

# Dimethylaminomicheliolide (DMAMCL) Inhibits Cell Proliferation and Increases Apoptosis and Efficacy of Gemcitabine via Annexin A2 in Pancreatic Adenocarcinoma

**Kaihui Liu**

Nankai University

**Jianshuang Guo**

Nankai University

**Ning Liu**

Nankai University

**Jiyan Wang**

Nankai University

**Mukuo Wang**

Nankai University

**Yanwei Zhang**

Nankai University

**Hao Jiang**

Nankai University

**Yanling Liu**

Dezhou University

**Fangzhong Hu**

Nankai University

**Wenzheng Fu**

PLA Strategic Support Force Medical Center

**Chunze Zhang**

PLA Strategic Support Force Medical Center

**Lianyong Li**

Tianjin Union Medical Center

**Mengyi Zhang**

Nankai University

**Jing Li** (✉ [jinglink@nankai.edu.cn](mailto:jinglink@nankai.edu.cn))

Nankai University

## Research Article

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# Abstract

**Background:** Pancreatic adenocarcinoma is one of the highest malignant tumors in digestive tract with extremely poor survival rate. Dimethylaminomicheliolide (DMAMCL) is a clinical developing anti-cancer agent, however, little is known regarding its effects in pancreatic cancer, and the mechanisms of DMAMCL are still not fully understood.

**Methods:** This study evaluated DMAMCL on three pancreatic cancer cell lines by cell viability assay, colony formation assay, and apoptosis assay. To identify the direct binding target of DMAMCL in pancreatic cells, a chemical proteomics approach, molecular docking and site-directed mutagenesis were performed.

**Results:** DMAMCL inhibits proliferation and promotes apoptosis of pancreatic cancer cells. Interestingly, using a chemical proteomics approach, we identify ANXA2 as a direct binding target of DMAMCL in pancreatic cells. Molecular docking and site-directed mutagenesis confirm that Cys132 (C132) of ANXA2 is the binding site for DMAMCL. The knockdown of ANXA2 largely decreases the inhibition activity of DMAMCL, indicating that the effect of DMAMCL is mainly mediated by ANXA2 in pancreatic cancer. In addition, the combination regimen of gemcitabine and DMAMCL exhibits synergistic effect on pancreatic cancer cell lines at both proliferation and pro-apoptosis level.

**Conclusions:** Thus, our findings elucidate the mechanisms of DMAMCL and may provide a potential strategy to enhancing the efficacy of gemcitabine in pancreatic adenocarcinoma.

## Background

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related deaths in the world [1]. Nowadays, gemcitabine has become the first-line therapy as single injection or combination with surgery and radiation [2-4]. However, due to the high degree of intrinsic or acquired resistance, the efficacy of gemcitabine still needs improved, with a response rate of about 5% [5]. Therefore, systematic chemotherapy with novel anti-cancer agents or molecular targeting agents is necessary [6].

Annexin A2 (ANXA2) is part of the Annexin family that bind anionic phospholipids in a calcium-dependent manner [7]. It is up-regulated in various cancer types (nasopharyngeal carcinoma, ovarian cancer, gliomas, hepatomas, pancreatic cancer, and breast cancer) and promotes cancer progression including proliferation, invasion, and metastasis [8-13]. In 1993, Vishwanatha *et al.* measured the levels of ANXA2 mRNA and protein in normal human pancreas, five pancreatic adenocarcinoma cell lines, three primary pancreatic tumors and one metastatic pancreatic tumor [14]. All pancreatic cancer cell lines examined had 5- to 15-fold higher levels of ANXA2 as compared to normal pancreas. Significant elevations (2- to 8-fold) of ANXA2 expression were observed in the primary and metastatic pancreatic tumors. Further immunocytochemical analysis indicated that the increased expression of ANXA2 was co-localized with cells that express proliferating cell nuclear antigen (PCNA), indicating the role of ANXA2 in cell proliferation of pancreatic cancer [15]. Using two-dimensional gel electrophoresis, Takano *et al.*

discovered that ANXA2 was a gemcitabine-resistant factor by comparing the protein profiling of gemcitabine-resistant (GEM-) and wild type (WT-) MIA PaCa-2 cell lines. In addition, immunohistochemistry of pancreatic cancer tissues demonstrated that the overexpression of ANXA2 was significantly associated with rapid recurrence after gemcitabine adjuvant chemotherapy [16]. Therefore, ANXA2 could be a potential drug target for enhancing the sensitivity of gemcitabine in pancreatic cancer treatment.

DMAMCL is a novel anti-cancer agent and has been under clinical trials in Australia for treatment of glioma tumors (trial number: ACTRN12616000228482). It suppresses glioma by regulating PI3K/AKT/mTOR signaling pathway, NF $\kappa$ B signaling pathway, ROS, and rewiring aerobic glycolysis [17-20]. In addition, DMAMCL can also inhibit leukemia cell and liver cancer cell growth [21, 22]. However, the potential effect of DMAMCL on pancreatic cancer and related mechanism has not yet reported.

In this study, we employed cell viability assay, colony formation assay, and apoptosis assay to demonstrate the effect of DMAMCL on cell viability and apoptosis in three pancreatic cancer cell lines (PANC-1, AsPC-1, BxPC-3). Chemo-proteomics, amino acid mutation, molecular modeling, and gene knock-down by RNAi were performed to verify that DMAMCL suppressed pancreatic cancer cells by targeting Cys132 (C132) of ANXA2. Moreover, we evaluate the synergistic effect between DMAMCL and gemcitabine on three pancreatic cancer cell lines. Overall, our results reveal that DMAMCL inhibits cell proliferation, promotes cell apoptosis, and increases the sensitivity of gemcitabine in pancreatic adenocarcinoma, which provides a novel strategy for the treatment of pancreatic cancer.

## Methods

### Chemicals

DMAMCL and its active metabolic form micheliolide (MCL) were synthesized as previously reported [23]. The positive probe (MCL-biotin) and the negative probe (MCL-S-biotin, NP) were synthesized as described [21]. Gemcitabine was obtained from Med Chem. Express (CAS: 95058-81-4).

### Cell culture

Human pancreatic cancer cell lines (PANC-1, AsPC-1, BxPC-3) were originally obtained from JIKAI GENE (NICR, 3111C0001CCC000023, 3111C0001CCC000214, 3142C0001000001478). PANC-1 cells were cultured in DMEM medium (Corning, 10-017-CVR) supplemented with 10% fetal bovine serum (BI, 04010-1A) and penicillin/streptomycin (Sigma, V900929) in a humidified incubator with 5% CO<sub>2</sub> at 37°C. AsPC-1 cells and BxPC-3 cells were cultured in RPMI1640 medium (Corning, 10-040-CVR) supplemented with 10% FBS in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

### Cell viability assay

For cell viability assay, human pancreatic cancer cell lines were seeded in 96-well-plates (4000 cells/well). Cells were treated with varying concentrations of DMAMCL or MCL for a certain time. After 48 or 72 h, 20  $\mu$ L MTT reagent (Solarbio, M8180) (5 mg/mL) was added to each well. After 4-h incubation, the liquid in the wells was replaced by 150  $\mu$ L dimethyl sulfoxide (DMSO). Cell viability was determined by optical density (OD) values at 570 nm.

