Triciribine Phosphate Monohydrate, an AKT Inhibitor, Enhances Gemcitabine Activity in Pancreatic Cancer Cells

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Abstract. *Background: Pancreatic cancer is a highly lethal cancer due to early metastasis and resistance to current chemotherapeutic agents. Abnormal protein kinase B (AKT) activation is an important mechanism of chemoresistance to gemcitabine, the most widely used agent in pancreatic cancer. Material and Methods: In the study, we tested the hypothesis that combining an AKT inhibitor with gemcitabine would augment anti-tumor activity. We treated human pancreatic cancer MiaPaCa-2 cells with gemcitabine and the AKT inhibitor triciribine, alone and in combination, and evaluated treatment effects using trypan blue assay, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium (MTT) assay, and cell death enzyme-linked immunosorbant assay. Colorimetric data of MTT assay were computationally analyzed for synergism of the combination therapy by CalcuSyn2 (Biosoft, Great Shelford, Cambridge, UK). Results: Both gemcitabine and triciribine inhibited cell growth in a dose-dependent manner. Triciribine synergistically enhanced the cytotoxic activity of gemcitabine. The combination index (CI) provides the synergistic, additive, or antagonistic effects of the two-drug combination. CI at the 50% effective dose at 1:500 ratio of gemcitabine to triciribine was 0.74, indicating the synergistic effect of the drugs. The combination treatment with the non-apoptotic dose of each agent distinctly induced apoptosis, with gemcitabine in combination with triciribine, synergistically inhibiting pancreatic cancer cell growth and inducing apoptosis. Conclusion: These findings support the use of triciribine to overcome activated AKT-mediated resistance of pancreatic cancer to gemcitabine.*

Pancreatic cancer is a dismal disease and is the fourth leading cancer death cause in the United States. Approximately 40,000 new cases and 36,000 deaths were estimated in 2013 (1). More than two-thirds of new patients present with an advanced stage of the disease at diagnosis and subsequently are not considered to be curative surgical candidates. Gemcitabine has been used for more than a decade as standard chemotherapy agent in monotherapy or combination therapy for inoperable advanced-stage disease (2). However, pancreatic cancer is a highly chemotherapy-resistant tumor.

More recently, the 5-fluorouracil, oxaliplatin, and irinotecan (FOLFIRINOX) regimen has been shown to be superior to gemcitabine alone (3). However, because of toxicity concerns associated with FOLFIRINOX, the regimen is only used for patients with good performance status. In 2013, nab-paclitaxel was approved by the Food and Drug Administration for use in combination with gemcitabine based on a large phase III study, which was compared to gemcitabine alone, and showed overall survival improvement of 1.8 months (4). The regimen seems to be better-tolerated than FOLFIRINOX and therefore is now commonly used in most patients who are not fit to receive FOLFIRINOX. Because of its better toxicity profile, most of the current studies in pancreatic cancer are still using gemcitabine alone or gemcitabine in combination with nab-paclitaxel as a backbone chemotherapy.

In pancreatic cancer, several molecular determinants have been reported to contribute to gemcitabine resistance. Hyperactivation of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway is one of the known resistance mechanisms (5). Therefore, targeting the activated PI3K-AKT pathway is a reasonable approach to sensitizing pancreatic cancer cells to gemcitabine. In addition, pre-clinically, it has been shown that AKT activation induces

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Table I. *Combination index (CI) for combinations of gemcitabine and triciribine at 1:100.*

Drug (ratio)	CI Values at					Dm	m	r
	ED_{75}	ED_{90}	ED_{96}	Mean	Rank			
MiaPaCa-2 with control vector (mean CI=0.224)								
Triciribine						5.558.27E-6	0.812	0.825
Gemcitabine						5.36E-6	0.955	0.845
Triciribine and gemcitabine (100:1)	0.235	0.222	0.215	0.224	****	69.11E-6	0.931	0.992
MiaPaCa-2 with myristoylated-AKT (mean CI=0.32)								
Triciribine						94.34E-6	1.204	0.872
Gemcitabine						1.6E-6	0.957	0.922
Triciribine and gemcitabine (100:1)	0.42	0.361	0.327	0.37	***	29.73E-6	1.326	0.976

ED: Effective dose; Dm: median-effect dose; m: slope; r: linear correlation coefficient. ****Strong synergism; ***synergism

