Investigational New Drugs (2021) 39:131–141
https://doi.org/10.1007/s10637-020-00998-z

PRECLINICAL STUDIES

# Pancreatic cancer drug-sensitivity predicted by synergy of p53-Activator Wnt Inhibitor-2 (PAWI-2) and protein biomarker expression

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Received: 13 July 2020 / Accepted: 4 September 2020 / Published online: 11 September 2020


## Summary

Today, pancreatic cancer (PC) is a major health problem in the United States. It remains a challenge to develop efficacious clinically useful PC therapies. New avenues, based on translational approaches and innovative validated biomarkers could be a preclinical option to evaluate PC drug candidates or drug combinations before clinical trials. Herein, we describe evaluation of combination therapies by incorporating a novel pathway modulator, **p**53-**A**ctivator **W**nt **I**nhibitor-**2** (PAWI-2) with other FDA-approved cancer drugs that have been used in PC clinical trials. PAWI-2 is a potent inhibitor of drug-resistant PC cells that has been shown to selectively ameliorate human pancreatic cancer stem cells (i.e., hPCSCs, FG$\beta_3$ cells). In the present study, we showed PAWI-2 produced therapeutic synergism with certain types of anti-cancer drugs. These drugs themselves oftentimes do not ameliorate PC cells (especially PCSCs) due to high levels of drug-resistance. PAWI-2 has the ability to rescue the potency of drugs (i.e., erlotinib, trametinib) and inhibit PC cell growth. Key molecular regulators of PAWI-2 could be used to predict synergistic/antagonistic effects between PAWI-2 and other anti-cancer drugs. Anti-cancer results showed potency could be quite accurately correlated to phosphorylation of optineurin (OPTN) in PC cells. Synergism/antagonism was also associated with inhibition of PCSC marker SOX2 that was observed in FG$\beta_3$ cells. Synergism broadens the potential use of PAWI-2 as an adjunct chemotherapy in patients with PC that have developed resistance to first-line targeted therapies or chemotherapies.

**Keywords** Pancreatic cancer cells · Cancer stemness · Drug resistance · Drug sensitivity · Drug synergy · Cell cycle arrest · PAWI-2

## Introduction

Pancreatic cancer (PC) is a major health problem in the United States and is the third leading cause of cancer-related deaths [1, 2]. PC is one of the most lethal diagnoses that oncology patients face. PC is known to be highly resistant to currently available treatments. Surgical resection with negative margins is the only potentially curative treatment for PC, but only 15%–20% of patients with PC are eligible for resection at initial diagnosis [3]. The remaining PC patients usually have metastatic or locally advanced disease that generally is considered incurable [4].

Most of the drugs approved by the United States Food and Drug Administration (FDA) for PC including capecitabine, erlotinib, 5-fluorouracil, gemcitabine, irinotecan, *nab*-paclitaxel, oxaliplatin, etc., are generally chemotherapies [5]. Unfortunately, PC oftentimes becomes resistant to these therapies. Increased administration of drugs is limited by somatic toxicity and serious side effects [6–8]. Combination of two or more standard therapies (i.e., chemotherapies) has the potential to revolutionize the treatment and care of PC. For example, a large number of PC patients do not respond to gemcitabine due to high levels of intrinsic and/or acquired chemo-resistance [9]. To improve clinical efficacy, gemcitabine-based therapy, is often combined with a second cytotoxic agent (e.g., platinum agents, fluoropyrimidine, etc.) [10–15].

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s10637-020-00998-z) contains supplementary material, which is available to authorized users.

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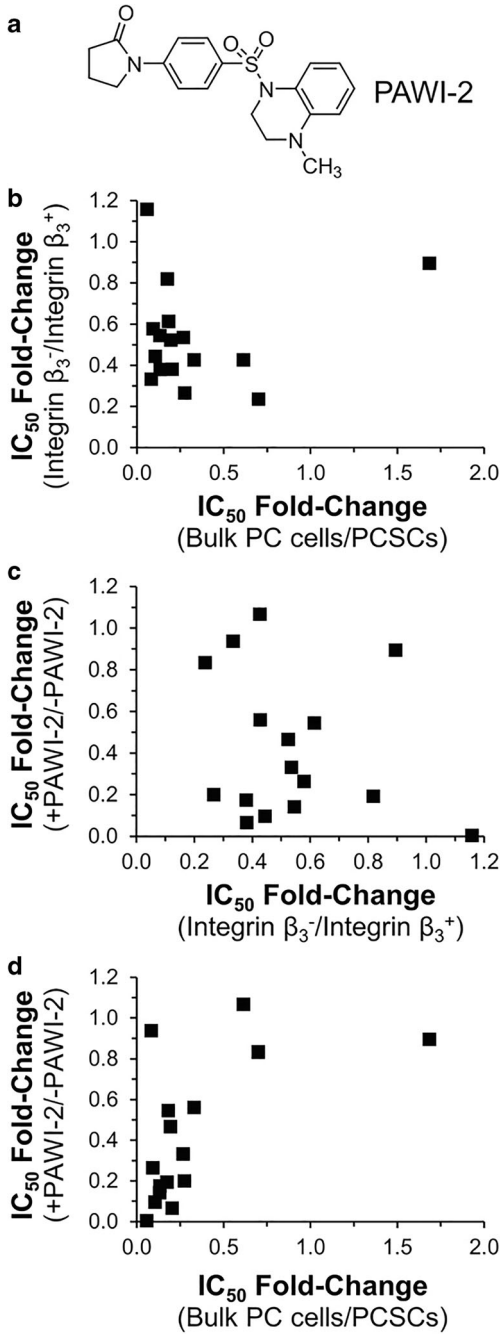