## Colony formation assay

For colony formation assay, human pancreatic cancer cell lines were seeded in 6-well-plate (2000 cells/well). Cells were treated with varying concentrations of DMAMCL or MCL for 14 days. After that, the culture medium was discarded, and cells were fixed with ice methanol for 15 min. Fixed cells were stained with crystal violet (Solarbio, G1063) for 15 min and washed 3 times with PBS. Finally, the plates were photographed by a digital camera.

## Apoptosis assay

The apoptosis assay was detected by FITC coupled with Annexin-V apoptosis detection kit (Beyotime, C1062). PANC-1 cells were seeded in 6-well-plates ( $2 \times 10^5$  cells/well). After cells adhered overnight, they were treated with different concentrations of DMAMCL or MCL, with or without gemcitabine. After 48 h treatment, cells were washed 3 times with PBS and digested with trypsin without EDTA (Gibco) in EP tubes, followed by centrifugation at 1000 g for 3 min. The cell pellets were washed twice with PBS, following by adding 100  $\mu$ L 1 $\times$ binding buffer to suspend the cells. For cell staining, 5  $\mu$ L FITC Annexin V and 5  $\mu$ L of PI were added to each tube and incubated at room temperature (keep in dark) for 15 min. Finally, each tube was supplemented with 400  $\mu$ L 1 $\times$ binding buffer. The samples were analyzed by FACSCalibur flow cytometer (Beckman, BD LSRFortessa), and the data was analyzed by FlowJo 7.6.1 software (10,0,0,0).

## Chemo-proteomics to identify MCL-bound proteins

Pull-down experiment was carried out according to the method of literature [24]. Briefly, BxPC-3 cells were plated on 10 cm<sup>2</sup> cell culture dish and grown to confluence for 24 h. Cells were harvested and lysed in NP40 lysed buffer containing 1 mM PMSF and protease inhibitors. MCL-biotin (Probe) or MCL-S-biotin (NC probe) was incubated with cell lysates overnight at 4°C, then the prewashed streptavidin beads (Thermo, 20349) were added to each sample and incubated overnight at 4°C. On the second day, the beads were washed six times with lysis buffer, and the bead-bound proteins were eluted and boiled in 2 $\times$ SDS loading buffer. The bead-bound proteins were separated by SDS-PAGE and visualized by silver staining. The protein-containing band in the gel was excised, followed by in-gel digestion and analysis by LC-MS/MS.

## Site-directed mutant assay

Site-directed mutant assay was constructed using QuikChange II XL site-directed mutagenesis kit (Stratagene, USA). In order to mutate the cysteine at position 132 of pETM3C-ANXA2 plasmid to glycine, primers were designed (forward, 5'-CTCATTGAGATCATCGGCTCCAGAAC-3'; reverse, 5'-CTCCTGAGAGAGTAA CTCTAGTAGC-3') and synthesized (GENEWIZ, China). According to the instructions of the site-directed mutagenesis kit, the mutated plasmid was obtained by PCR. Agarose gel electrophoresis was used to detect whether the target band of the PCR product is correct. If the target band was correct, added 1  $\mu$ L of DMT enzyme to the PCR product and mixed well. The mixture was kept in a 37°C water bath for 1 h to obtain digestion products. The digested product was purified by agarose gel DNA recovery kit (TIANGEN, DP209-03). The purified PCR product was transformed to *E. coli* strain DH5 $\alpha$ , and the plasmid was extracted according to the plasmid extraction instructions, and then sent to the company for sequencing. The finally confirmed target plasmid was transformed into *E. coli* strain BL21 (DE3) for the expression and purification of protein.

## Molecular docking

The molecular docking experiment was performed with Schrodinger software. The ANXA2 protein structure was downloaded from the UniProt database (<https://www.uniprot.org>), and the protein was optimized through Schrodinger software to obtain the best protein model. The three-dimensional structure of MCL was prepared using Chemdraw software. LigPrep tool was used to minimize the energy of the ligand to correct the coordinates, ionization, stereochemistry, and tautomeric substitution structure to obtain the proper conformation by adding or removing hydrogen bonds. Gliding agent ligand docking was used to dock MCL to the active site of ANXA2, and calculate the binding energy. All docking calculations used Glide XP (super precision) mode.

## Expression and purification of recombinant ANXA2

The pETM3C-ANXA2 plasmid was transformed into BL21 competent cells, coated on a kana-resistant solid medium plate, and placed in a 37°C incubator for 12-14 h. Monoclonal strains were picked into 5 mL of LB medium with kana resistance and shaken overnight at 37°C and 180 rpm constant temperature shaker. The bacterial liquid obtained above was transferred to 250 mL of LB medium with kana resistance, and expanded for 4 h at 37°C and 180 rpm constant temperature shaker. The bacterial solution was added to 25  $\mu$ L IPTG (1 mmol/L), and it was induced for 20 h at 18°C and 180 rpm constant temperature oscillator. The bacterial solution was collected, centrifuged at 4000 rpm, 4°C for 30 min, and the supernatant was discarded. The pellet was resuspended in an appropriate volume of 1 $\times$  bacteriolytic buffer and stored at -80°C for the next step of purification. The bacterial pellet was ultrasonically disrupted for 15 min, centrifuged at 4000 rpm, 4°C for 30 min, and the supernatant was collected. The supernatant was added to a nickel column equilibrated with 10 mM imidazole in advance, and repeated 2

times. Contaminated proteins were eluted with 30 mM imidazole until the collection solution detected by Coomassie Brilliant Blue G250 was not blue. The target protein was eluted with 250 mM imidazole and collected into 1.5 mL centrifuge tubes on ice until the collection solution detected by Coomassie Brilliant Blue G250 was not blue. 20  $\mu$ L of eluate were drawn from each tube, and 5  $\mu$ L of 5 $\times$ loading buffer was added to the suction fluid, mixed, and boiled to obtain protein samples. The protein purity was detected by SDS-PAGE and Coomassie Brilliant Blue G250 copying method. The high-purity protein collection solution was used to remove imidazole by ultrafiltration, and then the protein solution was obtained by adding an appropriate volume of physiological saline.

The same procedure was used to express and purify the pETM3C-ANXA2-C132G mutant.

## Lentiviral transduction

293T cells were seeded in a 6-well plate ( $3.5 \times 10^5$  cells/well) and cultured overnight in a humidified incubator with 5% CO<sub>2</sub> at 37°C. The medium was changed to serum-free medium (1 mL/well) before cell transfection. According to the instructions of JIKAI GENE, the lentiviral packaging systems corresponding to the three plasmids of ANXA2-RNAi (16427-1), ANXA2-RNAi (16423-1) and ANXA2-RNAi (16424-1) plasmids were obtained, and the system volume of each is 50  $\mu$ L. The lentivirus packaging system was added to 293T cells and incubated in a humidified incubator with 5% CO<sub>2</sub> at 37°C for 6 h. After 6 h, the mediums in the cells were replaced with new complete mediums (2 mL/well). After the cells were cultured in a humidified incubator with 5% CO<sub>2</sub> at 37°C for 48 h or 72 h, shRNA virus was obtained, and the shRNA virus was centrifuged to take the supernatant for use or stored at -80°C. For lentiviral transduction, AsPC-1 cells were cultured in serum-free medium containing lentivirus for 6 h, then replaced by new complete medium for 24 h. For generating stable cell lines, infected cells were selected with 1  $\mu$ g/mL puromycin (Sigma, 540411) for 72 h. Western blot experiments verified which plasmid has the best knockdown effect and used it for the next knockdown experiment.

