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Original Article

Gemcitabine Sensitivity Evaluation in Pancreatic Ductal Adenocarcinoma Following Inhibition of ABCG2 and Wnt/β-Catenin Pathway

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Abstract

Purpose: Pancreatic cancer is one of the most lethal malignancies worldwide. Gemcitabine based therapy is currently the first-line treatment and usually fails to treat the patients with advanced pancreatic ductal adenocarcinoma (PDA) because of rapid acquisition of resistance to chemotherapy. Thus, identification of agents that re-sensitize gemcitabine-resistance pancreatic cancer cells to gemcitabine and exploring more about molecular mechanisms of gemcitabine resistance are essential to develop new therapeutic approaches for pancreatic cancer.

Methods: In the present study, we report that FTC (an ABCG2 inhibitor) and IWR-1 endo (a Wnt/β-catenin signaling inhibitor) combination re-sensitize resistant AsPC-1 cells to gemcitabine as shown by MTT and western blot analysis. *Main findings:* IWR-1 and FTC co-incubation with different concentrations of gemcitabine (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 µmol) for 72 hours showed significant cell death effects, in which gemcitabine IC50 after IWR-1 endo or FTC added on AsPC-1GR (gemcitabine-resistance) cells were 1.35 and 1.58 (µmol), respectively, while cell's viability remarkably reduced following co-treatment of IWR-1endo plus FTC with gemcitabine (gemcitabine IC50=0.1 µmol) ($p \leq 0.05$).

Principal conclusion: Results of the current study suggest that gemcitabine co-treatment with combination of FTC and IWR-1 endo, could represent a novel therapeutic strategy for pancreatic cancer patients with gemcitabine resistant characterization.

Introduction

Pancreatic Ductal Adenocarcinoma (PDAC), is one of the most deadliest cancers, mainly occurs in elderly people (1). The annual statistic in the USA recently announced PDA as the fourth leading cause of cancer-related deaths in the world (2). In many of non-metastatic pancreatic cancer patients, nonsurgical therapy is not currently curative, and relapse after surgery commonly occurs (3). This is the result of intrinsic resistance to cytotoxic agents, knowing as the main characterization of pancreatic cancer (4). MDR tumors (Multi Drug Resistance) are the major barriers to achieve optimal treatment and suggested to be fundamental contributors to death

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by pancreatic cancer. The high priority of cancers with MDR characterization is understanding the molecular basis and developing strategies or clinical reagents to treat resistant tumors or prevent the occurrence of resistance (5). Because the resistance issue in PDAC has not been properly addressed, identifying responsible biomarkers for therapeutic resistance is crucial in PDAC patients (6). One of the main transporter protein involved in multidrug resistance is human ATP-binding cassette subfamily G member 2 (ABCG2) protein, a semi-membrane protein, which originally isolated from breast cancer cells. ABCG2 together with multidrug resistance protein (MRP) and P-glycoprotein belong to the ABC membrane transport proteins superfamily (7). In tumor cells, overexpression of ABCG2 is tightly associated with multidrug resistance. Indeed, high expression of ABCG2 has been indicated in many cancers like pancreatic cancer (8). ABCG2 overexpression in these tumor tissues is often accompanied by fast progression of malignancy, unsuccessful chemotherapy and poor prognosis (9). Another critical regulator of chemo-resistance in many tumors is Canonical Wnt signaling, mediated through â-catenin. Inhibition of Wnt/â-catenin signaling has been reported to increase chemotherapeutic drugs sensitivity in cancers (10). Also, the crucial role of dysregulation of Wnt/β-catenin signaling has been found in many recent pancreatic cancer chemo resistance studies (4). Since the sole approach treatment did not achieve absolute satisfaction, combination therapy concerned as a new and effective solution in cancer treatment in many investigations (11). The present study was designed to characterize the effect of combined Wnt/β-catenin and ABCG2 inhibitors on PDAC cells to re-sensitize resistant-gemcitabine cells to gemcitabine.

