Potentiation of gemcitabine by Turmeric Force™ in pancreatic cancer cell lines

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Abstract. Gemcitabine is a first line cancer drug widely used for the treatment of pancreatic cancer. However, its therapeutic efficiency is significantly limited by resistance of pancreatic cancer cells to this and other chemotherapeutic drugs. We have investigated the cytotoxic effect of Turmeric Force™ (TF), a supercritical and hydroethanolic extract of turmeric, alone and in combination with gemcitabine in two pancreatic carcinoma cell lines (BxPC3 and Panc-1). TF is highly cytotoxic to BxPC3 and Panc-1 cell lines with IC_{50} values of 1.0 and 1.22 μg/ml, respectively with superior cytotoxicity than curcumin. Gemcitabine IC_{50} value for both of these cell line is 0.03 μg/ml; however, 30-48% of the pancreatic cancer cells are resistant to gemcitabine even at concentrations >100 μg/ml. In comparison, TF induced cell death in 96% of the cells at 50 μg/ml. The combination of gemcitabine and TF was synergistic with IC_{90} levels achieved in both pancreatic cancer cell lines at lower concentrations. CalcuSyn analysis of cytotoxicity data showed that the Gemcitabine + Turmeric Force combination has strong synergism with combination index (CI) values of 0.050 and 0.183 in BxPC3 and Panc-1 lines, respectively at IC_{50} level. This synergistic effect is due to the increased inhibitory effect of the combination on nuclear factor-κ B activity and signal transducer and activator of transcription factor 3 expression as compared to the single agent.

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Abbreviations: CI, combination index; DMSO, dimethyl sulfoxide; ELISA, enzyme linked immunosorbent assay; GEM, gemcitabine; IL-8, interleukin-6; nuclear factor-κ B, NF-κB; PBS, phosphate-buffered saline; STAT3, signal transducer and activator of transcription factor 3; TF, Turmeric Force

Key words: pancreatic carcinoma, gemcitabine, Turmeric Force, cytotoxicity, synergism

Introduction

Pancreatic carcinoma is among the most aggressive forms of human cancer with a very high mortality rate. It represents the fourth leading cause of cancer death in United States with an annual mortality of 32,000. With a 5-year survival rate of only 3% and a median survival of <6 months, diagnosis of pancreatic adenocarcinoma carries one of the poorest prognoses of any form of cancer (1,2). Consequently, the management and treatment of this relatively common form of cancer is considered to be a major medical challenge for the 21st century. Pancreatic cancers are difficult to detect and diagnose because in the early stages of the disease, patients are generally asymptomatic or demonstrate signs and symptoms that mimic other more common illnesses (3). Consequently, at the time of diagnosis the disease has typically metastasized making treatment of pancreatic cancer extremely difficult. Chemotherapy alone or as adjuvant to surgery or radiation has not significantly contributed to the cure or prolongation of survival in pancreatic cancer patients (4). Among the recently approved drugs, gemcitabine (GEM) represents an important advance and is the first chemotherapy agent approved on the basis of clinical response instead of the traditional increase in survival time. Therefore, effective therapeutic drugs or drug combinations to improve survival time are yet to be identified.

Recent studies indicate that novel strategies for sensitizing pancreatic cancer cells with natural dietary chemopreventive agents would be beneficial for overcoming intrinsic tumor cell resistance (5-8). A review of the worldwide cancer incidence data show that India has one of the lowest incidences of pancreatic cancer with 1.8 cases per 100,000 persons as compared to 13 cases per 100,000 persons in United States and 11-12 cases per 100,000 persons in Europe, perhaps indicating a dietary or environmental factor associated with this low incidence (9). Among the most frequently consumed natural product in India, turmeric is a major ingredient in the daily diet with an average rate of consumption of ~1.8 g/day/person. Curcumin, one of the most active compounds in turmeric has been investigated for its effect against human cancers both *in vitro* and *in vivo*. While effective *in vitro*, the poor bioavailability of curcumin limits its use in cancer patients (10,11). Turmeric Force (TF) is a proprietary supercritical and hydroethanolic extract of turmeric, representing a relatively

complete spectrum of polar, semi-polar and non-polar compounds present in turmeric. These include the turmerones present in the non-polar fraction and curcuminoids (including curcumin) in the semi-polar fraction. Evidence suggests that this formulation possess better chemopreventive efficacy than curcumin (12). This liquid product in olive oil is sold as soft gel capsules in United States as a nutraceutical. We hypothesize that TF can be used as a single agent or synergistically with gemcitabine against pancreatic carcinoma. Therefore, we have investigated the cytotoxicity of TF alone as well as in combination with gemcitabine in pancreatic adenocarcinoma cell lines *in vitro*.

