

A humanized anti-IGF-1R monoclonal antibody (R1507) and/or metformin enhance gemcitabine-induced apoptosis in pancreatic cancer cells

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Abstract. Pancreatic cancer is a disease with a dismal prognosis and treatment options are limited. This study investigated the interaction of gemcitabine with R1507 and/or metformin and the induction of an inhibitor of apoptosis protein by this combination. Pancreatic cancer cells were treated with gemcitabine, R1507 and metformin alone or in combination. The effects of treatments were evaluated for cell proliferation, apoptosis, and the expression of genes related to inhibition of apoptosis and chemotherapy resistance. Combination of gemcitabine with R1507 and/or metformin additively interacted with the inhibition of cell proliferation in human pancreatic ductal adenocarcinoma cell lines, SUIT-2 and MIAPaCa-2 with differential gemcitabine resistance, and assessment of apoptosis demonstrated that drug associations increased the apoptotic index in both cell lines. Treatment with gemcitabine induced the expression of survivin and XIAP in both cell lines, indicating the induction of chemoresistance. In conclusion, these data demonstrate that the combination of gemcitabine with R1507 and/or metformin has an additive effect in pancreatic cancer cell lines with differential sensitivity to gemcitabine; however, gemcitabine may induce chemotherapy resistance.

Introduction

Pancreatic cancer is the fourth most common cause of cancer death in men and women in the US, with 5-year survival for all stages of disease <5% (1). Pancreatic cancer has no clear early warning signs or symptoms and is usually silent until

the disease is well advanced. Patients have a median survival of 4-8 months after diagnosis due in part to the advanced stage of disease by the time it is discovered and treatment is begun. It is therefore necessary to discover new therapies or therapeutic combinations to make significant impact on this deadly disease. The anti-metabolite agent gemcitabine is currently being employed to treat pancreatic cancer (2). While gemcitabine has shown a significant benefit in clinical applications, its ability to more than modestly affect pancreatic cancer is limited. It has been speculated that combinatory treatments using gemcitabine and other chemotherapeutics could improve survival rates (3).

The involvement of the insulin-like growth factor/insulin-like growth factor 1 receptor (IGF/IGF-1R) axis in tumorigenesis makes it an attractive target for anticancer therapeutics. R1507, a fully human IgG1 type monoclonal antibody directed against human IGF-1R, has been developed to antagonize IGF-1R signaling. R1507 binds the extracellular domain of IGF-1R with high affinity and selectivity, resulting in the displacement of bound IGF-1 and loss of receptor protein on the cell surface due to intracellular internalization and degradation. R1507 inhibits tumor proliferation of the prostate, lung, breast, pancreas, and sarcoma cell lines at concentrations <10 nmol/l and delays tumor growth in xenograft models (4-6). Recently we found pancreatic cancer cell lines expressed IGF-1R moderately and R1507 bound to the IGF-1R on membrane. Furthermore, R1507 showed significant survival effect in nude mouse intraperitoneal dissemination model with Suit-2 pancreatic cancer cell line (7). Metformin, a drug used in the treatment of diabetes for decades, decreases the growth of pancreatic ductal adenocarcinoma (PDAC) cell lines and could be a potential candidate in novel treatment strategies for PDAC patients (8). Metformin is an activator of AMP-activated protein kinase (AMPK) which inhibits protein synthesis and gluconeogenesis during cellular stress. The main downstream effect of AMPK activation is the inhibition of mammalian target of rapamycin (mTOR), a downstream effector of growth factor signaling. mTOR is frequently activated in malignant cells and is associated with resistance to anticancer drugs (9).

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, has previously been shown to be a prognostic

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marker for pancreatic cancer patients (10-12) and has also been implicated in cancer cell chemotherapy resistance (13). Many recent reports have demonstrated that inhibiting survivin expression by antisense oligonucleotides (14), dominant negative mutation (15,16), and ribozyme (17) can reduce cancer cell radio- and chemoresistance and may be important to resensitize these tumors to therapy. XIAP, a unique and best-characterized member of the IAP family, has been identified as a central regulator of caspase-dependent apoptosis. Whether the activation of apoptosis is initiated by events that perturb the mitochondria (via caspase-9) or progress directly from cell surface receptors (via caspase-8), the ability of XIAP to inhibit the downstream executioner caspases-3 and -7 makes it a potent and broad inhibitor of cell death and an important target for therapy (18).