Materials and Methods

Cell culture and cell line

Gemcitabine resistant AsPC-1 cells were selected from the PDA parental cell line grown in graduated concentrations (0.01 µmol to 1.0 µmol) of 4-hydroxy-Gemcitabine (Sigma Aldrich, Castle Hill, NSW, Australia) over six months (12). Cells were grown in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin in a humidified incubator at 37°C and 5% CO2. For maintaining, cells were passaged two times a week before reaching complete confluence; and were seeded into 96 well plates with 4000 cells/well; optimal cell confluence was based on doubling time.

Proliferation assay

Cell viability was measured using MTT Cell Proliferation Kit I (Roche, Basel, Switzerland) following the manufacturer's instructions. Briefly, AsPC-1 and AsPC-1GR cells were seeded into a 96-well plate with a concentration of 4000 cells in each well. After treatment with different drug concentrations for the final 72 hours, all cells were incubated with MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in a humidified atmosphere of 5% CO2 at 37°C for 4 hours. The absorbance was subsequently measured at 572 nm on a micro plate reader (SpectraMax M2), readouts averaged and adjusted accordingly.

Western blot analysis

The cells protein profiles were determined using western blot analysis. Cells were collected and washed three times with PBS (Phosphate Buffered Saline). Then, in the fresh RIPA protein lysis buffer (a lysis buffer used to lyse cells and tissue, for radio immune precipitation assay) containing 1% PMSF (phenyl methanesulfonyl fluoride), the cells were lysed on ice. BCA assay (bicinchoninic acid assay**)** was employed for the total protein concentration determination. After separation by SDS–PAGE electrophoresis, the samples were transferred to PVDF membrane (polyvinylidenedifluoride). Ponceau S staining was used to visualize protein blots. ABCG2 and Wnt/ â catenin primary antibodies were added for an overnight incubation at 4°C. The membrane was then incubated with the secondary antibody (1:10,000) at room temperature for 1 h and washed three times with TBST buffer. ECL chemi-luminescence reagent and Bio-Rad exposure apparatus were used for exposure (13). Membrane bound secondary antibodies were visualized by enhanced chemiluminescence (ECL) detection kits (Santa Cruz Biotechnology). Mouse monoclonal antibody b-actin (TA-09) (Zhongshan Goldenbridge

Biotechnology, diluted 1:2000) was used as a total protein internal control.

Statistical analysis

Viability values were converted to log 10 scale prior to statistical analysis. Graphsand their statistical comparisons were done using GraphPad Prism software with unpaired and two-tailed Student's t-test ($p \leq 0.05$).

Results

Development of gemcitabine-resistant cell line to create stable resistant pancreatic cancer cell

AsPC-1 cells were exposed to increase the concentrations of gemcitabine. Specifically, cells in log phase were first exposed to 10 n mol gemcitabine, which resulted in greater than 95% cell death. Once surviving cells reached 80% confluence, they were passaged twice in the same concentration of gemcitabine, the process was then repeated with intermittent increase in the doses of gemcitabine until the selected cell population demonstrated 50 folds greater IC 50 to gemcitabine. Resultant AsPC-1 cells were resistant to 1000 n mol gemcitabine. The resulting cell line was designated AsPC-1 GR. The gemcitabine-resistant phenotype has been stable over 20 passages during experiments.

Gemcitabine Effect on cell viability

MTT assay was performed on AsPC-1 and AsPC-1GR cells. Cells were exposed to different concentrations of gemcitabine (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 µmol) for 72 h. The gemcitabine IC50 for AsPC-1 and AsPC-1GR cells were 0.43 and 2.37µmol, respectively (Fig. 1A). Resistant cells morphology was different from their parent as shown