Materials and methods

Cell lines. Two pancreatic adenocarcinoma (BxPC3 and Panc-1) cell lines were used for the study. Both cell lines were initially purchased from American Type Culture Collection, Manassas, VA and maintained in the laboratory. BxPC3 and Panc-1 cell lines were grown in RPMI and DMEM media, respectively, supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) in a humidified 5% CO_2 incubator at 37˚C.

Cytotoxicity. Gemcitabine (GEM) and Turmeric Force™ (TF) were purchased from American Custom Chemicals, CA and New Chapter Inc., Brattleboro, VT, respectively. Stock solutions of TF (100 mg/ml) were prepared in dimethyl sulfoxide (DMSO) and dilutions were made in 10% DMSO containing phosphate-buffered saline (PBS). When TF is dissolved in DMSO and centrifuged at 4˚C for 10 min, olive oil was separated on the top of frozen DMSO that can be removed easily. GEM dilutions were prepared in PBS. BxPC3 and Panc-1 cells were plated at a density of 10,000 cells/well in 96-well plates with RPMI/DMEM medium and treated with GEM and/or TF at varying concentrations (0-200 μg/ml) and incubated in the CO_2 incubator at 37˚C for 72 h. The concentration of DMSO in the incubation medium was always kept below the cytotoxic level (<0.1%). Cell Proliferation assay kit I (MTT) from Roche Molecular Biochemicals (Indianapolis, IN) was used for cytotoxicity assays and the manufacturer's protocol was followed. The percentage of surviving cells over untreated control cells was calculated and plotted against drug concentrations. The IC_{50} value for individual cell lines was calculated from the dose-response curve (13).

Synergism between GEM and TF. To determine the synergism, cytotoxicity assay was performed with GEM and/or TF. In the case of combination protocol, drug concentrations were escalated keeping a constant ratio between the two drugs so as to conform to CalcuSyn software (Biosoft, Ferguson, MO). The fraction of surviving cells in each concentration was used for the analysis of synergism between GEM and TF by CalcuSyn software (14,15).

RT-PCR for NF-κB, Cox-2, STAT3 and IL-8 mRNA expression. BxPC3 and Panc-1 cells ($2x10^6$) were treated with GEM, TF or their combination in multiwell plates for 72 h at 37˚C in a CO_2 incubator. Cells were scraped off from the plates and washed with PBS twice. Total RNA was extracted from the cell pellet using TRIzol reagent (Sigma Chemical Co. St. Louis, MO) and DNA contamination in the RNA preparation was removed by treating with RNase-free DNase. Total RNA (5 μg) was reverse transcribed initially to obtain first-strand cDNA in a 20 μl reaction mix (Invitrogen, Carlsbad, CA) and ~0.025 μg cDNA was amplified using AmpiTaq DNA polymerase and gene-specific forward and reverse primers. NF-κB, IL-8 and G3PDH (control) primers were purchased from Bioscience International Inc., Camarillo, CA. The PCR products were separated on a 2% agarose gel and photographed.

NF-κB activity. BxPC3 and Panc-1 cells ($5x10^6$) were incubated with varying doses of GEM, TF or their combination for 72 h and nuclear proteins were extracted using the Nuclear extract kit from Active Motif, Carlsbad, CA according to manufacturer's instructions. NF-κB activity in the nuclear extract was determined by using the NF-κB activity ELISA kit as per the manufacturer's protocol (Active Motif).

STAT3 protein expression. Cellular protein extracts were prepared from drug-treated cells ($2x10^6$ cells) using 1 ml of cell extraction buffer containing 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_2P_2O_7$, 2 mM Na_2VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF and 1 protein inhibitor tablet/10 ml of extraction buffer. Cells were washed with PBS twice, incubated with extraction buffer on ice for 30 min, vortexed and centrifuged for 20 min at 4˚C. Protein concentration in the extract was estimated and 100 μg of protein extract was used for estimation of STAT3 using the ELISA kit from Biosource International, Inc., Camarillo, CA.