## Western blot analysis

Cell lines were lysed in NP40 lysis buffer with protease inhibitors and the total protein concentration was quantified by BCA assay (Thermo Fisher Scientific, 23227). The normalized samples were analyzed by SDS-PAGE and western blot using standard protocols and the following primary antibodies: anti-ANXA2 (1:2000 dilution, Immunoway, YT0236), anti-AKT (1:2000 dilution, Cell Signaling, 9272S), anti-pAKT (S473) (1:1000 dilution, Cell Signaling, 4060S), anti- $\alpha$ -tubulin (1:5000 dilution, TEL, KM9007), anti-GAPDH (1:5000 dilution, Cell Signaling, 2118S), goat anti-rabbit HRP-IgG (1:5000 dilution, Solarbio, SE134), goat anti-mouse HRP-IgG (1:5000 dilution, Solarbio, SE131).

## Synergy index calculation

Human pancreatic cancer cell lines were seeded in 96-well plates (4000 cells/well). The cells were treated with DMAMCL, gemcitabine and DMAMCL combined gemcitabine (concentration ratio 1:3) for 72 h, respectively. After 72 h, 20  $\mu$ L MTT reagent (Solarbio, M8180) (5 mg/mL) was added to each well. After 4 h incubation, the liquid in the wells was replaced by 150  $\mu$ L dimethyl sulfoxide (DMSO). Cell viability was determined by optical density (OD) values at 570 nm and the inhibition rate of each treatment was calculated. The inhibition effect and drug combination index of DMAMCL and gemcitabine was calculated by the Compusyn software (Dr. Dorothy Chou) using constant drug concentration ratio.

## Statistical analysis

Results are representative examples of three individual experiments. Statistical analysis and graphical presentation were using GraphPad Prism 5.0. *P* values were determined by a two-tailed Student's *t* test. All histogram data was presented as mean  $\pm$  SEM. \*, *P*< 0.05 and \*\*, *P*<0.01.

## Results

### DMAMCL inhibits proliferation and promotes apoptosis of pancreatic cancer cells

To investigate the effect of DMAMCL on the proliferation of pancreatic cancer cells, we performed cell viability assays on AsPC-1, BxPC-3 and PANC-1 cells (Table 1). Cells were treated with different concentrations of DMAMCL or its active metabolite MCL for 72 h, and then performed MTT assay. The results show that  $IC_{50}$  values of DMAMCL in AsPC-1, BxPC-3 and PANC-1 were  $37.76 \pm 1.58 \mu$ M,  $35.83 \pm 1.55 \mu$ M, and  $19.66 \pm 1.29 \mu$ M, respectively, and the  $IC_{50}$  values of MCL in AsPC-1, BxPC-3 and PANC-1 were  $18.79 \pm 1.27 \mu$ M,  $11.73 \pm 1.07 \mu$ M, and  $6.08 \pm 0.78 \mu$ M, respectively. Compared to AsPC-1 and BxPC-3, PANC-1 behaves more sensitive to DMAMCL and MCL. Furthermore, we investigated the effect of DMAMCL on the anchorage-independent growth of pancreatic cancer cells through colony formation assays. It was observed that the inhibition of DMAMCL on the foci formation of AsPC-1 and PANC-1 was enhanced in a dose-dependent manner, and MCL also has a similar effect (Figure 1A). These results indicated that DMAMCL could inhibit the proliferation of pancreatic cancer cells.

In order to explore the effect of DMAMCL on the apoptosis of pancreatic cancer cells, cell apoptosis was detected by flow cytometry analysis and further confirmed by western blot. PANC-1 cells were treated with different concentrations of DMAMCL or MCL for 48 h. PI-Annexin V analysis (Figure 1B) showed that DMAMCL and MCL promoted PANC-1 cell apoptosis in a dose-dependent manner. Compared to DMSO treatment group, both 20  $\mu$ M DMAMCL and 10  $\mu$ M MCL improved 20.4 % and 45.4 % apoptosis respectively. Western blotting (Figure 1C) showed that DMAMCL treatment promoted the activation of caspase-9 and the release of cytochrome C in PANC-1 cells, which is consistent with the study of DMAMCL-induced apoptosis in hepatocellular carcinoma by Shunnan Yao *et al* [25]. The above results

indicate that DMAMCL may promote apoptosis of pancreatic cancer cells in a caspase-dependent manner.

## MCL directly binds to Cys132 of ANXA2 in BxPC-3 cell

To identify the cellular targets of DMAMCL, we performed chemical proteomics assay in BxPC-3 cell. The main active metabolite of DMAMCL is MCL, which contains a typical  $\alpha$ -methylene- $\gamma$ -lactone group that can react with -SH of cysteine in accessible proteins, and induce a covalent modification [26]. Thus, using biotin-conjugated MCL (MCL-biotin, Probe) and biotin-conjugated inactive MCL (MCL-S-biotin, NC probe) as probes (Figure 2A), we explored the targets of DMAMCL in BxPC-3 cells. Cellular lysates were firstly incubated with Probe or NC probe, and the mixture was pulled down with streptavidin-coated agarose beads. Then precipitated proteins on beads were resolved by SDS-PAGE and detected by silver staining. Special proteins bands in Probe treatment was excised and confirmed by LC-MS/MS. Peptide mass fingerprinting data analysis revealed that 13 peptides of ANXA2 were detected in the mass spectrometry, which confirmed ANXA2 was one target of MCL. Immunoblotting was further used to monitor the presence of ANXA2 in the precipitates. The results showed that a single band with a molecular weight of about 38 kDa was clearly precipitated with Probe instead of NC probe, indicating ANXA2 was present in the precipitate (Figure 2B). Furthermore, the amount of ANXA2 in precipitate increased with the probe in concentration-dependent manner (Figure 2C). Using 20-fold excess MCL as a competitor, ANXA2 was disappeared in pull down precipitate of Probe (Figure 2D), which further suggested that MCL directly binds to ANXA2.

ANXA2 is highly expressed in pancreatic cancer cells and has four cysteine residues (Cys-8, Cys-132, Cys-261, and Cys-334) [7]. To further explore the site of MCL binding to ANXA2, molecular docking experiments and site-directed mutation experiments were performed. Molecular docking predicted that Cys132 (C132) of ANXA2 could be the binding residue for MCL with the lowest binding free energy as -3.084 (Figure 2E). Then we constructed plasmid containing C132G mutation of ANXA2 and expressed it in *E. coli*. Probe incubation and western blot assay showed that recombinant ANXA2 with C132G mutation displayed less binding signal with Probe (Figure 2F), which confirmed Cys132 was the main binding site of MCL.

## ANXA2 is upregulated in cancer patients and affects the inhibition activity of DMAMCL in pancreatic cancer cells

ANXA2 is a member of vertebrate annexins. To clarify its role in cancer, the expression of ANXA2 in different types of cancer patients was examined through the HPA database (<https://www.proteinatlas.org>). Among seventeen types of cancer examined, patients with pancreatic cancer has the highest expression of ANXA2 (Figure 3A). When compared the expression levels of ANXA2 between three primary tumors (glioma, colorectal cancer, pancreatic cancer) and corresponding normal

tissues, ANXA2 were up-regulated in all three examined tumors (Figure 3B). By correlating ANXA2 with overall survival in cancer patients, we founded that high levels of ANXA2 was associated with a low survival probability, whereas low ANXA2 expression predicted a relatively high survival probability (Figure 3C). These results indicated that ANXA2 could be a potential marker and drug target for cancer treatment.

