APR-246 enhances the anticancer effect of doxorubicin against p53-mutant AsPC-1 pancreatic cancer cells

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Abstract

Pancreatic cancer (PC) is a disease with a high mortality rate, and the early diagnosis of PC is still challenging. The 5-year relative survival rate remains below 8%, and therapeutic strategies are ineffective at increasing patient survival rates. In PC cells, resistance to treatment has been associated with genetic changes that initiate aberrant developmental signaling pathways; therefore, new strategies for treating PC are warranted. Here, we sought to explore the small molecule p53 reactivator, APR-246, either as a mono-drug or combined with doxorubicin (Doxo) in the p53 mutant (p53-mut) AsPC-1 PC cell line. Cytotoxicity of APR-246 and Doxo was studied alone or in combination with the AsPC-1 cell line with MTT assay. CalcuSyn software was used to calculate the combination index (CI), which was based on the Chou and Talalay technique. Apoptosis was assessed after staining with Acridine orange/ethidium bromide (AO/EB). Morphological alterations were examined under a 200x magnification by an inverted microscope. Gene expression was quantified using quantitative reverse transcription PCR assay (RT-qPCR). APR-246 alone exhibited modest anti-proliferative effects, while Doxo combined with APR-246 showed synergistic effects associated with morphological change; no more enhanced apoptosis was observed, as supported by NOXA gene levels. In conclusion, APR-246, whether monotherapy or combined with Doxo, was hindering the proliferation of p53-mut AsPC-1 PC cells, and further in vitro and in vivo research is warranted.

Keywords: APR-246, Combination, Doxorubicin, Pancreatic Cancer, p53 reactivator.

Introduction

One of the worst malignancies is pancreatic cancer, the seventh-highest cause of death from cancer globally. The disease has a poor prognosis and is currently in its mature stage 1. Several reasons contribute to this, including the absence of specific and sensitive tumor markers and non-specific clinical symptoms and, occasionally, negligible. In addition, gene mutations affecting specific genes and some malignant cells serving as cancer stem cells are decreasing the efficacy of chemotherapy and radiation therapy. Human malignancies, such as PC, have been found to harbor mutations in oncogenes and tumor suppressor genes 2. According to research, pancreatic cancer has been linked to many mutations, including Kirsten rat sarcoma viral oncogene homolog (KRAS), tumor protein p53 (TP53), breast cancer gene (BRCA), cyclin-dependent kinase inhibitor 2A (CDKN2A), and

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Mothers against decapentaplegic homolog 4 (SMAD4) 

About 50% of all malignancies have a TP53 mutation, while the same mutation is present in about 75% of PC patients. The p53 protein, encoded by the TP53 gene, binds to specific DNA regions and controls the transcription of downstream molecules. In addition to performing various biological functions, this protein plays a crucial role in maintaining stem cells, the cell cycle, energy metabolism, metabolic activity, and apoptosis.

Doxorubicin (also known as Adriamycin) is used to treat patients with various cancers, including breast cancer and leukemia; however, it is not a first-line medication for PC therapy. Doxo is a member of the anthracycline group of chemotherapeutic agents. Anthracyclines disrupt topoisomerase II, which causes double-strand breaks and inhibits DNA and RNA production by intercalating between base pairs of the DNA/RNA strands. Doxo is a potent inducer of p53 and reactive oxygen species (ROS), a topoisomerase inhibitor that intercalates into DNA. However, mutations in TP53 are associated with resistance against chemotherapies, including Doxo, challenging treatment as a single agent.