IL-8 protein expression. Both BxPC3 and Panc-1 ($1x10^6$) cells were treated with gemcitabine, TF or their combination for 72 h at 37˚C in the serum-free media. The IL-8 released into the medium by the cells was analyzed using the culture media and human IL-8 ELISA kit (Biosource International Inc., Camarillo, CA).

Statistical analysis. Single factor ANOVA was used to analyze the data on NF-κB activity, STAT3 and IL-8 protein expression.

Results

TF and GEM cytoxicity. The dose-effect curves of GEM, TF and their combination against BxPC3 and Panc-1 cell lines are given in Fig. 1. TF and GEM IC_{50} values are given in Table I. TF is cytotoxic in both cell lines with IC_{50} values of 1.0 μg/ml for BxPC3 and 1.22 μg/ml for Panc-1. This IC_{50} value is lower than that of curcumin (BxPC3 IC_{50} = 14.08 μg/ml: Panc-1 IC_{50} = 16.50 μg/ml) in pancreatic carcinoma cell lines (unpublished results).

GEM is one of the common drugs used for the treatment of pancreatic cancer patients. Even though this drug is cytotoxic to pancreatic carcinoma cell lines with an IC_{50} value

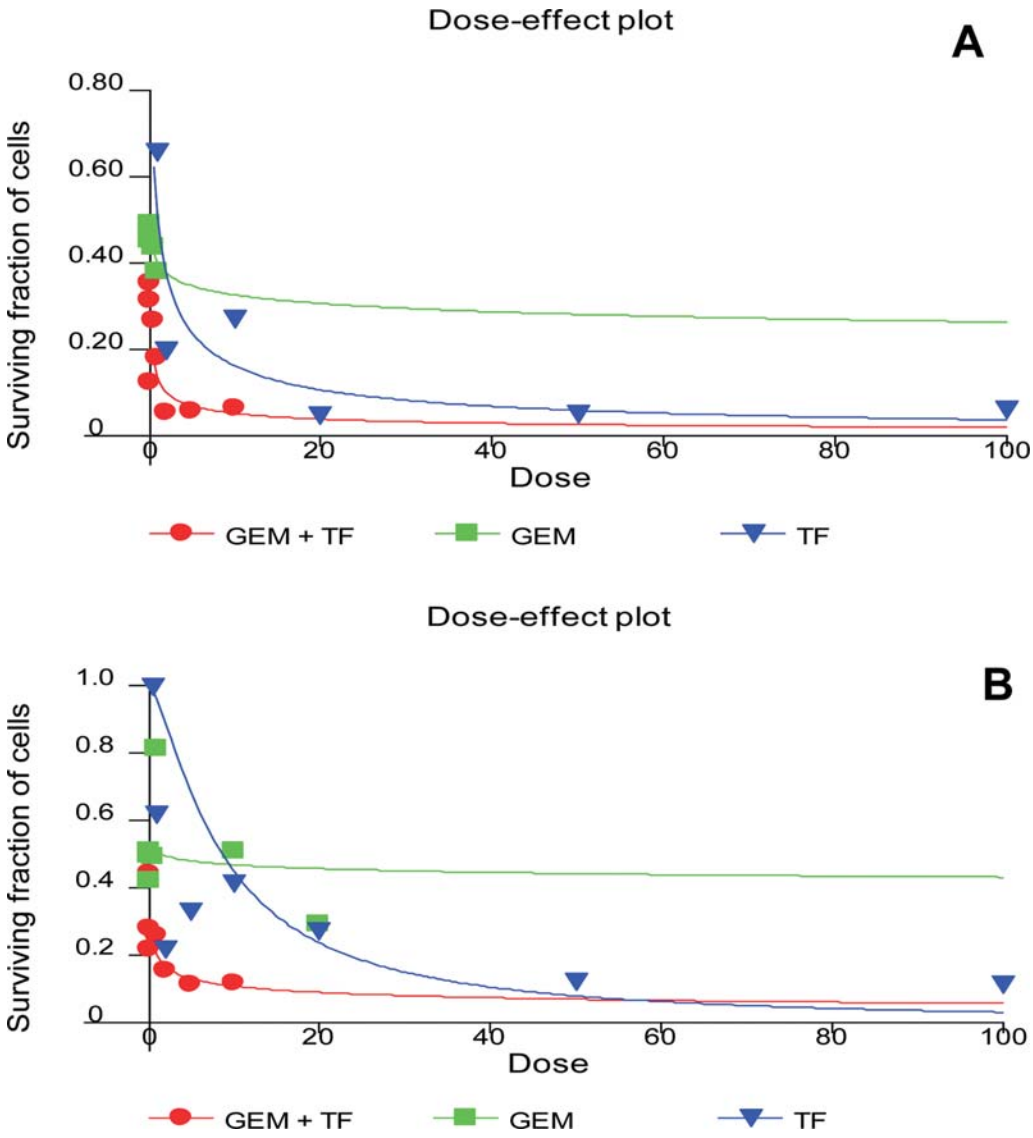

Figure 1. Dose-effect plots of gemcitabine (GEM), Turmeric Force (TF) and Gemcitabine+Turmeric Force (GEM+TF) in pancreatic carcinoma cell lines. (A) BxPC3; (B) Panc-1. The surviving cell fractions are plotted against drug doses in (μg/ml).

Table I. Cytotoxicity of gemcitabine and Turmeric Force in BxPC3 and Panc-1 pancreatic cancer cell lines.

Drug (μg/ml)	BxPC3 IC_{50} (μg/ml)	Panc-1 IC_{50} (μg/ml)
Gemcitabine	0.03±0.01	0.03±0.01
Turmeric Force	1.00±0.13	1.22±0.05

of 0.03 μg/ml (Table I), the highest concentration of 100 μg/ml was unable to induce >70 and 58% cell death in BxPC3 and Panc-1 cells, respectively, under a 72-h treatment schedule (Fig. 1). In comparison, TF produced higher levels of cytotoxicity than GEM at a lower concentration; 96% cytotoxicity at 50 μg/ml. The combination of GEM and TF treatments were more effective sepecifically at lower concentrations.

Table II. Combination index values between gemcitabine and Turmeric Force in BxPC3 and Panc-1 cell lines.

Cell line	CI value at IC_{50}	CI value at IC_{75}	CI value at IC_{90}
BxPC3	0.050	0.016	0.005
Panc-1	0.183	0.036	0.027

CI values: <0.1, very strong synergism; 0.1-0.3, strong synergism; 0.3-0.7, synergism; 0.8-0.9, moderate to slight synergism; 0.9-1.1, nearly additive; 1.1-1.45, moderate to slight antagonism; 1.45-3.3, antagonism; >3.3, strong antagonism.

Synergism between GEM and TF. The median effect plot and isobologram of GEM, TF and GEM+TF combination is shown in Fig. 2. The Combination Index values (CI) at IC_{50}, IC_{75} and IC_{90} are given in Table II. TF when combined with GEM showed increased inhibitory effects on tumor cell

