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Preclinical Studies of Apogossypolone, a Novel Pan Inhibitor of Bcl-2 and Mcl-1, Synergistically Potentiates Cytotoxic Effect of Gemcitabine in Pancreatic Cancer Cells

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Abstract

Objective—Overexpression of antiapoptotic Bcl-2 family proteins confers resistance to conventional therapy in pancreatic cancer patients. Apogossypolone (ApoG2) is an analogue of (–)-gossypol, exhibiting binding activity with K_i values of 35 nmol/L for Bcl-2 and 25 nmol/L for Mcl-1. The present study was designed to test our hypothesis whether inactivation of Bcl-2 family of proteins using ApoG2 could sensitize pancreatic cancer cells to the cytotoxic effect of gemcitabine.

Methods—Two pancreatic cancer cell lines were treated with ApoG2, gemcitabine, and their combination; cytotoxicity and apoptosis was confirmed by MTT and histone/DNA enzyme-linked immunosorbent assay. Coimmunoprecipitation experiments were performed to elucidate the mechanism of action of ApoG2. In vivo efficacy of ApoG2 was evaluated in a xenograft model to confirm its therapeutic benefit with gemcitabine.

Results—When ApoG2 was combined with gemcitabine, increased cytotoxicity and apoptosis was evident. Coimmunoprecipitation experiment revealed that ApoG2 blocks the heterodimerization of Mcl-1/Bax and Bcl-2/Bim in cells. Furthermore, administration of ApoG2 with gemcitabine resulted in a statistically higher antitumor activity compared with either ApoG2 or gemcitabine alone in a severe combined immunodeficiency mouse xenograft model.

Conclusions—Apogossypolone, which functions as a potent pan-Bcl-2 family inhibitor, seems therapeutically promising for future translational studies including the treatment of pancreatic cancer.

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Dajun Yang has competing interests with Ascenta Therapeutics Inc as senior vice president of research, shareholder, and cofounder. Ascenta Therapeutics Inc has licensed the technology related to apogossypolone from the University of Michigan.

Keywords

pancreatic cancer; nonpeptidic small-molecule inhibitor of Bcl-2 and Bcl-X_L; apogossypolone; chemosensitization

Pancreatic adenocarcinoma is one of the most lethal cancers in the world. For all stages combined, the 1-year survival rate is only 20%, and the 5-year survival rate is less than 5%. It is estimated that 42,000 new patients would have a diagnosis of pancreatic cancer and 33,000 deaths are expected for 2009 in the United States.¹ Its etiology is largely unknown, and pancreatic resection offers the potential for long-term cure in a select group of minority patient population. According to the American Cancer Society guidelines, the standard treatment options for locally advanced pancreatic cancer are chemotherapy with gemcitabine or its combination with radiation therapy and chemotherapy with gemcitabine alone. However, treatments now available are largely unsatisfactory and relatively ineffective for improving survival. Several vital survival molecules such as epidermal growth factor receptor (EGFR), survivin, nuclear factor- κ B, Bcl-2, Bcl-X_L, and Mcl-1 are highly expressed, and mutations in oncogenes such as K-*ras*, p53, p16, and DPC4 in this neoplasia confer de novo chemoresistance,²⁻⁴ suggesting that discovery of a novel therapeutic agent is urgently needed.

The Bcl-2 family members are important proteins that regulate programmed cell death (apoptosis) in mammalian cell lines. They include both death antagonists such as Bcl-2, Mcl-1, and Bcl-X_L and death agonists such as Bax, Bak, Bid, and Bad.⁵ The delicate balance between the amount of proapoptotic and antiapoptotic proteins dictates whether the cell will survive or undergo apoptotic death after an apoptotic stimulus such as chemotherapy.⁶ Clinical data showed that patients who have high expression of Bcl-2 and Bcl-X_L have a poor prognosis, whereas overexpression of the proapoptotic molecule Bax has a better prognosis.⁷⁻¹¹ Overexpression of Bcl-2 protein is also reportedly associated with increased metastatic potential in pancreatic cancer.¹² Mcl-1 is another antiapoptotic protein, which is overexpressed in 86% of pancreatic cancer.¹³ Therefore, Bcl-2, Mcl-1, and Bcl-X_L represent important molecular targets for the development of novel therapeutic strategies for pancreatic cancer.