**Fig. 1** **a** Structure of PAWI-2. **b**–**d** Correlations between $IC_{50}$s to inhibit cell viability of MIA PaCa-2, FG and FG$\beta_3$ cells by anti-cancer drugs. **b** There was no significant correlation between ratios of $IC_{50}$s for inhibition of cell viability by anti-cancer drugs in FG cells versus FG$\beta_3$ cells and the ratio of $IC_{50}$s for anti-cancer drugs in MIA PaCa-2 versus FG$\beta_3$ cells (Spearman's rank-correlation coefficient (ρ): −0.252, $P = 0.346$). **c** There was also no significant correlation between ratios of $IC_{50}$s for inhibition of cell viability by anti-cancer drugs in FG$\beta_3$ cells in the presence or absence of treatment with PAWI-2 (10 nM; <$IC_{50}$) and ratios of $IC_{50}$s for anti-cancer drugs in FG cells versus FG$\beta_3$ cells (ρ: −0.216, $P = 0.421$). **d** There was a significant correlation between ratios of $IC_{50}$s for inhibition of cell viability by anti-cancer drugs in FG$\beta_3$ cells in the presence or absence of treatment with PAWI-2 versus the ratio of $IC_{50}$s for anti-cancer drugs in MIA PaCa-2 versus FG$\beta_3$ cells (ρ: 0.542, $P = 0.0300$*). The 16 anti-cancer drugs were chosen from 22 cancer drugs because they were potent below 50 μM based on Table 1 (i.e., 5-FU, CAPE, OXP, Rib, Pal and Tra excluded). Correlations in **b**, **c**, **d** were analyzed by Spearman's rank-correlation test and $P < 0.05$ was considered a significant correlation

This has been extensively investigated and showed superior efficacy over single-agent treatment in several prospective clinical trials [9, 13–15]. However, due to increased treatment-related toxicity, most trials to date have failed to show a significant improvement in overall survival.

Lack of effective PC treatments underscores an inadequate understanding of the biological complexity of PC. It is still unclear why PC often becomes resistant to therapies that work against other types of cancer. Understanding the underlying mechanism and impact of inhibiting PC signaling pathways could lead to new treatment strategies. Combination therapy of two or more cancer drugs is a mainstay of anticancer treatment in PC, with optimal combinations producing synergistic anti-tumor responses. Currently, most PC agents work at combining different S-phase targeting agents affecting DNA synthesis or function [16, 17]. Additional incremental increases in survival benefit may be achieved by combining agents with established safety profiles and non-overlapping mechanisms of action. However, testing drug alternatives in clinical trials is challenging unless they work with standard treatments (e.g., gemcitabine) [16]. New approaches, based on translational work and innovative validated biomarkers could be a potential solution to address this problem.

Recently, characteristics of inherent resistance to cancer therapy have been linked to a small subpopulation of cells classified as cancer stem cells (CSCs). CSCs are believed to be the hallmark of cancer responsible for tumor initiation, maintenance, dormancy, metastasis and relapse [18–20]. Accordingly, we focused on a human pancreatic cancer stem cell (hPCSC) reported previously (i.e., FG$\beta_3$ cells; a validated hPCSC model [21–23]). Previously, we identified and developed a novel pathway modulator (**p**53-**A**ctivator **W**nt **I**nhibitor-**2**, PAWI-2; Fig. 1a) [24–28] that inhibited in vitro PC cell growth regardless of PC types [27]. PAWI-2 was shown to selectively ameliorate hPCSCs [29, 30]. Herein, we evaluate new combination therapies by incorporating PAWI-2 with other FDA-approved anti-cancer drugs that have been in PC clinical trials. We investigated whether key regulator(s) that are associated with the mechanism of action of PAWI-2 could be used to predict synergistic, additive or antagonistic effects between PAWI-2 and other anti-

cancer drugs. Effects of drugs and PAWI-2 were evaluated against FG$\beta_3$ and with other bulk PC cells (i.e., FG, MIA PaCa-2 cells) for comparison. Results showed that PAWI-2 produced potent synergism with certain types of anti-cancer drugs. Synergism was correlated to the phosphorylation of optineurin (OPTN) and also associated with inhibition of a CSC biomarker - Sex-determining region Y-Box2 (SOX2) that was observed in hPCSCs (i.e., FG$\beta_3$ cells).

## Materials and methods

### Cell lines

MIA PaCa-2 (CRL-1420), HPAC (CRL-2199), AsPC-1 (CRL-1682) and BxPC-3 (CRL-1687) PC cell lines were purchased from American Type Culture Collection (ATCC). 779E is a patient-derived, low-passage primary PC cell line from Dr. Andrew Lowy (UC San Diego) [31]. FG and FG$\beta_3$ cells were provided by Dr. David Cheresh (UC San Diego and the Scripps Research Institute) [21–23]. Commercial cell lines were grown according to ATCC recommendations and authenticated by short tandem repeat (STR) DNA profiling at ATCC. 779E cells were characterized and cultured as reported [31]. FG and FG$\beta_3$ cells were grown in DMEM with 10% FBS. After thawing, cell lines were cultured at 37 °C in a humidified 5% $CO_2$ atmosphere and routinely screened for mycoplasma contamination.

### Compounds

Synthesis and pharmaceutical properties of PAWI-2 (Fig. 1a) were reported previously [24, 25]. FDA-approved clinical cancer drugs used in this study were from the National Cancer Institute (NCI) DTP Plated Compounds (10 mM in DMSO) [32].

### Cell viability and apoptosis assays

Cells were seeded onto plates and treated with test compounds (vehicle: 0.5% DMSO; PAWI-2 or other drugs: 0.1 nM to 50 μM) for 72 h. Cell viability was quantified using CellTiter-Glo (Promega). Data were expressed as percentage of survival compared to survival of vehicle-treated cells. Chou-Talalay analysis (ComboSyn) was used to test synergy of PAWI-2 in the presence of clinical cancer drugs [33].

### Stem cell biomarker array

FG and FG$\beta_3$ cells were seeded onto plates and treated with test compounds (vehicle: 0.5% DMSO; PAWI-2: 50 nM) for 8 or 16 h. Stem cell marker array tests were carried out according to the manufacturer's protocol (R&D systems). Briefly, whole-cell extracts were obtained after lysis with non-denaturing buffer and incubated with array membranes (containing 15 different anti-stem cell marker antibodies) overnight at 4 °C. Signals were detected using detection antibody cocktail and chemiluminescence reagents after exposure. Densities of immunoblot spots were quantified using ImageJ (NIH).

### Immunoblotting

Immunoblot experiments were carried out as before [27]. Briefly, whole-cell extracts were obtained after lysis with RIPA buffer (Supplementary Materials and Methods). Protein extracts were resolved by SDS-PAGE followed by immunoblotting using antibodies specific for target proteins (Supplementary Materials and Methods). Densities of immunoblot bands were quantified using ImageJ (NIH).

### Statistical analysis

Statistical analyses and graphical plots were done using GraphPad Prism. $IC_{50}$ values were calculated using nonlinear regression analysis of the mean and standard deviation (SD) of at least triplicate samples for each biological assay. Student *t* tests were used to calculate statistical significance for comparison between two groups. *P*-values <0.05 were considered significant. Spearman's rank-correlation test was used to analyze the correlation between two ranked variables. The strength and direction of association between two ranked variables was defined by Spearman's Rank correlation coefficient (ρ). *P* value <0.05 was considered a significant correlation.

