Effects of 12-O-tetradecanoylphorbol-13-acetate in combination with gemcitabine on Panc-1 pancreatic cancer cells cultured in vitro or Panc-1 tumors grown in immunodeficient mice

XI ZHENG1,2, XIAO-XING CUI1, ZHI GAO3, MICHAEL VERANO1, MOU-TUAN HUANG1, YUE LIU1, ARNOLD B. RABSON4 and ALLAN H. CONNEY1,2,5

1Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA; 2Allan H. Conney Laboratory for Anticancer Research, College of Light Industrial and Chemical Engineering, Guangdong University of Technology, Guangzhou 510006; 3The Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, P.R. China; 4Child Health Institute of New Jersey, UMDNJ-RWJMS, New Brunswick, NJ 08901; 5Cancer Institute of New Jersey, New Brunswick, NJ 08903, USA

Received June 27, 2012; Accepted August 17, 2012

DOI: 10.3892/ijo.2012.1651

Abstract. In the present study, the effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) alone or in combination with gemcitabine on the growth of Panc-1 pancreatic cancer cells cultured in vitro or grown in NCr immunodeficient nude mice were investigated. Combinations of TPA and gemcitabine synergistically inhibited the growth and induced apoptosis in Panc-1 cells. The combination of TPA (0.16 nM) and gemcitabine (0.5 µM) induced a marked increase in phosphorylated c-Jun NH2-terminal kinase (JNK) in the Panc-1 cells. In animal experiments, NCr nude mice with established Panc-1 tumors received daily intraperitoneal (i.p.) injections of TPA (50 ng/g body weight/day) or gemcitabine (0.5 µg/g body weight/day) alone or in combination for 26 days. Treatment with daily i.p. injections of low doses of TPA or gemcitabine alone had a modest inhibitory effect on the growth of the tumors. However, the combination of low doses of TPA and gemcitabine more potently inhibited the growth of Panc-1 tumors than either agent used individually. Treatment with TPA or gemcitabine alone or in combination did not affect the body weight of the animals. Clinical trials with TPA alone or in combination with gemcitabine on patients with pancreatic cancer are warranted in order to confirm our results.

Introduction

Pancreatic cancer is one of the most lethal human malignancies (1). It was estimated that this disease caused more than 37,000 deaths in the United States in 2011 (2). Despite efforts in the past 50 years, conventional treatment approaches, such as surgery, radiation, chemotherapy, or combinations of these, have had little impact on the course of this aggressive neoplasm. Five-year survival rates remain at approximately 5% and a median survival of <6 months has remained unchanged for the last three decades (3-5). Surgical resection is still the only curative therapy, although in 80% of patients the tumor is already unresectable at diagnosis due to metastasis or local invasion (6). Even in 15 to 20% of patients undergoing potentially curative resection, the five-year survival is only 20% (6,7). Almost 100% of patients with pancreatic cancer develop metastases and succumb to the disease due to the debilitating metabolic effects of the unrestrained growth. Therefore, it is imperative to develop novel anticancer agents and effective combination therapies for the treatment of pancreatic cancer.

Gemcitabine (2,2-difluorodeoxycytidine) is a deoxycytidine-analog antimetabolite with broad activity against a variety of solid tumors and lymphoid malignancies (8). Gemcitabine is an important anticancer drug that has been approved for the treatment of non-small cell lung, pancreatic, bladder and breast cancers (9). This drug was approved as the standard of care in patients with pancreatic cancer over a decade ago (10). Treatment with gemcitabine improves clinical benefit responses, such as pain reduction, improvement in Karnofsky performance status and increase in body weight. However, the benefit of single-agent gemcitabine treatment in advanced and metastatic pancreatic cancer is inadequate. Combinations of gemcitabine and novel anticancer agents that target critical survival pathways in pancreatic cancer cells may have synergistic effects on growth inhibition and apoptosis and improve the therapeutic efficacy of gemcitabine.
The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) is a major active constituent of the seed oil of Croton tiglium L., a leafy shrub of the Euphorbiaceae family that is native to Southeastern Asia. In a preliminary study, our laboratory together with colleagues in China demonstrated pharmacological activity for intravenously administered TPA for the treatment of seriously ill myeloid leukemia patients refractory to other therapy (11). The results obtained in this study and data from a phase I trial with TPA at the Cancer Institute of New Jersey (12,13) indicated an acceptable toxicity profile. The study in the Cancer Institute of New Jersey using a different dosage than that used in China failed to find any positive effects of TPA on myeloid leukemia patients (12,13). In additional studies, we found that a low clinically achievable concentration of TPA (0.16 nM) in combination with all-trans retinoic acid (ATRA), 1α,25-dihydroxyvitamin D₃, sodium butyrate, or an NF-κB inhibitor (E)-3-[(4-methylphenyl)sulfonyl]-2-propenenitrile (BAY 11-7082) synergistically inhibited the growth and stimulated the differentiation of cultured HL-60 myeloid leukemia cells (14,15). Studies from our laboratory and from other investigators have shown that TPA inhibits growth and induces apoptosis in cultured pancreatic cancer cells (16-19). In a previous study, we also found that TPA alone or in combination with ATRA inhibited the growth of Panc-1 and BxPC-3 pancreatic tumor xenografts in immunodeficient mice (16).

