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.

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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-
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-diphenyltetrazoliumbromide, 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 (CombSyn, 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 (CombSyn, Inc.).

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

The pMID-USV-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), pMID-USV-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) membrane (Biorad, Hercules, CA, USA) and immunoblotted with anti-PARP-1, Bcl-2, 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 % events (Sub-G1 population).

2.8. Establishment of a mouse pancreatic cancer xenograft model

Female BALB/c nude mice (8–12 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 × 10⁶ 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 mm³, the mice were randomized into four groups (8 animals per group) and injected intraperitoneally with 200 μL of (i) phospho-buffered saline (PBS) with 10% Hydroxypropyl-p-Cyclodextrin (HP-p-CD) control group), (ii) AZD2281 (50 mg/kg) dissolved in PBS with 10% HP-p-CD once daily Monday through Friday (AZD2281 control group), (iii) GX15-070 (3 mg/kg) dissolved in PBS with HP-p-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-p-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 antigens 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 [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 slices. 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–biontin–peroxidase complex (Sigma–Aldrich) for 30 min. Reaction products were visualized with 3,3′-diaminobenzidine (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].
2.11. Statistical analysis

The in vitro data were expressed as mean values ± standard errors, while the in vivo data were expressed as mean values ± standard deviations. For the in vitro data, differences in AZD2281 IC_{50}s between GbX15-070 treated and vehicle control treated cells and differences in cell death/apoptosis between GX15-070 and AZD2281 treated (individually or combined) and vehicle control treated cells were compared using the paired two-sample t-test. Statistical analyses were performed with GraphPad Prism 5.0. For the in vivo data, statistical comparisons were performed with SPSS17.0 using analysis of variance (ANOVA) test, p < 0.05 was deemed to be statistically significant.

3. Results

3.1. PARP-1 and Bcl-2 family protein expression and inhibitor sensitivities in pancreatic cancer cell lines

To begin to determine if AZD2281 and GX15-070 cooperate to kill pancreatic cancer cells, we first determined PARP-1, Bcl-2, Bcl-xL, and Mcl-1 protein levels and IC_{50} values for each drug in six pancreatic cancer cell lines. The proteins were expressed at variable levels (Fig. 1A). The IC_{50} values for AZD2281 ranged from 16.18 μM (CFPAC-1) to 52.5 μM (MIAPaCa-2) and those for GX15-070 ranged from 219.98 nM (PANC-1) to 589.7 nM (AsPC-1, Fig. 1B and C and Table 1). The individual drug sensitivities did not appear to correlate with PARP-1, Bcl-2, Bcl-xL or Mcl-1 protein levels. Next, we treated BxPC-3 and PANC-1 cells with varying concentrations of AZD2281 (0–20 μM) or GX15-070 (0–2.5 μM) and assessed drug-induced apoptosis by PI staining and flow cytometry analyses and measuring the population with DNA fragmentation (sub-G1). Even at the highest concentration there was at most approximately 6% sub-G1 cells, indicating a minimal induction of apoptosis by either of the two agents (Fig. 1D and E).

3.2. AZD2281 and GX15-070 synergized to cause growth arrest and cooperated to induce non-apoptotic cell death

To determine if AZD2281 and GX15-070 synergize to induce pancreatic cancer cell growth arrest, the pancreatic cancer cell lines were treated simultaneously with varying concentrations of AZD2281 and 0 nM, 85 nM, or 170 nM GX15-070, clinically achievable concentrations [13], for 96 h and the AZD2281 IC_{50} values were determined using MTT assays. The AZD2281 IC_{50}s were...
significantly reduced by 2.7- (CFPAC-1) to 32.8-fold (HPAC). Additional to synergistic antitumor interactions were detected by calculating the combination index values for the combinations of AZD2281 with 85 nM GX15-070, while uniform synergistic antitumor interactions were detected for the combinations of AZD2281 with 170 nM GX15-070 (Fig. 2 and Table 1).

Next, we investigated whether or not AZD2281 and GX15-070 would cooperate to induce cell death. BxPC-3 and PANC-1 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ of GX15-070 (nM)</th>
<th>IC₅₀ of AZD2281 (μM) in the absence or presence of GX15-070 (nM)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>85</td>
</tr>
<tr>
<td>AsPC-1</td>
<td>589.7 ± 15.4</td>
<td>29.0 ± 1.8</td>
<td>13.7 ± 2.1 (0.62)</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>245.5 ± 9.7</td>
<td>25.4 ± 2.6</td>
<td>15.1 ± 1.0 (0.94)</td>
</tr>
<tr>
<td>CFPAC-1</td>
<td>527.7 ± 51.6</td>
<td>16.2 ± 1.1</td>
<td>10.8 ± 1.0 (0.83)</td>
</tr>
<tr>
<td>HPAC</td>
<td>322.8 ± 51.4</td>
<td>51.5 ± 3.0</td>
<td>17.9 ± 1.9 (0.61)</td>
</tr>
<tr>
<td>MIAPaCa-2</td>
<td>322.3 ± 26.6</td>
<td>52.5 ± 4.7</td>
<td>38.0 ± 3.3 (0.99)</td>
</tr>
<tr>
<td>PANC-1</td>
<td>220.0 ± 12.9</td>
<td>24.9 ± 4.2</td>
<td>6.6 ± 2.8 (0.66)</td>
</tr>
</tbody>
</table>