In order to verify whether DMAMCL or MCL inhibited pancreatic cancer proliferation by targeting ANXA2, we performed dose inhibition assays on AsPC-1-WT and AsPC-1-sh ANXA2 cells (Figure 3D). After treatment with MCL for 48 h, AsPC-1-shANXA2 cells showed less sensitivity to MCL, indicating that knockdown of ANXA2 partially abrogate the inhibition effect of MCL in AsPC-1 cells. Thus, ANXA2 was considered as one of the crucial drug targets for DMAMCL in pancreatic cancer cells.

## **DMAMCL enhances the inhibition effects of gemcitabine in pancreatic cancer cells**

Gemcitabine is one of the first-line chemotherapeutic agent for pancreatic cancer and its low clinical response is related to the high expression of ANXA2 [14, 16, 27]. Considering DMAMCL binds to ANXA2, we performed cell viability assays of gemcitabine with or without DMAMCL on three pancreatic cancer cells. The cells were treated with different concentrations of DMAMCL, gemcitabine or DMAMCL+ gemcitabine (mixed in proportion) for 72 h and then for MTT assay. Compared with DMAMCL or gemcitabine alone, DMAMCL combined gemcitabine significantly inhibited the proliferation of pancreatic cells (Figure 4A). In order to verify whether DMAMCL and gemcitabine produce a synergistic effect, we used CompuSyn software to calculate the combination index (CI) and dose reduction index (DRI) (Figure 4B, Table 2 and Table 3). The results showed that the CI value of DMAMCL and gemcitabine at ED<sub>50</sub>, ED<sub>75</sub> and ED<sub>90</sub> were 0.63, 0.69 and 0.77 in BxPC-3 cells, which exhibits the best synergistic effects in terms of reduction of cell viability based on the Chou–Talalay analysis. For MCL and gemcitabine, the best synergistic effect was found in PANC-1 cells, which is 0.43, 0.46 and 0.89 at ED<sub>50</sub>, ED<sub>75</sub> and ED<sub>90</sub>. Furthermore, under the treatment of DMAMCL at 5 μM, the IC<sub>50</sub> of gemcitabine was found decreased from 4.21 to 2.85 μM in AsPC-1 cells and from 2.33 to 0.75 μM in PANC-1 cells, indicated that DMAMCL could increase the efficacy of gemcitabine on pancreatic cancer cell proliferation (Table 4 and Figure 5).

## **DMAMCL enhances the pro-apoptosis effect of gemcitabine in pancreatic cancer cells**

In order to further verify whether DMAMCL enhance the pro-apoptosis effect of gemcitabine in pancreatic cancer cells, the early and late apoptosis was analyzed by flow cytometry analysis. Dual-labeled fluorescence activated cell sorting (FACS) analysis (Annexin V and PI) was used to measure early and late apoptosis. Annexin V detects early apoptosis, whereas both PI and Annexin V detect late apoptosis. By 48 h treatment, compared with single treatment (20 μM DMAMCL, 10 μM MCL, 2.5 μM gemcitabine),

combination regimen (20  $\mu$ M DMAMCL + 2.5  $\mu$ M gemcitabine ; 10  $\mu$ M MCL + 2.5  $\mu$ M gemcitabine) significantly increased cancer cell apoptosis, especially the early apoptosis (Figure 6A).

## **ANXA2 mediated PI3K/AKT inhibition contributes to the synergistic effect of DMAMCL and gemcitabine**

The phosphoinositide 3-kinase/Akt signalling pathway is a recognized key parameter in numerous cellular processes such as proliferation, cell cycle and angio-genesis, and is frequently activated in pancreatic cancer and especially in gemcitabine resistance [28]. Akt kinase can be activated by phosphorylation on Thr 308 or Ser 473 and active-Akt will promote cell growth and survival to apoptotic insults [29, 30]. It was also proved that DMAMCL has the ability to inhibit of PI3K/AKT and induce apoptosis in hepatocellular carcinoma [25]. To investigate whether the effect of DMAMCL on ANXA2 further influence the PI3K/AKT signal pathway, we detected the expression and the phosphorylation of Akt on ser473. Western blot results demonstrated that compared with DMAMCL or gemcitabine alone, DMAMCL + gemcitabine exhibited more potent inhibition of AKT signaling pathway (Figure 6B), which suggested that the synergistic effect of DMAMCL and gemcitabine could be partly mediated by ANXA2-AKT pathway.

## **Discussion**

Gemcitabine is the standard treatment for advanced pancreatic adenocarcinoma, but the efficacy of this reagent is still limited. ANXA2, as a tumor-associated protein, has been proved promoting cancer progression including proliferation, invasion, and metastasis in various cancer types [8-13]. In addition, ANXA2 was also reported to mediate gemcitabine resistance in pancreatic cancer [9, 16, 31, 32]. Thus, ANXA2-targeted agent is anticipated to be a novel treatment for pancreatic cancer.

DMAMCL has been shown to exert potent anticancer properties on multiple cancers, but has not yet in pancreatic cancer. Herein, we explored its effect on three pancreatic cancer cell lines and identified ANXA2 was a novel target for its property. Meanwhile, we found that gemcitabine in combination with DMAMCL could inhibit tumor growth and induce apoptosis more effectively than gemcitabine or DMAMCL alone.

To clarify the mechanisms of gemcitabine resistance induced by ANXA2 overexpression, Kagawa et al. analyzed the signaling pathways up-regulated in the gemcitabine-resistant cell lines with overexpressed ANXA2. Bio-Plex phosphorylation protein assay showed up-regulation of p-Akt in GEM-MIA PaCa-2 cells in which ANXA2 is highly expressed. Inhibition of p-Akt through mTOR inhibitor canceled gemcitabine resistance in GEM-MIA PaCa-2 cells [28], indicated ANXA2-mediated activation of Akt accounting for the low response of gemcitabine [15, 33-35]. Our previous work has demonstrated that DMAMCL can significantly inhibit the level of p-AKT in different cancer cells [20, 25]. In this study, we also observed that the inhibition of DMAMCL on p-AKT was significantly reduced in AsPC-1 (sh-ANXA2) cells. Thus, we

supposed that the synergistic mechanism of enhanced efficacy of gemcitabine may be ANXA2-AKT pathway inhibition induced by DMAMCL.

In conclusion, our work revealed that DMAMCL could suppress pancreatic cancer by targeting ANXA2. The binding of DMAMCL on C132 of ANXA2 might down-regulate p-AKT that offset the activation of p-AKT by gemcitabine. DMAMCL in combination with gemcitabine exhibits a synergistic cytotoxic effect, which may be used as a more effective treatment for pancreatic cancer.

## Abbreviations

DMAMCL, Dimethylaminomicheliolide; ANXA2, Annexin A2; PCNA, Proliferating cell nuclear antigen ; GEM-, Gemcitabine-resistant; WT-, Wild type; sh ANXA2, ANXA2 knock down; PI3K, Phosphoinositide 3-kinase; AKT, Protein kinase B; mTOR, mammalian target of rapamycin; NFkB, Nuclear factor kappa B; ROS, Reactive oxygen species; MTT, Thiazolyl Blue Tetrazolium Bromide; Cys, Cysteine; MCL, micheliolide; EDTA, Ethylene Diamine Tetraacetic Acid; C132G, Mutation of Cysteine132 to Glycine132; CI ,calculate the combination index; DRI, dose reduction index; PMSF, Phenylmethylsulfonyl fluoride; IPTG, Isopropyl-beta-D-thiogalactopyranoside.