Most Bcl-2 family proteins have conserved molecular structures, and Bcl-2, Bcl-X_L, and Mcl-1 can heterodimerize with the proapoptotic members Bad, Bak, Bid, and Bax in the BH3 domain. Hence, it is hypothesized that overexpression of antiapoptotic proteins will abrogate apoptotic stimuli by inhibiting the oligomerization of Bax protein and further inhibit the release of cytochrome *c* and caspase activation.^{14,15} By formulating a strong-affinity small-molecule inhibitor (SMI) of the BH3 domain, one can release the proapoptotic proteins and remove the stimuli for the antiapoptotic pathway. Results from several studies have demonstrated that it is possible to discover and design potent, nonpeptidic, SMIs that bind to the BH3 binding groove.¹⁶⁻¹⁸ Using multidimensional nuclear magnetic resonance methods, we have recently discovered an analogue of (–)-gossypol, Apogossypolone (ApoG2), which has conclusively shown to bind at the BH3 binding groove of Bcl-2 and Bcl-X_L.¹⁹

Because one major obstacle in the treatment of pancreatic cancer is loss of sensitivity to cell death, we speculate that a novel multiagent therapeutic approach relative to monotherapy will improve the cure rate of pancreatic cancer in patients and is warranted. In this context, we conceptualized that one possible hypothetical approach could be to lower the threshold of pancreatic cancer cells to apoptosis by using relatively nontoxic SMIs of Bcl2 family proteins that would be therapeutically important. Hence, this new compound, ApoG2, could

inhibit the antiapoptotic function of Bcl-2, Mcl-1, and Bcl-X_L and induce apoptosis in pancreatic cancer cells having overexpression of Bcl-2, Mcl-1, and Bcl-X_L. We report in this study a combination approach to test our hypothesis whether inhibition of Bcl-2, Mcl-1, and Bcl-X_L by ApoG2 could result in potentiation of cytotoxic effect of gemcitabine against pancreatic cancer cells in vitro and in vivo.

MATERIALS AND METHODS

Cell Culture and Reagents

The human pancreatic carcinoma cell lines BxPC-3, Panc-1, and Colo 357 were obtained from American Type Culture Collection (Manassas, Va). The cell lines were maintained in continuous exponential growth by twice-a-week passaging in Dulbecco modified Eagle medium (Life Technologies, Inc, Gaithersburg, Md) supplemented with 10% fetal bovine serum, 100-U/mL penicillin, and 10-mg/mL streptomycin. Apogossypolone was synthesized by modifying (–)-gossypol's 2 aldehyde groups and obtained from Dr Shaoming Wang, University of Michigan, Ann Arbor. Apogossypolone was dissolved in dimethyl sulfoxide at 1 mmol/L, diluted to the desired concentration, and used as a fresh sample. Gemcitabine, produced by Eli Lilly (Indianapolis, Ind), was obtained from our institute pharmacy, and fresh stock solution was used for each experiment. Antibodies were obtained from the following commercial sources: caspase-3, cytochrome *c*, and cytochrome *c* oxidase were purchased from Cell Signaling Technology (Beverly, Mass); anti-Bcl-X_L, Bcl-2, Bax, Bim, Bid, and Mcl-1 antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, Calif); and anti-polyadenosine diphosphate-ribose polymerase (PARP) antibody was from Biomol Research (Plymouth, Pa). Anti- α -actin antibody was from Sigma Chemical Co (St Louis, Mo).

Protein Extraction and Western Blot Analysis

Whole cell protein extracts were prepared by suspending the cells in radio immuno precipitation assay (RIPA) buffer and subjected to routine Western blot analysis as described earlier.²⁰ For cytochrome *c* release, after termination of the incubation period, cells were collected, washed with ice-cold phosphate buffered saline, lysed, and processed to obtain cytosolic and mitochondrial fraction for cytochrome *c* immunoblotting as described earlier.²⁰

Cell Viability Assay and Determination of Combination Index

Panc-1, BxPC-3, and Colo 357 cells were seeded at a density of 2 to 3 $\times 10^3$ cells per well in 96-well microtiter culture plates. After overnight incubation, fresh medium containing different concentrations of ApoG2 (0–10 μ mol/L) diluted from a 10-mmol/L stock was added to each well and incubated for 72 hours and then subjected to MTT assay and plotted as percent viable cells relative to the control in the absence of ApoG2. For determination of cell viability inhibition by gemcitabine, after initial attachment of the cells, the culture medium was replaced with fresh medium containing 5- μ mol/L ApoG2 and 50-nmol/L gemcitabine and exposed for an additional 72 hours. The effect of combination treatment on cell viability was examined by MTT and plotted as previously described. Combination index (CI) and isobologram for the combination treatment were calculated and plotted using the CalcuSyn software (Biosoft, Ferguson, Mo).

