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Inhibitory effects of (-)-epigallocatechin-3-gallate and pterostilbene on pancreatic cancer growth in vitro

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ABSTRACT

Background: It has been previously shown that the naturally occurring antioxidant (-)-epigallocatechin-3-gallate (EGCG), found in green tea, and pterostilbene, a stilbenoid derived from blueberries, inhibit pancreatic cancer in vitro when used individually. We hypothesized that the combination of EGCG and pterostilbene would reveal additive effects in vitro.

Methods: Using the pancreatic cancer cell lines MIA PaCa-2 and PANC-1, efficacy and synergism were evaluated for cell proliferation and viability (3-(4,5-dimethyltiazol-2-y1)-2,5-diphenltetrazolium bromide assays, cell cycle analysis) and mitochondrial apoptosis (mitochondrial depolarization, cytochrome C release, caspase-3/7 activity, cell death detection using enzyme-linked immunosorbent assay).

Results: Cell proliferation assays revealed significant additive antiproliferative effects with pterostilbene and EGCG in both cell lines at the later, 72-h, point ($P < 0.05$). MIA underwent S-phase arrest with the combination (10–12% increase); however, cell cycle arrest was not observed in PANC. The combination induced mitochondrial depolarization and upregulated cytochrome C ($P < 0.05$) in MIA, but these effects were not observed in PANC. EGCG increased caspase-3/7 in MIA; however, the combination did not significantly increase the activity in either cell line ($P < 0.05$). Apoptosis was only observed in PANC ($P < 0.05$). The reduction in proliferation in MIA in the 3-(4,5-dimethyltiazol-2-y1)-2,5-diphenltetrazolium bromide assays with the combination indicated that cell death occurs, possibly through another mechanism.

Conclusions: Our results are encouraging regarding the future use of EGCG and pterostilbene to improve traditional pancreatic cancer therapies. In conclusion, EGCG and pterostilbene have additive, antiproliferative effects in vitro and alter the apoptotic mechanisms in both cell lines by modulation at different points in the mechanism.

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1. Introduction

Current statistics in the United States have shown that 1 in 4 deaths are due to cancer [1]. People diagnosed with pancreatic cancer have a very poor prognosis. Pancreatic cancer is an extremely virulent form of cancer with few effective treatments [2]. It was estimated that there were 43,140 newly diagnosed cases of pancreatic cancer in 2010 and 36,800 deaths from pancreatic cancer [1]. Pancreatic cancer is rarely detected in its early stages, making it more difficult to treat effectively. The overall 5-year survival rate for pancreatic cancer patients is less than 5% [3]. The high mortality rate results from the high incidence of metastatic disease at the initial diagnosis, the aggressive clinical course, and the failure of systemic therapies [4]. Gemcitabine has been the reference regimen since its approval in 1996 [4]. Target therapies are currently being developed and tested to focus on reducing the molecular causes of pancreatic cancer. The usual course of treatment of pancreatic cancer is a combination of surgical resection, chemotherapy, and radiotherapy. Integrative oncology is a new focus in cancer research and treatment that focuses on natural compounds as nontoxic tools that can work with current cancer therapies in hope they can increase the efficacy of traditional treatments.

Pterostilbene, a naturally occurring stilbenoid, is present in blueberries and has been found to have antioxidant, anti-inflammatory, and antiproliferative properties [5]. Our laboratory has been investigating the effects and mechanistic properties of pterostilbene, including caspase-dependent apoptosis, for several years. We have found that pterostilbene induced a significant dose- and time-dependent decrease in MIA PaCa-2 and PANC-1 cell viability [6]. Treatment with pterostilbene leads to the inhibition of cell proliferation and/or cell death, cell cycle arrest, mitochondrial membrane depolarization, and activation of effector caspases [6].

The compound (-)-epigallocatechin-3-gallate (EGCG) is a biologically active antioxidant polyphenol flavonoid that is found in green tea. Several other catechins are found in green tea; however, it has been found that EGCG is the most abundant and the most effective. EGCG has been found to selectively inhibit cell growth and induce apoptosis in cancer cells without adversely affecting normal cells [7,8]. EGCG also inhibits angiogenesis, possibly through the inhibition of proangiogenic factors, including vascular endothelial growth factor [9]. EGCG has been found to be most effective in cancers of the gastrointestinal tract [10]. The molecular pathways of EGCG have been more widely studied than those of pterostilbene. We hypothesized that the combination of EGCG and pterostilbene would reveal additive effects in vitro.