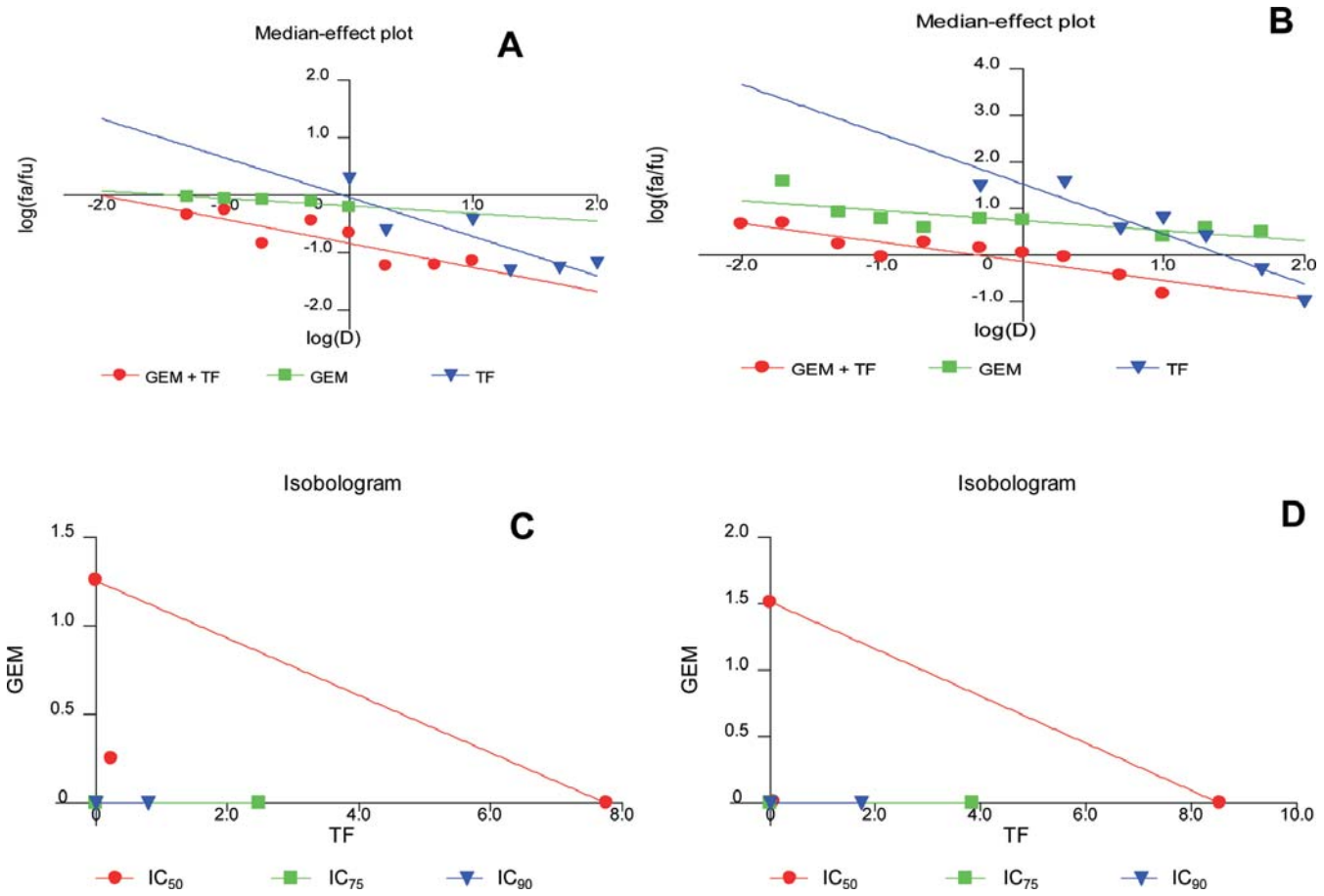

Figure 2. Medium effect plots (A and B) and isobolograms (C and D) of GEM, TF and GEM+TF in BxPC3 (A and C) and Panc-1 (B and D) cell lines. Surviving cell fractions were used for preparations of medium effect plots. The median effect plot, it is created by converting both dose and cytotoxicity values into log units using an equation x = log D vs. y = log (fraction affected/fraction unaffected). In this equation, slope yields the m value [an exponent signifying the sigmoidicity (shape) of the dose-effect curve] and the x-intercept gives log Dm value (the median effect dose signifying the potency). The different colored lines in isobolograms (C and D) represent diagonal lines for IC_{50}, IC_{75} and IC_{90} values.

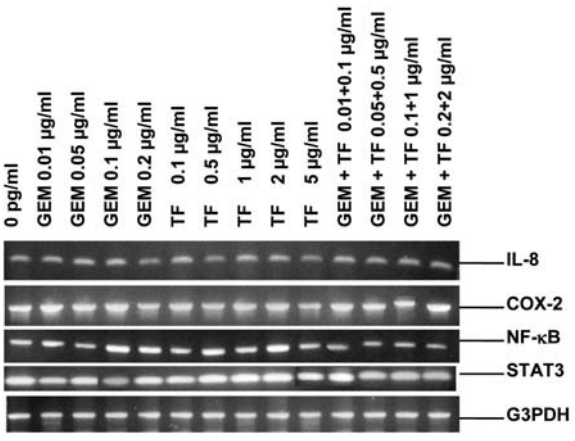

Figure 3. mRNA expression of IL-8, COX-2, NF-κB, STAT3 and G3PDH genes in BxPC3 cells treated with GEM, TF and GEM+TF analyzed by RT-PCR assay using gene-specific primers. No significant changes in mRNA levels were observed with drug treatments.