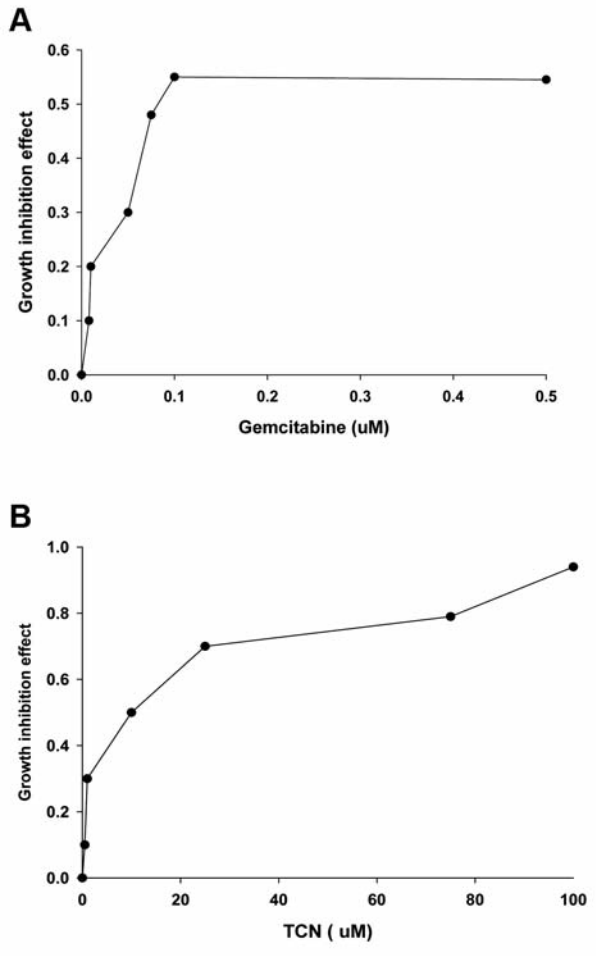

Figure 1. *Cell growth inhibition by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium assay in MiaPaca-2 cells after 72-h treatment with different concentrations of A: gemcitabine (Gem) and B: triciribine (TCN).*

malignant transformation and promotes cell survival, and that disruption of AKT pathways inhibits cell growth and induces apoptosis (6-8). Small-molecule inhibitors of AKT were previously identified in an evaluation of a chemical library of 1,992-compounds from the National Cancer Institute (the National Cancer Institute Diversity Set) for agents capable of inhibiting growth of AKT2-transformed but not parental NIH/3T3 cells. Out of 32 compounds that selectively inhibited growth of AKT2-transformed cells, the most potent was triciribine. The chemical properties of triciribine have been described elsewhere (9). Once inside cells, triciribine is converted to triciribine phosphate by adenosine kinase. Triciribine significantly inhibited AKT phosphorylation at both threonine-309 and serine-474, which are required for full activation of AKT (10).

In the present study, our aim was to evaluate whether the combination therapy of gemcitabine and triciribine in pancreatic cancer cell lines has synergistic anti-proliferative and pro-apoptotic effects.

Materials and Methods

Cell lines. MiaPaCa-2 cells, a human pancreatic adenocarcinoma cell line known to express activated AKT, were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA, USA), penicillin, and streptomycin (Invitrogen).

Stable transfection of MiaPaCa-2 cells: MiaPaCa-2 cells were stably transfected with pWZL retroviral empty vector only and vector containing constitutively active myristoylated AKT using transfection reagent (Addgene, Cambridge, MA, USA) as per the

