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# The glucosylceramide synthase inhibitor PDMP sensitizes pancreatic cancer cells to MEK/ERK inhibitor AZD-6244



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## ABSTRACT

Here we show that *D,L*-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), a glycosphingolipid biosynthesis inhibitor, increases the sensitivity of pancreatic cancer cells to the novel MEK–ERK inhibitor AZD-6244. AZD-6244 and PDMP co-administration induced massive pancreatic cancer cell death and apoptosis, more potently than either drug alone. We discovered that AZD-6244 induced ceramide production in pancreatic cancer cells, yet the excess ceramide was metabolically removed in the long-term (24–48 h). PDMP facilitated AZD-6244-induced ceramide production, and ceramide level remained elevated up to 48 h. Meanwhile, exogenously-added cell-permeable short chain ceramide (C2) similarly sensitized AZD-6244's activity, the two caused substantial pancreatic cancer cell death and apoptosis. At the molecular level, PDMP and AZD-6244 co-treatment inactivated ERK1/2 and AKT–mTOR signalings simultaneously in pancreatic cancer cells, while either agent alone only affected one signaling. In summary, PDMP significantly increased the sensitivity of AZD-6244 in pancreatic cancer cells. This appears to involve a sustained ceramide production as well as concurrent block of ERK and AKT–mTOR signalings.

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## 1. Introduction

Over the past decades, the incidence of pancreatic cancer has been increasing steadily worldwide [1,2]. It is arguably the most aggressive human cancer, with a median survival time of 3–6 months [1,2]. The five-year survival of pancreatic cancer is low (<5%) [1,2], and it is the fourth leading cause of cancer-related mortalities around the world [1–3]. Most pancreatic cancer patients present with tumors in advanced and/or incurable stages, often with metastases or local invasions, that are ineligible for curative surgical resection [3]. Yet, pancreatic cancers commonly display limited susceptibility, if not high resistance, to conventional chemo-drugs [3]. Clinically, gemcitabine is the only approved chemo-agent for pancreatic cancer therapy, with only limited value in promoting patients' survival [4]. Therefore, there is an urgent need for new therapies of pancreatic cancer [3].

**Abbreviations:** MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; PDMP, *D,L*-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; mTOR, mammalian target of rapamycin; MAPK, mitogen activated protein kinase; S6K1, p70 S6 kinase 1; PI3K, phosphatidylinositol-3-kinase; PARP, poly ADP ribose polymerase; PP, protein phosphatase.

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Many pancreatic cancers have molecular-based genetic mutations [3]. For example, activating mutations in *KRAS* occur in around 90% of all pancreatic cancers [5], causing constitutive activation of downstream signal transduction pathways including the MEK–ERK mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/AKT–mammalian target of rapamycin (mTOR) signaling cascades, both play significant roles in cancer cell proliferation, survival, migration and chemo-resistance [6]. AZD-6244 is a novel potent and selective MEK/ERK inhibitor. Its activity against pancreatic cancer has been tested *in vitro* and *in vivo* [7–9].

Ceramide is a main component of the programmed cell death (apoptosis) machinery triggered by different stimuli [10–12]. Many anti-cancer drugs are able to induce endogenous ceramide production to promote cancer cell apoptosis [11–13]. Ceramide homeostasis is controlled by several metabolic events leading to its deacylation, phosphorylation or glucosylation or to sphingomyelin synthesis [10–12]. *D,L*-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is a well-known inhibitor of glucosylceramide synthase [14]. The formation of glucosylceramide is drastically inhibited by PDMP, and a concomitant accumulation of ceramide may occur. In the current study, we found that PDMP facilitates AZD-6244-induced ceramide production to dramatically sensitize its anti-pancreatic cancer activity.