In the present study, we determined the effects of TPA alone or in combination with gemcitabine on the growth and apoptosis of Panc-1 pancreatic cancer cells cultured in vitro or Panc-1 tumors grown in immunodeficient mice. We also determined the effect of these drugs alone or in combination on the activation of c-Jun NH2-terminal kinase (JNK). We found that TPA in combination with gemcitabine more potently inhibited the growth and induced apoptosis in cultured Panc-1 cells than either agent alone. We also found that the combination of TPA and gemcitabine had a stronger inhibitory effect on the growth of Panc-1 tumors in immunodeficient mice than either agent alone.

Materials and methods

Cell culture and reagents. Panc-1 cells were obtained from Dr Pamela Crowell (Indiana University-Purdue University Indianapolis, Indianapolis, IN). TPA was obtained from Alexis Co. (San Diego, CA). Gemcitabine was provided by the Eli Lilly Co. (Indianapolis, IN). Propylene glycol, polysorbate 80, benzyl alcohol, ethanol and DMSO were purchased from Sigma (St. Louis, MO). Matrigel was obtained from BD Biosciences (Bedford, MA). Dulbecco’s modified Eagle’s medium (DMEM) tissue culture medium, penicillin-streptomycin, L-glutamine and fetal bovine serum (FBS) were from Gibco (Grand Island, NY). Panc-1 cells were maintained in DMEM culture medium containing 10% FBS that was supplemented with penicillin (100 U/ml)-streptomycin (100 µg/ml) and L-glutamine (300 µg/ml). Cultured cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and were passaged twice a week. Panc-1 cells were initially seeded at a density of 0.2x10⁵ cells/ml in 35-mm tissue culture dishes (2 ml/dish) for the proliferation and apoptosis assays, and seeded at a density of 1x10⁵ cells/ml of medium in 100 mm culture dishes (10 ml/dish) for the western blot analysis. TPA and gemcitabine were dissolved in DMSO, and the final concentration of DMSO in all experiments was 0.2%.

Determination of the number of viable cells. The number of viable cells after each treatment was determined using a hemacytometer under a light microscope (Nikon Optiphot, Hyogo, Japan). Cell viability was determined by the trypan blue exclusion assay, which was carried out by mixing 80 µl of cell suspension and 20 µl of 0.4% trypan blue solution for 2 min. Blue cells were counted as dead cells and the cells that did not absorb dye were counted as live cells.

Morphological assessment of apoptotic cells. Apoptosis was determined by morphological assessment in the cells stained with propidium iodide (20,21). Briefly, cytopsin slides were prepared after each experiment and the cells were fixed with acetone/methanol (1:1) for 10 min at room temperature, followed by 10 min with propidium iodide staining (1 µg/ml in PBS) and analyzed using a fluorescence microscope (Nikon Eclipse TE200; Nikon, Tokyo, Japan). Apoptotic cells were identified by classical morphological features including nuclear condensation, cell shrinkage and the formation of apoptotic bodies (20,21). At least 200 cells were counted in each sample and the percentage of apoptotic cells was presented.