## Declarations

## Acknowledgments

No applicable.

## Author contributions

J.L. and M.Y.Z. developed the concept and designed the study. K.H.L., J.S.G., N.L., J.Y.W. M.K.W., Y.W.Z. and H.J. carried the experiments. Y.L.L., W.Z.F., F.Z.H. C.Z.Z. and L.Y.L. provided technical support and conceptual advice. K.H.L. and M.Y.Z. wrote the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

# Ethics approval and consent to participate

Not applicable.

# Consent for publication

Not applicable.

# Competing interests

The authors declare that they have no known competing financial interests.

# Author details

<sup>1</sup>State Key Laboratory of Medicinal Chemical Biology, College of Pharmacy and College of Chemistry, Nankai University, Haihe Education Park, 38 Tongyan Road, Tianjin 300353, China

<sup>2</sup> College of Medicine and Nursing, Dezhou University, Dezhou, 253023, China.

<sup>3</sup> Department of Gastroenterology, PLA Strategic Support Force Medical Center, Beijing, 100101, China.

<sup>4</sup> Department of Colorectal Surgery, Tianjin Union Medical Center, Tianjin, 300121, China.

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## Tables

**Table 1**

Summary of IC<sub>50</sub> values of DMAMCL or MCL on AsPC-1, BxPC-3 and PANC-1 cells. Error bars, mean  $\pm$  s.e.m., n = 3 biological replicates.

IC <sub>50</sub> ( $\mu$ M)	AsPC-1	BxPC-3	PANC-1
<b>DMAMCL</b>	37.76 $\pm$ 1.58	35.83 $\pm$ 1.55	19.66 $\pm$ 1.29
<b>MCL</b>	18.79 $\pm$ 1.27	11.73 $\pm$ 1.07	6.08 $\pm$ 0.78

**Table 2**

The combination index (CI) and dose reduction index (DRI) of DMAMCL and gemcitabine in AsPC-1, BxPC-3 and PANC-1 cells.

		CI	DMAMCL DRI	Gemcitabine DRI
<b>AsPC-1</b>	<b>ED<sub>50</sub></b>	0.52639	4.61724	3.22777
	<b>ED<sub>75</sub></b>	0.96749	1.28252	5.32550
	<b>ED<sub>90</sub></b>	2.92089	0.35624	8.78654
<b>BxPC-3</b>	<b>ED<sub>50</sub></b>	0.62771	3.67495	2.81219
	<b>ED<sub>75</sub></b>	0.69377	3.3954.	2.50470
	<b>ED<sub>90</sub></b>	0.76703	3.13712	2.23083
<b>PANC-1</b>	<b>ED<sub>50</sub></b>	0.70755	2.72004	2.94199
	<b>ED<sub>75</sub></b>	1.39051	0.77215	10.4798
	<b>ED<sub>90</sub></b>	4.58897	0.21919	37.3302

**Table 3**

The combination index (CI) and dose reduction index (DRI) of MCL and gemcitabine in AsPC-1, BxPC-3 and PANC-1 cells.

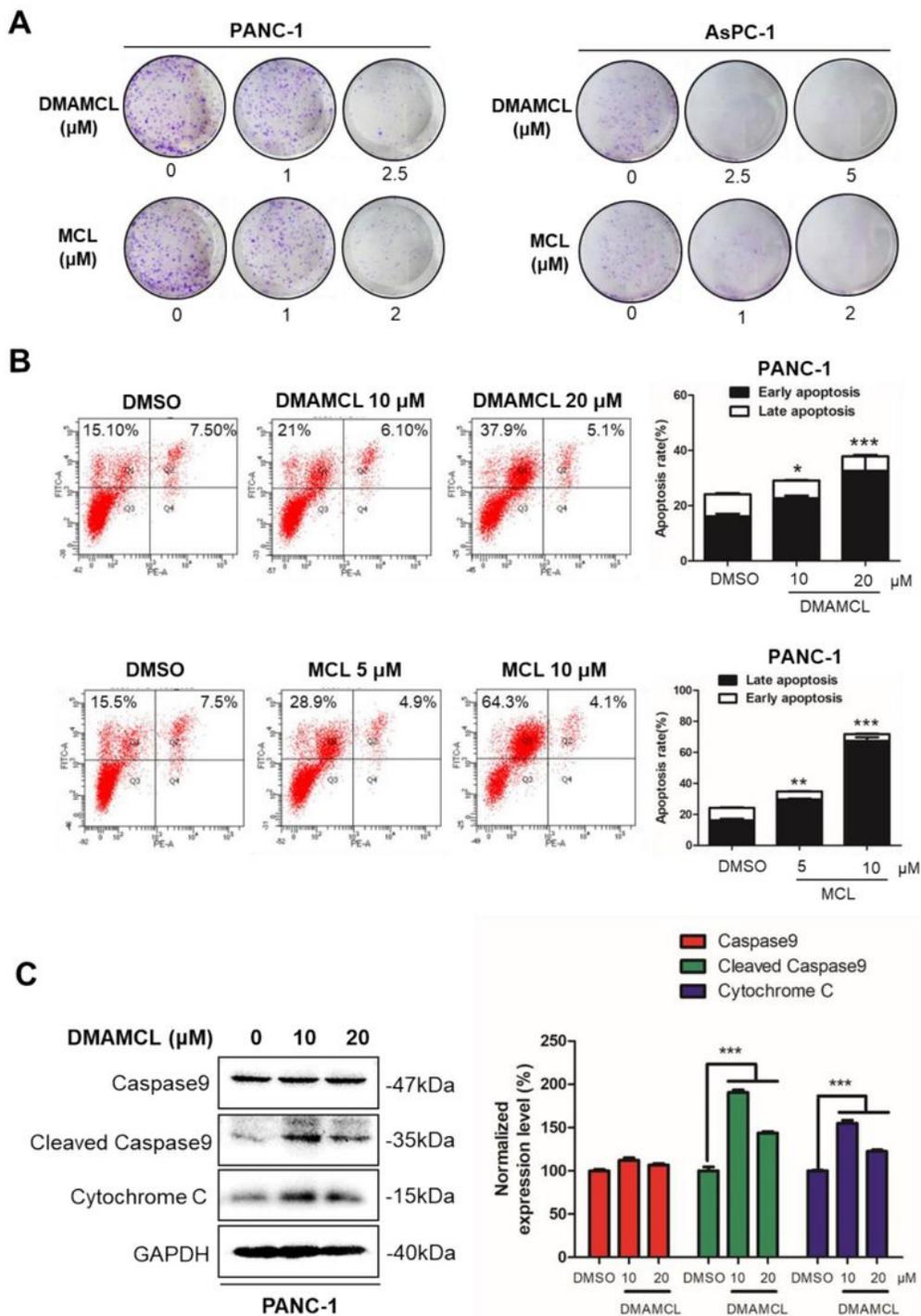
		CI	MCL DRI	Gemcitabine DRI
<b>AsPC-1</b>	<b>ED<sub>50</sub></b>	0.45723	5.9571	3.45589
	<b>ED<sub>75</sub></b>	0.84774	1.48603	5.72056
	<b>ED<sub>90</sub></b>	2.80320	0.37070	9.46929
<b>BxPC-3</b>	<b>ED<sub>50</sub></b>	1.07877	2.01259	1.71853
	<b>ED<sub>75</sub></b>	1.33391	1.35353	1.68039
	<b>ED<sub>90</sub></b>	1.70715	0.91029	1.64311
<b>PANC-1</b>	<b>ED<sub>50</sub></b>	0.43061	5.48189	4.02922
	<b>ED<sub>75</sub></b>	0.46706	2.50073	14.8865
	<b>ED<sub>90</sub></b>	0.89477	1.14078	55.0006

**Table 4**

Summary of IC<sub>50</sub> values of gemcitabine on AsPC-1 and PANC-1 cells when treated with or without 5 μM DMAMCL. Error bars, mean ± s.e.m., n = 3 biological replicates.