In the present study, we evaluated the combined effect of gemcitabine with R1507 and/or metformin on pancreatic cancer cell lines with differential sensitivity to gemcitabine and examined the induction of survivin and XIAP.

Materials and methods

Cell culture. SUIT-2 (19), MIPaCa-2 (20), BxPC-3, AsPC-1, Panc-1, H-48-N and TCC-PAN2 were derived from a human PDAC. All human PDAC cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 50 U/ml penicillin and 50 μ g/ml streptomycin in a humidified atmosphere under 5% CO₂ at 37°C. Immortalized human pancreatic ductal cell line HPDE6-E6E7-c7 (H6c7) is a generous gift from Dr Ming-Sound Tsao (University Health Network, Toronto, ON, Canada) (21,22). It was cultured in HuMedia-KG2 medium (Kurabo, Osaka, Japan) in a humidified atmosphere under 5% CO₂ at 37°C.

Drug sensitivity test. Human PDAC were seeded into 96-well plates (MIPaCa-2, 2.5×10^3 cells/50 μ l; SUIT-2, 1.25×10^3 cells/50 μ l) and allowed to attach for 24 h. Cells were treated with a) gemcitabine (0.01-100 μ M) (Toronto Research Chemicals, North York, ON, Canada); b) R1507 (0.1-300 nM) (kindly provided by Roche Diagnostics GmbH, Mannheim, Germany); c) metformin (0.5-100 mM) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h and 48 h in 50 μ l medium. At the end of drug exposure, 20 μ l 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, Madison, WI, USA) was added and cells were incubated in humidified 5% CO₂ atmosphere. After 2 h, spectrophotometric readings were taken for each sample according to the manufacturer's instructions. Cell growth inhibition was expressed as a percentage of the absorbance of control cultures measured at 490 nm with a microplate reader. The 50% inhibitory concentration of cell growth (IC₅₀) was calculated by a sigmoidal dose-response curve (GraphPad PRISM, San Diego, CA, USA). Drug interactions among gemcitabine, R1507, metformin or their combinations were assessed at a fixed concentration for 48 h exposure. The final concentration of each drug was set at $0.5 \times \text{IC}_{50}$. The IC₅₀ values after 48 h exposure were applied for gemcitabine and metformin treatment, whereas the IC₅₀ values after 24 h exposure were applied for R1507 treatment. Accordingly, R1507 was added twice at 0 and 24 h in the course of 48 h exposure.

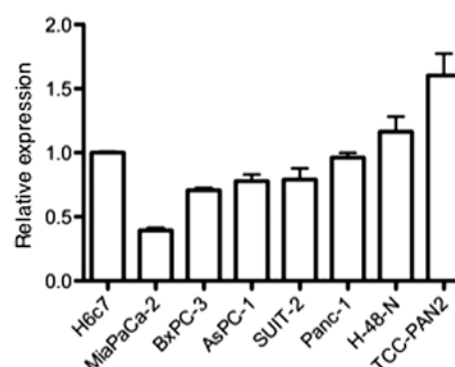


Figure 1. Relative expression of IGF-1R in PDAC cell lines. Total RNA was extracted from PDAC cell lines and an immortalized human pancreatic ductal cell line H6c7 cultured under normal conditions. Quantitative RT-PCR analysis was performed. Expression levels of IGF-1R were normalized with β -actin. The expression of H6c7 was set as one. N=3 for each experiment. Results are shown as the average \pm SD.