Histone/DNA Enzyme-Linked Immunosorbent Assay for Detecting Apoptosis

A cell apoptosis enzyme-linked immunosorbent assay (ELISA) detection kit from Roche (Palo Alto, Calif) was used to detect apoptosis in BxPC-3 and Colo 357 cells treated with ApoG2 and gemcitabine either singly or in combination according to the manufacturer's

protocol. The spectrophotometric absorbance of the samples was determined at 405 nm using ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC). The experiment was repeated twice with identical results.

Caspase-3 and -9 Activity Assay

Caspase-3 and -9 activities were measured in whole-cell lysates prepared using a commercially available assay kit (RandD Assay System, Minneapolis, Minn) according to the manufacturer's instruction.

Analysis of Cytochrome c Release

Cells were plated at a density of 1×10^6 cells in 100-mm dish and allowed to attach overnight. Apogossypolone was added directly to freshly replaced medium from stock at indicated concentrations. After termination of the incubation period, cells were collected and washed once with ice-cold phosphate buffered saline and lysed using Dounce homogenizer in 40 μ L of ice-cold lysis buffer (250-mmol/L sucrose, 1-mmol/L EDTA, 20-mmol/L Tris-HCl [pH 7.2], 1-mmol/L dithiothreitol, 10-mmol/L KCl, 1.5-mmol/L $MgCl_2$, and protease inhibitors [Complete Cocktail; Roche Molecular Biochemicals, Indianapolis, Ind]). Lysate was centrifuged at 500g for 5 minutes at 4°C. The supernatant containing mitochondria was collected and centrifuged again at 10,000g for 10 minutes to obtain the cytosolic extract and the pellet (fraction containing mitochondria). The pellet was resuspended in 50 μ L of homogenizing buffer and used for detecting mitochondrial cytochrome c (Cell Signaling Technology).

Clonogenic Assay

To test the survival of cells treated with ApoG2, gemcitabine, or the combination, BxPC-3 and Colo 357 cells were plated in a 6-well plate (50,000 cells per well) and incubated overnight at 37°C. After 72-hour exposure, the cells were trypsinized, and the viable cells were counted (trypan blue exclusion) and plated to determine the effects of treatment on clonogenic survival. The cells were then incubated for 10 to 12 days at 37°C in an incubator. The colonies were stained with 2% crystal violet and counted and expressed relative to the control.

Experimental Animals

Female ICR–severe combined immunodeficiency (SCID) mice were purchased from Taconic Farms (Germantown, NY). The mice were housed and maintained under sterile conditions and were used in accordance with Animal Care and Use Guidelines of Wayne State University. The mice received Lab Diet 5021 (Purina Mills, Inc, Richmond, Ind). BxPC-3 xenografts were developed as described previously.²¹ Each mouse received 10^7 BxPC-3 cells (in serum-free RPMI-1640) subcutaneously in each flank area. After formation of subcutaneous tumors, serial propagation was accomplished by excising the tumors, trimming extraneous material, and cutting the tumors into fragments of 20 to 30 mg, which were transplanted subcutaneously using a 12-gauge trocar needle into the flanks of a new group of mice.

Maximum Tolerated Dose and Efficacy Trial Design for ApoG2, Gemcitabine, and Their Combination

A dose-range-finding study of 3 dose levels (20, 40, and 120 mg/kg) of ApoG2 plus a vehicle-only control given intravenously (IV) daily for 5 consecutive days in non-tumor bearing animals was conducted. Animal survival was monitored for 3 weeks. The maximum tolerated dose (MTD) is defined as the dose that will lead to no deaths of any of the animals and not more than 10% loss of body weight during treatment, followed by weight gain.

Animal groups were ear-tagged and observed for immediate toxicity (first 30–60 min), then twice daily for the first 3 days (acute toxicity), and then daily for 2 weeks. The animals were weighed daily and monitored for activity, skin changes indicating dehydration (secondary to diarrhea), and any other physical or behavioral abnormalities.