2. Methods

2.1. Reagents

EGCG and pterostilbene were purchased from Sigma Aldrich (St. Louis, MO). Pterostilbene was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich), and EGCG was dissolved in pure de-ionized water (Millipore, Billerica, MA).

2.2. Cell lines

Two pancreatic cancer cell lines, MIA PaCa-2 and PANC-1, were purchased from the American Type Culture Collection (Manassas, VA). MIA PaCa-2 is described as a pancreatic carcinoma and PANC-1 as a pancreatic duct epithelioid carcinoma. The cells were cultured in Dulbecco's modified Eagle's medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (Fisher Scientific, Houston, TX), and 1% penicillin streptomycin (Sigma Aldrich). The cells were maintained in monolayers in T-25 flasks in a water-jacketed 5% carbon dioxide incubator (Fisher Scientific) at 37°C. The cells were harvested at 80–90% confluency for the experiments, using 0.25% trypsin in 0.1% EDTA (Fisher Scientific) to detach the cells. The cells were centrifuged for 5 min at 1000 rpm, resuspended in growth medium, and plated according to the experimental guidelines stated in the next section. The cells were plated overnight before treatment.

2.3. 3-(4,5-Dimethyltiazol-2-y1)-2,5-diphenltetrazolium bromide cell viability assay

The 3-(4,5-dimethyltiazol-2-y1)-2,5-diphenltetrazolium bromide (MTT) colorimetric assays were performed to detect cell viability after treatment after 24-, 48-, and 72-h incubations. The cells were seeded in 96-well flat bottom tissue culture plates (Fisher Scientific) at 10^4 cells per well. The cells were treated with EGCG (20, 30, 40 μM), pterostilbene (30 μM), or a combination (20, 30, or 40 μM EGCG plus 30 μM pterostilbene) for 24, 48, and 72 h. MTT (Sigma Aldrich), 5 mg/mL in minimum essential medium (Sigma Aldrich), was applied after removal of the medium. The plates were incubated at 37°C in the carbon dioxide incubator for 1 h. Mitochondrial dehydrogenase activity causes the yellow MTT dye to be reduced to purple formazan. MTT was aspirated off and replaced with 200 μL of DMSO, which solubilizes purple formazan. Absorbance was read at 570 nm on an enzyme-linked immunosorbent assay plate reader.

2.4. Cell cycle analysis

The cells were seeded at 3×10^5 cells per well into 6-well plates and incubated overnight at 37°C in the carbon dioxide incubator. The cells were then treated with EGCG (40 μM), pterostilbene (30 μM), or a combination (40 μM EGCG plus 30 μM pterostilbene) and allowed to incubate for 24, 48, or 72 h. The cells were washed with phosphate-buffered saline (PBS), trypsinized, and incubated in ice-cold ethanol for 2 h. After incubation, the cells were washed with PBS and resuspended in PBS plus 0.1% Triton X-100 plus 100 μg/mL RNase A (Sigma Aldrich) plus 40 μg/mL propidium iodide (MP Biochemicals, Salon, OH) for 30 min in the dark. The cells were run on a Coulter Elite Flow Cytometer. Propidium iodide, when bound to nucleic acids, has an excitation maximum at 535 nm and an emission maximum at 617 nm. ModFit LT, version 3.0,

software (Verity Software, Portland, ME) was used to analyze and categorize cell populations into cell cycle phases.

2.5. Mitochondrial depolarization

Cells were seeded at 3×10^5 cells per well into 6-well plates and incubated overnight at 37°C in the carbon dioxide incubator; 2 mM of JC-1 (Molecular Probes, Eugene, OR) was added to each well for 20 min at 37°C. The cells were then washed with PBS and treated with a DMSO control or with EGCG (40 μM), pterostilbene (30 μM), or the combination (40 μM EGCG plus 30 μM pterostilbene) for 30 min. The cells were trypsinized, resuspended in PBS, and run on the Coulter Elite Flow Cytometer. The excitation peak of JC-1 is 488 nm, and the approximate emission peak of the monometeric and J-aggregate forms is 529 and 590 nm, respectively.

