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Combination of AZD2281 (Olaparib) and GX15-070 (Obatoclax) results in synergistic antitumor activities in preclinical models of pancreatic cancer

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ABSTRACT

In this study, we explored the antitumor activities of the PARP inhibitor AZD2281 (Olaparib) and the pan-Bcl-2 inhibitor GX15-070 (Obatoclax) in six pancreatic cancer cell lines. While both agents were able to cause growth arrest and limited apoptosis, the combination of the two was able to synergistically cause growth arrest and non-apoptotic cell death. Furthermore, in an *in vivo* xenograft model, the combination caused substantially increased tumor necrosis compared to either treatment alone. Our results support further investigation of the combination of Bcl-2 and PARP inhibitors for the treatment of pancreatic cancer.

1. Introduction

Pancreatic cancer represents only 2% of all cancers in the US, but it has the highest mortality rate at 99% and the lowest 5-year survival rate of less than 5% [30,39]. There has been little improvement in the prognosis over the past 20 years partially due to the delay of diagnosis, with many cases being diagnosed at an advanced stage [10]. Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is the standard first-line drug for treating advanced pancreatic cancer [3]. However, its efficacy remains low, with a median survival of 5.7 months and 1-year survival rate of 18% [18,21]. Therefore, new therapies for this extremely aggressive disease are urgently needed.

PARP-1 is a DNA binding protein involved in apoptosis as well as DNA single- and double-strand break repair, and has been becoming a popular therapeutic target for many different malignancies [1,17,41]. PARP inhibitors have been demonstrated to have a strong synthetic lethal relationship with BRCA1/2 deficiency. The loss of PARP activity leads to more lesions needing to be repaired by the homologous recombination (HR) pathway, which can be lethal in a BRCA1/2 deficient background [24]. Based on this finding, PARP inhibitors have been widely used in recent clinical trials for treating BRAC1/2-deficient tumors including pancreatic cancer and have shown promising clinical activities [12,25,32,41]. The use of PARP inhibitors has recently been extended to tumors with other defects in the HR DNA repair pathway, as well as in combination with chemotherapy drugs and chemoradiotherapy [7,24,32]. Combining PARP inhibitors with agents that impair DNA damage repair to treat BRCA1/2 wild-type tumors could broaden the clinical use of these promising PARP inhibitors.

A common cause for chemotherapy resistance arises from the overexpression of the anti-apoptotic Bcl-2 family members. Bcl-xL and Mcl-1 are expressed in 88% whereas Bcl-2 is expressed in 23% of invasive ductal carcinomas [26]. Therefore targeting these proteins could be an effective treatment for pancreatic cancer. GX15-070 (Obatoclax) is a small molecule that binds to the BH3-binding site of Bcl-2, Bcl-xL, and Mcl-1 [9,20,28]. This pan-Bcl-2 inhibitor has been reported to directly induce apoptosis in a variety of cultured cancer cell lines and primary patient samples through the mitochondrial apoptotic pathway as well as non-apoptotic cell death [16,28,33]. Recent studies have found compelling evidence to suggest that the anti-apoptotic Bcl-2 family proteins can regu-

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late DNA double-strand break repair independent of their pro-survival functions [2,11,14,17,19,31,36–38,42,44]. We hypothesize that using a pan-Bcl-2 inhibitor may sensitize pancreatic cancer cells to PARP inhibitors.

In this study, we investigated the combination of the PARP inhibitor AZD2281 and the pan-Bcl-2 inhibitor GX15-070 in six pancreatic cancer cell lines harboring wild-type *BRCA1* and *BRCA2* genes. When combined simultaneously, the two agents caused additive to synergistic growth arrest and cooperatively induced non-apoptotic cell death in the pancreatic cancer cell lines. Our *in vivo* xenograft model studies revealed that the two drugs cooperate to induce substantial tumor necrosis, resulting in a cavity in the center of the tumors. These results provide support for the combination of PARP and Bcl-2 inhibitors in the treatment of pancreatic cancer.