For the subsequent drug efficacy trials, small fragments of the BxPC-3 xenograft were implanted subcutaneously and bilaterally into naive, similarly adapted mice, as previously described. The mice were checked 3 times per week for tumor development. Once transplanted, the BxPC-3 fragments developed into palpable tumors (60–100 mg); groups of 5 animals were removed randomly and assigned to different treatment groups. Using this model, the efficacy of ApoG2 (120 mg/kg of body weight, IV, $\times 5$ every day)/gemcitabine (75 mg/kg of body weight, IV, $\times 3$ every other day [QOD]) and their combination was studied. The mice were observed for measurement of subcutaneous tumors, changes in body weight, and adverse effects of the drugs. Subcutaneous tumors were measured 3 times per week.

Assessment of Tumor Response

The end points for assessing antitumor activity were according to standard procedures used in the National Cancer Institute and adopted in our laboratory. Tumor weight (milligram) = $(A \times B^2)/2$, where A and B are the tumor length and width (in millimeter), respectively. Tumor growth inhibition (T/C) is defined as the median tumor weight in the treated group (T) divided by the median tumor weight in the control group (C) at a time when the median tumor weight in the control group reached approximately 1200 mg. Tumor growth delay ($T-C$) was defined as the difference between the median time (in days) required for the treatment group tumor to reach 1200 mg and the median time (in days) for the control group tumor to reach the same weight. The total $\log_{10}$ tumor cell kill = $(T-C)/(3.32)(T_d)$. T_d was defined as the time in days required for the tumor to double its weight during the exponential growth phase.

Statistical Analysis

For in vitro studies, the experiments were done in quadruplicate for each of the observations. Each of the data represents the mean $\pm$ SD from 2 to 3 separate experiments. To analyze the statistical significance of the differences found in the data, the Student t test was performed. Statistical significance was assumed at $P < 0.05$.

For the comparison of tumor weight, the power to detect differences in the mean tumor weight at the completion of treatment between the treatment and control groups was calculated based on a sample of 5 mice containing a total of 10 tumors per group. Power calculations assume that the use of a 2-sided, 2-sample t test with equal variance and assume the difference between mean values to be a proportion of the SD of the outcome measurement.

RESULTS

Chemical Structure, Baseline Expression of Bcl-2 Family Proteins, and the Effect of ApoG2 on the Growth of Pancreatic Cancer Cell Lines

The chemical structure of ApoG2, an analogue of (–)-gossypol, is presented in Figure 1A. The baseline protein expression of 3 pancreatic cancer cell lines (Panc-1, BxPC-3, and Colo 357) shows a differential degree of expression for antiapoptotic and proapoptotic protein molecules Mcl-1, Bcl- X_L , Bcl-2, Bax, and Bim (Fig. 1B). As shown in Figure 1C, ApoG2 inhibited the percentage of metabolically viable pancreatic cancer cells BxPC-3 and Colo 357 in a concentration-dependent manner and showed significant loss of viable cells at 1,

2.5, and 5 $\mu\text{mol/L}$ after 72 hours ($P < 0.05$). Interestingly, both BxPC-3 and Colo 357 cells exhibited greater than 80% loss of viability when incubated with 10- $\mu\text{mol/L}$ ApoG2 for 72 hours. In contrast, Panc-1 cells lacking Bcl-2 were comparatively more stringent to loss of cell viability when exposed to identical concentrations of ApoG2 for a similar period. The median inhibition concentrations for BxPC-3 and Colo 357 were 5 and 6 $\mu\text{mol/L}$, respectively. Subsequent studies were undertaken to examine whether ApoG2 synergizes the cytotoxic effect of gemcitabine as concluded from cell viability assay and to confirm whether loss of cell viability results as a consequence of apoptosis induced by ApoG2 treatment.

Increased Loss of Cell Viability and Apoptosis Inducing Effect of ApoG2-and-Gemcitabine Combination in BxPC-3 and Colo 357 Cell Lines

The effects of ApoG2, gemcitabine, and their combination on cell viability and apoptosis in the ApoG2 sensitive cells was evaluated using MTT and histone/DNA ELISA apoptosis detection kit (Figs. 2A–C). As shown in Figures 2A and C, exposure of these cell lines to ApoG2 (5 $\mu\text{mol/L}$), gemcitabine (50 nmol/L), and their combination resulted in a significant loss of viable cells ($P < 0.01$) in a synergistic manner through apoptosis induction. Furthermore, to confirm synergism, we determined the CI values for the combination treatment groups. Combination index is a quantitative measure of the degree of drug interaction. A CI value less than 1 indicated synergism; greater than 1, antagonism; and 1, additive effect. Our results clearly demonstrate that ApoG2 is a potent agent in inducing apoptotic cell death and that it showed synergistic loss of cell viability when combined with gemcitabine (CI, 0.854 and 0.698; Fig. 2B).