growth. The dose modification factor indicates that the concentration of both TF and GEM can be significantly reduced in the combination. Moreover, it is quite feasible to achieve >95% cell death with the combination compared to only 70 and 58% cell death in BxPC3 and Panc-1 cells respectively, with GEM alone. When the cytotoxic data was analyzed with CalcuSyn software (Fig. 2), GEM+TF combination showed strong synergism with combination index (CI) values of 0.050 and 0.183 in BxPC3 and Panc-1 lines, respectively, at IC_{50} level. The CI values at IC_{75} and IC_{90} levels were also <0.1 indicating the very strong synergism between TF and GEM combinations (Table II).

mRNA expression. We have analyzed the mRNA expression of IL-8, COX-2, STAT3 and NF-κB genes along with the G3PDH control gene by RT-PCR. No significant changes in mRNA expression of IL-8, COX-2 or COX-2 were evident. NF-κB mRNA level showed substantial reduction in GEM+TF combination as compared to individual drugs (Fig. 3).

NF-κB activity. In both Bx-PC3 and Panc-1 cell lines, GEM inhibited NF-κB activity 43% initially and remained constant during escalating doses of GEM. TF on the other hand did not indicate a dose-dependent inhibition, although 30% inhibition was noticed with TF at 0.5 µg/ml dose in BxPC3 cells. When these two agents were combined, a clear dose-dependent inhibition was noted as compared to GEM or TF alone in both cell lines. The combined treatment of 0.2 µg/ml of GEM