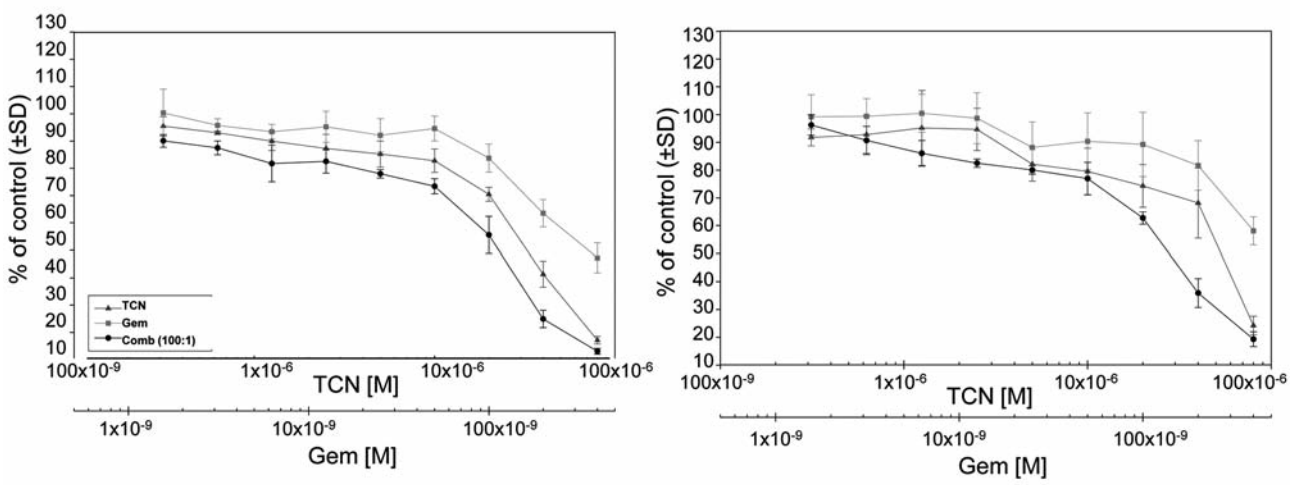

Figure 2. *Dose–effect curve of cell growth inhibition in monotherapy of triciribine (TCN) and gemcitabine (Gem) and their combination therapy. A: MiaPaca-2 cells transfected with control vector; B: MiaPaca-2 cells transfected with myristoylated-AKT.*

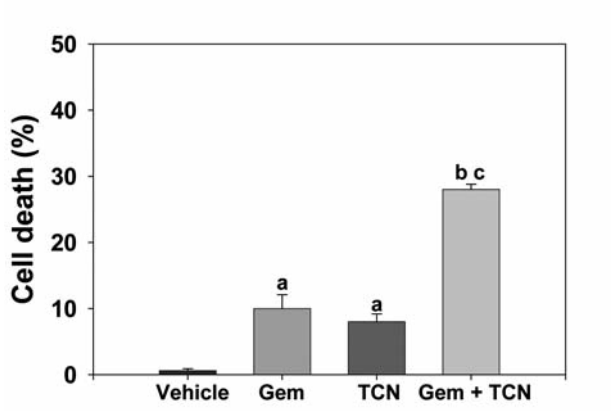

Figure 3. *Viable and non-viable cells by trypan blue assay after 72-hour treatment of MiaPaCa-2 cells with gemcitabine (Gem: 10 nM) and triciribine (TCN: 5 μM). The combination of gemcitabine and triciribine inhibited cell growth more greatly and induced more cell death ($^{b}p<0.001$) than did single-agent treatment ($^{c}p<0.05$) ; $^{a}p<0.05$ versus vehicle alone.*

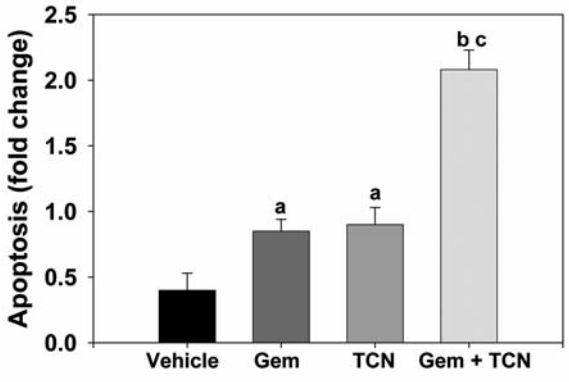

Figure 4. *Apoptotic cell death (fold change) after 72-hour treatment of MiaPaCa-2 cells with gemcitabine (Gem; 10 nM) and triciribine (TCN; 5 μM). Gemcitabine and triciribine significantly ($^{a}p<0.05$) induced apoptosis. The combination of both drugs induced significantly more apoptosis ($^{b}p<0.01$ versus vehicle alone, $^{c}p<0.02$ versus single agent).*

manufacturer's instructions. Cells were selected for two weeks with neomycin at a final concentration of 500 μg/ml.

Cell viability assay and evaluation of synergism. To quantify the effects of gemcitabine (Bedford Laboratories, Bedford, OH, USA) and triciribine (Moffitt Cancer Center, Tampa, FL, USA) alone and in combination in regard to MiaPaCa-2 cell proliferation, 3×10^3 cells were plated in triplicate onto 96-well plates for 24 h before addition of the drugs at different concentrations. Seventy-two hours later, viable cells in each well were quantified using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA). For evaluation of synergism, cells were treated with gemcitabine and triciribine in a fixed combination ratio of 1:500 at different concentrations. The data were computationally evaluated for synergism by CalcuSyn 2® (Biosoft, Great Shelford, Cambridge, UK).