2.6. Cytochrome C

A cytochrome C EIA kit (Assay Designs, Ann Arbor, MI) was used to determine the presence of cytosolic cytochrome C. Mitochondrial and cytoplasmic fractions of the cells were obtained. The cells were seeded at 1 million/mL and treated with EGCG (40 μM), pterostilbene (30 μM), or the combination (40 μM EGCG plus 30 μM pterostilbene) or DMSO (negative control) and allowed to incubate overnight at 37°C in the carbon dioxide incubator. Lysates were obtained at 7 million cells/mL. The cells were trypsinized and centrifuged (800 *g*), and the supernatant was discarded. The cell pellet was washed and resuspended in PBS. The cells were centrifuged (1000 *g*), and the supernatant was discarded. The cell pellets were resuspended with Digitonin Cell Permeabilization Buffer, vortexed, and incubated on ice for 5 min. The cells were centrifuged again (1000 *g*) for 5 min at 4°C. The supernatants were saved, because they contained the cytosolic fraction of cytochrome C. The pellets were resuspended with RIPA Cell Lysis Buffer 2 and incubated on ice for 5 min. Lysate was vortexed and centrifuged (10000 *g*) for 10 min at 4°C. The cytosolic fractions were added into wells in the microtiter strips, as were the dilutions of the lyophilized human cytochrome C standard. The plate was processed according to the product insert and read on a plate reader at 405 nm.

2.7. Caspase activity assay

Caspase-3/7 activity was determined using the Apo-ONE Homogeneous Caspase-3/7 Assay Kit (Promega Madison, WI). The cells were seeded into 96-well plates with opaque sidewalls at 10^4 cells per well and allowed to incubate at 37°C in the carbon dioxide incubator for 24 h for the cells to adhere. The cells were then treated with EGCG (40 μM), pterostilbene (30 μM), or the combination (40 μM EGCG plus 30 μM pterostilbene) and allowed to incubate for 24 h. The Apo-ONE Homogeneous Caspase-3/7 Assay substrate [rhodamine 110, bis-(N-CBZ-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110) was used to analyze the activity of caspase-3 and caspase-7, which are effector caspases that cleave intracellular protein substrates, triggering the apoptotic process [5]. Caspase-3/7 activity causes cleavage and removal of the DEVD peptides, and subsequent fluorescence. The amount of fluorescence produced is a direct marker of caspase-3/7 activity. After the addition of the proflourescent substrate, the plate was placed on a shaker (300–500 rpm) and incubated at room temperature for 1 h. Fluorescence was measured on a spectrofluorimeter (excitation wavelength 499 nm and emission maximum 521 nm).

2.8. DNA fragmentation assay

A Cell Death Detection ELISA^{PLUS} Kit (Roche Mannheim, Germany) was used to detect apoptosis in the cells. It is a sandwich enzyme immunoassay-based method used to detect the presence of nuclear DNA fragmentation. Mouse monoclonal antibodies directed against DNA and histones were used to recognize released nucleosomes after DNA nucleosomal fragmentation [11]. The cells were seeded into 96-well plates at 10^4 cells per well and allowed to incubate at 37°C in the carbon dioxide incubator for 24 h for the cells to adhere. The cells were then treated with EGCG (40 μM), pterostilbene (30 μM), or the combination (40 μM EGCG plus 30 μM pterostilbene) and allowed to incubate for 48 or 72 h. Lysis buffer was applied to the adherent cells, and the cells were centrifuged to produce a nucleosome-containing supernatant. The samples were transferred to a streptavidin-coated enzyme-linked immunosorbent assay microplate and incubated with anti-histone and anti-DNA antibodies. A peroxidase substrate was applied, causing a subsequent color change, proportional to the amount of nucleosomes captured in the antibody sandwich. The plates were read at 405 nm on a spectrophotometer.

2.9. Statistical analysis

The data are presented as the mean ± standard error of the mean. Statistical comparisons among groups were made using 1-way analysis of variance and Tukey's multiple comparison test run using GraphPad Software (San Diego, CA).