Caspase-3 Activity, PARP, and Clonogenic Survival Effect of ApoG2-and-Gemcitabine Combination

Caspases are important mediators of apoptosis induced by various agents. They are sequentially activated by proteolytic processing of their inactive procaspase forms, which in turn leads to cleavage of key cellular proteins including the DNA repair enzyme PARP. To validate that ApoG2 induces apoptosis in pancreatic cancer cells, we assessed the activation of caspase-3 spectrophotometrically and the status of PARP in ApoG2-and-gemcitabine-treated BxPC-3 and Colo 357 cells by Western immunoblotting (Figs. 3A, B). Our observations confirmed and attested the results obtained with MTT and ELISA for apoptosis by overall increased caspase-3 activity (Fig. 3A; $P < 0.01$) and by appearance of strong PARP cleavage band in the combination group. This was further substantiated by the results of clonogenic assay showing significantly reduced numbers of colonies formation in the combination treatment group (Fig. 3C).

Cytochrome *c* Release and the Activation of Apoptosis by ApoG2

In response to an apoptotic stimulus, cytochrome *c* release from the mitochondria is the first in the sequence of events leading to the activation of caspase cascade, triggering apoptosis. Therefore, we examined cytochrome *c*-related events from the mitochondria of the pancreatic cancer cells after incubation with ApoG2 for 72 hours. As depicted in Figure 4A, ApoG2 significantly reduced in a dose-dependent manner the mitochondrial cytochrome *c* and conversely increased the cytosolic level of cytochrome *c* in BxPC-3 and Colo 357 cells, suggesting its release from the mitochondria. These results suggest that ApoG2 initiates apoptosis via cytochrome *c* release from the mitochondria, subsequently leading to the caspase-dependent activation of events leading to initiation of apoptosis. We therefore evaluated the activation of caspase-3 and PARP in ApoG2- treated BxPC-3 and Colo 357 cells by Western immunoblotting and the activity of caspases 3 and 9 in BxPC-3 cells exposed to ApoG2 (0–5 $\mu\text{mol/L}$) for 72 hours. As shown in Figure 4B, a 4-fold ($P < 0.01$) increase in caspase-3 and -9 activation, after 72 hours of incubation with ApoG2, was

noticed. These findings were further confirmed by Western immunoblotting in both cell lines, wherein the expression of cleaved caspase-3 and PARP by ApoG2 treatment for 72 hours was noticeable (Fig. 4A), attesting caspase-dependent activation of apoptosis.

Inhibition of the Heterodimerization of Bax With Mcl-1

In addition to nuclear magnetic resonance study, our in vitro fluorescence polarization-based binding assay showed that ApoG2 binds potently¹⁹ to Mcl-1 with a K_i value of 25 nmol/L. On the basis of this observation, we hypothesized that ApoG2 might trigger apoptosis of BxPC-3 cells through inhibiting the heterodimerization of Mcl-1 with proapoptotic proteins such as Bax. Thus, to confirm our hypothesis, BxPC-3 cells were treated with varying concentrations (0–20 μ mol/L) of ApoG2 for 24 hours, and the total proteins from cell extracts were immunoprecipitated with Bax antibody and then probed with anti-Mcl-1 and Bcl-2 antibodies. As shown in Figure 5A, ApoG2 treatment of BxPC-3 at 20 μ mol/L clearly inhibited the binding between the antiapoptotic protein Mcl-1 and the pro-apoptotic protein Bax. The specificity was confirmed by using nonrelevant antibody (data not shown). There was no change in the total amount of Mcl-1 protein as detected by Western blot (data not shown). The previously described observation proves that ApoG2 inhibits the heterodimerization of Mcl-1 with at least 1 proapoptotic molecule, Bax, in BxPC-3 pancreatic cancer cells.

Inhibition of the Heterodimerization of Bcl-2 With Bim

We hypothesized that ApoG2 could also disrupt the heterodimerization of the proapoptotic Bim protein and antiapoptotic Bcl-2 protein. Indeed, as shown in Figure 5B, immunoprecipitation with Bcl-2 antibody and then probing with anti-Bim antibody showed a decrease in Bim expression after 24 hours. The specificity of binding was confirmed by using nonrelevant antibody (data not shown). These observations support the notion that ApoG2 can enter intact pancreatic cancer cells and inhibit the interaction between antiapoptotic and proapoptotic proteins of the Bcl-2 family.