Trypan blue assay. To quantify viable and non-viable cells after treatment, 5×10^4 cells were plated in triplicate onto 6-well plates. Seventy-two hours later, the cells were trypsinized and washed with phosphate-buffered saline. After being stained with 0.4% trypan blue (Sigma-Aldrich), the number of viable and non-viable cells were counted in a hemocytometer. To quantify apoptosis after treatment, 3×10^3 MiaPaCa-2 cells were plated in triplicate onto 96-

well microplate modules provided in the Cell Death enzyme-linked immunosorbant assay (ELISA) Plus kit (Roche, Indianapolis, IN, USA) 24 hours before treatment. Seventy-two hours after treatment, the cells were lysed, and histone-associated DNA fragments were detected by ELISA and quantified by an optometric plate reader. All experiments were performed in triplicate.

Cell death ELISA. To quantify apoptosis after treatment, 3×10^3 cells were plated in triplicate onto 96-well microplate modules provided in Cell Death ELISA Plus kit (Roche) 24 h before treatment. Seventy-two hours after treatment, the cells were lysed, and histone-associated DNA fragments were detected by ELISA and quantified by an optometric plate reader. All experiments were performed in triplicate.

Anchorage-independent growth assay. Colony formation in soft agar was determined. MiaPaCa-2 cells were cultured in full medium containing 0.3% agarose (BMA, Rockland, ME, USA) and seeded into six-well plates over a bottom layer containing 0.6% agarose. Gemcitabine, triciribine, or both were added to the top layer. Plates were incubated at 37˚C for two weeks. After staining with MTT at 37˚C for 2 hours, colonies were visualized and scanned. The number of colonies on the scanned image was manually counted. All experiments were performed in triplicate.

Statistical analysis. Data, expressed as means±SEM, were analyzed statistically using unpaired t-tests or one-way analysis of variance (ANOVA) where appropriate. ANOVA was followed by Duncan's multiple range tests using SAS statistical software (SAS, Cary, NC, USA) for comparison between different treatment groups. Statistical significance was set at $p<0.05$.

Results

Cell viability (MTT) assays. The IC_{50} is defined as the concentration of drug required for a 50% reduction in growth/viability. IC_{50} values are displayed in Table I. Gemcitabine and triciribine inhibited the cell growth of MiaPaCa-2 cells, a human pancreatic adenocarcinoma cell line, in a dose-dependent manner (Figure 1). When gemcitabine and triciribine were combined, MiaPaCa-2 stably transfected with pWZL retroviral empty-vector and with constitutively active myristoylated-AKT clearly demonstrated synergistic anti-proliferative effects at a fixed combination ratio of 1:100 (Figure 2).

Trypan blue staining. Trypan blue assay also showed consistent synergistic anti-proliferative effects of combined therapy, as was demonstrated by MTT assay above. Moreover, the combination induced more cell deaths than single-agent therapy with gemcitabine or triciribine alone. Cell Death ELISA Plus® showed more than double apoptosis levels when MiaPaCa-2 cells were treated by gemcitabine in combination with triciribine (Figures 3 and 4).

Anchorage-independent growth pathway. Figure 5 shows that single-agent therapy with gemcitabine (20 nM) and triciribine (5 μM) reduced colony formation in soft agar; the combination therapy further reduced colony formation (Figure 5).

Discussion

Activation of tumor survival and oncogenic pathways, such as the PI3K/AKT pathways, has been implicated in oncogenesis. In particular, the pathways that are mediated by AKT have been shown to be critical in many steps of malignant transformation, including cell proliferation, anti-apoptosis/survival, invasion and metastasis, and angiogenesis (11). Proof-of-concept of the involvement of AKT in oncogenesis has been demonstrated preclinically by showing that ectopic expression of AKT induces malignant transformation and promotes cell survival (12, 13) and that disruption of AKT pathways inhibits cell growth and induces apoptosis (14). In pancreatic cancer, earlier work has demonstrated that the presence of phosphorylated AKT was associated with poorly-differentiated tumors and worse prognosis in patients who underwent surgical resection (15). Furthermore, AKT overexpression and hyperactivation in pancreatic cancer were associated with poor prognosis, resistance to chemotherapy, and shortened survival time of patients with cancer (16). Therefore, we hypothesized that targeting activated AKT would augment the suppression of cancer cell growth and induction of apoptosis by chemotherapy.