## 2. Material and methods

### 2.1. Chemicals and reagents

PDMP was obtained from Sigma–Aldrich Chemicals (Sigma, St. Louis, MO). AZD-6244 was purchased from Selleck China (Shanghai, China). C2-Ceramide was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Anti-AKT1, ERK1/2, tubulin and p70 S6 kinase 1 (S6K1) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylate (p)-AKT (Ser-473), p-ERK1/2 (Tyr-202/Thr-204), p-S6K1 (Thr-389) and poly ADP ribose polymerase (PARP) antibodies were obtained from Cell Signaling Technology (Beverly, MA).

### 2.2. Cells

Human pancreatic cancer lines AsPC-1, PANC-1 or MIA-PaCa-2 (MIA) were maintained in DMEM/RPMI medium, supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and 4 mM L-glutamine, in a CO<sub>2</sub> incubator at 37 °C.

### 2.3. Cell viability detection

The cell viability was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plates at a density of  $1 \times 10^4$  cells/well. After treatment, 20  $\mu$ L of MTT (5 mg/mL, Sigma) was added for 2 h (Sigma–Aldrich, St. Louis, MO). Afterwards, the medium was rapidly removed, and the MTT crystals were solubilized using dimethyl sulfoxide (DMSO). The resulting absorbance was read in a plate reader at 490 nm wavelength. Absorbance readings were always subtracted from the value of blank wells.

### 2.4. Clonogenicity assay

A total of  $5 \times 10^3$  cells per well suspended in 150  $\mu$ L of Mix agar with 1.5 mL DMEM/10% FBS were plated in 30 mm plates overlying a 1% agar-DMEM/10% FBS (1:1) bottom layer. Cells were then maintained in drug-containing medium, which was renewed every two days for a total of 10 days. Afterwards, the number of remaining colonies were manually counted.

### 2.5. Trypan blue staining

After indicated treatment, dead cells were stained with trypan blue, which were counted in a Neubauer chamber, and the percentage (%) of cell death was calculated by trypan blue rates.

### 2.6. Annexin V apoptosis analysis

Apoptosis analysis was conducted using the Annexin V-FITC Apoptosis Detection Kit II (BD Biosciences, San Jose, CA) following the manufacturer's protocol. Briefly, after treatment, cells were washed twice with PBS, and  $1 \times 10^6$  cells were resuspended in 1 mL of  $1 \times$  Annexin V-binding buffer. Cells undergoing apoptotic cell death were analyzed by counting the cells that stained positive for Annexin V-FITC (fluorescein isothiocyanate) using FACSCalibur (BD Biosciences).

### 2.7. Caspase-3 activity assay

After treatment, cell lysates were cleared by centrifugation and assayed for caspase-3 activity using a DEVDpNA peptide substrate and incubated for 6 h at 37 °C. Caspase-3 activity was measured using Colorimetric Assay kits from R&D Systems (Minneapolis,

MN). The activities were quantified spectrophotometrically at a wavelength of 405 nm.

### 2.8. Intracellular ceramide measurement

Intracellular ceramide was measured as described previously [15,16]. After treatment, cells were washed in PBS and lysed using ceramide assay lysis buffer (50 mM Tris (pH-7.4) containing 0.4% IGEPAL CA 630, Sigma) by freeze and thaw method. The final concentration of IGEPAL CA 630 in the assay was 0.2%. The lysates were then heated at 70 °C for 5 min and centrifuged at 12000 rpm for 10 min at 4 °C. The reaction was started by mixing 10  $\mu$ L of supernatant with 10 ng recombinant human neutral ceramidase enzyme (10  $\mu$ L), and incubated for 1 h at 37 °C. After stopping the reaction by adding 55  $\mu$ L of stopping buffer (1:9, 0.07 M potassium hydrogen phosphate buffer: methanol), the released sphingosine was derivatized with ophthalaldehyde as described previously [15,16]. After incubation for 30 min at room temperature in the dark, an aliquot of 25  $\mu$ L was used for the ceramide analysis. High-performance liquid chromatography (HPLC) analyses were conducted using Waters 1525 binary pump system (Milford, MA). Waters XTerra RP18 (5  $\mu$ m,  $3 \times 250$  mm) column (Waters, Milford, MA) was equilibrated with a mobile phase (20% methanol, 80% 1:9 stopping buffer) at a flow rate of 0.5 mL/min. The fluorescence detector (Waters 2475) was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. OD value was utilized as an quantitative indicator of cellular ceramide level.