Maximum Tolerated Dose of ApoG2 and Its Efficacy, Alone and in Combination With Gemcitabine, Against BxPC-3 Xenograft Model

In our hands, the MTD of ApoG2 was not achieved because animals tolerated ApoG2 up to 800-mg/kg intravenous bolus, and we did not test beyond 800 mg/kg because of the limited ApoG2 quantity and other logistical issues. Previously, we have determined the MTD of gemcitabine to be 75 mg/kg, IV, $\times 3$ QOD. Because ApoG2 was nontoxic to animals, we did not reduce the MTD for gemcitabine. Animals at this dose of the combination treatment experienced less than 5% weight loss relative to the control and had scruffy fur; however, these animals fully recovered within 48 to 72 hours after completion of treatment. All animals tolerated the combination treatment very well.

For the subsequent efficacy trial, ApoG2 at 120 mg/kg of body weight injected IV ($\times 5$ every day), gemcitabine at 75 mg/kg of body weight injected IV ($\times 3$ QOD), and their combination were used. Animals received daily injections of 120 mg/kg of body weight of ApoG2 for 5 days. Gemcitabine was injected QOD for 3 days.

Although ApoG2 had minimal efficacy as a single agent, differences in the tumor progression from day 14 onward after tumor transplantation became evident. The combination arm showed the most dramatic tumor response with a significant delay in the progression of pancreatic cancer in our xenograft model (Figs. 6B, C). When testing the efficacy of ApoG2, gemcitabine, and their combination in the dose and schedule used in this study, gemcitabine showed significant antitumor activity against BxPC3. A TC value of 42% or less is considered significant antitumor activity by the Drug Evaluation Branch of

the Division of Cancer Treatment, National Cancer Institute. Gemcitabine showed borderline activity ($TC = 40\%$) compared with ApoG2 ($TC = 80\%$). However, when ApoG2 was given in combination with gemcitabine, it showed very high activity ($TC = 24\%$) and delayed tumor growth by 10 days with 1.00 $\log_{10}$ kill (Table 1).

DISCUSSION

Pancreatic cancer is a lethal disease with only less than 5% of patients surviving for 5 years.¹ Although attempts to improve the cure rate using prognostic combinations of a different class of chemotherapeutic regimens have been made, progress to date has been limited because of suboptimal therapeutic benefit. Targeted therapies including inactivation of the EGFR signaling pathway by the EGFR-related tyrosine kinase inhibitor erlotinib showed only a modest survival benefit in a large phase 3 clinical trial when combined with gemcitabine.²² We conceptualized targeting tumor cells with agents that can augment the apoptotic machinery of tumor cells, rendering them less chemoresistant as a promising alternative option toward pancreatic cancer therapy. Lately, such innovative approach with a compound ABT 737 showed a striking response in solid tumors.²³ Emerging evidence indicate that high expression of antiapoptotic proteins such as Mcl-1, Bcl-2, and Bcl-X_L may play a critical role in the progression of pancreatic cancer and augments resistance to a wide spectrum of chemotherapeutic agents.^{11,24} Hence, Mcl-1, Bcl-2, and Bcl-X_L have turned out to be attractive targets for the design of new anticancer drugs.^{25,26}

Apogossypolone, an analogue of (–)-gossypol, is a nonpeptidic SMI that is capable of antagonizing the function of pan-Bcl-2 family (Mcl-1, Bcl-2, and Bcl-X_L) and thus may have a therapeutic potential as an entirely new class of anti-pancreatic cancer agents. Recent laboratory research investigations reported by Zhang et al²⁷ indicate that ApoG2 was significantly more tolerable than (–)-gossypol in rats and mice, with an MTD dose at more than 2000 mg/kg in multidosing schedule. Furthermore, comparative evaluation on the efficacy of cisplatin and ApoG2 in inhibiting gastric cancer xenograft showed that ApoG2 alone results in modest tumor growth inhibition; in contrast, cisplatin given once a week for 3 weeks despite being more active was associated with significant weight loss.²⁷ Unfortunately, targeting only 1 pathway seems not to be sufficiently promising in achieving significant progress against pancreatic cancer. Because gemcitabine is the first-line conventional chemotherapy for pancreatic cancer, we hypothesized that ApoG2 in combination with gemcitabine might be more promising in pancreatic cancer therapy.