IC <sub>50</sub> (μM)	AsPC-1	PANC-1
<b>Gemcitabine</b>	4.21 ± 0.41	2.33 ± 0.50
<b>Gemcitabine (DMAMCL 5 μM)</b>	2.85 ± 0.39	0.75 ± 0.43

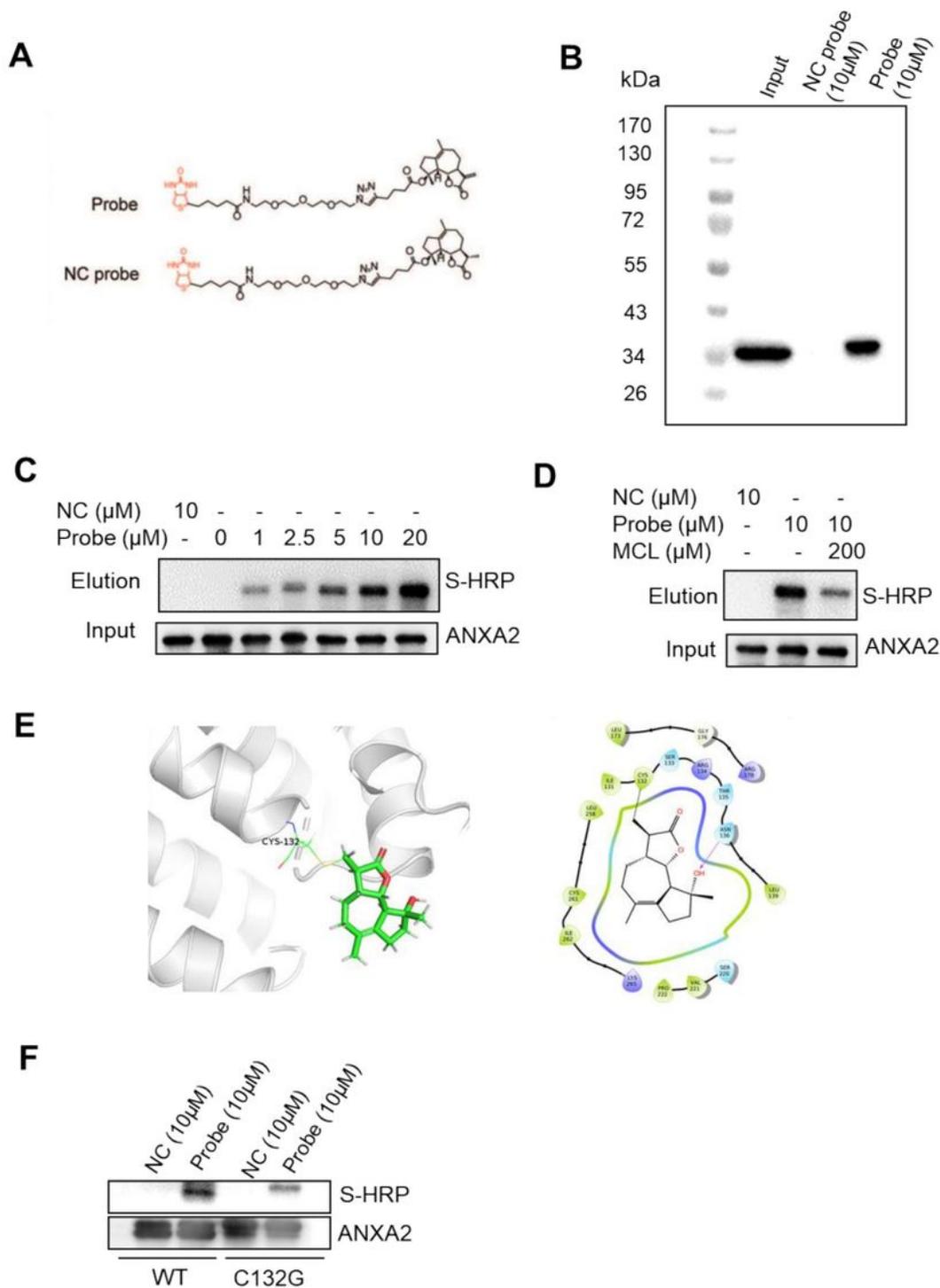
## Figures



**Figure 1**

DMAMCL inhibited foci formation and induced cell apoptosis in pancreatic cancer cells. (A) Colony formation assays of PANC-1 and AsPC-1 cells (2000 cells/well) treated with DMAMCL or MCL for 14 days. (B) Apoptosis assays of PANC-1 cells ( $2 \times 10^5$  cells/well) treated with different doses of DMAMCL or MCL for 48 h. (C) PANC-1 cells were treated with various doses of DMAMCL for 48 h, and then total cellular lysates were subjected to western blot analysis with cytochrome and caspase-9 antibodies.

GAPDH was used as a loading control. Error bars, mean  $\pm$  s.e.m., n = 3 biological replicates. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.



**Figure 2**

DMAMCL directly targeted Cys132 site of ANXA2. (A) Chemical structures of biotin-conjugated MCL (MCL-biotin,Probe) and biotin-conjugated single-bond MCL (MCL-S-biotin, NC probe). (B) Western blot detection of ANXA2 proteins from probe-cell lysis mixture. (C) NC probe and probes with different

concentrations were incubated with BxPC-3 cell lysates overnight at 4°C, and biotin was detected by western blot. (D) The BxPC-3 cell lysates were incubated with Probe in the absence or presence of a 20-fold excess of unlabeled MCL overnight at 4°C, and biotin was detected by western blot. (E) Molecular docking assays of MCL interacting with ANXA2. (F) ANXA2-WT and ANXA2-C132G mutation were incubated with probe overnight at 4°C, and biotin was detected by western blot.