### 2.9. Western blots

The cells were washed with ice-cold PBS before lysed with the lysis buffer (Beyotime, Shanghai, China). The lysates were separated by the 10% SDS–polyacrylamide gel, and were electro-transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Thereafter, membranes were blocked with 10% milk in PBS plus Tween-20 (0.5%) (TBST), incubated overnight at 4 °C with the primary antibody, and then incubated with HRP-conjugated secondary antibody (Santa Cruz). The detection was performed by Supersignal West Pico Enhanced Chemiluminescent (ECL, Pierce, Rockville, IL). The blot intensity was quantified by Image J software. The intensity of each phosphorylated band was normalized to the intensity of non-phosphorylated kinase band (the loading control).

### 2.10. Statistical analysis

Each experiment was performed three times with similar results obtained. Data presented were the mean  $\pm$  standard deviation (SD) of one representative experiment. Statistical analysis was performed using Graph pad Prism 5.0 by one-way ANOVA and Bonferroni post hoc test. A difference was considered significant when  $P < 0.05$ .

## 3. Results

### 3.1. PDMP sensitizes AZD-6244's activity against pancreatic cancer cells

A panel of human pancreatic cancer cell lines, including PANC-1, AsPC-1 and MIA-PaCa-2 cells, were maintained in 10% FBS to mimic the *in vivo* nutrition situation. MTT assay results in Fig. 1A–C showed that treatment with AZD-6244 (AZD) by itself only slightly inhibited the viability of above cancer cells. Whereas co-administration with D,L-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) caused a substantial viability reduction (Fig. 1A–C). Note that PDMP itself only had minor effect on pancreatic cancer cell viability (Fig. 1A–C). PDMP-mediated

AZD-6244-sensitization effect was most obvious in PANC-1 cells (Fig. 1A). AZD-6244 and PDMP co-administration also resulted in a dramatic increase in PANC-1 cell death (trypan blue staining), more potently than either agent alone (Fig. 1D). Results from the clonogenicity assay displayed that AZD-6244 and PDMP synergistically decreased the number of PANC-1 colonies, indicating growth inhibition (Fig. 1E). Drug concentration and time course were selected based on the previous publications and pilot experiments (same for all figures). Trypan blue staining and clonogenicity assay were also performed in other two other cancer cell lines, and similar results were obtained (data not shown).