3. Results

3.1. Reduction of cellular proliferation

Significant reductions in cellular proliferation were observed in the PANC-1 cell line at all treatment levels after 72 h of incubation ($P = 0.001$) and in the MIA PaCa-2 cell line at all treatment levels, except for 20 μM EGCG after 72 h of incubation ($P = 0.001$; Fig. 1). Data at 24 and 48 h of incubation in both cell lines are not shown graphically. After 24 h of incubation, no significant decreases were found in proliferation in PANC-1, although significant decreases in proliferation were observed with 20 μM EGCG, 40 μM EGCG, and with all 3 combination treatments. After the 48-h incubations, a significant decrease occurred in cell proliferation in PANC-1 with 30 μM EGCG plus 30 μM pterostilbene and 40 μM EGCG plus 30 μM pterostilbene and in MIA at all treatment levels. The combination of EGCG and pterostilbene caused a significant decrease in cell viability and cell proliferation in all but the 24-h PANC-1, where cell proliferation was reduced by 17.4% (0.622 ± 0.3742) in the group treated with 40 μM EGCG plus

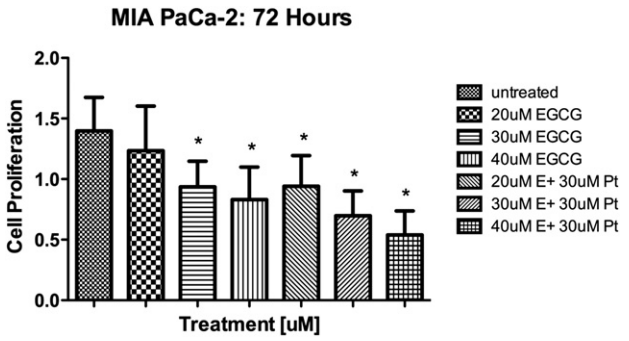

Fig. 1 – MTT cell viability assays showing reduction in cellular proliferation from treatment with EGCG alone, pterostilbene alone, and both agents together in PANC-1 and MIA PaCa-2 cell lines in vitro at 24, 48, and 72 h of incubation. Data presented as means ± standard deviation. *P < 0.001, by analysis of variance, compared with control.

30 μM pterostilbene ($P = 0.7234$). The combination treatment of 40 μM EGCG plus 30 μM pterostilbene reduced cell proliferation to a greater degree than with the other treatments. Cell proliferation was reduced in MIA when treated with 40 μM EGCG plus 30 μM pterostilbene by 58.22% at 24 h (0.401 ± 0.0884), 65.4% at 48 h (0.4986 ± 0.0995), and 61.4% at 72 h (0.539 ± 0.059). Proliferation was reduced in PANC when treated with 40 μM EGCG plus 30 μM pterostilbene by 36.7% (0.657 ± 0.362) at 48 h and by 71.6% (0.2926 ± 0.1395) at 72 h. The cell line PANC-1 had a time-dependent response, but responses were observed after 24 h in MIA. Additive effects were observed in both cell lines with the combination of both compounds: MIA at all points and PANC at 48 and 72 h.

3.2. *Cell cycle analysis*

The cell cycle analysis of PANC-1 and MIA shows that normal cell cycle progression was disrupted after 24 h and 48 h of incubation. As seen in Table 1, treatment with pterostilbene (30 μM) alone and combined with EGCG (40 μM) caused an increased percentage of MIA cells to arrest in the S phase compared with the control. A slight increase was seen in the percentage of PANC-1 cells in the G_0/G_1 phase. The data from the 24-h incubation (not shown) were similar to the 48-h data (Table 1).

3.3. *Mitochondrial depolarization*

Treated MIA and PANC cells were found to have rapidly depolarizing membranes within 30 min of treatment with JC-1 (Table 2). The combination treatment (40 μM EGCG plus 30 μM pterostilbene) in MIA caused an increase in the percentage of depolarized cells (26.2%) compared with the DMSO-treated controls (5.73%). There were slight, but not significant, increases in the percentage of depolarized cells in the cells treated with pterostilbene alone and with the combination treatment. In PANC, 30 μM pterostilbene caused a greater increase in the percentage of depolarized cells (47.6%) than the combination treatment compared with the DMSO-treated controls (21%). Slight increases were seen in the percentage of depolarized cells in the cells treated with EGCG alone and with the combination treatment.