The innovative therapy protocols for resistant malignancies are promising because they add selective inhibitors to traditional chemotherapies for cancerous cells. Small molecules known as p53 reactivators have been developed, which bind with mutant p53 and partially restore its tumor suppressor function to arrest tumor cells or cause apoptosis. APR-246 is one of these reactivators, also called PRIMA-1MET, and the clinical form is eprenetapopt. APR-246 is a methylated analog of PRIMA-1 that inhibits cellular proliferation in cells with mutant p53. Several cancer cells, including osteosarcoma, lung cancer, multiple myeloma, colon cancer, breast cancer, and PC, have responded favorably to PRIMA-1 and its methylated version, PRIMA-1MET. In phase I/II clinical trials, APR-246 was used to treat prostate cancer and hematological malignancies, and it has demonstrated efficacy and safety. Recently, a phase I trial was conducted on patients with TP53 mutation-positive acute myeloid leukemia (AML). The clinical versions of APR-246, eprenetapopt, azacitidine, and venetoclax were administered to the patients, and they demonstrated acceptable safety and encouraging activity.

In this work, our objective was to investigate the efficacy of the small molecule APR-246, which reactivates the p53 protein, either as a single medication or in combination with Doxo, in the AsPC-1 PC cell line with a mutant p53 gene. We assessed the effects of the drugs on cell viability, morphology, and apoptosis in vitro.

Materials and methods

Cell lines and drugs

In this investigation, pancreatic adenocarcinoma-derived AsPC-1 cells were employed; they express mutant p53 protein. The AsPC-1 cell line was generously provided by the postgraduate toxicology laboratory affiliated with the College of Pharmacy at Mustansiriya University. RPMI-1640 medium with L-glutamine (Capricorn, Germany) was used to cultivate AsPC-1 cells. Additionally, 1% penicillin/streptomycin solution (100x) and 10% fetal bovine serum (FBS) were added to the RPMI-1640 medium (Euroclone, Italy). In a humidified (95% humidity) CO2 incubator, cells were incubated at 37 °C. We bought Doxorubicin (Cat: ab120629) and APR-246, a p53 reactivator (Cat: Ab145974), from Abcam (USA). Dimethyl sulfoxide (DMSO) was used to dissolve the compounds to create a solution with a stock concentration of 10 mM for each inhibitor.

Cell viability assay

Using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, we evaluated the viability of AsPC-1 cells treated with mono-drugs (APR-246, Doxo) and their combinations. In brief, cells were seeded at a density of 7×10^4 cells/well in 96-well plates with a flat bottom, and they were incubated for 24 hours to ensure adhesion. The cells were then treated with APR-246 and Doxo for 72 hours. Once a 20μl MTT solution (5 mg/ml) was applied to each well, it was incubated at 37°C for three to four hours or until a violet precipitate was visible. An ELISA plate reader (BioTek, USA) was used to measure the absorbance of each well at 492 and 620 nm after adding 50μl of DMSO. The viability percentage is calculated as [(A test-A blank)/(A control-A blank)] x100, where absorbance is indicated by A. GraphPad Prism software version 6 (San Diego, CA, USA) was used to normalize the absorbance data as a percentage of
the control vehicle (CV). GraphPad Prism was used to determine the growth inhibitory concentration at which viability falls to 50% (GI50).

**Calculation of the CI values**

A combination of the two drugs (APR-246 and Doxo) was studied, and the cells were incubated with doses of each inhibitor in predetermined ratios (0.25x, 0.5x, 1x, 2x, 4x). The CalcuSyn software (Cambridge, UK) was used to calculate the CI using the Chou and Talalay approach. There is an additive effect when the CI is one, a synergistic effect when the CI is below one, and antagonism when the CI is greater than one.

**Observation of morphology under an inverted microscope**

The cellular morphology of AsPC-1 cells was studied after co-culture with APR-246 alone, Doxo alone, or both agents together. Plates with six wells and a flat bottom were used to plate AsPC-1 cells at a density of 5x10⁴ cells per well. The following day, APR-246 and Doxo were applied to the cells separately and together for 72 hours in an environment with 95% humidity, 37°C, and 5% CO2. A 200x inverted microscope (Meiji Techno, Japan) was used to examine the cells. A Canon camera and the EUS utility program were used to take the pictures.

