase activation (ZD55-TRAIL-IETD-Smac) and cyclin-dependent kinase (CDK) inhibitor SNS-032 will synergistically reinforce their anti-pancreatic cancer activities. The experiments *in vitro* demonstrated that SNS-032 enhances ZD55-TRAIL-IETD-Smac-induced apoptosis and causes marked pancreatic cancer cell death. Western blot assays suggested that the SNS-032 intensified ZD55-TRAIL-IETD-Smac-induced apoptosis of pancreatic cancer cells by affecting anti-apoptotic signaling elements, including CDK-2, CDK-9, Mcl-1 and XIAP. Additionally, animal experiments further confirmed that the combination of SNS-032 and ZD55-TRAIL-IETD-Smac significantly inhibited the growth of BxPC-3 pancreatic tumor xenografts. In conclusion, the present study demonstrated that SNS-032 sensitizes human pancreatic cancer cells to ZD55-TRAIL-IETD-Smac-induced cell death *in vitro* and *in vivo*. These findings indicate that combined treatment with SNS-032 and ZD55-TRAIL-IETD-Smac could represent a rational approach for anti-pancreatic cancer therapy.

Introduction

Pancreatic cancer is a fatal disease and remains consistently lethal with a 5-year survival rate of <5% (1). The disease generally causes few or no symptoms during early stages, thus the majority of patients are diagnosed at an advanced or terminal stage and few are suitable for curative surgical resection. Gemcitabine-based chemotherapy is the only option for treatment of advanced disease. Development of resistance to gemcitabine is a major concern. FLFIRINOX is an emerging treatment that offers a better survival outcome compared with gemcitabine. However, this chemotherapy regimen induces increased toxicity and exhibits only modest results (2,3). Thus, the development of novel methods for treating pancreatic cancer is urgently required.

Replication-selective oncolytic viruses (OVs) have emerged as promising therapeutic tools for cancer treatment with attractive advantages, including tumor-selective amplification and replication, thus inducing cancer cells lysis with minimal affect on normal tissues (4-9). Recently, >12 different oncolytic viruses are undergoing phase I-III clinical trials targeting different types of cancer (10). Oncolytic adenovirus is the first and most intensively investigated OV to date and H101 (a mutant with *E1B55K* gene deleted) is now authorized in China for treatment of head and neck cancer (Oncorine; Shanghai Sunway Biotech Co., Ltd., Shanghai, China) Our previous study developed a cancer-targeting dual-gene virotherapy (CTGVT-DG) strategy and generated a novel *E1B55K* gene deleted oncolytic adenovirus ZD55-TRAIL-IETD-Smac (ZD55-TIS) harboring tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and the second mitochondria-derived activator of caspase (Smac) genes joined by the four-amino acid isoleucine-aspartate-threonine-glutamate (IETD) linker (11). ZD55-TIS co-expressed TRAIL and Smac genes simultaneously and produced broad antitumor activity *in vitro*. Following intratumoral injection, the ZD55-TIS

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completely eradicated hepatocellar carcinoma cell xenograft tumors. However, the antitumor effects of ZD55-TIS against pancreatic cancer have not been evaluated.

The cyclin-dependent kinases (CDKs) are a family of serine/threonine kinases that control cell cycle events, and certain members are associated with transcriptional regulation. The tumor-specific deregulation makes the CDKs a major target for therapy (12,13). SNS-032 is a effective and selective inhibitor of CDK2, -7, and -9 (13). It has been reported that SNS-032 has antitumor activity in various tumors (14-17). Based on the report which suggesting that aberrant activation of CDKs and dysregulation of cell cycle progression is a feature of pancreatic cancer (18), the current study investigated whether SNS-032 is able to enhance the anticancer activity of ZD55-TRAIL-IETD-Smac against pancreatic carcinoma.

The present study examined whether the CDK inhibitor SNS-032 may enhance the antitumor activities of ZD55-TIS against pancreatic cancer. To the best of our knowledge, the present study is the first to demonstrate that ZD55-TRAIL-IETD-Smac co-operatively act on pancreatic cancer in vitro and in vivo for the first time. The present study indicated that combination therapy with ZD55-TRAIL-IETD-Smac and SNS-032 may be a practical novel strategy against pancreatic cancer in the future.

Materials and methods

Cell lines and viruses. Human pancreatic cancer cell lines PANC-1 and BxPC-3 and human embryonic kidney cell line HEK293 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated in a 5% CO_2 humidified incubator at 37°C.

Construction and production of recombinant oncolytic adenovirus ZD55-TIS were described previously (11). The amplification of recombinant adenovirus was performed by infecting HEK293 cells.

Cytotoxicity assay and quantitative analysis of synergy in vitro. SNS-032 (Selleck Chemicals, Houston, TX, USA) was prepared at 1 mg/ml in dimethyl sulfoxide, stored at -20°C, and then diluted as needed in cell culture medium. Cell viability was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, PANC-1 and BxPC-3 cells were plated in 96-well plates ($1x10^4$ density) in 100 μl culture medium. Cells were treated with different treatments (ZD55-TIS and SNS-032) at the indicated concentrations. After 48 or 72 h, MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) solution (10 μl, 5 g/l) was added to the cells which were then cultured for a further 4 h. Absorbance (570 nm) was measured using a DNA microplate reader (GENios model; Tecan, Maennedorf, Switzerland).