In our study, we observed synergistic effects in anti-proliferative and pro-apoptotic activities of gemcitabine when combined with triciribine. The combination inhibited the cell growth of MiaPaCa-2 cells, a human pancreatic adenocarcinoma cell line, in a dose-dependent manner. In addition, gemcitabine in combination with triciribine induced more apoptosis than gemcitabine and triciribine alone. We believe that that this is a very important finding, as hyperactivation of the PI3K/AKT pathway is one of the major resistance mechanisms for gemcitabine (5, 17). Therefore, triciribine has the potential to overcome chemoresistance with gemcitabine in pancreatic cancer.

Single-agent use of triciribine phosphate has been studied in a phase I trial of solid tumors (18). Although the study enrolled a small number of patients and neither clinical activity nor dose-limiting toxicity were observed, promising results were shown at a higher dose of triciribine phosphate, as there was preliminary evidence of pharmacodynamic activity as measured by decreased phosphorylated AKT.

The limitations of our study are the lack of *in vivo* evaluation and the usage of only one pancreatic cancer cell line. However, based on the single-agent phase I data and our *in vitro* data demonstrating triciribine inhibition of AKT phosphorylation and synergistic action with gemcitabine in this cultured human pancreatic cell line, it is reasonable to

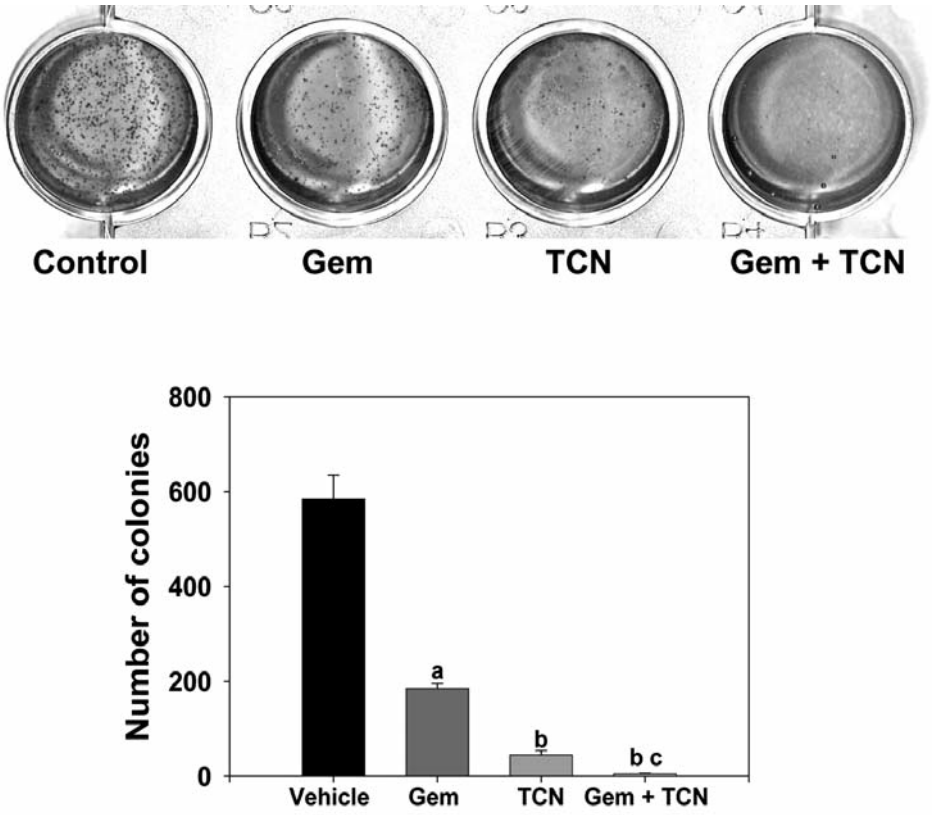

Figure 5. *Colony-formation assay after 2-week treatment of MiaPaCa-2 cells with gemcitabine (Gem; 20 nM), triciribine (TCN; 5 μM), and their combination. Gemcitabine ($^{a}p<0.02$), triciribine ($^{b}p<0.001$), and their combination ($^{b}p<0.001$) significantly inhibited colony formation compared to the vehicle alone. The combination of both drugs significantly inhibited colony formation ($^{c}p<0.01$) more than either drug alone.*

conduct a phase I trial with this combination. Since the standard treatment of advanced pancreatic cancer has now shifted to gemcitabine and nab-paclitaxel, targeting the AKT pathway with the new combination is warranted.

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Conflicts of Interest

None. Funding: None.

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