**Detecting apoptosis with dual AO/EB staining**

Following a prior study, AO dye permeates into both live and dead cells and reacts with DNA or RNA, emitting either a green or red color. Only dead cells display red (EB) due to the breakdown of RNA, emitting either a green or red color. Only dead cells display red (EB) due to the breakdown of RNA, emitting either a green or red color. Only dead cells display red (EB) due to the breakdown of RNA, emitting either a green or red color. Only dead cells display red (EB) due to the breakdown of RNA, emitting either a green or red color. Only dead cells display red (EB) due to the breakdown of RNA. Following that, the Ca

**RT-qPCR (real-time PCR)**

TRIzol™ Reagent (Invitrogen, USA) was used to extract total RNA from approximately 3 million AsPC-1 PC cells after a 24-hour treatment with CV, Doxo, and APR-246. Based on the manufacturer's instructions, extracted RNA's quantity and purity were determined using the QubitTM RNA HS Assay Kit (ThermoFisher®, USA, Cat: Q32852). This assay can handle contaminants such as salts, free nucleotides, solvents, detergents, or proteins and is highly reliable and selective for quantifying low-abundance RNA samples. Then, the ProtoScript® First Strand cDNA Synthesis Kit (NEB, UK, Cat: E6300S) was used according to the manufacturer's instructions. Total RNA was reverse transcribed to obtain complementary DNA (cDNA). The cDNA product quantification was also done through Qubit™RNA HS Assay Kit and stored until the second step (Relative quantitative PCR) was performed. This assay is highly specific for double-stranded DNA (dsDNA) over RNA. Following that, Luna Universal qPCR Master Mix (NEB, UK, Cat: M3003S) was used. It is an optimized 2X reaction mix designed to detect and quantify target DNA sequences using the SYBR®/FAM channel of most real-time qPCR instruments. Taq DNA polymerase and a passive reference dye are included in the kit, which can be used with various instruments. The 2−ΔΔCT method was employed to evaluate gene expression, with GAPDH as the reference gene. The primer sequences utilized in this study were as follows: NOXA, F: 5′-GAAGGGAGATGACCTGTGATTAG-3′, R: 5′-GAGTCAGTTGGAACACTGAAA-3′; and GAPDH, F: 5′-GTGGTGGAACCATGAGTATGA-3′, R: 5′-GAGTCCTTCCACAGTACCAAAG-3′.

**Statistical analysis**

Microsoft Excel 2019 and GraphPad Prism were used for the statistical analysis. There were usually one to three biological replicates in most experiments. An unpaired student's t-test was used in this study to compare mean values. A p-value below the threshold of 0.05 is deemed statistically
significant for all data reported in terms of the standard error of the mean (SEM).

Results

**APR-246 and Doxo mono-treatment inhibit proliferation**

We examined the effects of increasing concentrations of APR-246 and Doxo on the viability of a mutant p53 (AsPC-1) PC cell line using the MTT assay. After incubation for 72 hours, both single drugs decreased the viability of cells in a dose-dependent manner (Figure 1). AsPC-1 was moderately sensitive to APR-246 (Mean GI$_{50}$ = 79 μM) (Figure 1A); in contrast, it was more sensitive to Doxo (Mean GI$_{50}$ = 1.58 μM) (Figure 1B). Accordingly, individual drugs reduced the proliferation of AsPC-1 but it was more sensitive to Doxo.

![AsPC-1 (72hr)](image)

**Figure 1.** Sensitivity curves for AsPC-1 cells cultured for 72 hours at escalating concentrations of APR-246 (A) and Doxo (B). Utilizing the MTT test, cell viability was evaluated. The data is the mean survival percentage ± standard error of the cells that treated with CV. The GI$_{50}$ value is shown in red.

**Co-treatment of APR-246 with Doxo has synergistic effects**

The CalcuSyn program was employed to compute CI values to ascertain the synergistic effects of APR-246 and Doxo combination on AsPC-1 cells. AsPC-1 cells were cultured with CV, fractions of GI$_{50}$ of APR-246 alone, Doxo alone, or both combined for a duration of 72 hours, as shown in the viability dose-response curves (Figure 2A). Interestingly, the simultaneous use of the drugs exhibited synergistic effects (CI<0.9) at effective dose 50 (ED50) (Mean CI=0.82 ± 0.09), ED75 (Mean CI=0.83 ± 0.11) and ED90 (Mean CI=0.85 ± 0.12) (Figure 2B).