3.4. *Cytochrome C*

As seen in Fig. 2, statistically significant changes were seen in cytochrome C activity in both cell lines ($P < 0.0001$). There was a statistically significant upregulation of cytochrome C activity (pg/mg) in PANC when the cells were treated with 30 μM pterostilbene (83.3%, 99,899 ± 5731), with a dramatic increase when the cells were treated with 40 μM EGCG (213.3%, 1.217×10^6). Treatment with 40 μM EGCG plus 30 μM pterostilbene caused a slight increase in activity. Statistically significant increases in activity were also observed in MIA when the cells were treated with EGCG (40.6%, 7,77,778

Table 1 – Cell cycle analysis with EGCG and pterostilbene.

Treatment	G_0/G_1	S	G_2/M
MIA PaCa-2			
Control	63	13	25
DMSO	61	13	27
30 μM Pterostilbene	49	25	26
40 μM EGCG	66	11	22
40 μM EGCG plus pterostilbene	55	22	23
PANC-1			
Control	48	36	14
DMSO	54	31	14
30 μM Pterostilbene	46	33	19
40 μM EGCG	43	37	15
40 μM EGCG plus pterostilbene	53	30	15

EGCG = (-)-epigallocatechin-3-gallate; DMSO = dimethyl sulfoxide.
Data presented as percentage of cells in given cell cycle phase.
Cell cycle analysis of PANC-1 and MIA PaCa-2 cell lines when treated with EGCG alone, pterostilbene alone, and both agents together.

Table 2 – Mitochondrial depolarization with EGCG and pterostilbene.

Treatment	Normal (%)	Depolarized (%)
MIA PaCa-2		
Positive control	78.8	28.4
DMSO	94.4	5.73
30 μM Pterostilbene	91.0	11.3
40 μM EGCG	87.8	13.6
40 μM EGCG plus pterostilbene	80.9	26.2
PANC-1		
Positive control	42.5	72.1
DMSO	74.3	21.0
30 μM Pterostilbene	60.0	47.6
40 μM EGCG	76.3	26.8
40 μM EGCG plus pterostilbene	73.4	30.3

EGCG = (-)-epigallocatechin-3-gallate; DMSO = dimethyl sulfoxide.
Data presented as percentage of normal and depolarized cells.
Mitochondrial depolarization of JC-1–labeled PANC-1 and MIA PaCa-2 cells after 30-min treatments.

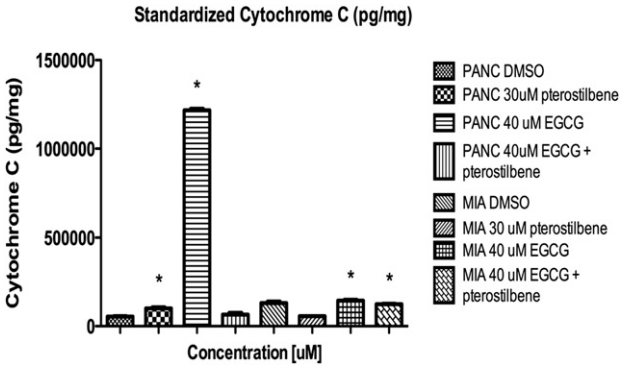

Fig. 2 – Cytochrome C activity in PANC-1 and MIA PaCa-2 cell lines in vitro standardized in pg/mg when treated with EGCG alone, pterostilbene alone, and both agents together. Data presented as mean ± standard deviation. *P < 0.001, by analysis of variance, compared with control.

± 2,11,559) and when they were treated with the combination (31.06%, 90,320 ± 154,707).