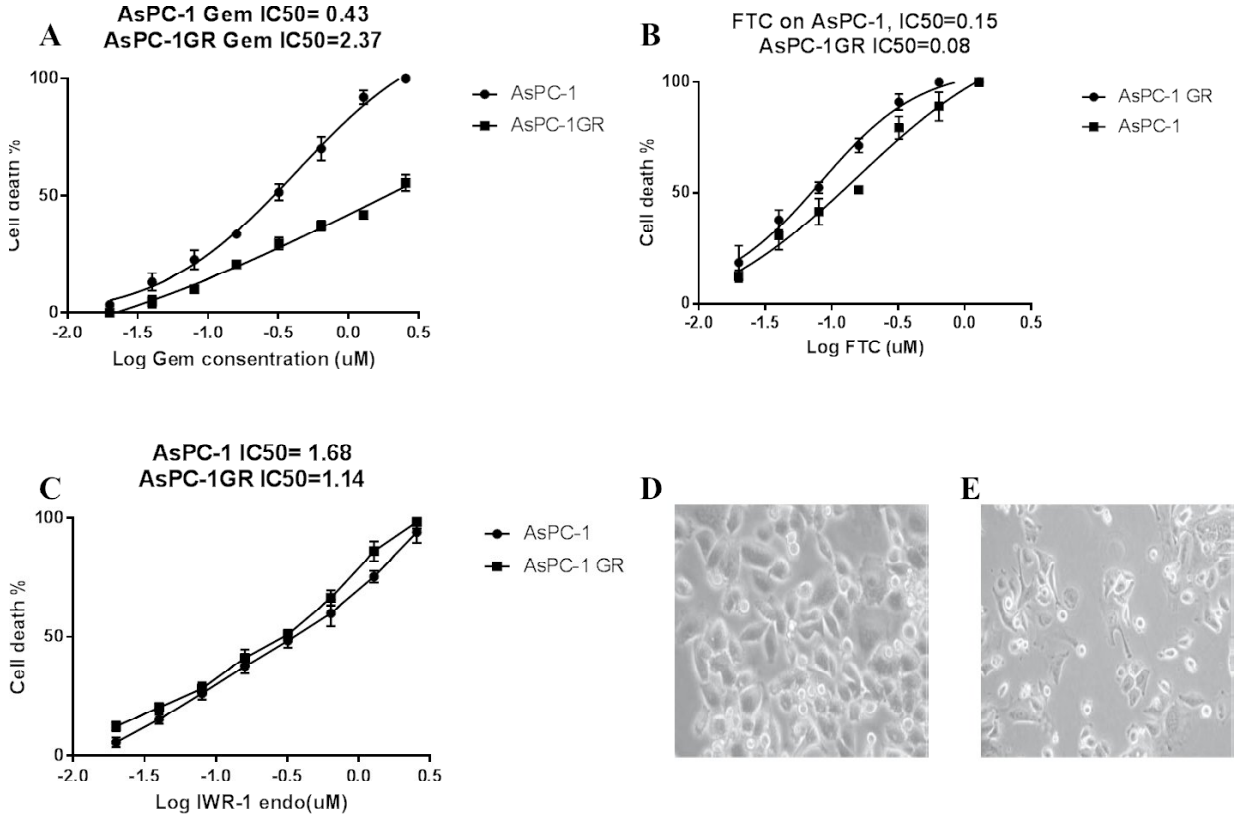

Fig. 1 : MTT proliferation assay on normal and resistant AsPC-1 cells. A: Viability of parental AsPC-1 and AsPC-1 GR (gemcitabine resistant) cells treated with increasing doses of Gemcitabine (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 µmol), cell media was used as control.The IC50 of FTC (C) and IWR-1 endo (B) was measured on AsPC-1 and AsPC-1GR cells. D: AsPC-1 parenteral and E: AsPC-1 GR cells morphology. The values shown are the Mean±Standard deviation (S.D.) of three different experiments measured simultaneously. The measured IC 50 is based on (µmol) and the P value was calculated using two-tailed Student's t-test ($p \leq 0.05$) (A, B and C).