Hoechst 33342 staining assay. Hoechst 33342 staining was used to detect morphological features of cell apoptosis. PANC-1 cells were treated with SNS-032 or ZD55-TRAIL-IETD-Smac, or

the combination of SNS-032 and ZD55-TRAIL-IETD-Smac. After treatment for 72 h, 1 mg/ml (5 μl) Hochest 33342 (Sigma-Aldrich, Merck KGaA) was added in the cells for 30 min and morphology was observed under an inverted fluorescence microscope. Untreated cells served as a control.

Flow cytometry analysis. PANC-1 cells were treated with ZD55-TRAIL-IETD-Smac [8 multiplicity of infection (MOI)], SNS-032 (160 ng/ml), or ZD55-TRAIL-IETD-Smac (8 MOI) plus SNS-032 (160 ng/ml). After 48 h, apoptotic cells were detected by using Annexin V- fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining or PI staining alone following the manufacturer's instructiona. Cell apoptosis and cell cycle were examined using the FACStar cytofluorometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. PANC-1 cells were collected and washed twice with PBS, then lysed in radioimmunoprecipitation assay buffer (4°C, 30 min). Protein concentrations were determined by bicinchoninic acid assay (Thermo Fisher Scientific, Inc.). Protein samples (10 μg) were separated by 12% SDS polyacrylamide-gel and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% bovine serum albumin (cat. no. P0007; Beyotime Institute of Biotechnology, Haimen, China), in a 10 mmol/l Tris-HCl pH 8.0, 150 mmol/l NaCl and 0.05% Tween-20 buffer overnight at 4°C, and then incubated with the corresponding primary antibodies at 1:1,000 dilution overnight at 4°C. Following incubation with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature, signals were detected by enhanced chemiluminescence (ECL) with BeyoECL reagents (cat. no. P0018; Beyotime Institute of Biotechnology). Antibodies targeting Adenovirus-5 E1A (cat. no. sc-374663), caspase-3 (cat. no. sc-271759), poly (ADP-ribose) polymerase (PARP; cat. no. sc-56197), caspase-8 (cat. no. sc-166596) and GAPDH (cat. no. sc-47724) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies targeting CDK-2 (cat. no. 2546S), CDK-7 (cat. no. 2916), CDK-9 (cat. no. 2316S), BCL2 apoptosis regulator (Bcl-2; cat. no. 4223S), BCL2 family apoptosis regulator (Mcl-1; cat. no. 4572), X-linked inhibitor of apoptosis (XIAP; cat. no. 2042), TRAIL (cat. no. 3219S) and Smac (cat. no. 2954S) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The secondary antibodies (cat. nos. 70-GAR007 for anti-rabbit and 70-GAM007 for anti-mouse) were purchased from MultiSciences (Hangzhou, China).

Animal experiments. Animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and in accordance with institutional standards. Male BALB/C nude mice (32 total) aged 5 weeks were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Science (Shanghai, China). The mice were adapted to animal housing at 25°C. BxPC-3 cells ($7x10^6$ cells in 100 μl serum-free DMEM/mouse) were subcutaneously injected into the right flank of male nude mice. Mice were checked three times per week for tumor development. Once

subcutaneous tumors reached ~100-150 mm³, the nude mice were randomly divided into four groups (n=7 per group). Subsequently, mice were injected with ZD55-TIS, SNS-032, a combination of the virus and the drug, or PBS. ZD55-TIS ($1x10^9$ plaque-forming units per mouse) was delivered via an intratumor injection, while SNS-032 was intraperitoneally injected into the mice at a dose of 30 mg/kg body weight or 100 μl PBS as control by intratumor injection for three times of continuous injection once every other day. Tumor volume was measured with a vernier caliper every 4 days as calculated as follows: Tumor volume (mm³)=(AxB²)/2; A and B are the tumor length and width (in mm), respectively. The tumor volumes were used the produce tumor volume growth curves. The mice were sacrificed at 60 days post-tumor cell injection.