2. Materials and methods

2.1. Drugs

GX15-070 (Obatoclax) and AZD2281 (Olaparib) were purchased from Selleck Chemicals LLC (Houston, TX, USA). Both agents were dissolved in DMSO and stored at −80 °C, as recommended by the supplier.

2.2. Cell culture

The BxPC-3, HPAC, MIAPaCa-2, PANC-1, AsPC-1 and CFPAC-1 human pancreatic cancer cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All of these cell lines harbor wild-type *BRCA1* and *BRCA2* genes [8,40]. The cell lines were cultured in Dulbecco's Modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA, for HPAC, MIAPaCa-2 and PANC-1), RPMI1640 medium (Invitrogen, for AsPC-1 and BxPC-3), or Iscove's Modified Dulbecco's medium (IMDM, Invitrogen, for CFPAC-1) with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Labs, Logan, UT, USA) plus 100 U/mL penicillin and 100 μg/mL streptomycin in a 37 °C humidified atmosphere containing 5% CO2/95% air. Cell lines were authenticated by the University of Arizona Genetics Core Facility (Tucson, AZ, USA).

2.3. In vitro cytotoxicity assays

In vitro AZD2281 or GX15-070 cytotoxicities of pancreatic cancer cell lines were measured by using MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium-bromide, Sigma–Aldrich, St Louis, MO, USA) reagent, as previously described [45,46]. The extent and direction of GX15-070 and AZD2281 antitumor interactions were evaluated using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). Drug interactions were quantified by determining the combination index (CI), where CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively [4].

2.4. Colony formation assays

Colony formation assays were carried out as previously described [43]. Briefly, 500 BxPC-3 cells were seeded into 100 mm dishes in complete RPMI1640, cultured for 24 h, and then treated with the indicated concentrations of GX15-070, AZD2281, or AZD2281 plus GX15-070 for 48 h. The cells were then washed twice with drug-free RPMI1640 and cultured in complete RPMI1640 for up to 3 weeks. Colonies were visualized by coomassie blue staining and counted. The extent and direction of interactions between GX15-070 and AZD2281 in suppressing colony formation were determined using CompuSyn software (ComboSyn, Inc.).

2.5. shRNA knockdown of PARP-1 in PANC-1 cells

The pMD-VSV-G and delta 8.2 plasmids were gifts from Dr. Dong at Tulane University. The transfection was carried out by using Lipofectamine and Plus reagents (Life Technologies, Carlsbad, CA, USA), as previously described [6,45,46]. Briefly, lentivirus vector (PARP-1 or non-target control shRNA construct from the RNAi Consortium, Sigma–Aldrich), pMD-VSV-G and delta 8.2 were co-transfected into TLA-HEK293T cells and the culture medium was harvested 48 h post-transfection. PANC-1 cells were transduced by adding 1 mL of virus supernatant and 4 μg of polybrene. After selection with puromycin, a pool of infected cells was expanded and tested for PARP-1 expression by Western blotting. A pool of cells from the non-target control transduction was used as the negative control.

2.6. Western blot analysis

Western blotting was performed using polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Inc., Rockford, IL, USA) and immunoblotted with anti-PARP-1, -Bcl-2, -Mcl-1, -Bcl-xL, -cleaved caspase3, -CDK1, -CDK2, cyclin B1, -caspase 3 (Cell Signaling Technology, Beverly, MA, USA), or -β-actin (Sigma–Aldrich) antibody, as described previously [6]. Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA), as described by the manufacturer.

2.7. Cell death, apoptosis, and cell cycle progression

Cells were treated as indicated and cell death was determined by trypan blue exclusion. The remaining cells were fixed with ice-cold 80% (v/v) ethanol for 24 h. After centrifugation at 200×*g* for 5 min, the cell pellets were washed with PBS (pH 7.4) and resuspended in PBS containing propidium iodide (PI, 50 μg/mL), Triton X-100 (0.1%, v/v), and DNase-free RNase (1 μg/mL). DNA contents were determined by flow cytometry analysis, as previously described [43]. Cell cycle analysis was performed with Multicycle software (Phoenix Flow Systems, Inc., San Diego, CA, USA). Apoptotic events were recorded as PI^+ events (Sub-G1 population).