In this study, we used the 2 human pancreatic cancer cell lines BxPC-3 and Colo 357. Both cell lines have high Mcl-1 and Bcl-X_L but low Bcl-2 expressions (Colo 357). Apogossypolone has a K_i value of approximately 35 nmol/L to Bcl-2, 660 nmol/L to Bcl-X_L, and 25 nmol/L to Mcl-1 as deduced from a cell-free assay system.^{19,20} Studies have revealed that ApoG2 binds directly to the groove in Bcl-2 family proteins and blocks their subsequent heterodimerization with a proapoptotic member, which results in the loss of function and induction of apoptosis. On the basis of our findings, 5- μ mol/L ApoG2 was capable of inducing significant growth inhibition and apoptosis in both BxPC-3 and Colo 357 cells as detected by MTT assay and histone/DNA ELISA, respectively. These results reiterate that blocking the biological function of antiapoptotic proteins is sufficient to trigger apoptosis in pancreatic cancer cells overexpressing these molecules. Nevertheless, because ApoG2 is targeting only the Bcl-2 family protein, we currently presume that this may not be sufficiently effective in inhibiting 100% of the apoptotic pathway. Nonetheless, our results are consistent with studies showing that the parent compound (–)-gossypol, which blocks Bcl-2 and Bcl-X_L, induces apoptotic cell death in lymphoma and pancreatic cancer.^{28,29} This also partly explains that cell lines lacking Bcl-2 (Panc-1 cells) but instead containing

another important antiapoptotic molecule, Bcl-X_L, succumb to the effect of ApoG2 in inducing loss of viable cells and induction of apoptosis.

To further elucidate the mechanism of action, we carried out a coimmunoprecipitation experiment to determine the effect of ApoG2 on disrupting the heterodimerization of antiapoptotic proteins Mcl-1 and Bcl-2 with proapoptotic proteins Bax and Bim using BxPC-3 pancreatic cancer cells as our investigative model. We found that exposure of BxPC-3 cells with ApoG2 (0, 5, 10, and 20 μ mol/L) resulted in a decreased binding of Mcl-1 and Bcl-2 with proapoptotic proteins Bax and Bim, without appreciable alterations in the total amount of Bcl-2, or actin proteins in the same cellular lysates, indicating that ApoG2 treatment does not affect the levels of proapoptotic and antiapoptotic proteins. Previously, we had similar results where the parent compound, (–)-gossypol, did not affect the levels of Bcl-2, Bcl-X_L, Bax, or Bak.²⁹ These results support the notion that ApoG2 could enter intact BxPC-3 pancreatic cancer cells with the ability to inhibit the interaction between proapoptotic and antiapoptotic proteins. This study strongly suggests that one potential mechanism by which ApoG2 induces apoptosis is, at least, partially mediated through the inhibition of Mcl-1/Bax and Bcl-2/Bim heterodimerization. These findings also draw parallel with current concepts of mitochondria-mediated apoptosis related to the Bcl-2 family of proteins.^{30–32} According to Adams and Cory,³³ SMIs work in cells with Bak⁺Bax⁺ and do not induce apoptosis in Bak[–]Bax[–] cells.

Gemcitabine is the only chemotherapeutic agent that has shown survival benefit in treating pancreatic cancer patients. Hence, any agent that synergistically potentiates therapeutic advantage over that of gemcitabine might prove beneficial in pancreatic cancer therapy. Our in vitro study implies that ApoG2 in combination with gemcitabine caused a superior effect relative to monotherapy in causing cytotoxicity and apoptosis in BxPC-3 and Colo 357 cell lines. However, the main challenge in developing a novel therapeutic agent needs to demonstrate efficacy using a human pancreatic cancer xenograft model. In our BxPC-3–SCID xenograft model, the combination regimen of ApoG2 and gemcitabine produced a statistically significant ($P < 0.05$) delay in tumor progression. Hence, these results suggest that ApoG2 represents a promising new agent that should be developed for the treatment of pancreatic cancer. Our results also indicate that targeting the antiapoptotic pathway can cause both increased cytotoxicity and apoptosis in pancreatic cancer and that other oncologic diseases should also be tested to see if this approach could lead to better drug development.

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FIGURE 1.