Hematoxylin and eosin (H&E) staining, immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. Tumor tissues were fixed in 4% paraformaldehyde and embedded in paraffin wax. The sections from tumors were stained with H&E for histological analysis. For immunohistochemistry, formalin-fixed, paraffin-embedded tissue sections were cut at a thickness of 4 μm. For heat-induced epitope retrieval, tissue sections were deparaffinized and rehydrated in 0.01 M pH 6.0 citrate buffer 3 times at 90°C for 5 min using a microwave oven. Immunohistochemical staining was performed using the streptavidin-biotin immunoperoxidase technique. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol for 15 min, and nonspecific immunoglobulin binding was blocked by incubation with 5% normal rabbit serum (cat. no. ZLI-9025; ZSGB-BIO Beijing, China) for 10 min. Sections were incubated at 4°C overnight with primary antibodies at 1:50 dilution. Antibodies targeting CDK-2 (cat. no. 2546S), CDK-7 (cat. no. 2916), and CDK-9 (cat. no. 2316S) were from Cell Signaling Technology, Inc., and antibodies targeting E1A (cat. no. sc-374663), TRAIL (cat. no. sc-4547), and Smac (cat. no. sc-136302) were from Santa Cruz Biotechnology, Inc. The sections were rinsed and incubated for 30 min at room temperature with biotinylated secondary antibody at 1:100 dilution (cat. no. PV8000; ZSGB-BIO). Following washing, the sections were incubated for another 30 min with horseradish peroxidase-conjugated streptavidin (cat. no. ZLI-9017; ZSGB-BIO), and finally treated with 3,3'-diaminobenzidine tetrahydrochloride as a substrate for 10 min. For the TUNEL assay, an *in situ* cell apoptosis detection kit (Roche Diagnostics, Indianapolis, IN, USA) was used, according to the manufacturer's instructions. PBS-treated tissue sections were used as a negative control. Hematoxylin was used as counterstain. Bright field microscopy was used to examine sections.

Statistical analysis. The experimental results are expressed as the mean ± standard deviation. Statistical significance was analyzed by GraphPad 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). For comparison of two groups, two-tailed unpaired *t*-test was used. For comparison of more than two groups, one-way analysis of variance followed by Tukey's post hoc test was used. $P<0.05$ was considered to indicate a statistically significant difference.
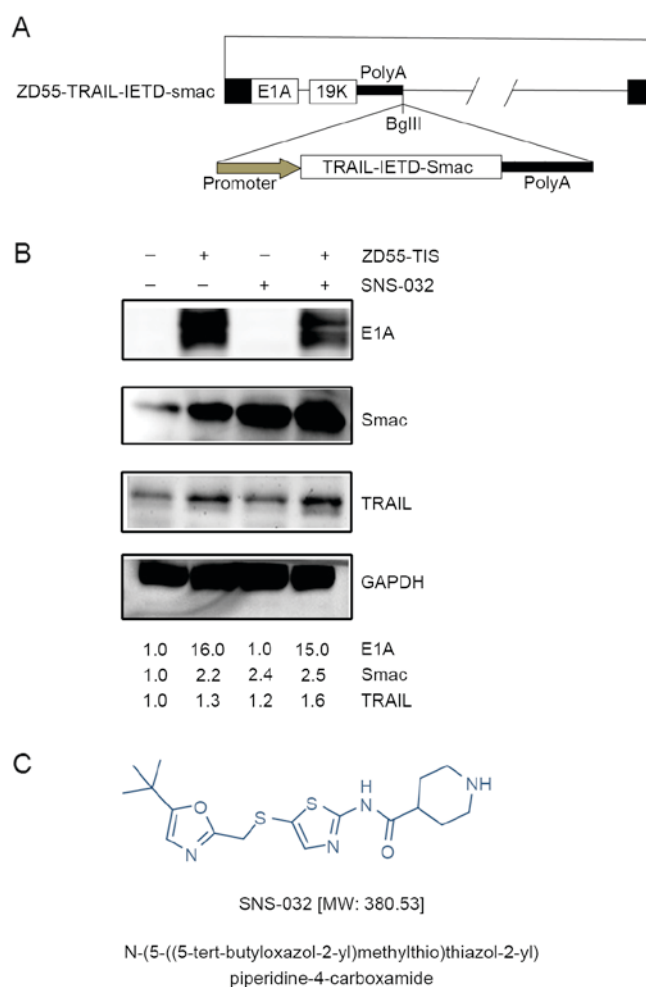

Figure 1. Characterization of oncolytic adenovirus ZD55-TIS and structure of SNS-032. (A) Schematic diagram of the oncolytic adenovirus ZD55-TIS. (B) Identification of recombinant adenovirus by western blot analysis. ZD55-TIS (8 MOI), SNS-032 (160 ng/ml), or ZD55-TIS plus SNS-032 was used to treat PANC-1 cells for 48 h. Cell proteins were collected to determine the expression of E1A, Smac and TRAIL. Untreated cells were used as a control group. Differences in protein expression level is semi-quantitatively determined by densitometry and expressed as a value relative to untreated cells. GAPDH was used as a protein loading control. (C) Schematic diagram of SNS-032 ($C_{17}H_{24}N_4O_2S_2$). ZD55-TIS, ZD55-TRAIL-IETD-Smac; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IETD, isoleucine-aspartate-threonine-glutamate; Smac, second mitochondria-derived activator of caspase; MW, molecular weight.