2.8. Establishment of a mouse pancreatic cancer xenograft model

Female BALB/c nude mice (18–22 g) were purchased from Vital River Laboratories (Beijing, China). The animal study was conducted following internationally recognized guidelines and was approved by the Animal Research Committee of Norman Bethune College of Medicine, Jilin University. Log phase BxPC-3 cells were digested with trypsin, adjusted to 2×10^7 cells/ml with matrigel (BD Biosciences, San Jose, CA, USA), and inoculated subcutaneously in the right side axillae of BALB/c mice to generate a xenograft (100 μl per mouse). When the xenografts reached an average volume of 75.6 ± 36.5 mm^3, the mice were randomized into four groups (8 animals per group) and injected intraperitoneally with 200 μl of (i) phosphate-buffered saline (PBS) with 10% Hydroxypropyl-β-Cyclodextrin (HP-β-CD) (control group), (ii) AZD2281 (50 mg/kg) dissolved in PBS with 10% HP-β-CD once daily Monday through Friday (AZD2281 group), (iii) GX15-070 (3 mg/kg) dissolved in PBS with HP-β-CD once daily Monday through Wednesday (GX15-070 group), or (iv) AZD2281 five times a week and GX15-070 three times a week dissolved in PBS with HP-β-CD (combination group), for 3 weeks. Tumor diameters were measured with a caliper every 3–4 days. Blood samples were taken via retro-orbital bleed under anesthesia on day 28 post drug treatment initiation. The whole blood was collected into sterile Ep tubes and centrifuged at 3000 rpm for 10 min, and serum was collected for detection of carbohydrate antigen 19-9 (CA19-9). The mice were then sacrificed and the tumors were removed, weighed and fixed in 10% formalin for hematoxylin and eosin (H&E) and immunohistochemical staining.

2.9. Detection of CA 19-9 in blood serum

Serum CA 19-9 was measured by using a CA19-9 [I^{125}] IRMA kit (3 V Biosciences, Weifang, China), according to the manufacturer's instructions.

2.10. H&E and immunohistochemical staining

Tumors from 3 mice in each treatment group were analyzed by H&E and immunohistochemical staining. All specimens were fixed in 10% formalin, embedded in paraffin, and cut into 4 μm-thick slides. The slides were dewaxed and stained with H&E for histological assessments. Center necrosis areas were quantified by using the BI-2000 Medical Image Analysis System (TME Technology Co., Chengdu, China).

For immunohistochemical staining, the endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide solution in methanol for 20 min. Epitope retrieval was performed by treating the slides with 10 mM sodium citrate buffer (pH 6.0) and heating twice in a microwave oven at high power for 6 min each. Non-specific binding was prevented by blocking with goat serum (Dingguo Biosciences, Beijing, China) (1:10 in PBS) for 10 min. Immunostaining of proliferating cell nuclear antigen (PCNA) and CD34 was performed using mouse anti-human monoclonal antibodies (Abgent Inc., San Diego, CA, USA). After incubation with the primary antibody (1:100 in PBS) for 60 min, the slides were incubated with a biotinylated goat anti-mouse IgG (H + L) (Beyotime, Shanghai, China) at 37 °C for 30 min, followed by incubation with a 1:200 streptavidin–biotin–peroxidase complex (Sigma–Aldrich) for 30 min. Reactive products were visualized with 3,3′-diaminobenzidene (DAB) as the chromogen, and the slides were counterstained with hematoxylin and coverslipped. Sections previously known to express PCNA or CD34 were included in each run, receiving either the primary antibody as the positive control, or a mouse IgG as the negative control. The stained slides were analyzed with a microscope, and brown staining was scored using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA). The microvessel density (MVD) was calculated using the "Hot spot" method: CD34 positive vascular dense areas, or "hot spots", were identified at 40× magnification. The discrete microvessels in a 200× field were then counted. Three separate hotspots were assessed to give a mean MVD value for each tumor [22].