3.5. *Caspase activity*

To determine whether the biologic activity of EGCG and the combination of EGCG and pterostilbene involved effector caspases, an assay was performed to analyze the activity of 2 enzymes involved in the effector phase of apoptosis: caspase-3 and caspase-7. Caspase activity was measured through fluorescence activity. As seen in Fig. 3, PANC-1 showed a significant ($P < 0.0217$) decrease in caspase-3/7 activity compared with the DMSO-treated control (46,063 ± 12,218) when treated with 40-μM EGCG (65.2%, 16,034 ± 1191), 30 μM pterostilbene (65.2%, 16,012 ± 1805), and the combination treatment of 40 μM EGCG plus 30 μM pterostilbene (63.2%, 16,943 ± 810.5). MIA showed a significant ($P < 0.0186$) increase (187%, 25,421 ± 1026) in caspase activity when treated with EGCG and slight, but not statistically significant, increases in caspase activity when treated with pterostilbene (31%, 15,437 ± 1020) and the combination treatment (115.7%, 25,421 ± 1026) compared with the DMSO-treated controls (11,781 ± 799.2).

3.6. *DNA fragmentation assay*

Programmed cell death is characterized by chromatin condensation, membrane blebbing, internucleosomal degradation of DNA, and apoptotic body formation. To investigate whether cytotoxic effects of pterostilbene were due to necrosis or apoptosis, we performed an assay of released nucleosomes (Fig. 4). Statistically significant increases and decreases in the number of released nucleosomes were observed in both cell lines with treatment. Statistically significant increases in released nucleosomes when MIA was treated with pterostilbene (110.6%, 1.646 ± 0.01050) and EGCG (42.4%, 1.113 ± 0.0015), and a statistically significant ($P < 0.0001$) decrease was observed when the cells were treated with the combination treatment (25%, 0.586 ± 0.017). A statistically significant decrease ($P < 0.0011$) was seen in released nucleosomes when PANC cells were treated with pterostilbene (9.27%, 0.137 ± 0.00), although statistically significant increases were observed when the cells were treated with EGCG (15.9%, 0.1738 ± 0.0028) and the combination treatment (10.9%, 0.1675 ± 0.0005). At 48 h, no statistically

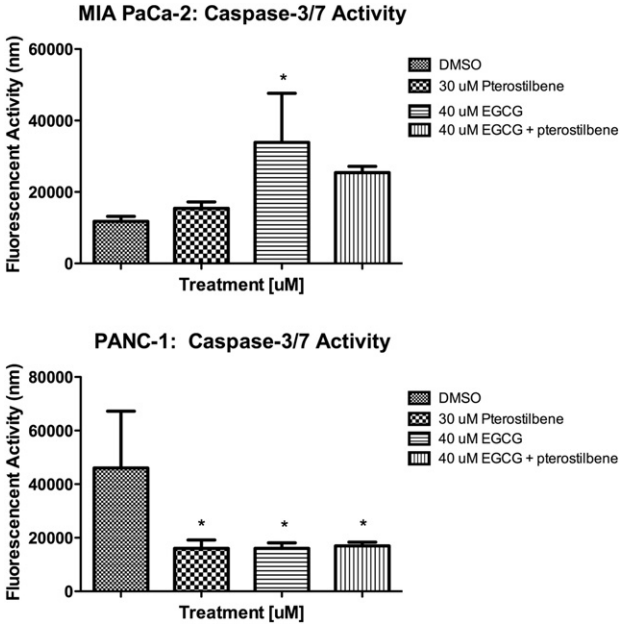

Fig. 3 – Caspase-3/7 activity increases in PANC-1 and MIA PaCa-2 cell lines in vitro when treated with EGCG alone, pterostilbene alone, and both agents together. Data presented as mean ± standard deviation. *P < 0.001, by analysis of variance, compared with control.

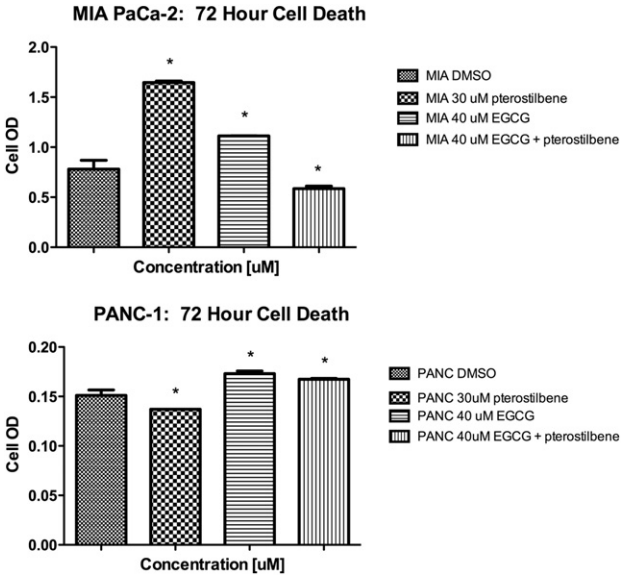

Fig. 4 – Cell death detection using enzyme-linked immunosorbent assay at 72 h in PANC-1 and MIA PaCa-2 cell lines in vitro when treated with EGCG alone, pterostilbene alone, and both agents together. Data presented as mean ± standard deviation. *P < 0.001, by analysis of variance, compared with control.