## Results

### Effect of clinically used cancer drugs on cell viability in different PC cells

Integrin $\beta_3$/KRAS-driven cancer stemness and drug resistance have been reported [21–23]. FG$\beta_3$ cells were generated by stable transfection of fast-growing (FG) human PC cells with human integrin $\beta_3$/pcDNA3.1. FG$\beta_3$ cells have been thoroughly documented as an aggressive cell line showing CSC-like properties and cancer drug resistance [21–23]. In order to systematically characterize stem-like properties of these cells (i.e., FG$\beta_3$ cells), a stem cell biomarker array test was applied. FG$\beta_3$ cells and parental FG cells were compared. Several stem cell biomarkers were observed overexpressed in FG$\beta_3$ cells (i.e., Oct-3/4, SOX2, Nanog) but expressed less in FG cells (Supplemental Fig. S1A,B). This showed FG$\beta_3$ cells possessed stem-like properties.

**Table 1** Cell viability of PC cells in the presence of 22 anti-cancer drugs ± PAWI-2

| Drugs | Abbreviation | $IC_{50}$ values[a] (nM) | | | |
|---|---|---|---|---|---|
| | | MIA PaCa-2 | FG | $FG\beta_3$ | $FG\beta_3$ (with PAWI-2)[b] |
| 5-Fluorouracil | 5-FU | 14,000 | 6000 | 31,000 | >50,000[c] |
| Capecitabine | CAPE | 15,000 | >50,000[c] | >50,000[c] | >50,000[c] |
| Oxaliplatin | OXP | 19,000 | >50,000[c] | >50,000[c] | >50,000[c] |
| Irinotecan | IRI | 4600 | 3200 | 7500 | 8000 |
| Mitomycin C | MMC | 200 | 180 | 190 | 170 |
| Epirubicin | Epi | 40 | 280 | 480 | 450 |
| Gemcitabine | Gem | 21 | 7.1 | 30 | 25 |
| Erlotinib | Erlo | 5300 | 9900 | 26,000 | 1700 |
| Sirolimus | SLM | 220 | 4400 | 3800 | 12 |
| Sorafenib | SORA | 3300 | 3200 | 12,000 | 2400 |
| Sunitinib | Sun | 1300 | 8100 | 14,000 | 3700 |
| Ceritinib | Cer | 860 | 3600 | 8100 | 780 |
| Ribociclib | Rib | 14,000 | >50,000[c] | >50,000[c] | >50,000[c] |
| Palbociclib | Pal | 5200 | 21,000 | >50,000[c] | >50,000[c] |
| Trametinib | Tra | 1.7 | 50,000 | >50,000[c] | 4100 |
| Cabazitaxel | CBZ | 1.6 | 5.4 | 8.8 | 4.8 |
| Docetaxel | DTX | 2.7 | 3.5 | 8.2 | 4.6 |
| Paclitaxel | PTX | 4.1 | 11 | 21 | 9.8 |
| Vinblastine | VLB | 2.9 | 12 | 22 | 3.1 |
| Vinorelbine | NVB | 77 | 360 | 440 | 85 |
| Vorinostat | rINN | 670 | 1900 | 5000 | 870 |
| Bortezomib | Bor | 7.5 | 15 | 28 | 9.3 |

[a] $IC_{50}$ is the mean of three independent determinations

[b] Concentration of PAWI-2 was 10 nM; $<IC_{50}$

[c] Compound was not potent up to 50,000 nM

We used these cells to investigate how PAWI-2 re-sensitized clinical cancer drugs that normally have limited effects to inhibit PC cell viability. First, $IC_{50}$ values were determined for 22 clinical anti-cancer drugs examined in three cell lines (i.e., MIA PaCa-2, FG and $FG\beta_3$ cells; Table 1). The ratios of $IC_{50}$ values represent the loss of drug-sensitivity between bulk PC cells to PCSCs (i.e., the ratio of $IC_{50}$s in MIA PaCa-2 versus $FG\beta_3$ cells or the ratio of $IC_{50}$s in FG cells versus $FG\beta_3$ cells). These ratios were plotted against one another. There was no significant correlation between $IC_{50}$ ratios for drugs in FG cells (integrin $\beta_3^-$ cells) versus $FG\beta_3$ cells (integrin $\beta_3^+$ cells; hPCSCs) and $IC_{50}$ ratios for drugs in MIA PaCa-2 cells (bulk PC cells) versus $FG\beta_3$ cells (Fig. 1b; Spearman's rank-correlation coefficient (ρ): −0.252, $P = 0.346$). This showed that $IC_{50}$ values for this cohort of drugs did not possess a similar trend from drug-sensitive PC cells (low $IC_{50}$ values) to drug-resistant PC cells (large $IC_{50}$ values). Second, we quantified $IC_{50}$ values of 22 drugs in the presence of PAWI-2 (co-treated with 10 nM PAWI-2; < $IC_{50}$) in $FG\beta_3$ cells (Table 1). The $IC_{50}$ ratios (i.e., the ratio of $IC_{50}$s in FG versus $FG\beta_3$ cells or the ratio of $IC_{50}$s in MIA PaCa-2 versus $FG\beta_3$ cells) for 22 drugs were plotted versus $IC_{50}$ ratios in the presence and absence of PAWI-2 (Fig. 1c, d). The result showed no significant correlation between $IC_{50}$ ratios in the presence or absence of PAWI-2 (+PAWI-2/-PAWI-2) in $FG\beta_3$ cells and the ratio of $IC_{50}$s in FG cells (integrin $\beta_3^-$) versus $FG\beta_3$ (integrin $\beta_3^+$) cells (Fig. 1c; ρ: −0.216, $P = 0.421$). This data showed the ability of PAWI-2 to facilitate sensitization of anti-cancer drugs was not mainly associated with overexpression of integrin $\beta_3$ in $FG\beta_3$ cells. In contrast, there was a significant correlation between $IC_{50}$ ratios in the presence or absence of PAWI-2 (+PAWI-2/-PAWI-2) in $FG\beta_3$ cells and $IC_{50}$ ratios for drugs in MIA PaCa-2 cells (bulk PC cells) versus $FG\beta_3$ cells (Fig. 1d; ρ: 0.542, $P = 0.03$). The data showed the ability of PAWI-2 to overcome anti-cancer drug resistance in PCSCs was mainly correlated to recover the sensitivity of these clinical anti-cancer drugs in PCSCs. This effect could rescue the drug sensitivity back to a comparable level as that in bulk PC cells (i.e., MIA PaCa-2).