Analysis of apoptosis. Human pancreatic cancer cell lines were cultured in slide chambers to no more than 50-60% confluence and treated with gemcitabine, R1507, metformin or their combinations, as described in the 'drug sensitivity test'. After 48-h treatment, apoptosis was assessed using the *in situ* cell death detection kit (Roche Applied Science, Mannheim, Germany), following the manufacturer's recommendations. Briefly, cells were washed with PBS once, fixed with 4% buffered paraformaldehyde for 1 h, washed again with PBS, and permeabilized on ice with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min. Slides were rinsed twice with PBS and then incubated for 60 min at 37°C with terminal deoxynucleotidyl transferase enzyme in reaction buffer. The slides were rinsed three times with PBS and mounted with PermaFluor (Thermo Fisher Scientific, Waltham, MA, USA). Samples were analyzed by fluorescence microscopy. TUNEL-positive nuclei were detected by a bright color in condensed or ruptured nuclei. The rate of apoptosis was calculated as the ratio of the number of apoptotic cells to the total number of cells (both apoptotic and non-apoptotic cells).

Quantitative real-time PCR. Total RNA was extracted from pancreas cancer cell lines treated with anti-cancer drugs using an RNeasy RNA isolation kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. cDNA was transcribed using a 20- μ l volume with the ThermoScript RT-PCR System and a random hexamer (Invitrogen) according to the manufacturer's instructions. All PCR reactions were carried out in a final volume of 25 μ l and were performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Sequence-specific primers are quoted from internet site 'PrimerBank' (23) and summarized in Table I. The reaction mix consisted of SYBR Premix Ex Taq (2x) (Takara Bio, Otsu, Japan) 12.5 μ l, ROX Reference Dye (x50) (Takara) 0.5 μ l, 0.2 μ M of each specific forward and reverse primer, and 9 μ l diluted cDNA (equivalent to 0.03-2.85 ng total RNA). Amplifications were performed under standard conditions (10 sec at 95°C followed by 40 cycles of 5 sec at 95°C and 31 sec at 60°C). The number of PCR cycles needed to reach the

Table I. Gene-specific primer pairs for real-time RT-PCR.

Human gene	Primer sequence (5'-3')	Length (bp)
IGF-1R	Sense: AGGATATTGGGCTTTACAACCTG Antisense: GGCTTATTCCTCCCAATGTAGTT	145
Survivin	Sense: AGGACCACCGCATCTCTACAT Antisense: AAGTCTGGCTCGTTCTCAGTG	118
XIAP	Sense: ATAGTGCCACGCAGTCTACAA Antisense: AGATGGCCTGTCTAAGGCAAA	101
β -actin	Sense: CATGTACGTTGCTATCCAGGC Antisense: CTCCTTAATGTCACGCACGAT	250

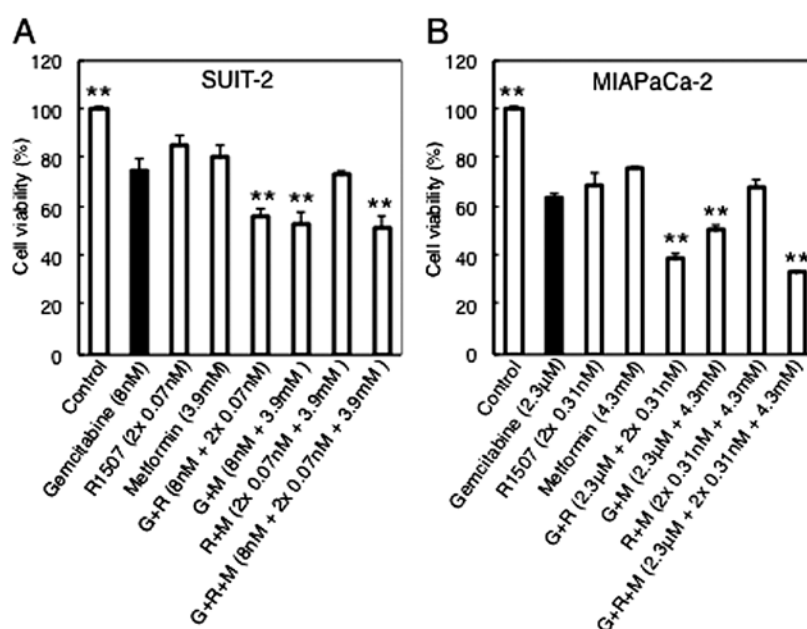


Figure 2. Combination treatment of gemcitabine with R1507 and/or metformin on the viability of SUIT-2 (A) and MIAPaCa-2 (B) cells. Cells were treated with gemcitabine, R1507, metformin or their combinations at a fixed concentration. Cell growth inhibition was expressed as a percentage of the absorbance of control cultures measured at 490 nm with a microplate reader. N=3 for each experiment. Results are shown as the average \pm SD. * $p < 0.05$, ** $p < 0.01$ vs. gemcitabine treatment.