### 3.2. PDMP facilitates AZD-6244-induced pancreatic cancer cell apoptosis

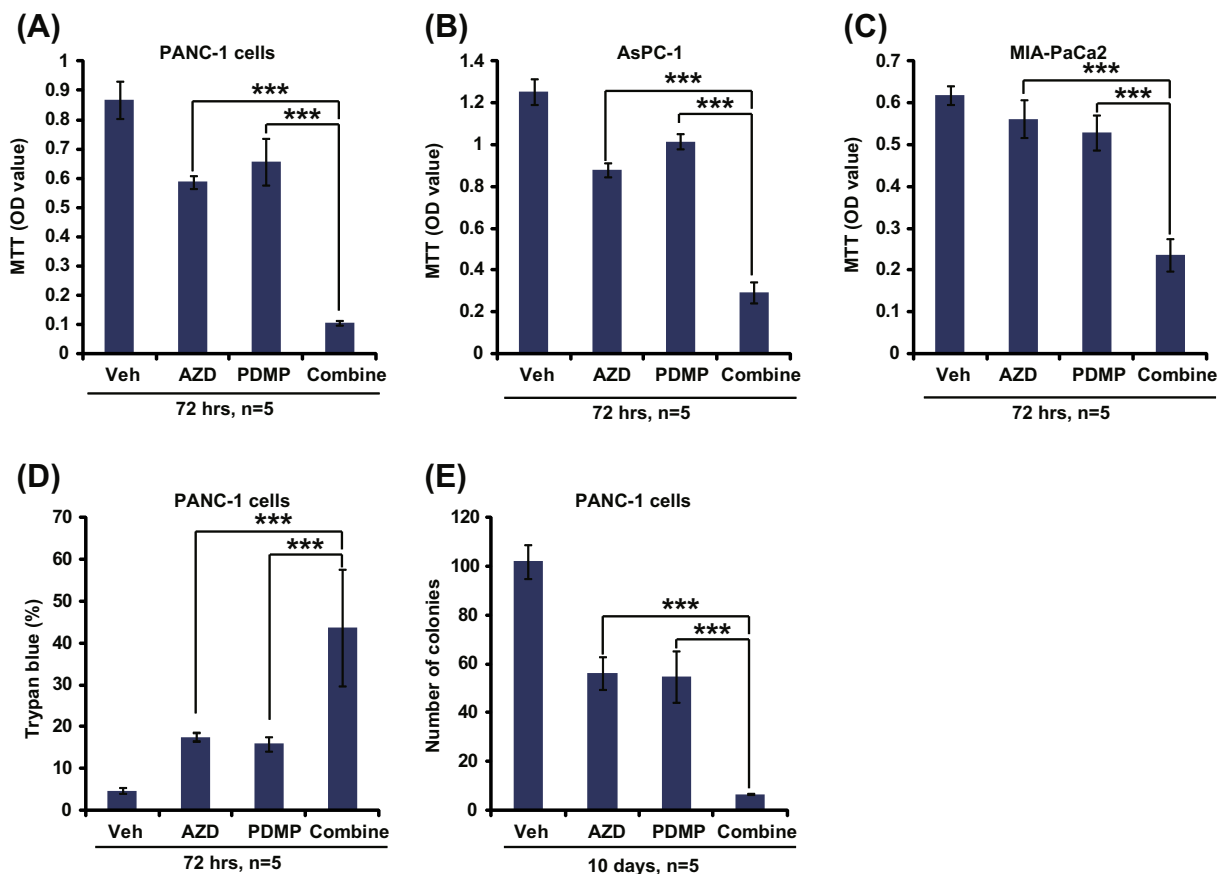
Next, we tested the effect of PDMP on AZD-6244-induced pancreatic cancer cell apoptosis, which was tested by three independent assays: Annexin V FACS assay, caspase-3 activity assay and Western blots testing cleaved-PARP. Results from all these assays showed that AZD-6244 as a single agent only slightly increased apoptosis in PANC-1 cells (Fig. 2A–D), co-administration with PDMP significantly enhanced AZD-6244-induced PANC-1 cell apoptosis, evidenced by dramatic increases in Annexin V percentage (Fig. 2A and B), caspase-3 activity (Fig. 2C) and PARP cleavage (Fig. 2D). Similar results were also seen in AsPC-1 cells (Fig. 2E–G) and MIA-PaCa-2 cells (data not shown). Note that PDMP alone also induced moderate apoptosis in tested pancreatic cancer cells (Fig. 2). Thus, PDMP significantly facilitates AZD-6244-induced pancreatic cancer cell apoptosis.