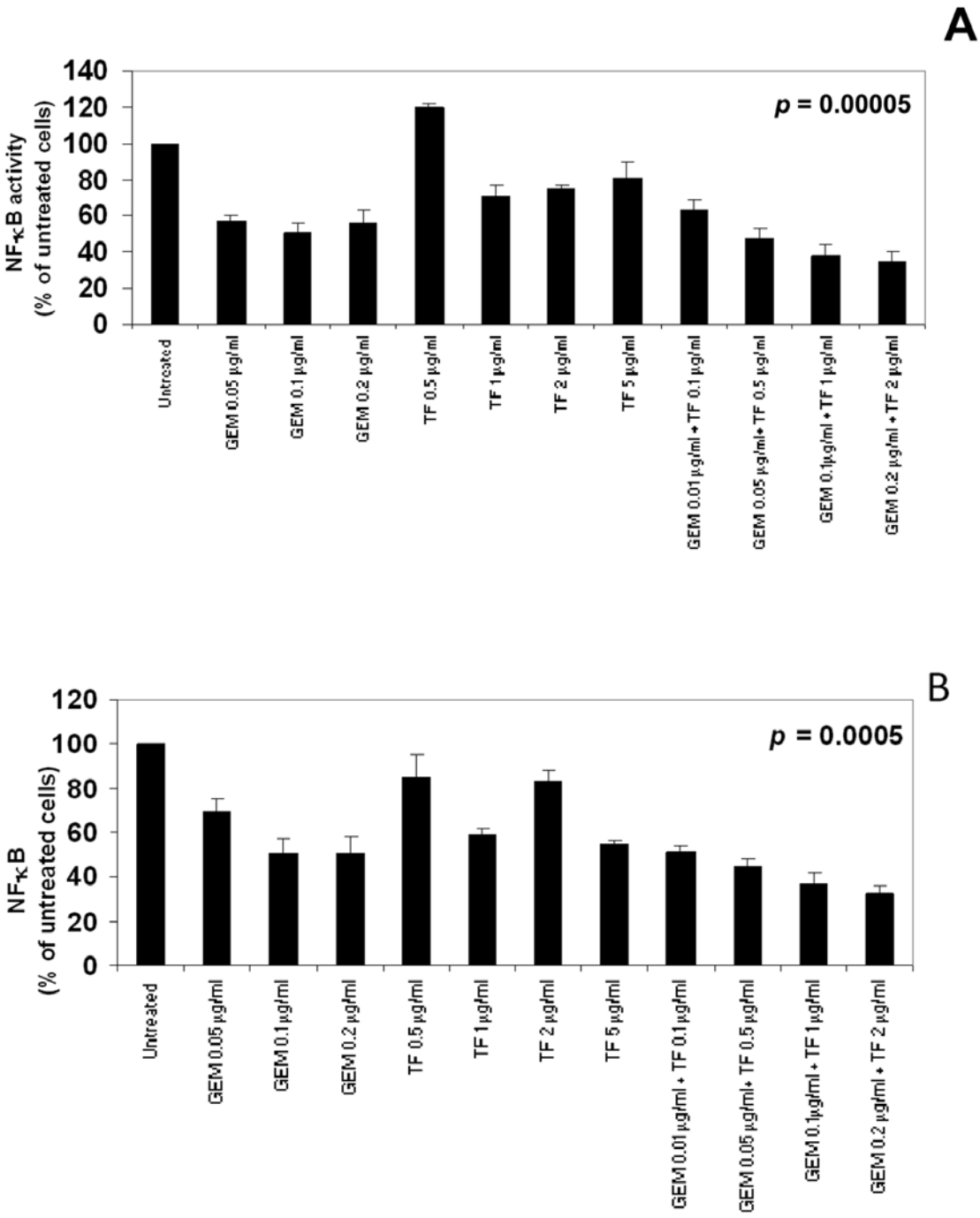

Figure 4. NF-κB activity in BxPC3 (A) and Panc-1 (B) cells treated with GEM, TF and GEM+TF analyzed using ELISA kit from Active Motif, CA. P-values calculated by single factor ANOVA.

and 2 μg/ml of TF significantly reduced the NF-κB activity to 34% of untreated BxPC3 cells (Fig. 4).

STAT3 protein expression. Both GEM and TF showed inhibitory effects on STAT3 protein expression in BxPC3 cell line (Fig. 5). A dose-dependent inhibition was clearly evident with both agents. However, when GEM and TF were combined at lower doses, the effect was significantly better than single agents ($P<0.05$).

IL-8 protein expression. The effect of GEM and/or TF on IL-8 protein expression is presented in Fig. 6. GEM, TF and their combination failed to show any significant inhibitory effect on IL-8 expression in either pancreatic cancer cell line ($P>0.05$).

Discussion

Pancreatic cancer is one of the lethal human malignancies with a high mortality rate. Even though gemcitabine either alone or with other agents is the standard treatment for advanced pancreatic cancer, the outcome has been very disappointing (17-19). Recently, a phase II clinical trial combining curcumin with gemcitabine was completed at M.D. Anderson Cancer