fluorescence threshold was determined in triplicate for each cDNA, averaged, and then normalized to a reference gene (β -actin). A standard curve was generated with serial 3-fold dilutions of representative cDNA. For all assays tested, the PCR reaction was linear over the range studied (20-40 cycles of amplification). All RT-PCR reactions gave a single band when analyzed by gel electrophoresis.

Statistical analysis. Results are reported as the average \pm SD. Group comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparison test. Differences were considered significant at $p < 0.05$.

Results

Relative expression of IGF-1R, the target of the humanized monoclonal antibody (R1507), was investigated in PDAC

cell lines. An immortalized human pancreatic ductal cell line H6c7 was used as a control. The results indicate that all PDAC cell lines express IGF-1R at levels comparable to that of H6c7 (Fig. 1).

The cytotoxicity of gemcitabine, R1507 and metformin against pancreatic cancer cell lines was examined by the MTS assay and the values of IC_{50} were calculated. Gemcitabine, R1507 and metformin inhibited the growth of the SUIT-2 and MIAPaCa-2 cell lines in a concentration-dependent manner, and the IC_{50} values are summarized in Table II. Our results indicate that MIAPaCa-2 is 300-fold more resistant to gemcitabine than SUIT-2. While the IC_{50} of R1507 and metformin were comparable between the two cell lines. Next the drug interaction of gemcitabine with R1507 and/or metformin was assessed at a fixed concentration for 48 h treatment. As a single treatment of R1507 was more potent at 24 h exposure than at 48 h exposure, we planned to add R1507 at 0 and 24 h referring to the values of IC_{50} for 24 h exposure.

Table II. *In vitro* cytotoxicity of gemcitabine, R1507 and metformin on human pancreatic cancer cells, SUIT-2 and MIAPaCa-2.

Cell name	Exposure	IC ₅₀		
		Gemcitabine (μ M)	R1507 (nM)	Metformin (mM)
SUIT-2	24 h	0.0023	0.13	5.6
	48 h	0.0015	>100	7.8
MIAPaCa-2	24 h	0.71	0.61	10.9
	48 h	0.45	>100	8.5