Results

SNS-032 enhances ZD55-TRAIL-IETD-Smac-mediated growth inhibition in pancreatic cancer cells. The oncolytic adenovirus vector, ZD55, was constructed by deleting the E1B 55-kDa gene of adenovirus 5, which can selectively replicate in various tumor cells. Based on the Cancer Targeting Gene-Viro-Therapy, ZD55 was used to package the dual therapeutic gene TRAIL and Smac linked via a caspase-8 cleavage site (IETD) to obtained the novel recombinant oncolytic adenovirus, ZD55-TRAIL-IETD-Smac (Fig. 1A). To validate the characterization of ZD55-TRAIL-IETD-Smac, western blot analysis was performed to detected the protein expression of the adenovirus E1A, and therapeutic genes TRAIL and Smac. The strong expression of E1A, TRAIL and Smac in PANC-1 cells infected with ZD55-TRAIL-IETD-Smac indicated that
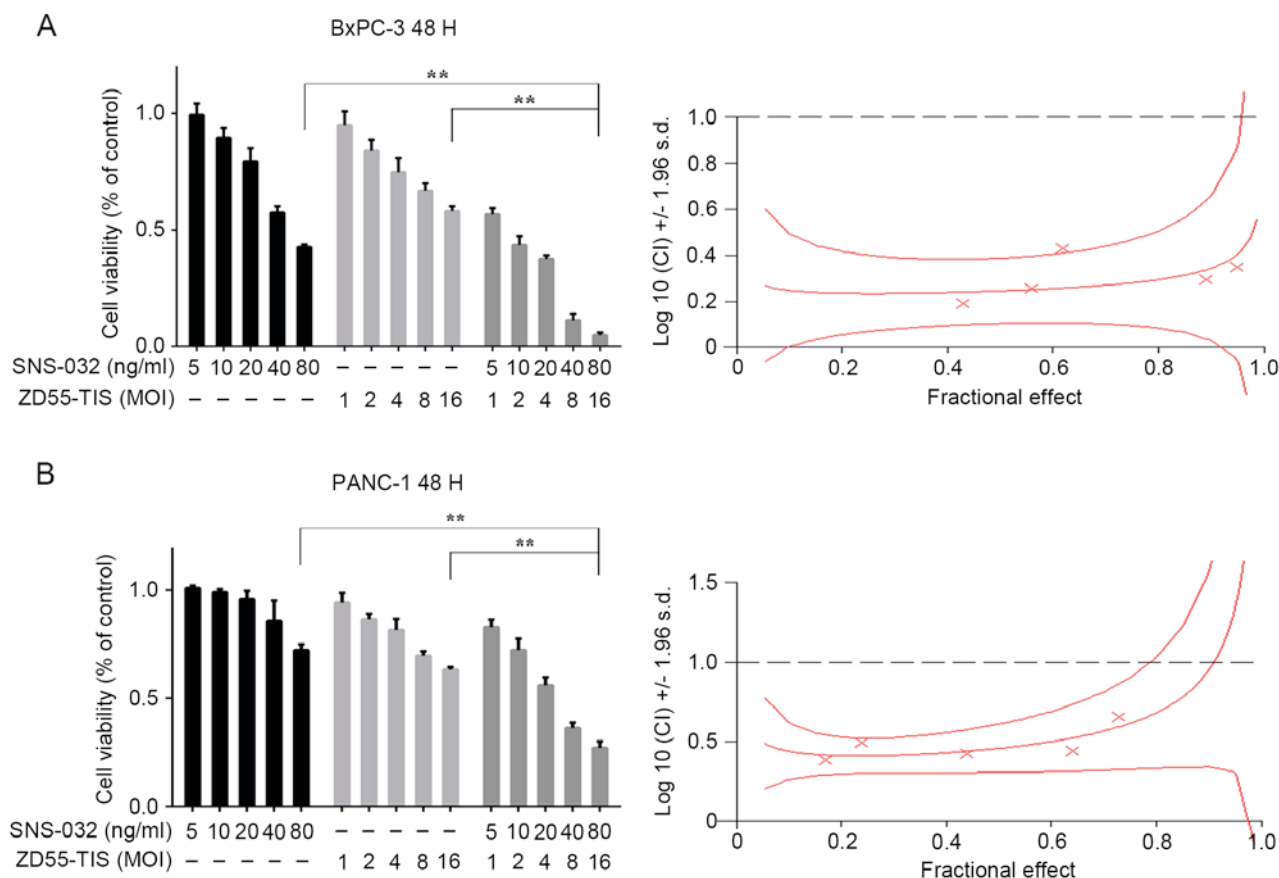
Figure 2. Combination of ZD55-TIS and SNS-032 enhances suppression of tumor cell proliferation. Pancreatic cancer cells (A) PANC-1 and (B) BxPC-3 were treated with ZD55-TIS (1, 2, 4, 8 and 16 MOI), SNS-032 (5, 10, 20, 40 and 80 ng/ml), or ZD55-TIS plus SNS-032 for 48 h. The image represents three independent experiments. Cell viability was evaluated by MTT assay and the synergistic effect of ZD55-TIS combined with SNS-032 on PANC-1 and BxPC-3 was quantified by CI analysis and expressed as log (CI) vs. fractional effect. Where calculable, 95% confidence intervals are shown. **P<0.05. ZD55-TIS, ZD55-TRAIL-IETD-Smac; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IETD, isoleucine-aspartate-threonine-glutamate; Smac, second mitochondria-derived activator of caspase; CI, combination index.

ZD55-TRAIL-IETD-Smac can selectively replicate at high levels in pancreatic cancer cells (Fig. 1B).