### 3.3. AZD-6244 induces ceramide production, facilitated by PDMP

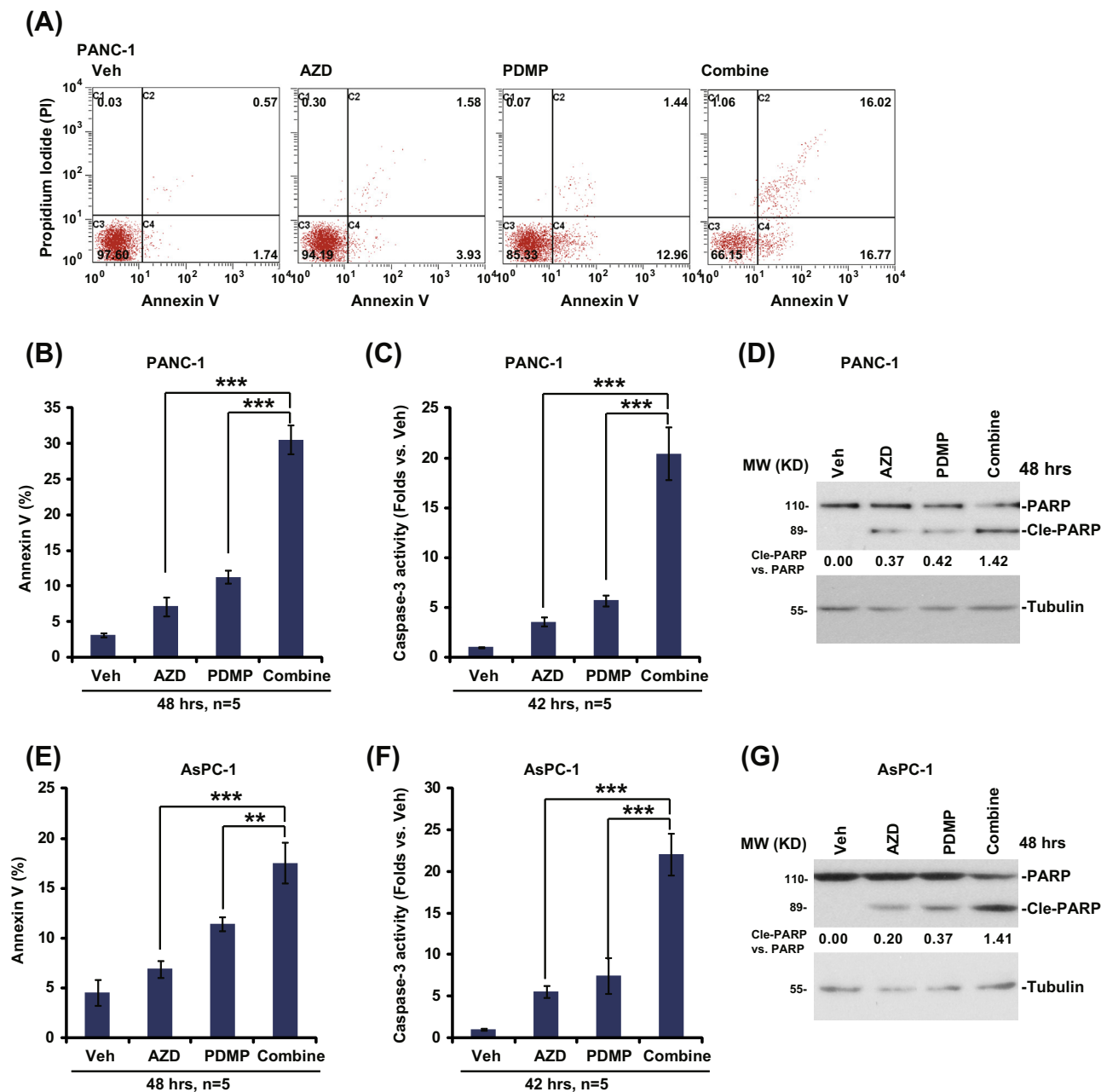
PDMP is a potent inhibitor of sphingolipid glucosylation [17]. PDMP could increase ceramide level by facilitating its synthesis and inhibiting its metabolism [18,19]. We thus tested the level of ceramide in PANC-1 cells treated with PDMP and/or AZD-6244. We discovered here that AZD-6244, the novel MEK/ERK inhibitor, could also induce a short-term (12 h) ceramide production in PANC-1 cells, yet excessive ceramide went down to control level after 24–48 h (Fig. 3A). Significantly, co-administration with PDMP resulted in a sustained (last at least for 48 h) and potent ceramide increase in PANC-1 cells (Fig. 3A). PDMP alone also slightly increased cellular ceramide level (Fig. 3A). Based on these results, we speculate that AZD-6244-induced ceramide production is probably subjected to metabolic clearance (i.e. glucosylation) in PANC-1 cells, where inhibition of sphingolipid glucosylation by PDMP hampers this ceramide clearance to maintain cellular ceramide at a high level, causing substantial cell apoptosis. Correspondingly, exogenously adding a cell-permeable short chain ceramide (C2) similarly sensitized AZD-6244's activity, causing massive PANC-1 cell death and apoptosis (Fig. 3B–D). Note that same results in this figure were also repeated in AsPC-1 and MIA-PaCa-2 cells (Data not shown).