To confirm the synergistic effect, we also investigated the morphology of AsPC-1 cells under an inverted microscope after exposure to CV, APR-246 (10μM), Doxo (1μM), or both drugs simultaneously for 24 and 72 hours (Figure 2C). After 24 hours, only a slight reduction in cell number was observed in Doxo and dual treatment and the morphology of cells was nearly similar in all treatments (Figure 2C, top panel). However, the cell number was remarkably reduced in dual drug treatment after 72 hours compared to single agents. Moreover, the cells were elongated or floating in Doxo and mixture treatments, and the effect was more potent in combination treatment (Figure 2C, bottom panel). These results indicate a potentially synergistic effect on the viability and morphology of PC cells.
Figure 2. The combination of APR-246 and Doxo is synergistic. (A) AsPC-1 cells were incubated with CV, fractions of GI\textsubscript{50} APR-246 alone, Doxo alone, or both drugs simultaneously. Cell viability was determined using the MTT assay. (B) Based on CalcuSyn software, CI values were calculated at ED50, ED75, and ED90. (C) Representative images of AsPC-1 exposed to CV, APR-246 (10μM), Doxo (1μM), or a combination of both drugs for 24 (top panel) and 72 hours (bottom panel), an inverted microscope was used. Data represent the average of two biological replicates. Ten and one micromolar were considered the GI\textsubscript{50} values for APR-246 and Doxo, respectively.

Effect of APR-246 co-treatment with Doxo on apoptosis and NOXA gene expression

After staining with AO/EB, fluorescent microscopy was used to assess the percentage of apoptosis induction in the AsPC-1 cells subjected to single and combined drugs. The cells were co-cultured with APR-246 (10μM), Doxo (1μM), or both drugs for 72 hours (Figure 3A, B). Remarkably, AsPC-1 PC cells cultured with Doxo either by itself or in conjunction with APR-246 exhibited a substantial rise in apoptosis (early plus late apoptosis). On the other hand, compared to cells treated with CV, cells treated with APR-246 monotherapy did not exhibit a statistically significant increase in apoptosis. (Figure 3A). Nevertheless, no further increase in the percentage of apoptotic cells was observed in the dual treatment compared to the Doxo treatment. Similarly, no significant increase was seen in the ratio of necrotic cells in different treatments compared to CV. The morphology and coloration of 100 cells were used to classify them into different phases of apoptosis (Figure 3B).
Figure 3. APR-246 and Doxo mixture affect apoptosis and NOXA expression in APC-1 cells. (A) AsPC-1 cells were exposed to CV, APR-246 (10μM), Doxo (1μM), or both drugs for 72 hours before apoptosis detection. (B) Representative images of two biological replicates of AsPC-1 stained with AO/EB and examined under a fluorescence microscope at a magnification of 400x. (C) Additionally, after 24 hours of treatment, the expression of NOXA gene in AsPC-1 cells was identified. After being incubated with the indicated treatments, the RT-qPCR bars show the fold change over CV; as a reference gene, GAPDH was used. Compared to the equivalent control (CV) bar, asterisks indicate significant changes in the displayed bars. The bar graphs (A, C) with error bars represent the mean ± SEM of two replicates, * p<0.05; *** p<0.001; ns=not significant. (B) Green arrows are viable cells, yellow arrows are early apoptotic cells, and orange arrows are late apoptotic cells.

To further understand the mechanism of synergy, we investigated the NOXA gene mRNA level by RT-qPCR in AsPC-1 cells incubated with APR-246/Doxo combination for 24 hours (Figure 3C). Noxa is a pro-apoptotic protein that mediates apoptosis induction downstream of the p53 pathway through the mitochondrial route. Interestingly, NOXA expression was significantly reduced in cells treated with single and combination treatments compared to cells incubated with CV (Figure 3C). As a result, Doxo alone induced apoptosis; however, when combined with APR-246, no further increase was obtained, which was confirmed at the NOXA gene level.

