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The small-molecule IAP antagonist AT406 inhibits pancreatic cancer cells *in vitro* and *in vivo*

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ABSTRACT

In the present study, we tested the anti-pancreatic cancer activity by AT406, a small-molecule antagonist of IAP (inhibitor of apoptosis proteins). In established (Panc-1 and Mia-PaCa-2 lines) and primary human pancreatic cancer cells, treatment of AT406 significantly inhibited cell survival and proliferation. Yet, same AT406 treatment was non-cytotoxic to pancreatic epithelial HPDE6c7 cells. AT406 increased caspase-3/-9 activity and provoked apoptosis in the pancreatic cancer cells. Reversely, AT406′ cytotoxicity in these cells was largely attenuated with pre-treatment of caspase inhibitors. AT406 treatment caused degradation of IAP family proteins (cIAP1 and XIAP) and release of cytochrome C, leaving Bcl-2 unaffected in pancreatic cancer cells. Bcl-2 inhibition (by ABT-737) or shRNA knockdown dramatically sensitized Panc-1 cells to AT406. *In vivo*, oral administration of AT406 at well-tolerated doses down-regulated IAPs (cIAP1/XIAP) and inhibited Panc-1 xenograft tumor growth in severe combined immunodeficient (SCID) nude mice. Together, our preclinical results suggest that AT406 could be further evaluated as a promising anti-pancreatic cancer agent.

1. Introduction

Pancreatic cancer remains a lethal disease to the majority affected patients [1–3]. It is characterized by rapid disease progression without significant clinical symptoms, causing early detection and/or treatment extremely difficult [1–3]. The five-year overall survival of the pancreatic cancer patients is dismissal (less than 3%) [1–3]. The prognosis for those with advanced or recurrent pancreatic cancer is even worse [1–3]. Gemcitabine and it-based combination treatment are currently utilized for pancreatic cancer patients [4]. Yet, the response is far from satisfactory [1–3]. Therefore, the development of alternative anti-pancreatic cancer agents is vital [3,5,6].

The inhibitor of apoptosis proteins (IAPs), including the X-linked IAP (XIAP) and cellular IAP1/2 (cIAP1/cIAP2), are a family of key apoptosis inhibitor proteins [7]. Of which, XIAP directly binds to and potently inhibits at least three caspases: caspase-3 and -7, and -9 [7,8]. cIAP1 and cIAP2 were originally identified by their ability to associate with tumor necrosis factor associated factor 2 (TRAF2) and TNF receptor-1/-2, therefore inhibiting caspase-8 activation [7–9]. By inhibiting these caspases, IAPs play a pivotal role in apoptosis inhibition [7–9]. Studies have confirmed over-expression of IAPs in pancreatic cancers, which are important for apoptosis inhibition and chemo-resistance [10].

A recent study by Cai et al., has characterized a novel, potent and orally bio-available antagonist of IAPs [11]. This compound, named AT406, could directly bind to and inhibit several key IAPs, including XIAP, cIAP1 and cIAP2 [11]. The aim of the present study is to evaluate its potential anti-cancer activity against human pancreatic cancer cells.

2. Materials and methods

2.1. Reagents, chemicals and antibodies

AT406 and ABT-737 (the Bcl-2 inhibitor [12]) were purchased from Selleck (Shanghai, China). The caspase-3 specific inhibitor Ac-DEVD-CHO, the caspase-9 inhibitor Ac-LEHD-CHO and the pan

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caspase inhibitor Ac-VAD-CHO were purchased from Calbiochem (La Jolla, CA). All the antibodies utilized in the study were obtained from Cell Signaling Tech (Shanghai, China). Cell culture reagents were obtained from Gibco (Shanghai, China).

2.2. Cell culture

Established human pancreatic cancer cell lines, Panc-1 and MiaPaCa-2, were purchased from the Cell Bank of Shanghai Institute of Biological Science, Chinese Academy of Science (Shanghai, China). Cells were cultured in RPMI1640 medium, supplemented with 10% heat-inactive fetal bovine serum (FBS). The pancreatic epithelial cell line HPDE6c7 (non-cancerous line) was provided by Dr. Shang's group [13]. HPDE6c7 cells were cultivated in DMEM supplemented with 10% FBS and necessary antibiotics.

2.3. Primary culture of human pancreatic cancer cells

As described [13], surgery-removed fresh pancreatic cancer specimens from informed-consent patients were thoroughly washed and minced. Resolving cancer tissues were then digested via Collagenase I (Sigma, Shanghai, China). Single-cell suspensions were then pelleted and washed. Primary cancer cells were then cultured in the medium described early [13]. The protocol was approved by the Institutional Review Board and Ethics Committee of all authors' institutions. All research were conducted in accordance with the principles expressed in the Declaration of Helsinki.