significant changes were seen in MIA (data not shown). In PANC at 48 h, a statistically significant decrease was seen in nucleosome release when the cells were treated with pterostilbene and statistically significant increases when the cells were treated with EGCG and with the combination treatment (data not shown).

4. Discussion

The present study has demonstrated the in vitro anticancer activity of EGCG and pterostilbene on 2 pancreatic cancer cell lines. Integrative oncology is the use of alternative treatments, such as natural compounds, in conjunction with traditional therapies, with the hope they will enhance the efficacy of the traditional treatment. Previous studies have proved the anticancer activity of pterostilbene, including its activation of caspase-dependent apoptosis in pancreatic cancer [6]. EGCG is the most widely studied catechin in green tea and has been found to inhibit the cell cycle and induce apoptosis [10,12]. In addition, EGCG has been found to modulate through a wide variety of molecular pathways in pancreatic cancer, including inhibition of Hsp90 [3], induction of CDK inhibitors [13], and inhibition of FAK and insulin-like growth factor-1 receptor [14]. We hypothesized that the naturally occurring antioxidant EGCG will inhibit pancreatic cancer cell growth in vitro alone and when combined with pterostilbene. It should be noted that the DMSO-treated MIA and PANC cells were used as a control in all experiments, because pterostilbene was dissolved in DMSO and EGCG was dissolved in de-ionized water. Previously, in an in vivo study, pterostilbene dissolved in DMSO was well-tolerated and effective when given to nude mice [15].

Our results indicate that EGCG and pterostilbene have an additive, antiproliferative affect in vitro (Fig. 1). MIA PaCa-2 and PANC-1 have been shown to have time- and concentration-dependent antiproliferative effects when subjected to treatment with EGCG and pterostilbene alone [6,13]. Current research studying the limitations of MTT and MTS-based assays for measurements of antiproliferative effects of catechins, including EGCG, have shown that these assays actually result in an underestimation of antiproliferative effects as a result of increased activity of mitochondrial dehydrogenase [16]. No observable arrests were seen in the cell cycle in PANC; however, S phase arrests were observed with the combination treatment and with pterostilbene alone in MIA (Table 1). EGCG has been found to cause growth arrest in the G_1 phase through regulation of cell cycle proteins, such as p21, p27, cyclin D1, and cdk4 and cdk6 [12]. EGCG has also been found to suppress the protein levels of basic fibroblast growth factor and vascular endothelial growth factor in colorectal cancer, causing a reduction in angiogenesis and a subsequent reduction in tumor growth and metastasis [9]. Pterostilbene has been shown to cause cell cycle arrest in the S phase in breast cancer [11].

The examination of the mechanisms leading up to apoptosis showed that the combination does not always elicit the strongest response, if at all, compared with EGCG or pterostilbene alone. Our study considered the mitochondrial-derived apoptotic pathway. EGCG and pterostilbene have previously been shown to affect this pathway. The combination of EGCG and pterostilbene causes an increase in mitochondrial depolarization in MIA, and pterostilbene treatment alone causes a greater amount of depolarization than the combination in PANC (Table 2). The mitochondrial depolarization in MIA is consistent with results from previous studies in which EGCG-induced depolarization in MIA [17]. The data are also consistent with the studies of pterostilbene in MIA and PANC, in which mitochondrial depolarization was increased in both cell lines [6]. Mitochondrial depolarization is a precursor to cytochrome C release. Cytochrome C was upregulated in PANC with both pterostilbene and EGCG treatment alone but was significantly upregulated with combination treatment and with EGCG treatment in MIA (Fig. 2). Both EGCG and pterostilbene have been found to upregulate cytochrome C activity in a wide variety of cancers [18,19]. The large upregulation of cytochrome C in PANC with treatment of EGCG alone did not follow our mitochondrial depolarization data. Caspase-3/7 activity was only increased significantly in MIA with treatment with EGCG alone (Fig. 3). The results did not follow those of previous studies. Caspases have been found to be activated by both pterostilbene and EGCG [6,12]. Apoptosis, as a result of the combination treatment, was only detected in PANC (Fig. 4). Mannal *et al.* [6] found that apoptosis as a result of treatment with pterostilbene was only detected in MIA, not in PANC. The observed conflicting results can be explained by the different doses and timing. Additional studies are needed to evaluate these findings.