To assess whether the CDK inhibitor SNS-032 (Fig. 1C) enhances ZD55-TRAIL-IETD-Smac-induced cell death in pancreatic cancer cells, MTT assay was performed. PANC-1 and BxPC-3 pancreatic cancer cell lines were infected with ZD55-TRAIL-IETD-Smac and/or treated with SNS-032. The results suggest that the combination of ZD55-TRAIL-IETD-Smac and SNS-032 has an enhanced cytotoxic effect on pancreatic cancer cells compared with either ZD55-TRAIL-IETD-Smac or SNS-032 alone (Fig. 2A and B).

SNS-032 enhances ZD55-TRAIL-IETD-Smac-induced apoptosis and cell cycle arrest in pancreatic cancer cells. Subsequently, Hoechst 33342 staining was performed to observe the morphological alterations of PANC-1 cells treated with ZD55-TRAIL-IETD-Smac and/or SNS-032. The results in Fig. 3A demonstrated that compared with ZD55-TRAIL-IETD-Smac treatment alone, co-treatment with SNS-032 led to marked apoptosis characterized by chromatin condensation, nuclear fragmentation and apoptotic body formation.

Western blot analysis demonstrated that ZD55-TRAIL-IETD-Smac activated the caspase-dependent pathway, including activation of caspase-9 and caspase-3, and cleavage

of PARP, and this effect was further enhanced by co-treatment with SNS-032 and ZD55-TRAIL-IETD-Smac compared with either treatment alone (Fig. 3B).

To quantify the effects of SNS-032 on ZD55-TRAIL-IETD-Smac-induced apoptosis, Annexin V-FITC/PI double staining was used to analyze cell apoptosis (Fig. 3C). The results demonstrated that the apoptotic rate of PANC-1 cells co-treated with ZD55-TRAIL-IETD-Smac and SNS-032 was 40.93%, which was nearly 4-fold more than that of ZD55-TRAIL-IETD-Smac treatment alone (9.67%).

Furthermore, to determine whether the anti-proliferative effects of ZD55-TRAIL-IETD-Smac and SNS-032 may also cause cell cycle arrest, cell cycle analyses were performed on PANC-1 cells treated for 48 h. As demonstrated in Fig. 3D, when used individually, treatment with ZD55-TRAIL-IETD-Smac or SNS-032, 12.9 or 16.7% cells, respectively, were in the G2 cell cycle phase. However, treatment with a combination of ZD55-TRAIL-IETD-Smac and SNS-032 arrested a greater number of cells in the G2 phase (38.9%).