Note: The pancreatic cancer cell lines were treated with variable concentrations of AZD2281 for 96 h in the absence or presence of variable concentrations of GX15-070 administered simultaneously. Viable cells were measured by MTT assays and the extent and direction of antitumor interactions between AZD2281 and GX15-070 were determined by using Compusyn software. The numbers in the parentheses represent the combination index (CI) values, where CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively. The data are presented as mean values ± standard errors from at least 3 independent experiments.

a IC₅₀s were estimated using GraphPad Prism 5.0.
were treated with 5 or 1.25 μM AZD2281, 85 nM GX15-070, or in combination for 72 h and percentages of dead cells were determined with the trypan blue exclusion assay. Simultaneous treatment resulted in a significantly increased percentage of dead cells compared to the individual drug treatments (Fig. 3A and B). We did not detect cleavage of caspase-3 or PARP-1, indicating that the increase in cell death was due to non-apoptotic cell death (Fig. 3C). Bcl-xL and Mcl-1 protein levels were largely unchanged.
and a small decrease in Bcl-2 was detected after the combined drug treatment compared to vehicle control treated cells. Cell cycle analysis in BxPC-3 cells revealed a small significant increase in G2/M phase after treatment with AZD2281 alone, which was further significantly increased by the addition of GX15-070 and accompanied by a decrease in CDK1 protein levels (Fig. 3D and F, \( p < 0.05 \)). In the PANC-1 cells, we detected a small significant increase in S phase after individual drug treatments, which was further significantly increased when the drugs were administered simultaneously and accompanied by a minor decrease in CDK2 protein levels (Fig. 3E and F, \( p < 0.05 \)). Cyclin B1 protein levels remained unchanged in the BxPC-3 cells, while there was an increase in the AZD2281 treated PANC-1 cells. Consistent with the lack of cleavage of caspase-3 and PARP-1 in the cells treated with AZD2281 or GX15-070, alone or combined, no increase of sub-G1 population in the combined treatments could be detected by PI staining and flow cytometry analyses (data not shown).

To provide further evidence that PARP inhibition is required for the enhanced non-apoptotic cell death for the combination of AZD2281 and GX15-070, we performed lentiviral shRNA knockdown of PARP-1 in PANC-1 cells (designated PARP-shRNA, Fig. 3G). The cells were treated with or without 85 nM GX15-070 for 72 h and percentage of dead cells were determined using the trypan blue exclusion assay. After treatment with GX15-070, the PARP-shRNA cells had a significantly higher percent of dead cells compared to the NTC-shRNA control cells (Fig. 3H).

Cell death was further confirmed by colony formation assays in BxPC-3 cells. AZD2281 or GX15-070 treatment alone caused dose-dependent decrease in colony formation (Fig. 4A and B). Combined AZD2281 and GX15-070 treatment showed a synergistic and significant decrease in colony formation compared to the individual drug treatments (CI = 0.69, Fig. 4C and D).

3.3. In vivo antitumor efficacy of AZD2281 and GX15-070 in a xenograft model

To evaluate the effects of AZD2281 and GX15-070 on pancreatic tumor growth in vivo, we used a mouse BxPC-3 xenograft model. Individual and combined drug treatments were well tolerated, indicated by the lack of a significant loss of body weight for the drug treatment groups compared to the vehicle control group (Fig. 5A). Surprisingly, neither the individual nor the combined drug treatments resulted in significant delay of externally measurable tumor growth during the three week drug treatment period (Fig. 5B). Based on these results, the mice were sacrificed on day 28 post drug treatment initiation. On that day, body weight and tumor weight were essentially the same among the four treatment groups (Fig. 5C and D), however, serum CA19-9 levels (a pancreatic cancer biomarker that is used to monitor chemotherapy response [23,27,29,47]) in the combination group were significantly lower compared to that for the other treatment groups, indicating treatment response (Fig. 5E).

To investigate the in vivo effects of these drug treatments, tumors from 3 mice in each group were analyzed with H&E and immunohistochemical staining. Interestingly, H&E staining revealed substantially increased necrosis in the tumors for the drug treated groups compared to the vehicle control group (Fig. 5F). Quantification showed mean 5.4 ± 2.3%, 10.6 ± 2.5%, 34.0 ± 16.8%, and 55.2 ± 10.6% center necrosis areas for the vehicle control, AZD2281, GX15-070, and the combination groups, respectively (Fig. 5G). The difference in the center necrosis areas between the combination group and the vehicle control group or AZD2281 group was statistically significant, while that between the combination group and the GX15-070 group was not, probably due to the large variation of center necrosis areas within the GX15-070 group and the small sample size. There was no obvious invasion of the tumor into other tissues (data not shown).
Analogous to the results from H&E staining, immunohistochemical staining showed substantially decreased expression of PCNA and CD34 in the combination group compared to the other groups (Fig. 5H and I). The proliferation index values of the combination group (18.3 ± 6.6%) were significantly lower than that in the vehicle control group (56.5 ± 10.5%, Fig. 5H). Further, the MVDs of the combination group (25.6 ± 8.8%) were significantly lower than the vehicle control group (60.3 ± 17.1, Fig. 5I).