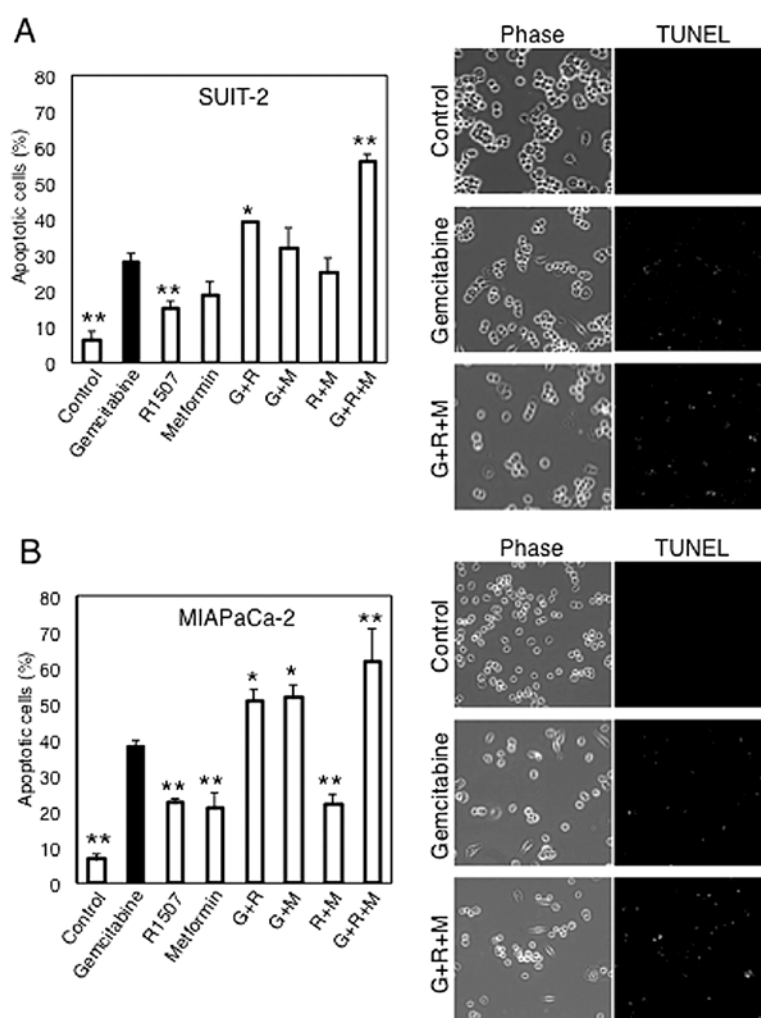


Figure 3. Combination treatment of gemcitabine with R1507 and/or metformin on the induction of apoptosis in SUIT-2 (A) and MIAPaCa-2 (B) cells. Cells were treated with gemcitabine, R1507, metformin or their combinations at a fixed concentration, as shown in Fig. 1. The rate of apoptosis was calculated as the ratio of the number of apoptotic cells to the total number of cells. N=3 for each experiment. Results are shown as the average \pm SD. * p <0.05, ** p <0.01 vs. gemcitabine treatment (left). Representative microscopic photos of control, gemcitabine-treated and gemcitabine/ R1507/ metformin-treated PDAC cell lines (right).

Consequently, the combinations of gemcitabine with R1507 and/or metformin were more potent than gemcitabine, R1507 or metformin alone in both cell lines, indicating an additive effect, while there was no additive effect with the combination of R1507 and metformin (Fig. 2).

To explore the reason for the additive cytotoxic effects of gemcitabine, R1507 and/or metformin, the induction of

apoptosis was analyzed by TUNEL assay. Upon exposure to gemcitabine, R1507, metformin, and their combinations, pancreatic cancer cells presented typical apoptotic morphology with cell shrinkage, nuclear condensation and fragmentation, and cellular rupture into debris. The occurrence of apoptosis was significantly higher in gemcitabine-treated cells than R1507- and/or metformin-treated cells (Fig. 3). R1507 and metformin