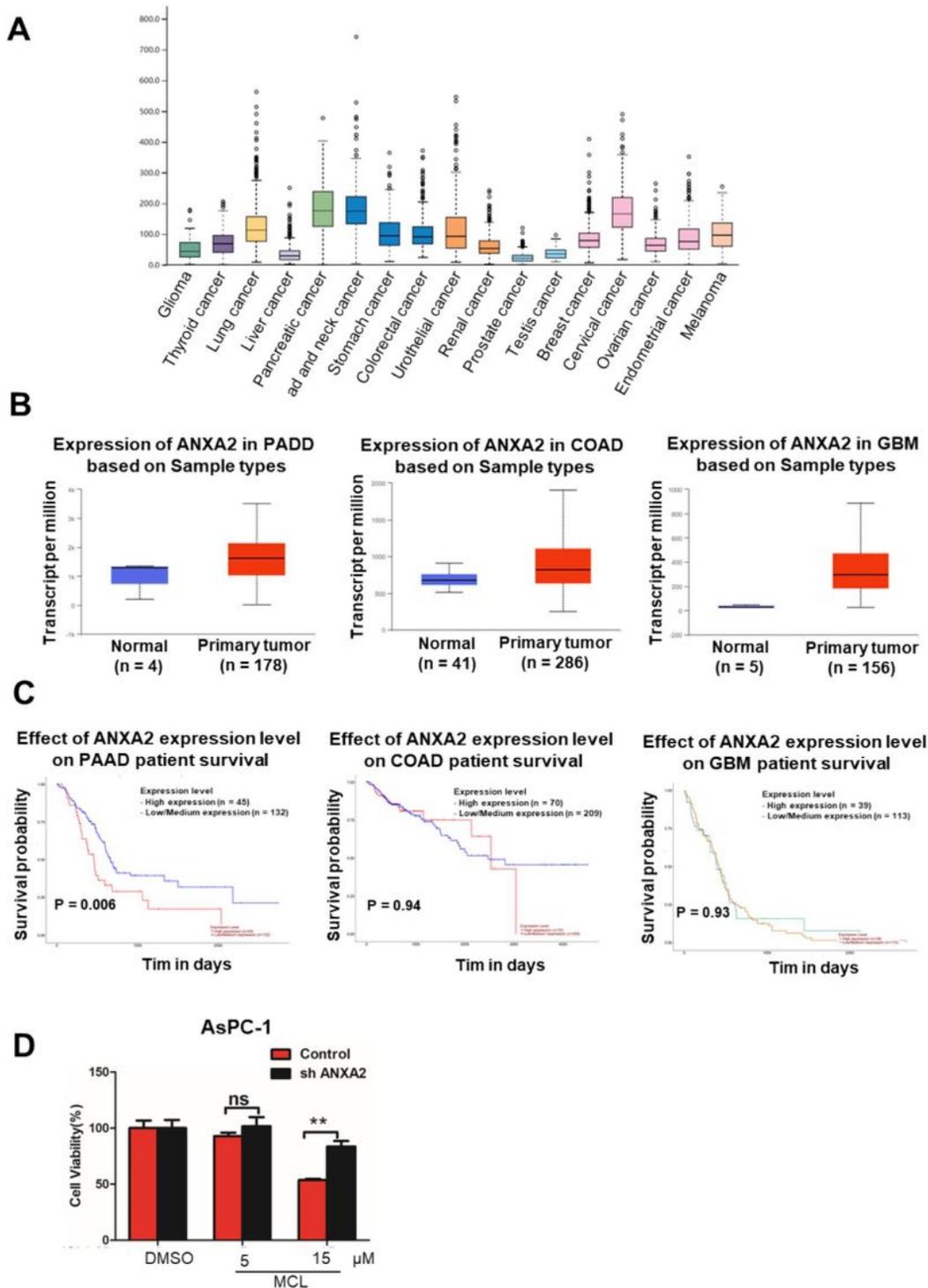
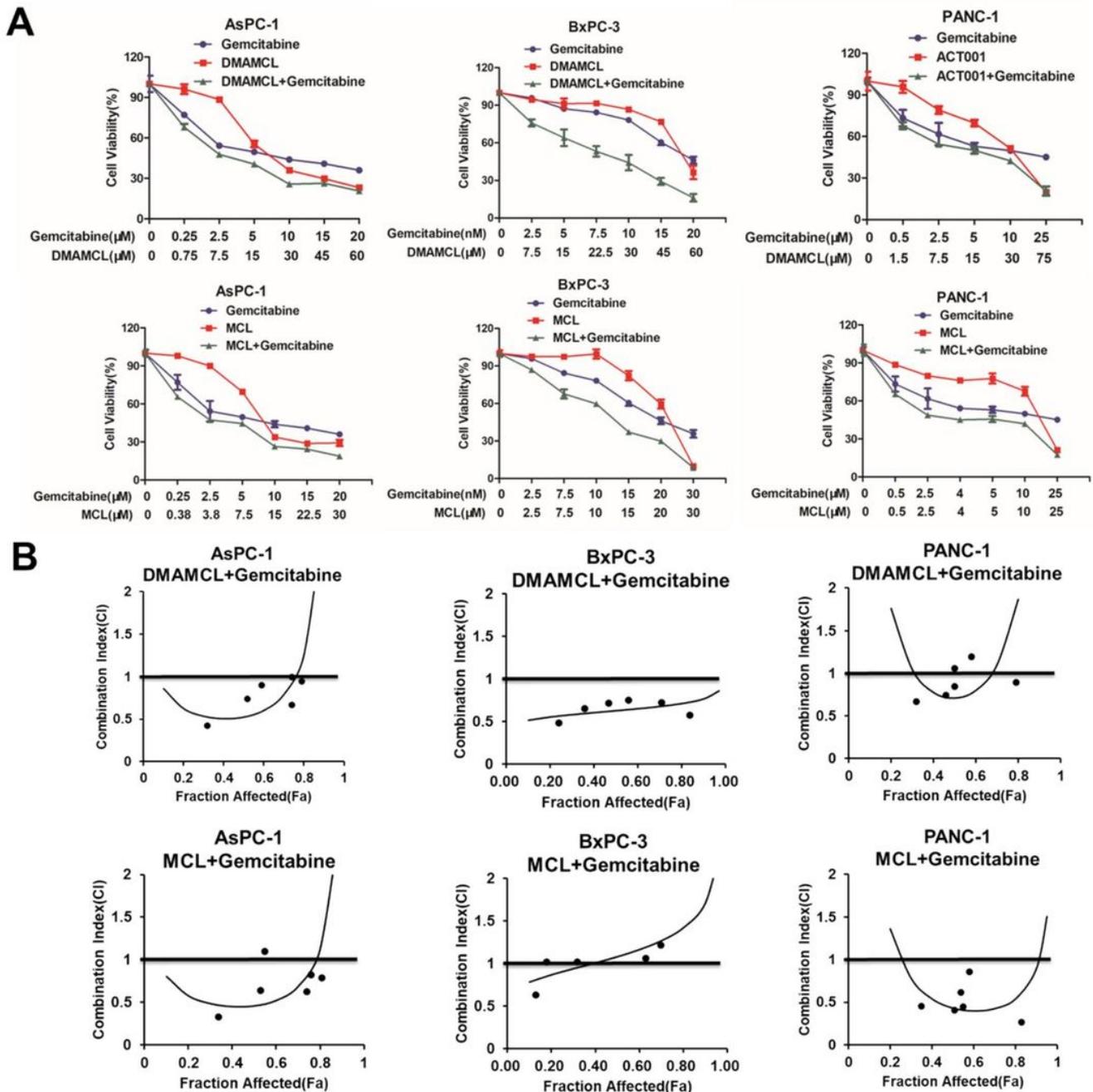