## Effect of PAWI-2 on PC cell viability in the presence of clinically used anti-cancer drug classes

Many of the drugs used in this study have been used in clinical trials of PC treatment [16, 34]. Drugs were separated into different classes for evaluation. Their ability to overcome drug-resistance was determined by testing the effect of each drug to inhibit FG$\beta_3$ cell viability in the presence of PAWI-2 (10 nM; <$IC_{50}$). We expanded this study to include class-specific clinically used drugs (i.e., 55 drugs; Fig. 2). This list included DNA repair/damage agents, epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), vascular endothelial growth factor receptor (VEGFR)-TKI, breakpoint cluster region protein (BCR)/Abelson murine leukemia viral oncogene homolog 1 (ABL) inhibitors, other kinase inhibitors (i.e., mTOR, CDK4/6, MEK, PI3K, ALK), microtubule (MT) disturbance agents and other pathway inhibitors (i.e., HDAC, PARP, Proteasome, Hedgehog pathway, Bcl-2). PAWI-2

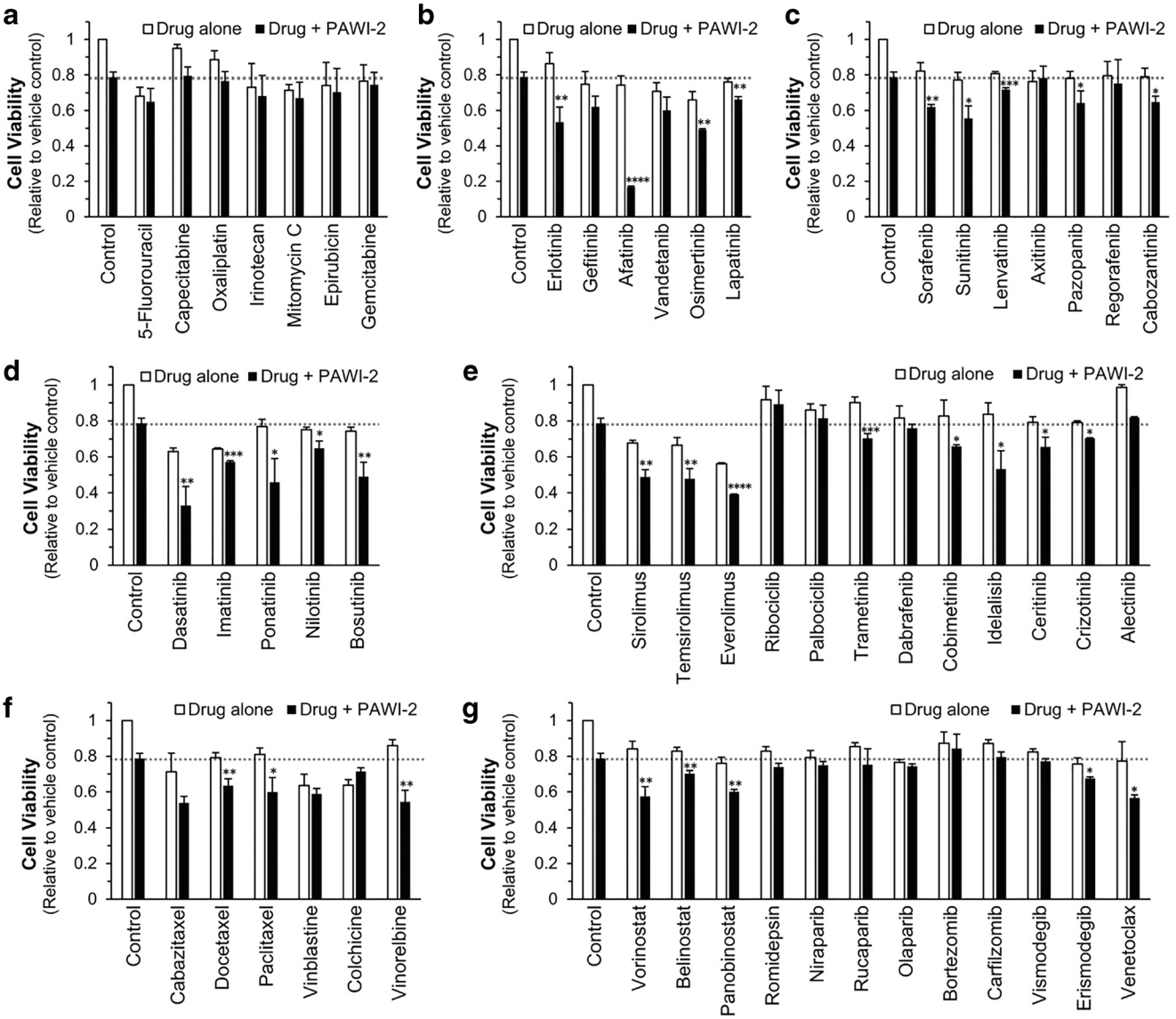


**Fig. 2** Percent inhibition of FG$\beta_3$ cell viability by clinically used anti-cancer drugs in the presence of PAWI-2 compared to single agent treatment. **a** DNA repair/damage agents, **b** EGFR-TKIs, **c** VEGFR-TKIs, **d** BCR/ABL inhibitors, **e** other kinase inhibitors (i.e., mTOR, CDK4/6, MEK, PI3K, ALK), **f** microtubule disturbance agents and **g** other pathway inhibitors (i.e., HDAC, PARP, Proteasome, Hedgehog pathway, Bcl-2). Concentration of PAWI-2 used was 10 nM. A dashed baseline in each figure stands for the level of % inhibition on cell viability by the treatment of PAWI-2 alone; treatment time used was 72 h; vehicle control (0.5% DMSO). Data are mean ± SD ($n = 3$); *P*-values were estimated by Student *t* tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$)

significantly enhanced ($P < 0.05$) inhibition of FG$\beta_3$ cell viability in the presence of certain classes of inhibitors (Fig. 2). Included in this list are most EGFR-TKIs (Fig. 2b), VEGFR-TKIs (Fig. 2c), mTOR inhibitors (Fig. 2e), MT disturbance agents (Fig. 2f), HDAC inhibitors (Fig. 2g). PAWI-2 also significantly enhanced ($P < 0.05$) inhibition of cell viability in the presence of Bcl-2 inhibitor Venetoclax (i.e., currently the only FDA-approved Bcl-2 inhibitor, Fig. 2g). The data for Venetoclax was consistent with previous results that showed PAWI-2 consistently acted at antagonizing a pro-survival role of Bcl-2 and Bcl-2-like proteins (i.e., Bcl-xL, Mcl-1) [27, 28].