Discussion
The p53 protein is widely postulated to be responsible for most of the "hallmarks of cancer" while also being implicated in other cellular processes, such as apoptosis and control of the cell cycle. Therefore, restoring p53 activity is a promising therapeutic strategy, primarily focusing on specific disease hallmarks. Interestingly, selective small molecules have gained popularity with recent advancements as a treatment for various cancers, including PC. Additionally, 50–70% of PC patients carry mutations of the TP53 gene, which may make patients more resistant to chemotherapeutics like Doxo. Consequently, the protein encoded by the TP53 gene, whether in its wild-type or mutant form, represents a promising target for therapeutic intervention. Reactivating p53 has the potential to counteract the development of resistance to chemotherapy. Thus, this study aimed to determine whether the p53 reactivator APR-246 enhances the activity of chemotherapy treatment (doxorubicin) using human-derived PC cells with mutant p53 protein.

PRIMA-1.MET, also known as APR-246, is a compound that functions as a reactivator of the p53 protein; it restores the wild-type functionality of mutant TP53-encoded proteins. Interestingly, APR-246 causes cell cycle arrest and apoptosis in several cancer types; moreover, it has shown clinical efficacy in treating AML and Myelodysplastic syndromes (MDS). Given this encouraging result, we examined the effects of APR-246 monotherapy and Doxo monotherapy on PC cells (AsPC-1) with mutant p53 status. Viability results indicated a mild response after mono-treatment of AsPC-1 cells with APR-246. A possible explanation for the mild effect is an increase in the expression of the p53 inhibitor MDM2 in response to a remarkable drug-mediated restoration of the activity of mutated p53. In addition, APR-246 treatment (10μM) can not significantly alter the morphology of AsPC-1 cells. In contrast, the data reported by Yoshikawa et al. indicated that the use of elevated doses of APR-246 alone (50-100μM) in epithelial ovarian cancer cells was associated with significant morphological changes. Interestingly, the GI50 value for Doxo in AsPC-1 cells was comparable to that shown in another study.

Interestingly, several preclinical studies showed a synergistic effect after adding the p53 reactivator (APR-246, PRIMA-1.MET, PRIMA-1) to Doxo in several cancer types like multiple myeloma, ovarian cancer, and Neuroblastoma. Moreover, the APR-246 plus liposomal pegylated doxorubicin (LPD) regimen implemented in phase 1b clinical study showed encouraging efficacy in high grade serious ovarian cancer (HGSOC) patients. Therefore, we investigated the potential synergistic impact after combining APR-246 with Doxo on the viability of p53-mut AsPC-1 PC cells, and a synergistic effect was obtained, in addition to changes in cellular morphology. Interestingly, Mohell and co-authors found that adding APR-246 to Doxo reverted resistance and re-sensitized (reduced the GI50 of Doxo) Doxo-resistant p53 mutant ovarian cancer cell lines (A2780ADR and the parental cell line A2780) to Doxo. Another study speculated two potential scenarios behind the enhanced effect of the APR-246/Doxo combination. The first mechanism is via reactivation and the refolding process of mutant p53 protein by APR-246, thereby improving the impact of chemotherapeutic agent (Doxo) or by the accumulation of altered p53 protein by Doxo (DNA-damaging agent), hence, potentiates the effect of the p53 reactivator compound (APR-246).