4. Discussion

Emerging evidence has demonstrated that Bcl-2, Bcl-xL and Mcl-1 possess non-apoptotic functions, in addition to their traditional roles in the mitochondrial apoptosis pathway, and are involved in the DNA damage response [5,19,38,44]. PARP inhibitors have demonstrated efficacy in cells with impaired HR due to BRCA1/2 mutations, as well as mutations in other HR-related genes [7,24,32]. Thus, targeting Bcl-2, Bcl-xL, and/or Mcl-1 may impair the DNA damage response and potentiate BRCA1/2 wild-type pancreatic cancer cells to PARP inhibitors.

In this study, we combined GX15-070 and AZD2281 in vitro and in vivo to determine if they cooperate to induce growth arrest and cell death. Based on the traditional roles Bcl-2, Bcl-xL, and Mcl-1 play in the mitochondrial apoptosis pathway, GX15-070 would be expected to cause apoptotic cell death [16,33]. However, at clinically achievable concentrations of GX15-070 we detected minimal

Fig. 5. In vivo antitumor efficacies of AZD2281 and GX15-070 alone or in combination in a BxPC-3 xenograft model. Panels A–C: Body weights (panels A and C) and tumor volumes (panel B) were measured every 3–4 days. Tumor volume was calculated according to the following formula: \( m_1^2 \times m_2 \times 0.5236 \) (m1: short diameter; m2: long diameter). Panel D: The mice were sacrificed on day 28 post drug treatment initiation and the tumors were removed and weighed. Panel E: The serum CA19-9 levels were detected using a CA19-9 IRMA kit. Panel F: Tumor specimens were fixed in 10% formalin, embedded in paraffin, and cut into 4 μm-thick slides for H&E, PCNA, and CD34 staining. Panel G: Center necrosis areas were quantified by using the BI-2000 Medical Image Analysis System. Necrosis was defined as tumor area with loss of clearly defined cell boundaries and without pyknotic nuclei. Panel H: The proliferation index was calculated as proliferation index = PCNA positive cells/observed cells \( \times 100\% \) and graphed. Panel I: Microvessel density was calculated and graphed as described in the Materials and Methods. The data are presented as mean values ± standard deviations. * Indicates \( p < 0.05 \), while ** indicates \( p < 0.01 \).
apoptosis, but significant growth arrest, indicating a non-classical mechanism underlying the cytotoxic effects of GX15-070 ([Fig. 1C and E]). Our results demonstrate that these two drugs induce pancreatic cancer cell growth arrest in an additive to synergistic manner in all of the cell lines tested ([Fig. 2 and Table 1]). Interestingly, the combination also caused a significant increase in non-apoptotic cell death ([Figs. 3 and 4]), potentially due to decrease in Bcl-2 protein levels. This was accompanied by significantly enhanced S or G2/M cell cycle arrest compared to the individual drug treatments ([Fig. 3]), possibly due to the decrease in CDK1 or CDK2 protein levels. These results further indicate that GX15-070 potentiates the cytotoxic effects of AZD2281 through non-classical mechanisms.

Surprisingly, in our in vivo studies, externally measuring tumor volume of pancreatic cancer xenografts in nude mice indicated a lack of response to the drug treatments. However, the combined drug treatment caused a significant decrease in serum CA19-9 levels compared to the other treatment groups, indicating treatment response. Upon further investigation, H&E staining revealed that the tumors from the combined drug treatment had larger center necrosis areas compared to the individual drug treatments, while only minimal was detected in the vehicle control group. Furthermore, immunohistochemical examination of PCNA showed that the combined drug treatment group had lower PCNA expression than the other treatment groups, indicating that the two drugs cooperated in suppressing proliferation of pancreatic cancer cells in the tumors. Analogous results were obtained with CD34 immunohistochemical staining, indicating that GX15-070 and AZD2281 cooperated in suppressing angiogenesis in the tumors, resulting in the appearance of a necrotic cavity in the center of the tumor. Indeed, there are data to suggest that Bcl-2, Bcl-xl and PARP-1 are involved with the process of angiogenesis ([15,34,35]).

Taken together, our results demonstrate cooperative antitumor effects of GX15-070 and AZD2281 on preclinical models of pancreatic cancer in vitro and in vivo. Although we do not have direct evidence, we suspect that GX15-070 predominantly targets the non-apoptotic functions of Bcl-2, Bcl-xl and/or Mcl-1 to potentiate the cytotoxic effects of the PARP inhibitor AZD2281 in pancreatic cancer cells. Further studies are necessary to determine the molecular mechanisms of non-apoptotic cell death induced by the combination. Our novel findings suggest that there might be a clinical benefit for using the combination of GX15-070 and AZD2281 to treat pancreatic cancer.

Conflict of Interest

The authors declare no competing financial interests.

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References