SNS-032 synergized the anti-pancreatic cancer effect of ZD55-TRAIL-IETD-Smac by changing the expression of cell apoptosis signaling elements. In order to further investigate the potential mechanism of the synergistic effect of SNS-032 combined with ZD55-TRAIL-IETD-Smac, CDK-2,
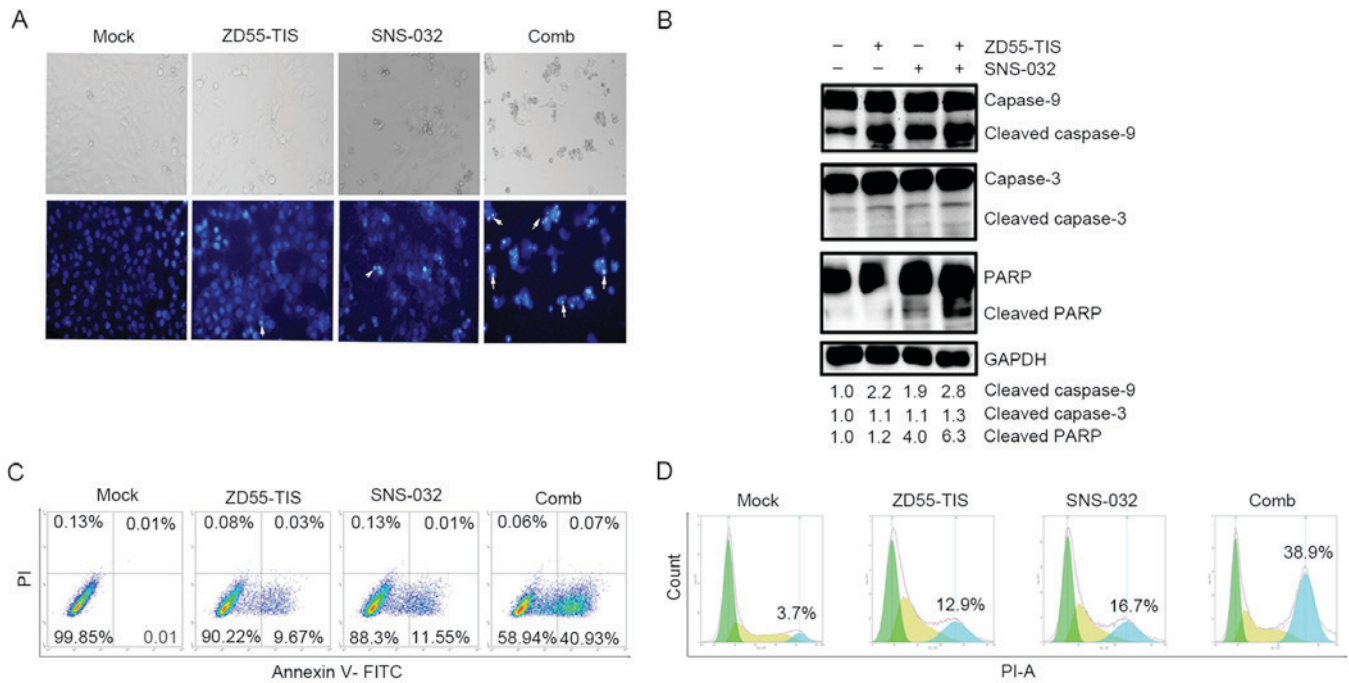
Figure 3. SNS-032 enhances ZD55-TIS-induced apoptosis and cell cycle arrest in pancreatic cancer cells. PANC-1 were infected with ZD55-TIS (8 MOI), treated with SNS-032 (160 ng/ml) or ZD55-TIS (8 MOI) plus SNS-032 (160 ng/ml). (A) After 72 h, PANC-1 cells were stained with Hoechst 33342 and observed under an inverted fluorescence microscope. White arrows indicate positive apoptotic cells. Original magnification, x200. (B) Western blotting was performed to detect cleaved caspase-9 and -3, and PARP. GAPDH was used as the loading control and the level in the untreated group was set as 1. At 48 h after treatment, (C) apoptosis and (D) the cell cycle were analyzed by flow cytometry and the % of cells in the G2 phase was calculated. Uninfected cells served as control. ZD55-TIS, ZD55-TRAIL-IETD-Smac; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IETD, isoleucine-aspartate-threonine-glutamate; Smac, second mitochondria-derived activator of caspase; Comb, combination. PI, propidium iodide; FITC, fluorescein isothiocyanate; PARP, poly(ADP-ribose) polymerase.
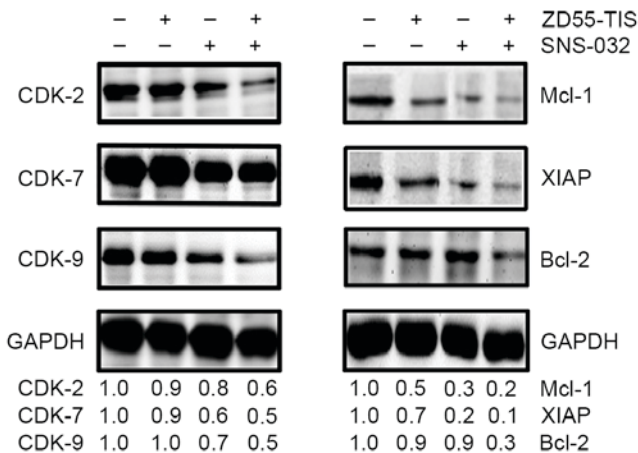

Figure 4. Effect of SNS-032 combined with ZD55-TIS on pro-apoptotic signaling elements in PANC-1 cells. PANC-1 cells were treated with ZD55-TIS (8 MOI) or/and SNS-032 (160 ng/ml). The combination of ZD55-TIS and SNS-032 downregulated the protein expression of CDK-2, -7, and -9, and XIAP, Mcl-1 and Bcl-2. ZD55-TIS, ZD55-TRAIL-IETD-Smac; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IETD, isoleucine-aspartate-threonine-glutamate; Smac, second mitochondria-derived activator of caspase; CDK, cyclin-dependent kinase; Mcl-1, Bcl-2 family apoptosis regulator; XIAP, X-linked inhibitor of apoptosis; Bcl-2, Bcl-2 apoptosis regulator.

-7 and -9, and other apoptosis-associated proteins (Mcl-1, XIAP and Bcl-2) were analyzed by western blotting. The results are presented in Fig. 4. Compared with the control, the expression levels of CDK-2, -7 and -9, and Mcl-1, XIAP

and Bcl-2 were downregulated by treatment with SNS-032 (160 ng/ml) or ZD55-TRAIL-IETD-Smac (8 MOI) treatment alone in PANC-1 cells. Downregulation of CDK-2, -7, and -9 were not notable when treated with ZD55-TRAIL-IETD-Smac alone, the effects were marked when combined treatment with SNS-032 (160 ng/ml) was performed. These results indicated that SNS-032 has the synergistic effect on ZD55-TRAIL-IETD-Smac-induced apoptosis in pancreatic cells by affecting anti-apoptotic regulators, including CDK-2, CDK-9, Mcl-1 and XIAP.