**Table 2** Combination index (CI) values for inhibition of cell viability by 22 anti-cancer drugs ± PAWI-2 in hPCSCs FG$\beta_3$ cells

| Drug + PAWI-2[a] | Abbreviation | Combination Index[b] (CI) | | | |
|---|---|---|---|---|---|
| | | $ED_{50}$[c] | $ED_{75}$[c] | $ED_{90}$[c] | $ED_{95}$[c] |
| 5-Fluorouracil | 5-FU | 1.32 | 1.65 | 2.16 | 2.63 |
| Capecitabine | CAPE | 1.00 | 1.15 | 1.69 | 1.91 |
| Oxaliplatin | OXP | 1.11 | 1.03 | 0.99 | 0.97 |
| Irinotecan | IRI | 1.57 | 1.75 | 2.02 | 2.25 |
| Mitomycin C | MMC | 1.44 | 1.47 | 1.89 | 2.12 |
| Epirubicin | Epi | 1.68 | 2.16 | 2.50 | 2.64 |
| Gemcitabine | Gem | 1.35 | 1.26 | 1.60 | 2.14 |
| Erlotinib | Erlo | **0.68** | **0.45** | **0.32** | **0.25** |
| Sirolimus | SLM | **0.72** | **0.68** | **0.70** | **0.71** |
| Sorafenib | SORA | **0.64** | **0.79** | **0.81** | **0.84** |
| Sunitinib | Sun | 1.02 | 1.13 | 1.18 | 1.19 |
| Ceritinib | Cer | 1.02 | **0.91** | **0.66** | **0.66** |
| Ribociclib | Rib | 1.72 | 2.07 | 2.54 | 2.93 |
| Palbociclib | Pal | 2.68 | 2.90 | 3.15 | 3.35 |
| Trametinib | Tra | **0.93** | **0.76** | **0.61** | **0.46** |
| Cabazitaxel | CBZ | **0.68** | **0.56** | **0.50** | **0.49** |
| Docetaxel | DTX | **0.65** | **0.68** | **0.69** | **0.65** |
| Paclitaxel | PTX | **0.78** | **0.73** | **0.68** | **0.65** |
| Vinblastine | VLB | **0.22** | **0.85** | 3.31 | 8.36 |
| Vinorelbine | NVB | **0.34** | **0.55** | **0.68** | **0.72** |
| Vorinostat | rINN | **0.86** | **0.83** | **0.80** | **0.78** |
| Bortezomib | Bor | 1.15 | 1.56 | 2.56 | 2.98 |

[a] Anti-cancer drug/PAWI-2 were kept constant over the dose-dependent treatment for individual drug-PAWI-2 combinations; those ratios were determined based on the $IC_{50}$ values of anti-cancer drug alone in FG$\beta_3$ cells from Table 1 and the $IC_{50}$ of PAWI-2 alone in FG$\beta_3$ cells (i.e., 15 nM) [29]

[b] Combination Index (CI) values were calculated based on the Chou-Talalay method [33]. Values of CI < **1**, = 1 and > 1 indicate **synergism**, additive and antagonism, respectively; bold values show synergy

[c] $ED_{50,\ 75,\ 90,\ 95}$ represent CI values calculated at 50%, 75%, 90% and 95% inhibition of cell viability by the treatment of anti-cancer drug plus PAWI-2

## Chou-Talalay analysis of synergism/antagonism of PAWI-2 with 22 clinically used anti-cancer drugs

Twenty-two drugs that have been used in clinical trials of PC treatment (including DNA repair/damage agents, EGFR-TKI, VEGFR-TKI, mTOR, CDK4/6, MEK, ALK inhibitors, MT disturbance agents and HDAC, Proteasome inhibitors, as shown in Table 2) [16, 34] were examined in synergism analysis. Chou-Talalay analysis was done based on dose-dependent studies with PAWI-2. Chou-Talalay analysis (in vitro) is a well-established synergism analysis method based on comparison of dose-dependent inhibition of cell viability for drugs alone and drugs in combination [33]. Synergism or antagonism between drugs was defined by combination index (CI) values [33].

In FG$\beta_3$ cells, PAWI-2 synergized (CI < 1, Table 2) EGFR-TKI (i.e., Erlotinib), VEGFR-TKI (i.e., Sorafenib), mTOR inhibitor (i.e., Sirolimus), MEK inhibitor (i.e., Trametinib), ALK inhibitor (i.e., Ceritinib), MT disturbance agents (i.e., Cabazitaxel, Docetaxel, Paclitaxel, Vinorelbine) and HDAC inhibitor (i.e., Vorinostat). PAWI-2 antagonized (CI > 1, Table 2) DNA repair/damage agents examined (i.e., 5-Fluorouracil, Capecitabine, Oxaliplatin, Irinotecan, Mitomycin C, Epirubicin, Gemcitabine), CDK4/6 inhibitors (i.e., Ribociclib, Palbociclib) and proteasome inhibitor (i.e., Bortezomib). PAWI-2 strongly antagonized MT destabilizer - Vinblastine (CI at $ED_{95}$, >8). Strong antagonism was also observed when FG$\beta_3$ cells were co-treated with PAWI-2 and another MT destabilizer – Colchicine (CI at $ED_{95}$, >15). The antagonism between PAWI-2 and MT destabilizer may be attributed to the fact that PAWI-2 binds to a similar binding site as MT destabilizer [26].

The effect of PAWI-2 on Chou-Talalay synergism/antagonism of 22 clinically used anti-cancer drugs was evaluated in the bulk PC cell line (i.e., MIA PaCa-2). The results showed comparable trends on CI values for different classes of clinically used anti-cancer drugs as that observed in FG$\beta_3$ cells (Supplemental Table S1). However, in MIA PaCa-2 cells, PAWI-2 synergized additional drugs (e.g., Mitomycin C, Gemcitabine, Palbociclib; see Supplemental Table S1). In a parallel study in PCSCs (i.e., FG$\beta_3$ cells), gemcitabine only showed good synergism with 5 of 21 drugs tested (Supplemental Table S2).