To further understand the synergistic effect seen in AsPC-1 cells, the impact of APR-246 plus Doxo combination was also investigated on apoptosis induction and NOXA gene level, a component of the p53-dependent apoptosis pathway. In line with our previous studies, APR-246 monotherapy did not induce significant apoptosis as supported by the NOXA gene level. The suppression of NOXA might be attributed to mitogen-activated protein kinase (MEK) inhibition by APR-246, as indicated by prior reports. However, significant apoptosis accompanied by a reduction in NOXA gene expression was observed in AsPC-1 cells treated with Doxo; this may indicate that Doxo-induced apoptosis is mediated via p53-independent pathways. Although significant apoptosis was seen in both Doxo and combination treatments, the results implicated nearly similar percentages of apoptotic cells (at an early stage). Hence, the synergistic effect of dual therapy can be ascribed to cell cycle arrest besides apoptosis. Cell cycle arrest and apoptosis are the primary consequences of p53 activation; therefore, the reactivation of mutant p53 by APR-246 has been established by eliciting these effects, as observed in wild-type p53. To this end, a recent study found that APR-246 and Doxo dual drugs are
synergistic in p53-mut neuroblastoma cells, and the synergy was associated with the activation of the cell cycle inhibitor CDKN1A. As a direct target of p53 after p53 activation, transcription of p21/CDKN1A is significantly increased, leading to the induction of many target genes, which regulate cell cycle progression and eventually induce cell cycle arrest. Consequently, further studies on p53-mut and p53 wild-type PC cells are necessary to confirm the synergism obtained in this combination. Unfortunately, the biggest obstacle in this study was the lack of financial support.

Conclusion

In conclusion, about two-thirds of PC patients have mutant p53 proteins and are less likely to respond to chemotherapy. APR-246 benefits various cancer types by restoring p53 activity and reverting chemoresistance. Our data showed modest anti-proliferative activity of APR-246 as a monotherapy; however, when combined with Doxo, a synergistic effect associated with morphological changes but no further induction of apoptosis was observed in p53 mutant PC cells. This may indicate that this combination is synergistic via induction of cell cycle arrest. Hence, further preclinical and clinical studies are warranted to determine the efficacy of APR-246 and Doxo as a potential targeted cancer therapy in PC patients with mutant p53.

Authors’ Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Al-Nahrain.

Authors’ Contribution Statement

AA, designed the study. All authors performed material preparation and data collection. AA, analyzed Figures 1-3b, and SA, analyzed Figure 3c. AA, and SA, wrote the main manuscript text, and all authors reviewed the final draft.

References

يعزز التأثير المضاد للسرطان لعقار الدوكسوروبيسين ضد خلايا سرطان البنكرياس APR-246 المتحولة p53 AsPC-1
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الخلاصة
سرطان البنكرياس هو مرض ذو معدل وفيات مرتفع، ولا يزال التشخيص المبكر لسرطان البنكرياس يمثل تحديًا. يظل معدل البقاء النسبي لمدة 5 سنوات أقل من 8%，والاستراتيجيات العلاجية غير فعالة في زيادة معدلات بقاء المريض على قيد الحياة. في خلايا سرطان البنكرياس، ارتبطت مقاومة العلاج بالانحرافات الجينية التي تؤدي إلى ظهور مسارات خلوية شاذة؛ ولذلك، هناك ما يبرر ايجاد استراتيجيات جديدة لعلاج هذا المرض. هنا، سعينا لاستكشاف مُنشط p53 APR-246، إما كدواء أحادي أو مدمج مع الدوكسوروبيسين، دوائي p53-mut المحادي (p53-mut) AsPC-1 في خط خلايا p53-mut AsPC-1 ذات بروتين p53-mut AsPC-1، تم دراسة السمامة الخلوية لدوائي APR-246 ودوائي p53-mut AsPC-1، باستخدام اختبار MTT وحيال سبيكة. تم حساب مؤشر معامل قياس التأزمر (Combination Index) باستخدام برنامج CalcuSyn لدالة نهج Chou و Talalay. في الختام، APR-246 كأثر مضاد للتكاثر، في حين أظهر الدوكسوروبيسين تأثيرات تآزرية مرتبطة بالعوامل المورفولوجية. لم يلاحظ تأثير APR-246 على نسب موت الخلايا المبرمج في الخلايا المعالجة بالدوائي APR-246 و p53-mut AsPC-1. أظهر Clone 11 APR-246، علاج تآزر، دوائي APR-246، سرطان البنكرياس، سرطان، APR-246، APR-246، APR-246.