SNS-032 enhances ZD55-TRAIL-IETD-Smac-mediated pancreatic tumor growth suppression in vivo. To determine the therapeutic effects of combination treatment with SNS-032 and ZD55-TRAIL-IETD-Smac *in vivo*, animal experiments were performed using a pancreatic tumor xenograft model established using BxPC-3 cells. Compared with the PBS group, SNS-032 group or ZD55-TRAIL-IETD-Smac group, SNS-032 plus ZD55-TRAIL-IETD-Smac significantly suppressed tumor growth (P<0.0001 compared with ZD55-TRAIL-IETD-Smac; P<0.0001 compared with SNS-032) in Fig. 5A. The average tumor volume in the mice receiving combination therapy was 682 mm^3 at the end of the experiment (day 60). Whereas, the average volumes of mice injected with ZD55-TRAIL-IETD-Smac, SNS-032 and PBS were 1129.07 mm^3, 1394.18 mm^3 and 2805.36 mm^3, respectively (Fig. 5A). Additionally, all the tumors masses were removed for imaging and to compare the volume changes among different treatment groups (Fig. 5B).
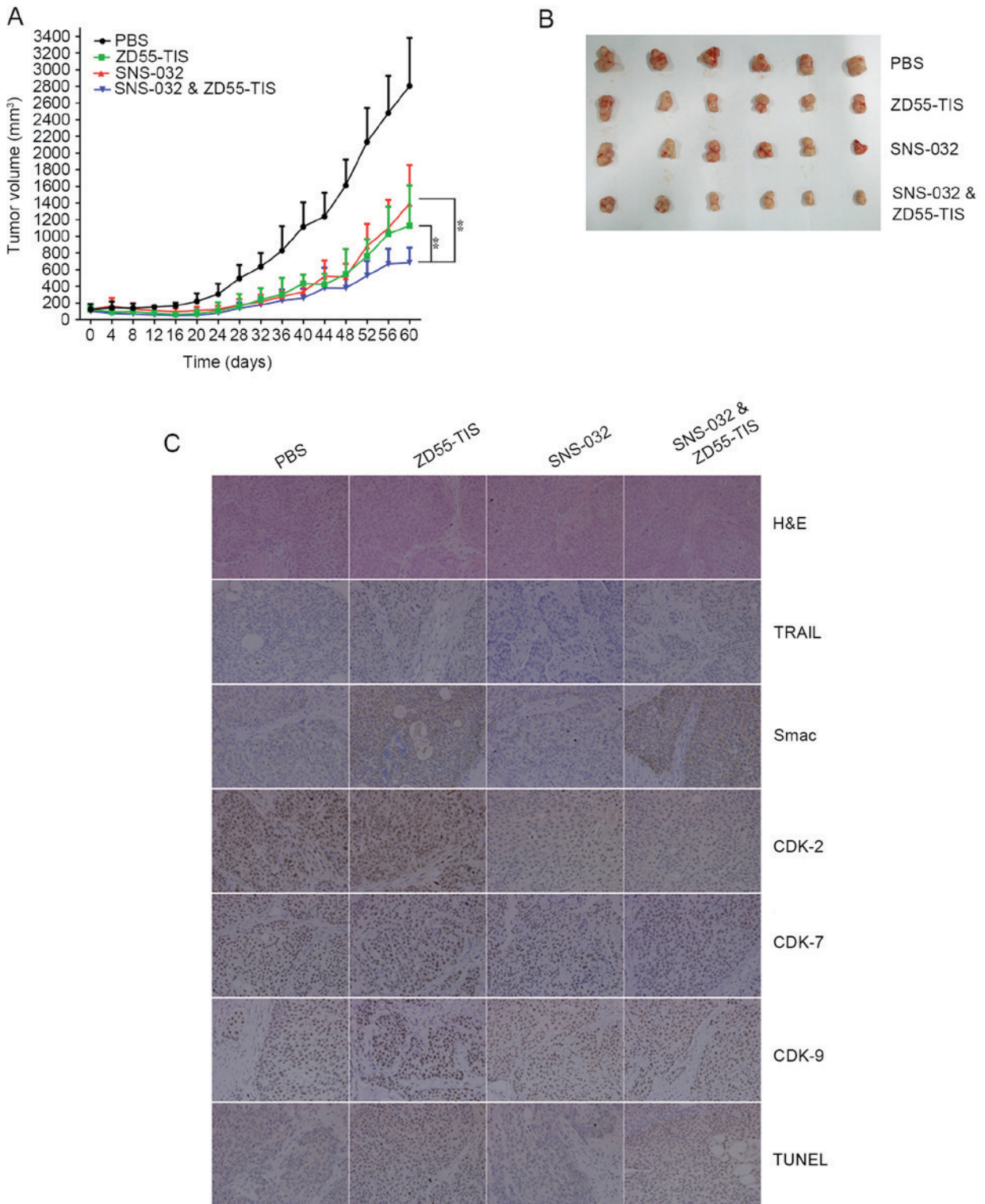
Figure 5. Synergistic effects of ZD55-TIS and SNS-032 *in vivo*. (A) Tumor volume was measured at different times after treatment. Data are presented as the mean ± standard deviation (n=7). **P<0.05, with comparisons indicated by lines. (B) The image presents inhibitory effect of each group on tumor growth at the last time point (day 60) when the mice were sacrificed. (C) Subcutaneous BxPC-3 tumors were collected 7 days after injection, and sections were analyzed by different methods. H&E staining showed that tumor tissues treated with the combination of ZD55-TIS and SNS-032 exhibited the strongest cell death (original magnification, x400). Immunohistochemistry analysis demonstrated there was strong expression of TRAIL and Smac in xenografts treated with ZD55-TIS and the combined therapy group. However, the combined therapy group had a more obvious downregulation of CDK-2, -7 and -9 expression compared with SNS-032 alone. TUNEL assay revealed enhanced cell apoptosis in the group treated with ZD55TIS plus SNS-032. ZD55-TIS, ZD55-TRAIL-IETD-Smac; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IETD, isoleucine-aspartate-threonine-glutamate; Smac, second mitochondria-derived activator of caspase; H&E, hematoxylin and eosin; CDK, cyclin-dependent kinase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Cytopathic effect of combination therapy on tumor growth inhibition. H&E staining verified that treatment of