## Correlation between combination index (CI) values and biomarker protein expression

The expression of biomarker proteins involved in the mechanism of action of PAWI-2 in FG$\beta_3$ cells includes modulation of OPTN and phosphorylation of OPTN at Ser177 (P-OPTN), modulation of p62/Sequestosome-1 (p62/SQSTM1, abbreviated as p62) and phosphorylation of p62 at Ser403 (P-p62), modulation of serine/threonine kinase Tank-binding kinase 1

(TBK1, IκB kinase (IKK)-related kinase) and phosphorylation of TBK1 at Ser172 (P-TBK1), modulation of cyclin-dependent kinase inhibitor p21 (also known as $p21^{WAF1/Cip1}$, abbreviated as p21) and modulation of cyclin D3 (CCND3) [29]. After individual administration of 22 clinically used anti-cancer drugs to FGβ3 cells, PAWI-2 modulated biomarker protein expression P-OPTN, P-OPTN/OPTN, P-p62, P-p62/p62, P-TBK1, p21, and CCND3 (Supplemental Fig. S2). Fold-change of protein biomarker expression in FGβ3 cells treated with anti-cancer drugs and PAWI-2 compared to drug alone were plotted versus CI values calculated for combination treatment (as shown in Table 2). Results showed a significant correlation between CI values and fold-change for phospho-Ser177-OPTN (P-OPTN) (Fig. 3a and Table 3; ρ: −0.929, $P < 0.0001$ for CI values at $ED_{90}$) and relative phosphorylation of OPTN (P-OPTN/OPTN) (Fig. 3b and Table 3; ρ: −0.916, $P < 0.0001$ for CI values at $ED_{90}$). In FGβ3 cells, data showed that in the presence of anti-cancer drugs greater synergism with PAWI-2 (lower CI values) was associated with increased phosphorylation of OPTN. In MIA PaCa-2 cells, CI values showed similar trends as that observed for FGβ3 cells for different classes of clinically used anti-cancer drugs (Supplemental Table S3; ρ: −0.842, $P < 0.0001$ for P-OPTN/OPTN versus CI values at $ED_{90}$). The phosphorylation of OPTN (expression level of P-OPTN or the ratio of P-OPTN/OPTN) induced by the 22 anti-cancer drugs examined in the presence or absence of PAWI-2 was useful for predicting drug synergism sensitivity in PC cells. In addition, observations about phosphorylation of OPTN was also very useful because it was broadly predictive of synergy by PAWI-2 regardless of PC cell types (Supplemental Fig. S3). In contrast, other relevant protein biomarkers (i.e., p62 and P-p62, TBK1 and P-TBK1, p21, CCND3), showed no significant correlation between biomarker expression ratios and CI values (Table 3; ρ: −0.30 to 0.48, $P > 0.05$).

As determined by a stem cell biomarker array test (Supplemental Fig. S1) and further confirmed by immunoblots of individual biomarkers (Supplemental Fig. S2), PAWI-2 primarily inhibited expression of three stem cell biomarkers (i.e., Oct-4A, Nanog, and SOX2). Similarly, fold-changes of stem cell biomarker expression in FGβ3 cells treated with combinations of anti-cancer drug and PAWI-2 compared to drug alone were plotted versus CI values calculated for anti-cancer drug combination treatment (as shown in Table 2). There was no significant correlation between fold-changes of stem cell biomarker expression (i.e., Nanog and Oct-4A) for the 22 drugs examined and the CI values for synergism or antagonism (Table 3; ρ: 0.01 to 0.18, $P > 0.4$). However, a significant correlation was found between expression level ratios of SOX2 and CI values of drugs synergized or

**Table 3** Correlation analysis between combination index (CI) values for $ED_{50,75,90,95}$ and fold-changes of protein biomarker expression for 22 anti-cancer drugs ± PAWI-2 in hPCSCs FGβ3 cells

| Protein Biomarkers[a] | Spearman's correlation coefficient (ρ)[b] | | | | *P*-value[c] | | | |
|---|---|---|---|---|---|---|---|---|
| | $ED_{50}$[d] | $ED_{75}$[d] | $ED_{90}$[d] | $ED_{95}$[d] | $ED_{50}$[d] | $ED_{75}$[d] | $ED_{90}$[d] | $ED_{95}$[d] |
| P-OPTN | −0.482 | −0.747 | −0.929 | −0.926 | 0.023* | < 0.0001 **** | < 0.0001 **** | < 0.0001 **** |
| P-OPTN /OPTN | −0.488 | −0.740 | −0.916 | −0.914 | 0.021 * | < 0.0001 **** | < 0.0001 **** | < 0.0001 **** |
| P-p62 | 0.123 | 0.006 | −0.211 | −0.189 | 0.585 ns | 0.980 ns | 0.345 ns | 0.399 ns |
| P-p62/p62 | −0.044 | −0.171 | −0.301 | −0.290 | 0.846 ns | 0.446 ns | 0.173 ns | 0.191 ns |
| p21 | −0.216 | −0.214 | −0.302 | −0.307 | 0.335 ns | 0.339 ns | 0.171 ns | 0.164 ns |
| CCND3 | 0.365 | 0.484 | 0.382 | 0.398 | 0.095 ns | 0.023 * | 0.080 ns | 0.066 ns |
| Nanog | 0.176 | 0.106 | 0.020 | 0.058 | 0.434 ns | 0.638 ns | 0.928 ns | 0.797 ns |
| Oct-4A | 0.014 | 0.141 | 0.119 | 0.133 | 0.950 ns | 0.530 ns | 0.599 ns | 0.554 ns |
| SOX2 | 0.519 | 0.777 | 0.905 | 0.884 | 0.013 * | < 0.0001 **** | < 0.0001 **** | < 0.0001 **** |

[a] Fold-changes of protein biomarker expression were determined by the relative ratio of protein density in the cells treated with "anti-cancer drug + PAWI-2" versus "anti-cancer drug alone". P-OPTN/OPTN and P-p62/p62 stand for relative phosphorylation of OPTN and p62, respectively

[b] Spearman's correlation coefficient (ρ) defines the strength and direction (positive or negative) of association between CI values and fold-changes of protein biomarker expression in Spearman's rank-correlation test; the closer ρ is to +1/−1 indicates the stronger positive/negative correlation relationship between the two ranked variables

[c] *P*-value <0.05 was considered a significant correlation: ns (not significant, $P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$

[d] $ED_{50,\ 75,\ 90,\ 95}$ represent ρ values or *P*-values calculated by the relevant correlations using CI values at 50%, 75%, 90% and 95% inhibition of cell viability (as shown in Table 2)