A, Chemical structure of ApoG2. Apogossypolone is an analogue of (–)-gossypol, which was synthesized in the laboratory of Dr Wang. B, Comparative expression levels of proapoptotic and antiapoptotic cellular protein expression in human pancreatic cancer cell lines (Panc-1, Colo 357, and BxPC-3). Proteins (30 μ g per lane) obtained from cell lysates were separated through 12.0% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. C, Inhibition of cell viability in BxPC-3, Colo 357, and Panc-1 pancreatic cancer cell lines by MTT assay. Cells were seeded into 96-well plates and exposed to 0- to 10- μ mol/L ApoG2 for 72 hours and percent viable cells determined as described in Materials and Methods. * P < 0.05; ** P < 0.01 compared with untreated control.

FIGURE 2.

Loss of cell viability (A) and induction of apoptosis (C) by ApoG2, gemcitabine, and their combination in BxPC-3 and Colo 357 cells after 72 hours of treatment as described in Materials and Methods. The middle panel (B) represents isobologram plots for combination treatments with ApoG2 and gemcitabine in the doses shown in the upper panel. * $P < 0.05$; ** $P < 0.01$ compared with control.

FIGURE 3.

A, Spectrophotometric activity assay for caspase-3 in BxPC-3 and Colo 357 cells exposed to either 0- or 5-μmol/L ApoG2, 100-nmol/L gemcitabine, and combination of ApoG2 and gemcitabine for 72 hours. * $P < 0.05$; ** $P < 0.01$ compared with control. B, Western immunoblot showing PARP cleavage in BxPC-3 and Colo 357 cells. C, Clonogenic survival of treated cells showing colony number counted per 3 fields. * $P < 0.05$; ** $P < 0.01$ compared with control.

FIGURE 4.

A, Western immunoblot showing loss of cytochrome *c* release from mitochondria and increase in cytosolic cytochrome *c* content after ApoG treatment and activation of cleaved caspases and PARP in BxPC-3 and Colo 357. B, Spectrophotometric activity assay for caspases 3 and 9 on BxPC-3 cells exposed to ApoG2 (0–5 μ mol/L) for 72 hours. One hundred micrograms of proteins from cell lysates were incubated in triplicates with the corresponding substrates for caspases 3 and 9, and caspase activity was measured as per the manufacturer's instructions.

FIGURE 5.

A, Coimmunoprecipitation experiment to study the effect of ApoG2 on disrupting the heterodimerization of Mcl-1 and Bax with proapoptotic protein in BxPC-3. BxPC-3 cells were treated with ApoG2 (0, 5, 10, and 20 $\mu\text{mol/L}$). Total cell lysates were immunoprecipitated with Bax-specific antibody and then immunoblotted with Mcl-1-specific antibody. Western blot of Bax shows a dose-dependant increase in Bax expression but no change in B-actin expression. B, Coimmunoprecipitation experiment to study the effect of ApoG2 on disrupting the heterodimerization of Bim and Bcl-2 with proapoptotic protein in BxPC-3 cells. BxPC-3 cells were treated with ApoG2 (0, 5, 10, and 20 $\mu\text{mol/L}$). Total cell lysates were immunoprecipitated with Bcl-2-specific antibody and then

immunoblotted with Bim-specific antibody. Western blots of Bcl-2 and B-actin showed no change in protein expression.

FIGURE 6.

A, Schematic representation of treatment protocol and schedule of treatments. B, Tumor growth rate expressed as tumor weight (in milligram; mean $\pm$ SD) of BxPC-3 tumor-bearing SCID in the control (diluent), ApoG2, gemcitabine, and ApoG2 plus gemcitabine. Tumor weight decreased significantly in mice that received ApoG2-and-gemcitabine combination. All animals were treated 10 days after tumor transplantation. * P 0.05; ** P 0.01. C, Comparison of the tumor weights in each experimental group on the day when all mice in the control group were killed (day 26), showing that ApoG2 + gemcitabine was highly significant compared with either ApoG2 or gemcitabine alone (** P 0.01).

TABLE 1

Activity of ApoG2, Gemcitabine, and Their Combination Against BxPC3-Bearing SCID Xenografts

Agent	<i>T/C</i> , %	<i>T-C</i> , d	Log ₁₀ Kill, net
Diluent (Control)	100	0.0	0.0
ApoG2	80	2	0.20
Gemcitabine	40	6	0.60
ApoG2+Gem	24	10	1.00

 $\text{Log}_{10} \text{ kill} = (T-C)/(3.32)(Td).$