ZD55-TRAIL-IETD-Smac plus SNS-032 resulted in marked cell death and tumor mass necrosis, whereas minimal or

no death/necrosis was observed in tumor tissue from the SNS-032, ZD55-TRAIL-IETD-Smac or PBS groups.

Furthermore, analysis of tumors by immunohistochemical staining using anti-TRAIL, anti-Smac, anti-CDK-2, anti-CDK-7 and anti-CDK-9 antibodies revealed that there was a strong expression of TRAIL and Smac in xenografts treated with ZD55-TRAIL-IETD-Smac and the combined therapy group. However, there was a downregulation of CDK-2, -7 and -9 in xenografts treated with SNS-032 and in the combined therapy group.

To investigate the potential mechanisms underlying tumor growth inhibition induced by ZD55-TRAIL-IETD-Smac combined with SNS-032, a TUNEL assay was used to verify whether the action of the combination therapy can lead to a pro-apoptotic effect. As demonstrated in Fig. 5C, ZD55-TRAIL-IETD-Smac plus SNS-032 caused marked cell death in the tumor.

Discussion

TRAIL, a member of the tumor necrosis factor super family, can selectively induce apoptosis in various tumor cells, but almost no toxicity in normal cells (19). The TRAIL apoptotic signaling pathway inhibitory regulation has several levels. Inhibitors of apoptosis (IAPs) can inhibit caspase activation. XIAP can directly bind and inhibits caspases-3, -7 and -9 (20,21). IAP overexpression has been demonstrated to be associated with tumor resistance to apoptosis-inducing agents (22,23). Hence, reducing IAP inhibition of apoptosis may be pivotal for sensitizing cancer cells to anti-cancer drugs. Additionally, by eliminating the IAP inhibition of caspases 12 and -13, Smac and the murine homolog DIABLO promote caspase activation. In response to apoptotic stimuli, Smac is released from mitochondria into the cytosol and binds to XIAP, thereby reduced the XIAP inhibition of caspases (24). Our previous studies have demonstrated that overexpressed Smac rapidly enhances sensitivity and promotes apoptosis of hepatocellular carcinoma (HCC) cells to TRAIL and complete regression of HCC could only be achieved by the combined treatment of Smac and TRAIL (25). Furthermore, our previous study generated a novel E1B-55 K deleted oncolytic adenovirus, ZD55-TRAIL-IETD-Smac, that harbors both TRAIL and Smac genes (11). In this vector a caspase-8 cleavage site (IETD) was introduced between the genes, allowing the production of TRAIL and Smac following activation of caspase-8 in virus-infected cells. Several *in vitro* and *in vivo* experiments have demonstrated the antitumor effects of ZD55-TRAIL-IETD-Smac in models of hepatocellular, cervical, lung, breast and colorectal cancer.

Combination targeted therapy is necessary for cancer treatment because tumors are genetically diverse and resistance seems inevitable (26,27). Therefore, it is important to identify combinations of two or more therapeutic agents, which function by different mechanisms with synergistic effects without increasing adverse effects. Oncolytic virotherapy for cancer is a novel treatment strategy. However, oncolytic virotherapy has not been effective in preclinical animal tumor models and clinical trials (28). Several clinical studies have illustrated that combination of oncolytic adenoviruses with chemotherapy (29-31) or radiation therapy (32) may enhance and have synergistic antitumor activity. Oncolytic adenoviruses combined with cytotoxicitic chemotherapies may enhance the potential of oncolytic adenovirus and optimize treatment.

The aim of this study was to investigate the possibility of combining ZD55-TRAIL-IETD-Smac with SNS-032, a CDK inhibitor that may be a novel therapeutic agent for patients with pancreatic carcinoma (18,33,34), and thereby enhance their antitumor activities. The MTT analysis demonstrated that combination of SNS-032 with ZD55-TRAIL-IETD-Smac was obviously superior to SNS-032 or ZD55-TRAIL-IETD-Smac alone.

In conclusion, to the best of our knowledge, the present study is the first to demonstrate that SNS-032 sensitizes human pancreatic cancer cells to ZD55-TRAIL-IETD-Smac-induced cell death *in vitro* and *in vivo*. These findings indicate that the combined treatment with SNS-032 and ZD55-TRAIL-IETD-Smac could represent a rational approach for anti-pancreatic cancer therapy.

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