in Fig. 1D and E; the resistant cells were more rounds, larger, flatter and exhibited more mesenchymal phenotype than the parental cell line AsPC-1. Fumitremorgin C (FTC, an ABCG2 inhibitor) and IWR-1 endo (a Wnt/β-cateninin inhibitor) (Sigma Aldrich, Castle Hill, NSW, Australia) were treated with resistant and non-resistant cells to evaluate the cell-death effect. There were not significant differences between their IC50 in normal or resistant cancer cells. As shown in Fig. 1, the IC50 for FTC and IWR-1 endo in AsPC-1 cells determined as 1.68 and 0.15 µmol respectively, while their IC50 on AsPC-1GR for FTC was 1.14 µmol (Fig. 1C) and for IWR-1 endo was 0.08 µmol (Fig. 1B). These results showed that the inhibitors alone could not increase cell death in resistant cells as much as when they co-treated with gemcitabine.

Western blotting of β-catenin and ABCG2 protein expressions

Western blotting was performed to determine relative β-catenin and ABCG2 protein expressions in both cell lines. As expected, β-catenin and β-actin were detected as single bands at 92 and 42KDa, respectively. ABCG2 is a 72 KD as it shown in Fig. 3. The β-catenin and ABCG2 expressions were higher in AsPC-1 GR than those in normal AsPC-1cells. As it shown in Fig. 2, gemcitabine separately co-treatment with FTC or IWR-1 endo, decreased ABCG2 and β-catenin expression, however their all combination treatment on resistant cells decreased both protein expression significantly (Fig. 2B).