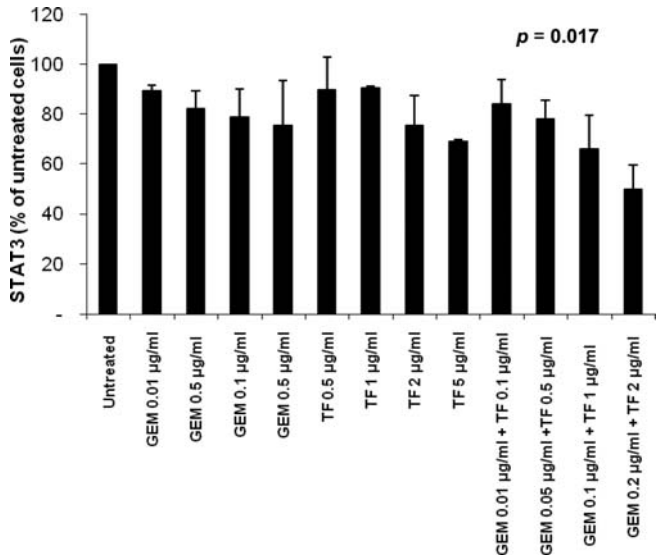

Figure 5. Inhibition of STAT3 expression in BxPC3 cells by GEM, TF and GEM+TF combination analyzed using ELISA kit. P-value calculated by single factor ANOVA.

Center, Houston, USA (16). This study showed only minimal benefit with 1 out of 21 patients showing stable disease for >18 months. Also a phase II clinical trial combining curcumin and gemcitabine on advanced pancreatic cancer is progressing at Rambam Healthcare Campus, Israel (ClinicalTrials.gov identifier: NCT00192842). However, the poor biological availability of curcumin to achieve an effective therapeutic plasma level is the major limitation for its use in cancer patients (11). Studies to date have indicated that poor absorption and rapid metabolism of curcumin severely curtail its bio-availability. Because of this limitation on the therapeutic use of curcumin, structural modifications of this agent has been attempted to increase its absorption and bioavailability (20-22). However, it is too early to predict their experimental and clinical success.