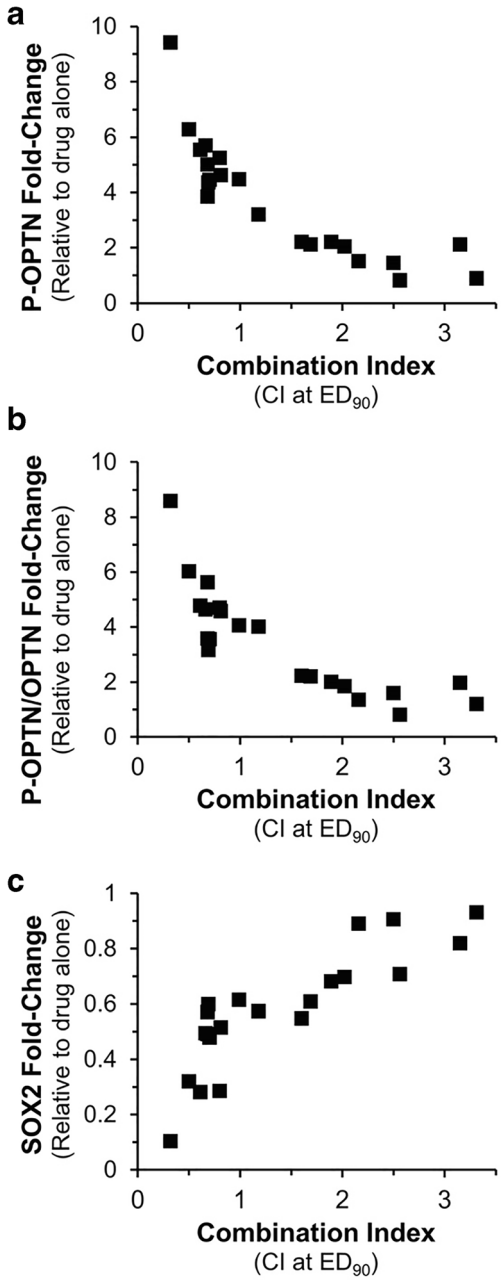


**Fig. 3** Correlations between fold-change of protein biomarkers ± PAWI-2 and combination index (CI) values for 22 anti-cancer drugs in FG$\beta_3$ cells. There was a significant correlation for fold-change of **a** phospho-S177-optineurin versus CI (P-OPTN; ρ: −0.929, $P < 0.0001$****), **b** relative phosphorylation of optineurin versus CI (P-OPTN/OPTN; ρ: −0.916, $P < 0.0001$****), **c** SOX2 (ρ: 0.905, $P < 0.0001$****) versus CI values. Fold-change of biomarkers in **a**, **b**, **c** where the ratio of protein levels in cells with co-treatment of 22 anti-cancer drugs and PAWI-2 was expressed relative to that in cells treated with anti-cancer drugs alone. These ratios represent an enhanced inhibition or activation of each specific protein biomarker by comparing combination of PAWI-2 with tested clinically used anti-cancer drugs to drugs alone. CI values in **a**, **b**, **c** were calculated at $ED_{90}$. Synergistic, additive or antagonistic effects of cancer drugs ± PAWI-2 (i.e., CI values of <1, = 1 or > 1, respectively) were examined in FG$\beta_3$ cells. Correlations in **a**, **b**, **c** were analyzed by Spearman's rank-correlation test and $P < 0.05$ was considered a significant correlation (****$P < 0.0001$)

antagonized by PAWI-2 (Fig. 3c and Table 3; ρ: 0.905, $P < 0.0001$). This suggested that certain types of stem cell markers could also be used to predict drug-sensitivity in PC cells with stem-like properties. However, this was only observed in FG$\beta_3$ cells and not in bulk PC cells (i.e., FG cells had 2-fold less expression and MIA PaCa-2 cells had undetectable expression of those stem cell markers).

Finally, there was no significant correlation between biomarker expression ratios (i.e., P-OPTN, P-p62, CCND3 and SOX2) and CI values evaluated by the same analysis of gemcitabine (Supplemental Table S4; ρ: −0.3 to 0.2, $P > 0.1$). In summary, increases in drug-sensitivity predicted by correlation of CI values and phosphorylation of OPTN or SOX2 expression is anti-cancer drug class specific and apparently unique for PAWI-2.

## Discussion

Although chemotherapies (i.e., gemcitabine-based therapy, often combined with a second cytotoxic agent such as platinum agents [10], fluoropyrimidine [11, 12] or a targeted cytotoxic agent [13–15]) is still the standard of care for patients with PC, recent clinical studies have not shown striking results with combination therapies involving two or more drugs [9, 35]. For example, only a combination of erlotinib with gemcitabine improved patients' survival, albeit not in a clinically meaningful way [13]. Considering the pivotal role of CSCs in the processes of tumorigenesis, progression, invasion, and metastasis, several CSC regulatory agents have been developed as PC therapies. While these agents initially showed encouraging results in clinical studies (e.g., tarextumab) they eventually failed due to a lack of patient benefit [34]. Targeting PCSCs by CSC regulatory agents applied in combination PC therapies (i.e., napabucasin with gemcitabine and nab-paclitaxel) also failed to significantly benefit patients [34]. Therefore, further studies are needed to identify optimal agents or best combination regimens for efficient therapies for PC especially for highly aggressive drug-resistant PC types (with stem-like properties).

In the present study, most of the 22 clinically used anti-cancer drugs for PC trials and used herein showed 5- to 20-fold less potent $IC_{50}$ values in PCSCs than that observed in bulk PC cells. In one case, the $IC_{50}$ value of Trametinib (i.e., a MEK1/2 inhibitor) in FG$\beta_3$ cells was $>10^4$ fold-less potent than that in MIA PaCa-2 cells (Table 1). This illustrates the fact that FG$\beta_3$ cells are relatively more drug-resistant than other PC cells. We observed that poorly potent drugs against PCSCs were "rescued" by PAWI-2. In some cases, drug potency was returned to that observed in bulk PC cells. This effect was not specifically related to stemness induced by overexpression of integrin $\beta_3$ because the ability of PAWI-2 to facilitate increased sensitization of anti-cancer drugs was

not correlated to drug-resistance specifically induced by over-expression of integrin $\beta_3$ (shown in Fig. 1c). The conclusion is that PAWI-2 does not work mainly through affecting dysregulated KRAS-NFκB signaling as we reported previously [29]. PAWI-2 is not dependent on one specific PCSC signaling pathway. Thus, the breadth of mechanism for PAWI-2 for inhibition of stem-like properties broadens the use of PAWI-2 in combination therapy applications to overcome drug-resistance of PCSCs.