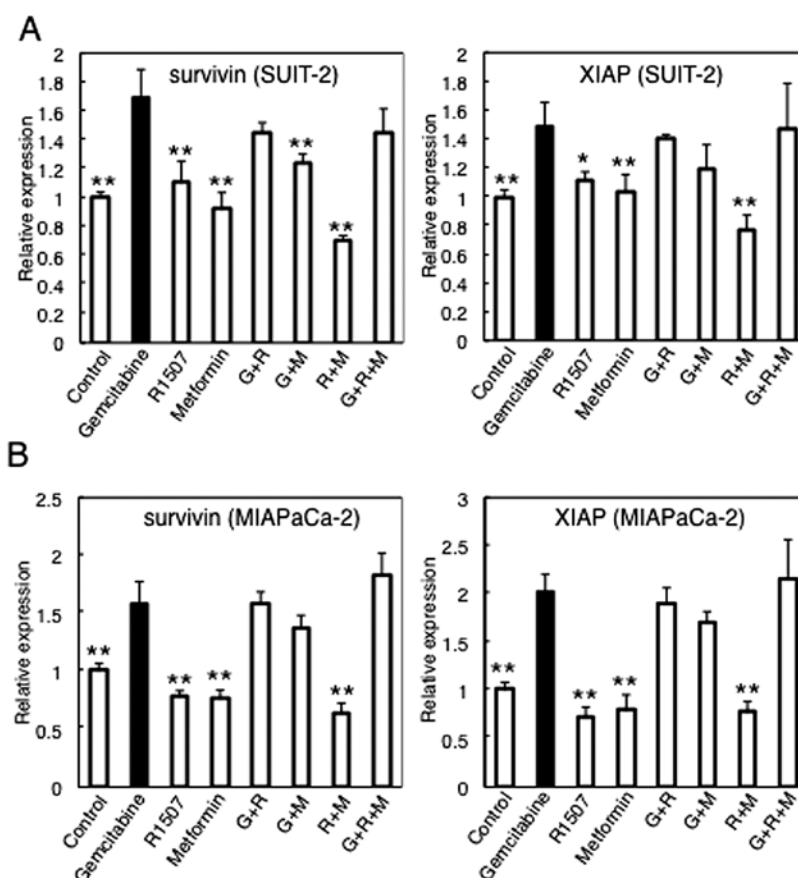


Figure 4. Quantitative RT-PCR analysis of survivin and XIAP mRNAs in SUIT-2 (A) and MIAPaCa-2 (B) cells. Cells were treated with gemcitabine, R1507, metformin or their combinations at a fixed concentration, as shown in Fig. 1. Expression levels of survivin and XIAP were normalized with β -actin. N=4 for each experiment. Results are shown as the average \pm SD. * $p < 0.05$, ** $p < 0.01$ vs. gemcitabine treatment.

had additive effects on gemcitabine-induced apoptosis in both cell lines (Fig. 3).

We investigated the expression levels of the IAP family, survivin and XIAP during chemotherapy treatments by quantitative real-time PCR. Treatment with gemcitabine induced the expression of survivin and XIAP significantly in both cell lines, while treatment with R1507 and metformin did not affect the expression of survivin and XIAP in either cell line (Fig. 4).

Discussion

Pancreatic cancer remains one of the most difficult malignancies to treat. Even patients who are eligible for curative surgical resection gain only modest benefits. Furthermore, the lack of effective chemotherapeutic and targeted agents highlights the urgent need to better understand pancreatic cancer biology to guide the identification and development of novel treatment strategies. The nucleoside analog gemcitabine provided encouraging results in both antitumor effects and its impact on parameters of clinical benefit for patients with pancreatic cancer such as, decreased pain severity, decreased requirement for opioid analgesics, increased appetite and weight gain (24). In direct comparison on locally advanced pancreatic cancer and metastatic pancreatic cancer, gemcitabine treatment resulted in a 5.56 month overall survival compared to a 4.41 month overall survival using 5-FU (25). As a result, gemcitabine has become

widely accepted for unresectable pancreatic cancer. However, the combination treatment using gemcitabine with other agents has not been successful in increasing the overall survival. These disappointing results call for novel combination therapies to improve the survival outcome of pancreatic cancer patients.

The aim of the current study was to evaluate the combined effect of gemcitabine with R1507 and/or metformin on pancreatic cancer cell lines. The growth inhibitory effects of R1507 and metformin may be mediated through inhibition of the IGF-1R pathway and the downstream mTOR pathway (26). Whereas gemcitabine is a nucleoside analogue metabolized to triphosphate moiety by deoxycytidine kinase and is incorporated into the nascent DNA and blocks DNA replication (27). We combined the drugs with two different modes of action for the treatment of two pancreatic cancer cell lines with differential sensitivity to gemcitabine. Consequently, the combination of gemcitabine with R1507 and/or metformin was more potent than gemcitabine, R1507 or metformin alone, indicating an additive effect in both cell lines. While there was no additive effect with the combination of R1507 and metformin, which suggest the majority of the growth inhibitory effects of R1507 and metformin are mediated through inhibition of the IGF-1R pathway and the downstream mTOR pathway. Supporting the cytotoxicity assay, the R1507 and/or metformin have additive effects on gemcitabine-induced apoptosis in both cell lines. However, our data suggest that treatment with gemcitabine may induce chemotherapy resistance in both cell lines through

the induction of survivin and XIAP, while treatment with R1507 and metformin does not. Survivin has been pursued as a therapeutic target, given its role in chemoresistance and its preferential expression in malignant cells over normal cells (28,29). Both antisense and chemical XIAP inhibitors were developed as therapeutic agents for the treatment of malignancy (30). Their combination with IAP inhibitors might be effective against chemoresistance caused by gemcitabine treatment. In conclusion, the combination of gemcitabine with R1507 and/or metformin has an additive effect in pancreatic cancer cells with differential sensitivity to gemcitabine; however, gemcitabine may induce chemotherapy resistance.

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