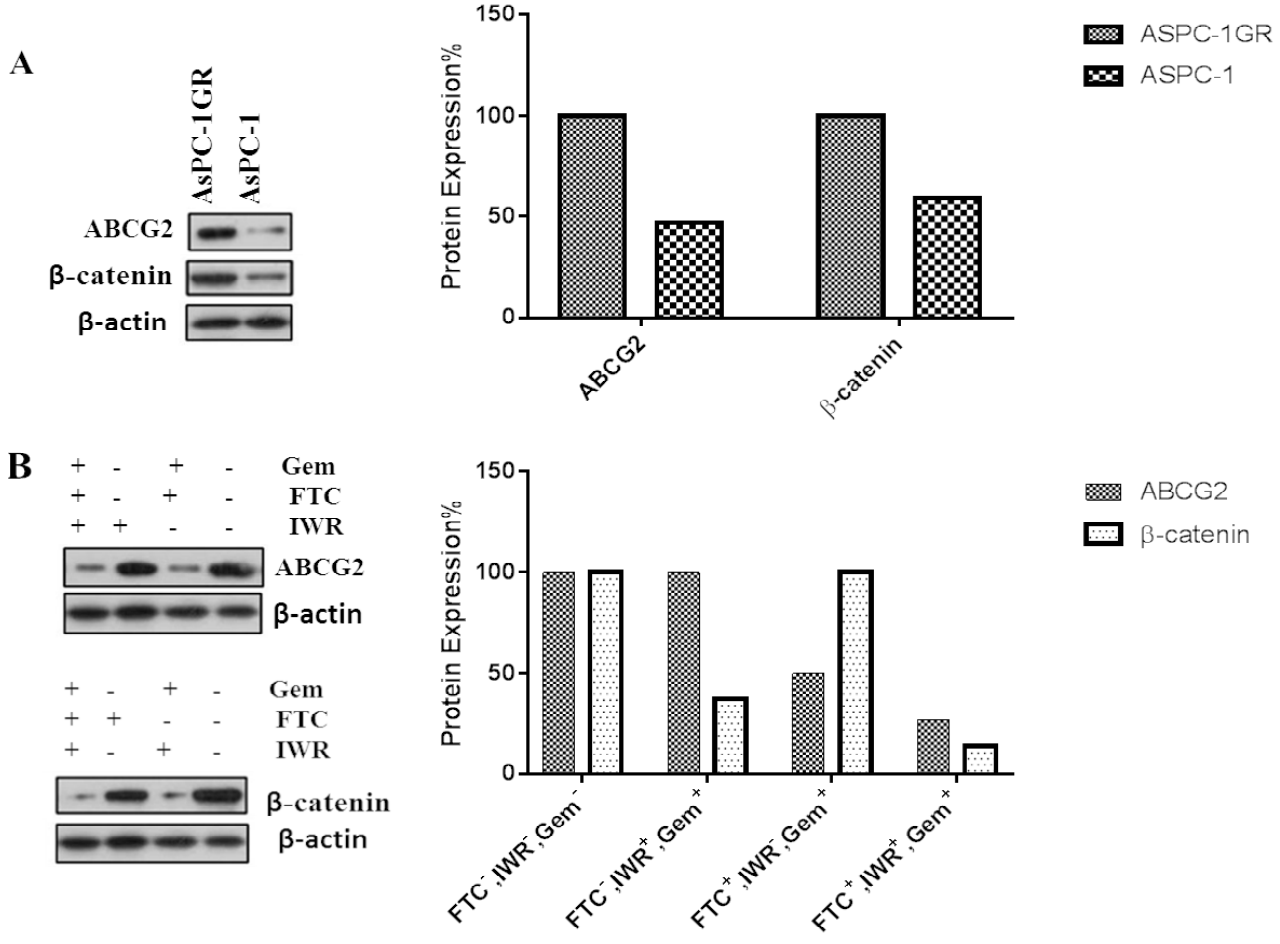

Fig. 2 : α-catenin and ABCG2 protein expression by western blot analysis in AsPC-1 and AsPC-1GR cells. A: β-catenin and ABCG2 protein expressions were significantly higher in resistant cell line than those in AsPC-1 cells. B: β-catenin and ABCG2 protein expressions were decreased after IWR-1 and FTC added in response to their inhibition effect in gemcitabine-resistant cells. The combination of inhibitors and gemcitabine effects were notable on ABCG2 and β-catenin expression in resistant cells. All graph's results normalized with β-actin as control.

ABCG2 and Wnt/β-catenin inhibitors alone and their combination significantly re-sensitize gemcitabine-resistant pancreatic cancer cells to gemcitabine.

Treatment with 1 µmol of gemcitabine induced death in ~ 70% of AsPC-1 cells; it also reduced cell viability in AsPC-1 GR cells (Fig. 1) however, it was remarkably lower than normal cancer cells. AsPC-1 GR cells were treated with different concentrations of gemcitabine (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 µmol) and co-incubated with ABCG2 inhibitor, Wnt/β-catenin inhibitor and these inhibitors combination. The results confirmed that ABCG2 and Wnt/â-catenin inhibitors alone stimulated gemcitabine-induced cell death in AsPC-1 GR cells. Gemcitabine IC50 after 72 h was 1.58 µmol and 1.35 µmol in order following addition of FTC and IWR-1 endo. These inhibitors combination co-incubation with gemcitabine resulted in further increase cell death due to gemcitabine re-sensitization in resistant cells, which was confirmed by gemcitabine IC50=0.14 µmol (Fig. 3). As it was shown in Fig. 2 B and C, these inhibitors had not significant effect on cell death without gemcitabine, while their combination with gemcitabine improved gemcitabine cell-death effect. These results indicated that ABCG2 and Wnt/β-catenin inhibitors alone and their combination notably inhibit the activated signaling pathway and specific proteins with crucial role in the gemcitabine-resistant cells.