Herein, the effect of PAWI-2 to induce anti-PC cell growth interactions was evaluated by Chou-Talalay synergism analysis with 22 clinically used anti-cancer drugs. These data showed PAWI-2 selectively synergized almost half (10 of 22) of these first-line therapies. Synergism between PAWI-2 and other clinically used drugs was class-specific but not single-drug specific (Fig. 2), indicating the effect may be attributed to cross-talk between multiple pathways and not an exclusive effect for certain drugs. Compared to FG$\beta_3$ cells, bulk PC cells (i.e., MIA PaCa-2 cells) showed similar sensitivity to some but not all classes of drug combinations. The data showed that PAWI-2 could re-sensitize inhibition of clinically used PC drugs and overcome certain types of drug resistance in highly aggressive PC types (i.e., PCSCs). In a parallel study of gemcitabine, less apparent synergism was observed (6 of 21 drugs). Chou-Talalay analysis showed there was only limited additive effects between gemcitabine and erlotinib in aggressive hPCSCs (i.e., FG$\beta_3$ cells, Supplemental Table S2). This may help explain the modest effect of gemcitabine to increase overall survival in combination regimens for the treatment of PC in the clinic.

Molecular targeting of specific signaling pathways have proven to be a useful strategy to overcome drug resistance. For example, increased expression of thymidine phosphorylase (TP) was reported to correlate with a poor response to 5-FU-based treatment in PC patients [36]. Additionally, gemcitabine-resistance is associated with elevated levels of cytidine deaminase (CDA) [37]. Development of efficient combination therapies based on certain protein expression profiles could be a helpful strategy for selecting agents that are likely to synergize and maximize the response to treatment. Our studies of the mechanism of PAWI-2 showed that certain proteins (e.g., P-OPTN, P-p62, P-TBK1, p21, CCND3, etc.) are associated with sensitivity to PAWI-2 [29]. To evaluate whether any changes occurred in cells that initiate/induce drug synergy or antagonism, we also analyzed expression levels of proteins associated with the mechanism of PAWI-2. The present data suggest that elevated levels of phosphorylation of OPTN, coupled with low expression of SOX2 (a stem cell biomarker) may be important determinants for developing combination regimens with PAWI-2.

As reported previously, phosphorylation of conserved OPTN residue (Ser177) induced by PAWI-2 promotes OPTN translocation into the nucleus and causes G2/M arrest [29]. Concomitantly, OPTN phosphorylation induced by PAWI-2 has negative feedback regulation of TBK1 functional activity to reverse tumor stemness and drug resistance in FG$\beta_3$ cells. Herein, phosphorylation of OPTN was observed to be broadly associated with determining control of PAWI-2 in increasing drug-sensitivity of PC. This effect was observed for PAWI-2 but not for other standard treatments (i.e., gemcitabine, Supplemental Table S4). In addition, certain biomarkers may also explain the differential response of PAWI-2 in synergizing other drugs in different PC types. For example, low stimulation of phosphorylation of OPTN by PAWI-2 in BxPC-3 cells (Supplemental Fig. S3) may account for less apparent synergism observed previously [27].

Transcription factor SOX2 is involved in CSC maintenance and dysregulation of SOX2 expression is highly associated with the capacity of cancer cell proliferation and metastasis. Moreover, there is evidence that SOX2 mediates resistance towards established cancer therapies. However, SOX2 is aberrantly expressed in PC and the function of SOX2 in PC is unclear [38]. Recently, SOX2 was shown to contribute to PC stemness/de-differentiation via regulation of G1/S transition. SOX2 overexpression promotes S-phase entry and cell proliferation associated with cyclin D3 induction [39]. SOX2 expression is also associated with increased levels of other PCSC biomarkers (e.g., ALDH1, ESA and CD44) [39]. Targeting and negatively regulating expression of SOX2 by PAWI-2 could further contribute to the ability of PAWI-2 to overcome cancer stemness in PCSCs. SOX2 protein expression could also be a useful biomarker for predicting drug-sensitivity of clinical drugs with PAWI-2 for SOX2-positive PC types (i.e., PCSCs).

Genetic heterogeneity of PC confers characteristics of invasion, metastatic migration and multiple treatment resistance [40, 41]. PC has been described as a complex molecular landscape with no predominant phenotype and several core molecular pathways (e.g., DNA damage repair, cell cycle regulation, etc.) [42]. These molecular pathways may be considered druggable but only affect a limited percentage of patients with PC. Personalized therapeutic strategies have not made a significant improvement for PC [43, 44]. In the preclinical setting, results herein suggest that a combined evaluation and quantification of several protein markers could help develop individualized medicines by exploring biomarker expression patterns in patients with different PC genotypes.

In conclusion, PAWI-2 is a potent drug for treating drug-resistant PCs. PC drug sensitivity can be predicted by correlations between synergism induced by PAWI-2 and biomarker expression (i.e., P-OPTN, SOX2). No cross-relationship for this type of correlation for other drugs (e.g., gemcitabine) was observed pointing to the novelty of PAWI-2. The results suggest that PAWI-2 could be used alone or as adjunct chemotherapy in patients with PC that have developed resistance to one of the first-line chemotherapies or targeted therapies. In

addition, quantitative analyses of certain biomarkers in different PC types point to a potent strategy for developing individualized chemo-therapeutic regimens.

**Acknowledgements** We thank Dr. Andrew Lowy of the University of California, San Diego for 779E cells. We also thank Dr. David Cheresh of the University of California, San Diego and The Scripps Research Institute for FG and FG$\beta_3$ cells. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official view of CIRM or any other agency of the State of California.

**Authors' contributions** J. C. and J. R. C. conceived the study. J. C. conducted and carried out all cell-based studies, data analysis and statistical analysis. Both authors contributed to drafting and revising the manuscript. Both authors approved the manuscript.

**Funding** This work was supported by an Inception Award from the California Institute for Regenerative Medicine (CIRM) (DISC1–10583; J. R. Cashman) and by funds from the Human BioMolecular Research Institute and ChemRegen, Inc. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official view of CIRM or any other agency of the State of California.

**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Compliance with ethical standards

**Conflicts of interest** The authors declare that there are no conflicts of interest.

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Code availability** Not applicable.

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