2.4. Cell viability assay

A CellTiter-Glo luminescent cell viability assay kit was applied to evaluate cell viability according to the manufacturer's instructions (Promega, Shanghai, China). Cells were seeded at 5×10^3/well onto 96-well plates. Following treatment of cells, the CellTiter-Glo reagent (100 μL/well) was added and incubated for 10 min. Luminescence was recorded by a Fluorescence/Multi-Detection Microplate Reader (Synergy 2, BioTek, Winooski, VT).

2.5. Clonogenic assay

Panc-1 cells were seeded at 3000/well onto 6-well plates. Following indicated AT406 treatment, cells were then allowed to grow for a further 10 days before fixation with methanol and staining with crystal violet (0.5% solution). The number of viable colonies were then counted manually.

2.6. Cell proliferation assay

Cell proliferation was tested via a BrdU assay kit (Cell Signaling Technology, Shanghai, China) according to the manufacturer's protocol. Briefly, cells were seeded at 5×10^3/well onto 96-well plates. After applied treatment, BrdU (10 μmol/L) was added and cells were incubated for additional 3–4 h. Afterwards, the BrdU absorbance was tested at 450 nm via the above multi-detection microplate reader.

2.7. Caspase activity assay

After the applied AT406 treatment, cytosolic proteins (25 μg per treatment) [14] were incubated with the caspase assay buffer (312.5 mM HEPES, pH 7.5, 31.25% sucrose, 0.3125% CHAPS) with corresponding caspase substrate (Ac-LEHD-AFC for caspase-9, Ac-IETD-AFC for caspase-8 and Ac-DEVD-AFC for caspase-3). The released AFC was measured via the above multi-detection microplate reader with excitation of 400 nm [14].

2.8. Mitochondrial depolarization assay

The mitochondrial depolarization, an early sign of cell apoptosis [15], was detected via the JC-10 dye (Invitrogen, Shanghai, China). Briefly, following the applied AT406 treatment, cells were immediately washed and stained with JC-10 dye (1 mg/mL, Invitrogen) for 10 min at 37 °C. Afterwards, cells were washed, and JC-10 green fluorescence intensity, the indicator of mitochondrion depolarization ($\Delta\Psi m$), was measured via the above multi-detection microplate reader at 485 nm [16,17].

2.9. TUNEL staining assay of apoptosis

Cell apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) In Situ Cell Death Detection Kit (Roche, Shanghai, China). TUNEL ratio (TUNEL/DAPI × 100%) was recorded under a fluorescence microscope (Zeiss, 1: 100 magnification). For each condition, a total of ten random views containing at least 500 cells were included to count TUNEL ratio.

2.10. Annexin V assay of apoptosis

After the applied AT406 treatment, FITC-conjugated Annexin V (Bender, Burlingame, CA) was added to cells. Afterwards, Annexin V intensity was detected in fluorescence channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm, via the above multi-detection microplate reader. Annexin V fluorescence intensity was recorded as a quantitative measurement of cell apoptosis [18].

2.11. Western blot assay

The detailed protocol for Western blot assay was described in other studies [13]. In brief, equal amounts of cytosol protein extracts (30 μg per sample) were resolved by SDS-PAGE and analyzed by Western blot. The antibody-antigen binding was visualized via the Super-Signal West Pico ECL Substrates (Pierce). Band intensity (total gray) was quantified via the Image J software.

2.12. Bcl-2 shRNA knockdown

The two non-overlapping lentiviral Bcl-2-shRNAs ("-1/-2") were provided by Dr. Cui's group [19]. Cells were seeded onto the polybrene (Sigma)-coated 6-well plate with 50% confluence, which were then infected with the lentiviral shRNA for 24 h. Afterwards, cells were cultured in fresh medium, and were subjected to puromycin (1.0 μg/mL, Sigma) selection for 12–14 days. Bcl-2 down-regulation in stable cells was confirmed by Western blot assay. Control cells were infected with same amount of scramble nonsense control shRNA lentivirus (Santa Cruz).

2.13. Mouse xenograft model

Studies on Panc-1 xenografts were approved by the IACUC and Ethics Committee of all authors' institutions. Briefly, Panc-1 cells (1×10^7 per mouse) were injected *s.c.* into the left flank of male severe combined immunodeficient (SCID) nude mice (Animal Research Centre, Shanghai, China). After 10–14 days when established tumors were around 0.1 cm^3 in volume, mice were randomized into three groups. Ten mice per group were treated with vehicle (Saline, oral gavage) or AT406 (5 and 25 mg/kg body weight, oral gavage, at Day-1, 2, 3, 8, 15 and 22). Tumor volume was calculated using the formula: $(A \times B^2)/2$, where A is the longest and B is the shortest perpendicular axis of an assumed ellipsoid