Western blot analysis. After treatment, Panc-1 cells were washed with ice-cold PBS and lysed with 800 µl of lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 µM sodium orthovandate, 2 mM iodoacetic acid, 5 mM ZnCl₂, 1 mM phenylmethylsulfonyl fluoride and 0.5% Triton X-100). The homogenates were centrifuged at 12,000 x g for 15 min at 4°C. The protein concentration of whole cell lysates was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Equal amounts (20 µg) of protein were then resolved on a 10% Criterion precast gel (Bio-Rad) and transferred onto PVDF membrane using a semi-dry transfer system. The membrane was then probed with anti-phosphorylated JNK primary antibodies (Cell Signaling Technology, Beverly, MA). After hybridization with primary antibody, the membrane was washed with Tris-buffered saline three times, then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and washed with Tris-buffered saline three times. Final detection was performed with enhanced chemiluminescent reagents. The extent of protein loading was determined by blotting for β-actin. The membrane was incubated in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl at pH 6.7) at 50°C for 30 min with occasional agitation before incubating in blocking buffer and re-probing using anti-β-actin antibody (Santa Cruz Biotechnology).

Panc-1 tumor xenografts in NCr nude mice. Female NCr nude mice (6-7 weeks old) were obtained from Taconic Farms Inc. (Germantown, NY). The animals were housed in sterile filter-capped microisolator cages and provided with sterilized food and water. For subcutaneous tumor xenografts, Panc-1 pancreatic cancer cells (2x10⁵ cells/0.1 ml/mouse) suspended in 50% Matrigel (Collaborative Research, Bedford, MA) in
DMEM medium were injected subcutaneously into the right flank of the mice (22). When the tumors reached a moderate size (0.6-1.0 cm wide and 0.6-1.0 cm long), the mice received daily intraperitoneal (i.p.) injections with TPA (50 ng/g body weight/day), gemcitabine (0.5 µg/g body weight/day) or a combination of TPA (50 ng/g/day) and gemcitabine (0.5 µg/g/day) for 26 days. Each group consisted of eight animals. In all the experiments, animals in the different experimental groups received the same amount of the vehicle (5 µl/g body weight) which consisted of propylene glycol, polysorbate 80, benzyl alcohol, ethanol and water (40:0.5:1:10:48.5) (21). Tumor size (length x width) and body weight were measured three times a week. The animal experiments were carried out under an Institutional Animal Care and Use Committee (IACUC)-approved protocol.

Statistical analyses. The potential synergistic effect of TPA and gemcitabine was assessed by the isobole method (23), using the equation \( \frac{A_c}{A_e} + \frac{B_c}{B_e} = \text{combination index (CI)} \). Ac and Bc represent the concentration of drug A and B used in the combination, and Ae and Be represent the concentration of drug A and B that produced the same magnitude of effect when administered alone. If the CI is <1, then the drugs are considered to act synergistically. If the CI is >1 or =1, then the drugs are considered to act in an antagonistic or additive manner, respectively. The analysis of variance (ANOVA) model with Tukey-Kramer adjustment (24) was used for the comparison of tumor size and body weight among the different treatment groups at the end of the treatment period.

Figure 1. Effects of TPA or gemcitabine on growth and apoptosis of Panc-1 cells. Panc-1 cells were seeded at a density of 0.2x10^5 cells/ml of medium and incubated for 24 h. The cells were then treated with TPA (0.16 to 1.6 nM) or gemcitabine (0.1 to 1.0 µM) alone for 96 h. The number of viable cells was determined by a trypan blue exclusion assay (16). Apoptotic cells were determined by morphological assessment (21). Each value is the mean ± SE from at least three experiments.

Results

Effects of TPA and gemcitabine on growth and apoptosis of Panc-1 pancreatic cancer cells. The in vitro effects of TPA and gemcitabine alone or in combination on the growth and apoptosis of pancreatic cancer cells were determined using the Panc-1 human pancreatic cancer cell line. In our experiments, Panc-1 cells were treated with TPA or gemcitabine alone or in combination for 96 h. As shown in Fig. 1, the treatment of Panc-1 cells with TPA (0.16-1.6 nM) or gemcitabine (0.1-1.0 µM) alone resulted in a concentration-dependent decrease in the number of viable cells. Combinations of TPA and gemcitabine more potently inhibited the growth of Panc-1 cells than either agent alone (Table I). The CI for IC_{50} was calculated as 0.83, indicating the synergistic effect of TPA and gemcitabine in combination in the inhibition of the growth of cultured Panc-1 cells. Treatment with TPA (0.16-1.6 nM) or gemcitabine (0.1-1.0 µM) resulted in 3-25 and 3-32% apoptosis, respectively (Fig. 1). Various combinations of TPA and gemcitabine at different concentrations all had stronger effects on the stimulation of apoptosis than either agent alone (Table I). The CI for 50% apoptosis was calculated as 0.69. Our results indicated that a combination of TPA and gemcitabine had synergistic effects on growth inhibition and apoptosis stimulation in Panc-1 cells.