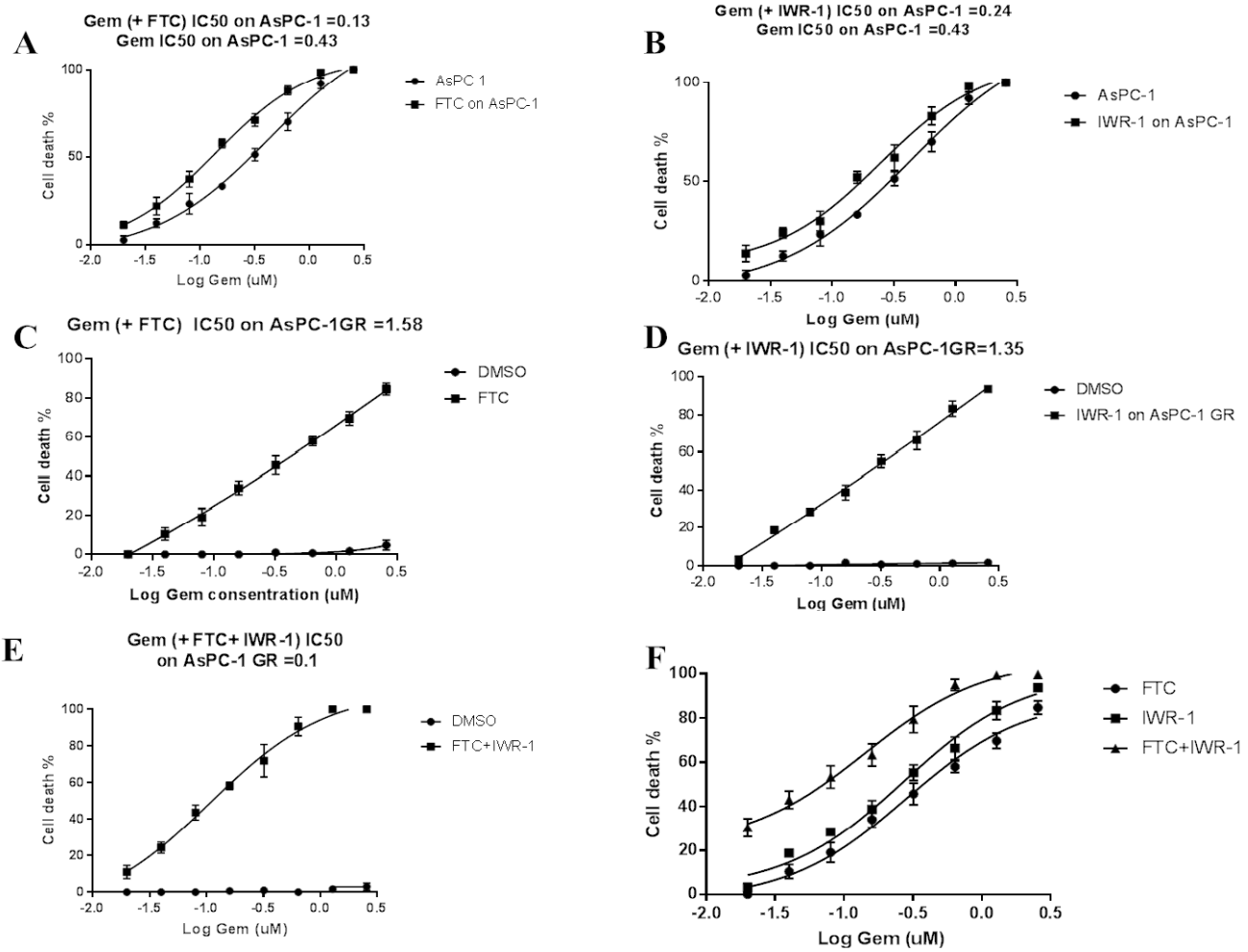

Fig. 3 : The inhibitory effects of Wnt inhibitor (IWR-1 endo) and ABCG2 inhibitor (FTC) on proliferation of AsPC-1 GR cells. A: Gemcitabine IC50 on AsPC-1 cells with and without FTC, B: Gemcitabine 1C50 on AsPC-1 cells with and without IWR-1 endo; C: Gemcitabine 1C50 on AsPC-1GR cells co-treated with FTC, D: Gemcitabine 1C50 on AsPC-1GR cells co-treated with IWR-1 endo . E: Gemcitabine IC50 on AsPC-1GR cells co-treated with IWR-1 endo and FTC combination, and F: Gemcitabine IC50 on AsPC-1GR cells co-treated with FTC alone, IWR-1 endo alone and their combination in comparison. The measured IC 50 is based on (µmol) and the P value was calculated using two-tailed Student's t-test ($p \leq 0.05$).