### 3.4. PDMP and AZD-6244 co-administration in-activated AKT, ERK and S6K1 in pancreatic cancer cells

Studies have shown that combined block AKT and ERK activation will result in a better inhibition of pancreatic cancer cells. As a matter of fact, AZD-6244 displayed higher efficiency against pancreatic



**Fig. 1.** PDMP sensitizes AZD-6244's activity against pancreatic cancer cells—The viability of PANC-1 (A), AsPC-1 (B) and MIA-PaCa-2 (C) cells, treated with AZD-6244 (100 nM) and/or PDMP (20  $\mu$ M) for 72 h, was tested by MTT assay. PANC-1 cells, stimulated with AZD-6244 (100 nM) and/or PDMP (20  $\mu$ M), were subjected to trypan blue staining assay (D, 72 h) and clonogenicity assay (E, 10 days). The data were expressed as mean  $\pm$  S.D. of one representative experiment (same for all figures). Experiments in this and all following figure were repeated three times, and similar results were obtained. "Veh" stands for 0.1% DMSO. "hrs" stands for hours (all figures). \*\*\* $P$  < 0.001.

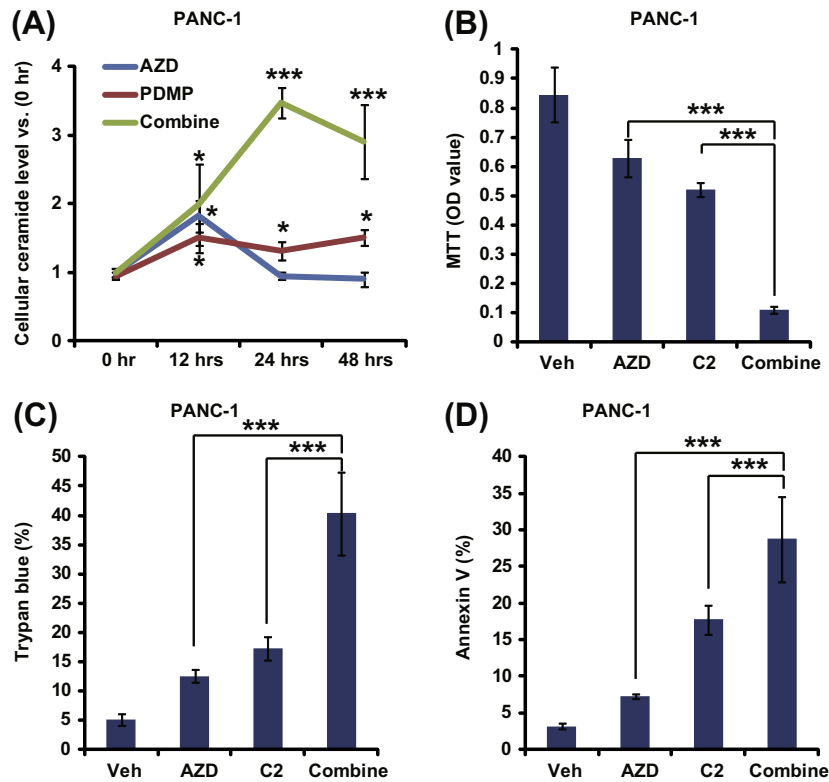


**Fig. 2.** PDMP facilitates AZD-6244-induced pancreatic cancer cell apoptosis—PANC-1 cells or AsPC-1 cells, stimulated with AZD-6244 (100 nM) and/or PDMP (20  $\mu$ M), were subjected to Annexin V FACS assay (A, B, and E) or caspase-3 activity assay (C and F) to test cell apoptosis. Expression of PARP and tubulin in above cells 24 h after treatment was also shown (D and G). PARP cleavage was quantified. “Veh” stands for 0.1% DMSO. \*\* $P < 0.01$ . \*\*\* $P < 0.001$ .

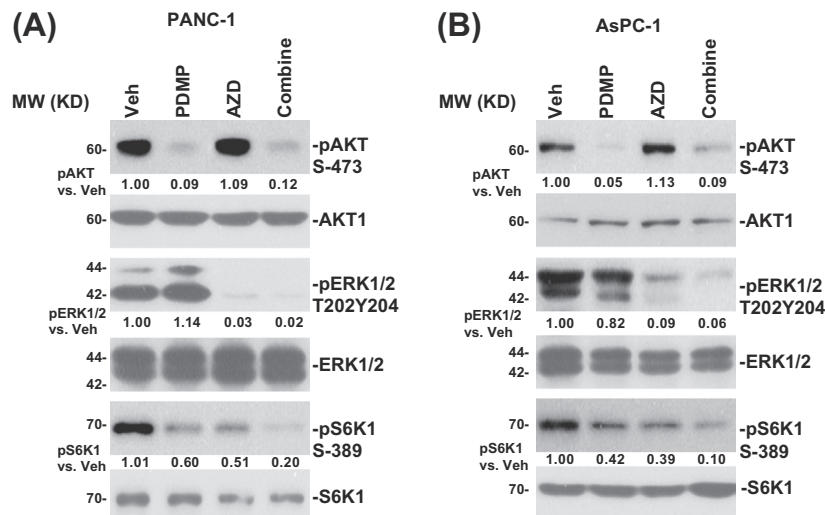