These results indicate that EGCG and pterostilbene modulate at different points in the apoptotic mechanism and that MIA, although clearly undergoing a reduction in proliferation as a result of the combination treatment (Fig. 1), does not undergo cell death as a result of apoptosis. Shankar *et al.* [11] found that EGCG induces mitochondrial depolarization in MIA through the induction of the Bcl-2 family of genes (Bax, Bac, PUMA) causing a disruption in mitochondrial homeostasis. This might be an example of an instance in which the 2 compounds can affect each other negatively, outweighing the other's positive effects.

Although our results and other current research did not indicate a difference in proliferative pathway expression between the 2 cell lines, it is an area of interest for future research. Both MIA and PANC have been found to have the common K-*ras* oncogene point mutation at codon 12, with MIA at 12C and PANC at 12V [20]. In addition to the K-*ras* oncogene mutation, 3 other mutations are commonly found in pancreatic cancers: functional inactivation of the tumor suppressor genes *p53*, *p16*, and *DPC4* [21,22]. MIA and PANC have *p53* mutations, resulting in a change in the amino acid product, and a mutation of *p16*, causing homozygous deletion and a subsequent absence of the final product [23]. MIA and PANC have both been observed to have wild-type *DCP4* [23].

The health benefits of green tea polyphenols and other natural compounds have been touted for centuries but have not begun to be understood from a scientific perspective until more recently. Although it is apparent that these compounds have effects in vitro, it is important to work to translate the effective in vitro dosages to effective in vivo models.

Understanding the pharmacokinetics of natural compounds such as EGCG and pterostilbene is an important step toward being able to determine the effective dosages and possible interactions with adjuvant cancer therapies for future treatment of cancer patients. The pharmacokinetics of EGCG have been more widely studied than those of pterostilbene. A recent study comparing the bioavalibility of pterostilbene in rats to that of its analog resveratrol found that when equimolar doses were administered, the plasma concentration of pterostilbene was 3–4 times greater than that of resveratrol [24]. A rapid decline in the plasma concentration of pterostilbene was also observed for 24 h [24]. McCormack *et al.* [15] observed a significant decrease in pancreatic tumor volume (MIA PaCa-2 cells) using oral gavage pterostilbene treatment in nude mice. EGCG is now being tested in Phase I human trials to determine the systemic bioavailability of the compound and its half life. Although large variations were observed in the systemic availabilities between the 2 studies, it was determined that 800 mg is an effective dose of EGCG and that persistent treatment leads to greater levels of free EGCG in the plasma, despite its rapid decline in availability during a 24-h period [25].

To further investigate how these compounds affect the mitochondrial apoptotic pathway, SMAC/Diablo and reactive oxygen species generation should be studied. The study of additional molecular pathways will provide useful information to determine whether MIA is undergoing cell death through a nonmitochondrially derived pathway when treated with the combination of ECGC and pterostilbene. Shankar *et al.* [18] found that EGCG inhibits growth and induces apoptosis through the mitogen-activated protein kinase pathways in MIA. In the future, we also hope to study the in vivo effects of EGCG and pterostilbene and their effects when combined with conventional cancer therapy.

5. Conclusions

We have demonstrated, for the first time, that the combination of EGCG and pterostilbene have an additive, antiproliferative effect in pancreatic cancer. We have shown that the combination has a wide variety of effects on the mitochondrial apoptotic pathway, including the induction of apoptosis. The presence of EGCG and pterostilbene in everyday foods, in addition to their innocuous properties, make them a promising target for cancer therapy and possible cancer prevention.

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