Discussion

Although gemcitabine based treatment is the main approach for pancreatic ductal adenocarcinoma, survival remains poor because of resistance acquisition to gemcitabine in pancreatic cancer cells. Combination therapy is one of the best ways to over drug resistance. In this survey for the first time we identified significant effect of combining ABCG2 and Wnt/β catenin pathway inhibitors on AsPC-1 gemcitabine resistant cell line *in vitro*. This is the first report to show that FTC and IWR-1 endo combination re-sensitizes gemcitabine-resistant cultured pancreatic cancer cells to gemcitabine-induced cell death. Using gemcitabine-resistant cells is an important aspect of this study, which was established by gradually increasing exposure to gemcitabine, for drug screening and the following analysis. Cultured resistant cell line have been mainly used for such screening before (14). Resistant cell analyzing has provided new insights into finding new treatments for pancreatic cancer (15). Similarly, our screening method found that FTC and IWR-1 endo combination has high potential as a useful biochemical tool to achieve better cancer treatment results. Several studies showed effective pancreatic cancer treatment by combination therapy; for example positive combination effects of sorafenib which is a multikinase inhibitor and an isothiocyanate found in broccoli, named sulforaphane, reduced pancreatic cancer cells drug resistance (16). Moreover, significant increased sensitivity of pancreatic cancer cells to different chemotherapeutic drugs including gemcitabine with sulforaphane found by Kallifatidis (17). Zhou et al, showed synergistic effects of sulforaphane and quercetin that target pancreatic cancer stem cells, which their role in pancreatic cancer drug resistance have been proved in many studies (18). In the present study, treatment of AsPC-1GR cells with IWR-1 endo resulted in pronounced inhibition of Wnt/ â catenin pathway due to higher activity of this pathway in resistant cells (4). Since pancreatic cancer cell re-sensitization to gemcitabine induced by IWR-1 endo and gemcitabine co-incubation in AsPC-1GR cells was not remarkable, it was assumed that inhibition of different ways which are involve in pancreatic cells drug resistance may give more significant and decisive results. In this regard, FTC an inhibitor of ABCG2 was added to complex of IWR-1 endo and gemcitabine. Different gemcitabine concentrations (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 µmol) co-incubated with IWR-1 and FTC. The gemcitabine IC50 in AsPC-1GR cells were 1.35 and 1.58 µmol, respectively, while gemcitabine IC50 following addition of IWR-1 endo + FTC combination on these cells was 0.14 µmol. This means that combinatory effects of these inhibitors in re-sensitizing pancreatic cancer cells to gemcitabine were several folds more than FTC and IWR-1 endo alone co-treatment with gemcitabine. The main aspect of present study is demonstration of effective re-sensitization of pancreatic tumors cells to gemcitabine with FTC plus IWR-1 endo co-administration with gemcitabine compared to gemcitabine alone or gemcitabine with just one of these inhibitors. Therefore, suggesting that combined treatment may serve as a best choice to target gemcitabine resistance pancreatic cancer cells. ABCG2 overexpression in AsPC-1 cells promotes migration and invasion; however the mechanism by which this transporter becomes overexpressed is not clearly understood (19). Moreover, one of the main characterizations of pancreatic cancer stem cells which have a vital role in tumor resistance is ABCG2 overexpression, which determines stem cell chemosensitivity after its inhibition (20). On the other hand, Wnt/β catenin pathway inhibition could reverse multi-drug resistant in many solid tumors like pancreatic cancer (21). ABC transporters including ABCG2 downregulation by inhibiting Wnt- signaling pathway has been reported in many studies (22-24). In a recent one, a combinatorial treatment (celecoxib and imatinib) re-sensitized K562 imatinib resistant cells through downregulation of several ABC transporters such as MDR1, MRP1-5, ABCA2 and ABCG2 by inhibiting Wnt/β catenin pathway signaling (25). In conclusion, our data demonstrated that FTC, an inhibitor of ABCG2, in combination with IWR-1 endo, a Wnt/β catenin pathway inhibitor, could represent a novel therapeutic strategy for pancreatic cancer patient with gemcitabine resistant characterization.

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