cancer cells when co-administrated with AKT–mTOR inhibitors [7]. We thus tested activation of ERK and AKT–mTOR in pancreatic cancer cells treated with AZD-6244 and/or PDMP. As expected, AZD-6244 blocked phosphorylation/activation of ERK1/2, but not AKT, in both PANC-1 and AsPC-1 cells (Fig. 4A and B). On the other hand, PDMP inhibited AKT activation without affecting ERK activation in two tested cell lines (Fig. 4A and B). AZD-6244 and PDMP co-administration thus inhibited AKT and ERK activation simultaneously (Fig. 4A and B). More importantly, phosphorylation of S6K1, an indicator of mTOR activation, was dramatically inhibited by the co-administration, while either agent unaccompanied had a less potent effect (Fig. 4A and B). In summary, co-administration of AZD-6244 and PDMP inhibits activation of ERK, AKT and mTOR signaling simultaneously in pancreatic cancer cells.

#### 4. Discussions

Standard gemcitabine-based chemotherapies only showed limited value in improving pancreatic cancer patients' survival [1,20,21]. Novel approaches are urgently needed [1,20,21]. Recent research focuses have been on targeting signaling pathways that are over-activated in pancreatic cancers. Major interests are on the KRAS–MEK–ERK signaling [5,6,22]. The constitutive activation of MEK–ERK caused by KRAS mutations is important for many aspects of pancreatic cancer cell progression [5,6,22]. However, AZD-6244, the novel and potent MEK/ERK inhibitor only showed moderate activity against pancreatic cancer cells in preclinical models [23]. Similar observations were also obtained in the current study.