Figure 3

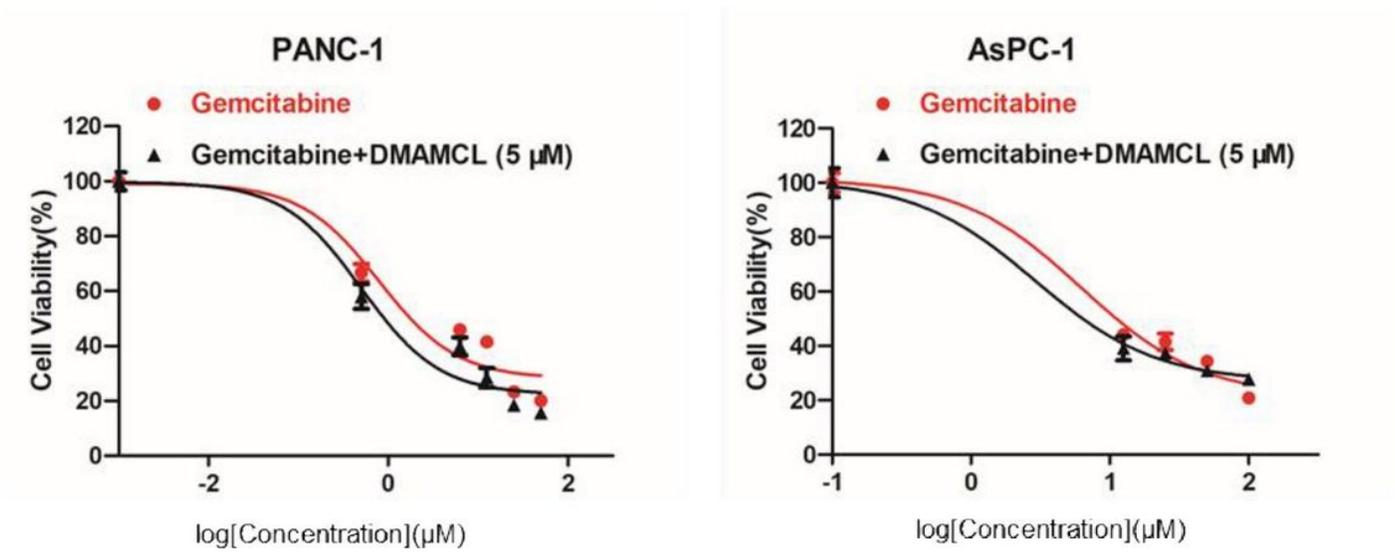
HPA database analysis and dose inhibition assays. (A) Expression analysis of ANXA2 in all types of cancer. (B) Expression analysis of ANXA2 in normal and glioma (GBM), colorectal (COAD) and pancreatic cancer (PADD) patients. (C) Survival analysis of ANXA2 in patients with glioma (GBM), colorectal (COAD) and pancreatic cancer (PADD). (D) Dose inhibition assays on AsPC-1-WT and AsPC-1-sh ANXA2. Error bars, mean  $\pm$  s.e.m., n = 3 biological replicates. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001



**Figure 4**

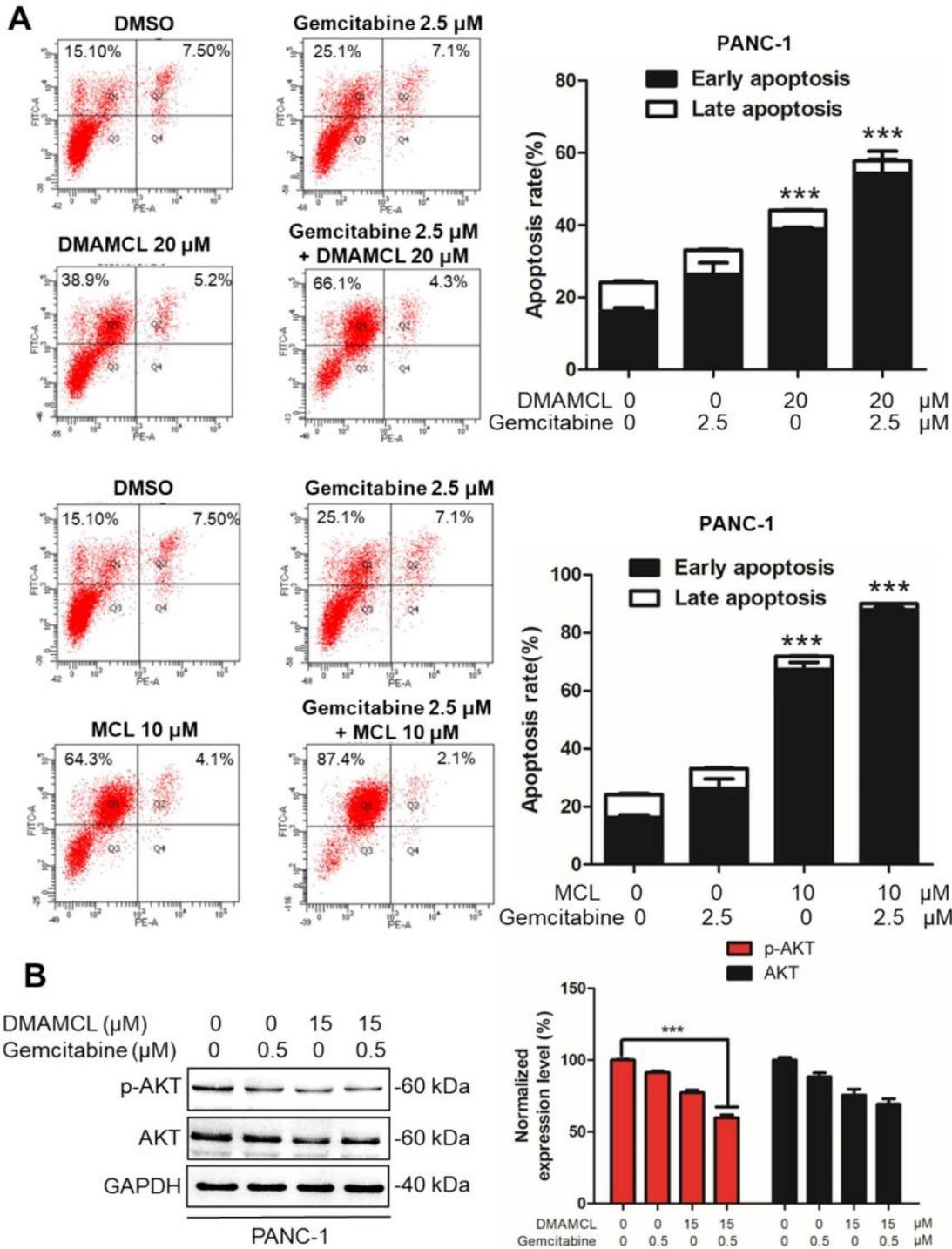
Synergistic combination effects between DMAMCL (MCL) and gemcitabine. (A) MTT assays of the AsPC-1, BxPC-3 and PANC-1 cells (4000 cells/well) were treated with different concentrations of DMAMCL

(MCL), gemcitabine or DMAMCL (MCL) + gemcitabine (mixed in proportion) for 72 h. (B) The Chou – Talalay analysis of AsPC-1, BxPC-3 and PANC-1 cells treated with DMAMCL (MCL) and gemcitabine for 72 h. Error bars, mean  $\pm$  s.e.m., n = 3 biological replicates.



**Figure 5**

DMAMCL enhances the sensitivity of gemcitabine in pancreatic cancer cells. MTT assays of AsPC-1 and PANC-1 cells (4000 cells/well) treated with gemcitabine or gemcitabine + DMAMCL (5 µM) for 72 h.



**Figure 6**

DMAMCL combined with gemcitabine inhibit the AKT pathway and induce apoptosis in pancreatic cancer cells. (A) PANC-1 cells were treated with DMAMCL (MCL), gemcitabine or DMAMCL (MCL) + gemcitabine for 48 h and cellular apoptosis was measured by FITC coupled with Annexin-V apoptosis detection kit. (B) The protein levels of AKT/p-AKT in PANC-1 cells analyzed by western blot. Error bars, mean  $\pm$  s.e.m., n = 3 biological replicates. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.