Activation of JNK in Panc-1 cells treated with TPA or gemcitabine alone or in combination. The effect of TPA and
factors may also be involved. gated the effect of TPA and gemcitabine on apoptosis, other by the activation of JNK. Since SP600125 only partially abro the synergistic effect of TPA and gemcitabine on growth and apoptosis of Panc-1 cells. To the best of our knowledge, this is the first study indicating inhibition of JNK. Panc-1 cells were seeded at a density of 1x10⁶ cells/ml of medium and incubated for 24 h. The cells were then treated with TPA (0.8 nM) or gemcitabine (0.5 µM) alone or in combination for 96 h. The number of viable cells was determined by a trypan blue exclusion assay (16). Apoptotic cells were determined by morphological assessment (21). Each value is the mean ± SE from three experiments.

Table I. Effects of TPA and/or gemcitabine on the growth and apoptosis of Panc-1 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viable cells (% of control)</th>
<th>Apoptosis (% of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>TPA (0.16 nM)</td>
<td>94.5±1.9</td>
<td>3.3±0.5</td>
</tr>
<tr>
<td>Gemcitabine (0.1 µM)</td>
<td>96.7±1.2</td>
<td>3.0±1.1</td>
</tr>
<tr>
<td>TPA (0.16 nM) + gemcitabine (0.1 µM)</td>
<td>77.5±3.7</td>
<td>12.8±1.7</td>
</tr>
<tr>
<td>TPA (0.80 nM)</td>
<td>73.7±4.4</td>
<td>17.6±2.3</td>
</tr>
<tr>
<td>Gemcitabine (0.5 µM)</td>
<td>73.3±6.5</td>
<td>19.4±2.3</td>
</tr>
<tr>
<td>TPA (0.80 nM) + gemcitabine (0.5 µM)</td>
<td>31.6±3.2</td>
<td>50.7±3.3</td>
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Inhibitory effect of TPA or gemcitabine alone or in combination on the growth of Panc-1 tumor xenografts in NCr nude mice. Male NCr nude mice with subcutaneous Panc-1 tumors were treated with i.p. injections of TPA or gemcitabine alone or in combination once a day as described in Fig. 4. As shown in Fig. 4A, tumor growth was observed in the vehicle-treated control group. Treatment with i.p. injections of low concentrations of TPA or gemcitabine had a modest inhibitory effect on the growth of the tumors (Fig. 4A). The combination of low concentrations of TPA and gemcitabine more potently inhibited the growth of Panc-1 tumors than either agent used individually (Fig. 4A). Statistical analysis using ANOVA with the Tukey-Kramer multiple comparison test showed that the difference in the percentage of initial tumor size at the end of the experiment between the control group and the combination group was statistically significant (p<0.001), and that the differences between the TPA-treated group and the combination group (p<0.01) or between the gemcitabine-treated group and the combination group were also statistically significant (p<0.05). As shown in Fig. 4B, treatment with TPA or gemcitabine alone or in combination did not affect the body weight of the animals. Statistical analysis using ANOVA with the Tukey-Kramer multiple comparison test showed that the difference in the percentage of initial body weight between any two groups was not statistically significant (p>0.05).

Discussion

In the present study, we demonstrate that TPA in combination with gemcitabine synergistically inhibits the growth and stimulates apoptosis in cultured Panc-1 human pancreatic cancer cells. We also show that a combination of low concentrations of TPA and gemcitabine more potently inhibits the growth of Panc-1 tumors than either agent used individually. To the best of our knowledge, this is the first study indicating the synergistic effect of TPA and gemcitabine on growth and apoptosis in human pancreatic cancer cells.