**Fig. 3.** AZD-6244 induces ceramide production, facilitated by PDMP—Cellular ceramide level in PANC-1 cells with AZD-6244 (100 nM) and/or PDMP (20  $\mu$ M) treatment was tested as described (A). PANC-1 cells, stimulated with AZD-6244 (100 nM) and/or C2 ceramide (30  $\mu$ M), were subjected to MTT cell viability assay (B, 72 h), trypan blue staining assay (C, 72 h) and Annexin V FACS assay (D, 48 h). “Veh” stands for 0.1% DMSO. \* $P$  < 0.05, \*\*\* $P$  < 0.001 vs. 0 h group (A). \*\*\* $P$  < 0.001 (B–D).



**Fig. 4.** PDMP and AZD-6244 co-administration in-activated AKT, ERK and S6K1 in pancreatic cancer cells-PANC-1 cells or AsPC-1 cells were stimulated with AZD-6244 (100 nM) and/or PDMP (20  $\mu$ M) for 24 h, expression of list proteins was tested by Western blots (A and B). Kinase phosphorylation was quantified, and normalized to “Veh” group. “Veh” stands for 0.1% DMSO.

Ceramide plays an important role in mediating cancer cell apoptosis by a number of anti-cancer drugs [11,13]. Here we discovered that AZD-6244 treatment could also increased intracellular ceramide production in pancreatic cancer cells. Interestingly, however, the excess ceramide was metabolically removed at long-term (24–48 h). This short-lived ceramide production might explain the moderate activity of AZD-6244 in promoting pancreatic cancer apoptosis here. Different groups have shown that use of PDMP could

augment the activity of several anti-cancer agents [18,19,24]. Here, we observed that PDMP co-combination facilitated AZD-6244-induced ceramide production, and ceramide level remained elevated up to 48 h. Thus, PDMP sensitizes the activity of AZD-6244 against pancreatic cancer cells, probably through facilitating ceramide production. To support this, we found that exogenously-added C2 ceramide similarly sensitized AZD-6244's activity, resulting in substantial pancreatic cancer cell death and apoptosis.



Due to mutations in *KRAS* and many growth factor receptors, MEK–ERK pathway and AKT pathway are concurrently activated in pancreatic cancer cells [25–27]. A number of studies have shown that combined inhibition of MEK–ERK and AKT signalings could result in a robust activity against pancreatic cancer cells [7,23,28]. As expected, we found that AZD-6244 only blocked MEK–ERK signaling, but had no effect on AKT activation in pancreatic cancer cells. Significantly, PDMP and AZD-6244 co-administration in-activated AKT and ERK, which might explain its superior activity. Following its activation, AKT is de-phosphorylated/in-activated through protein phosphatase (PP) 2A and PP1 phosphatases. Ceramide is known to activate PP1 and PP2A thus inhibiting AKT kinase [29]. Therefore, AKT in-activation by PDMP or PDMP plus AZD-6244 combination here could be the result of increased ceramide production, see similar results in other studies [18,30].

Activation of mTOR is another important contributor of pancreatic cancer progression [26,27]. Interestingly, we found that S6K1 phosphorylation, the indicator of mTOR activation, was inhibited by PDMP or AZD-6244 alone. Combination of the two resulted in further S6K1 activation. These results indicate that activation mTOR (S6K1) might be downstream event of AKT and MEK–ERK in tested pancreatic cancer cells, and combined inhibition of AKT and MEK–ERK signalings by the co-administration resulted in robust mTOR inhibition [31]. In summary, we conclude that combined administration of PDMP and AZD-6244 synergistically induces pancreatic cancer cell death and apoptosis. This appears to involve a sustained ceramide production as well as concurrent ERK and AKT–mTOR in-activation.

## Conflict of interest

No conflict of interests by any co-author.

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