TF is a supercritical and hydroethanolic extract of turmeric that has similar anticancer properties of curcumin. TF has 45% tumerones and 11% curcuminoids, with both classes of compounds contributing to its cytotoxic effects. In the present study, we have seen that TF is cytotoxic in pancreatic cancer cell lines and TF IC_{50} values are much lower than that of curcumin (23,24). Perhaps TF may perform better than curcumin in clinical trials because of lower IC_{50} values.

The cytotoxic curve presented in Fig. 1 also indicates that TF can cause death in 96% tumor cells at 50 μg/ml dose. GEM on the other hand induced only 70 and 58% tumor cell death in BxPC3 and Panc-1 cells respectively, which necessitates combination therapy with other drugs. The combination studies indicated that GEM can be combined with TF and the combination has very strong synergistic cytotoxicity as revealed by the CalCusyn analysis. Moreover, IC_{75} and IC_{90} levels are attained with this combination unlike GEM alone. The mechanistic studies also indicated that the genes associated with carcinogenesis and inflammation (NF-κB , IL-8 and COX-2) are not inhibited at mRNA level. However, their protein levels were altered by GEM and TF either singly or in combination which may indicate that the inhibitory effect is at translational level. In conclusion, our results indicate that TF may be developed as a single agent or as an adjuvant in combination with GEM for therapeutic management of human pancreatic cancer. However, these laboratory results have to be confirmed in animal studies and clinical trials. The results of the present study also reinforce the traditional wide use of turmeric in India where we have one of the lowest incidence of pancreatic cancer compared to Western countries.

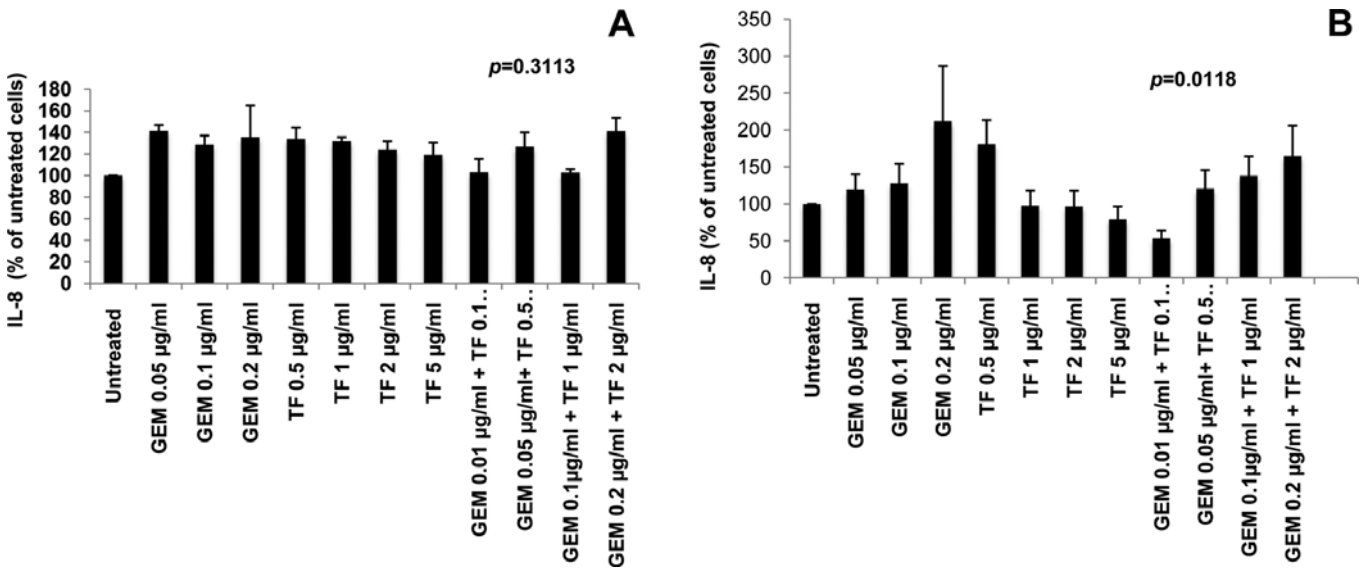

Figure 6. IL-8 expression in BxPC3 (A) and Panc-1 (B) cell lines treated with GEM, TF and GEM+TF analyzed by using ELISA kit. P-values calculated by single factor ANOVA.

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