TPA is an irritant and inflammatory agent that has been used in previous studies as a tumor promoter (usual dose = 5-16 nmol twice a week) on the skin of mice previously initiated with 7,12-dimethylbenz[a]anthracene or other polycyclic aromatic
In contrast to the irritant effects of TPA at high concentrations on mouse skin, at a 10,000-fold lower concentration TPA is a potent stimulator of differentiation of cultured myeloid leukemia cells (28-30). Previous studies from our laboratory have demonstrated that TPA at clinically achievable concentrations inhibits the growth of cultured human prostate and pancreatic cancer cells (16,21). The peak blood concentrations of TPA ± SD in several patients who received an intravenous infusion of TPA (0.125 mg/m²) was 1.75 ± 0.55 ng/ml and ranged between 0.3 and 5.2 ng/ml (13,31). The concentrations of TPA used to obtain a synergistic effect in the present study (0.1-1 ng/ml; 0.16-1.6 nM) are clinically achievable (13,31).

Gemcitabine is the standard first-line chemotherapeutic agent for the treatment of advanced pancreatic cancer. The majority of pancreatic cancer patients treated with gemcitabine do not have an objective response to treatment and only a minority obtain stabilization of disease or a partial response (9,32). Due to the inadequate benefit and significant toxic side-effects of gemcitabine as a single-agent (9,32), efforts have been devoted to developing effective combinations of gemcitabine with other anticancer agents (33). However, the combination of other chemotherapeutic agents with gemcitabine has not resulted in a meaningful improvement in survival (34-38). Combinations of gemcitabine and novel anticancer agents that target critical survival pathways in pancreatic cancer cells may have synergistic effects on growth inhibition and apoptosis. Our previous study demonstrated that a combination of TPA and ATRA (16) enhanced the anticancer activity of TPA. The present study demonstrates that low concentrations of gemcitabine in combination with TPA synergistically inhibit the growth and increase apoptosis in Panc-1 pancreatic cancer cells. These results indicate that the anticancer activity of gemcitabine may be increased while the toxic side-effects of gemcitabine may be reduced when a low dose is used in combination with TPA.

The mechanisms for the synergistic effect of TPA and gemcitabine on pancreatic cancer Panc-1 cells are not yet clear.
TPA is known to activate protein kinase C (PKC) leading to the activation of JNK and apoptosis in several experimental systems (25,39-42). In the present study, we found that TPA strongly activated JNK in Panc-1 cells, and that the addition of a selective inhibitor of JNK partially abrogated the effect of TPA on growth inhibition and apoptosis stimulation. These results suggest that JNK activation is required for TPA-induced growth inhibition and apoptosis stimulation in cultured Panc-1 cells. The main action of gemcitabine is thought to be the competitive incorporation of gemcitabine diphosphate and triphosphate into DNA, after which DNA polymerase is able to add only one more nucleotide before DNA fragmentation and cell death occurs (43,44). This so-called ‘masked chain termination’ prevents exo-nuclease recognition and the excision of gemcitabine. Gemcitabine induces an S-phase arrest (45,46) and stimulates apoptosis in cancer cells (47,48). The activation of JNK is required for gemcitabine-induced apoptosis in human lung cancer cells (49). Gemcitabine alone or in combination with other agents has been shown to induce the activation of JNK in pancreatic cancer cells (50,51). In the present study, we found that the treatment of Panc-1 cells with a low dose of gemcitabine resulted in a small increase in the level of phosphorylated-JNK. A combination of low doses of TPA and gemcitabine further increased the level of phosphorylated-JNK in the cells. Moreover, we found that the addition of a selective JNK inhibitor partially abrogated the stimulatory effect of TPA in combination with gemcitabine on apoptosis in Panc-1 cells. These results suggest that apoptosis in Panc-1 cells induced by TPA in combination with gemcitabine is at least partially mediated by the activation of JNK.

In conclusion, in the present study, we show that TPA in combination with gemcitabine synergistically inhibits growth and induces apoptosis in Panc-1 human pancreatic cancer cells. The concentrations of TPA and gemcitabine required for these effects are clinically achievable. In addition, we found that the treatment of NCr nude mice with a combination of TPA and gemcitabine inhibited the growth of Panc-1 tumor xenografts. Clinical studies with TPA in combination with gemcitabine on patients with pancreatic cancer are warranted in order to confirm our results.

Acknowledgements

The present study was supported by departmental funds from the Department of Chemical Biology in the Ernest Mario School of Pharmacy at Rutgers University. The authors thank Ms. Annette Dionisio for her excellent assistance in the preparation of this manuscript.

References


