Pomegranate Extract Induces Cell Cycle Arrest and Alters Cellular Phenotype of Human Pancreatic Cancer Cells

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Abstract. *Background: Pomegranate extract (PE) is a standardized whole-fruit extract of pomegranate, a fruit with known anticancer properties. Materials and Methods: PANC-1 and AsPC-1 human pancreatic cancer cells were used as in vitro models to test the effects of PE. Results: PE treatment induced cell cycle arrest and inhibited cell proliferation in PANC-1 cells. PE treatment increased the proportion of cells lacking CD44 and CD24 expression, which are associated with increased tumor-initiating ability, demonstrating that PE altered cell phenotype. PE was more effective in inhibiting the proliferation of PANC-1 cells than the clinically used dose of paclitaxel. Similar results were obtained in the AsPC-1 cell line. Individual pomegranate phytochemicals were only modestly effective in inhibiting cell proliferation, suggesting that unidentified phytochemicals are responsible for the inhibitory effect of PE. Conclusion: These data suggest that PE is a promising candidate for further preclinical testing for treatment of human pancreatic cancer.*

Pancreatic cancer, one of the most lethal forms of cancer, is the fourth leading cause of cancer death in both genders. The poor prognosis of pancreatic cancer patients is due to often late-stage diagnosis, and the ineffectiveness of current chemotherapeutic regimens. 5-fluorouracil and gemcitabine are the only chemotherapeutic agents that have been successful in the treatment of pancreatic cancer, but their efficacy is low and they cause serious side-effects (1). Therefore, the identification and development of alternative medicines for the treatment and prevention of pancreatic cancer is needed. Dietary sources are a promising source of new therapeutic options. Pomegranate (*Punica granatum*) is a fruit used in many cultures (the genus name, *Punica*, is derived from the Roman name for Carthage, where the best pomegranates were known to grow). The pomegranate tree is native to the region of Persia and is now cultivated over the entire Mediterranean area, Asia, and America. Pomegranate extract (PE) consists of a mixture of various phytochemicals, including the punicalagins, a class of tannins unique to pomegranates, which have been shown to possess free radical-scavenging properties (2, 3). Pomegranate has been valued in many cultures for millennia for its therapeutic attributes, including anti-inflammatory, anti-hypertensive, and anti-diabetic properties (4). Recent studies have shown that pomegranate is a potent anticancer agent that causes the induction of apoptosis and cell cycle arrest in cancer cells, inhibition of multiple signaling pathways in cancer cells, inhibitionof tumorigenesis in animal models of various carcinomas (5-8). These qualities make pomegranate a promising source of novel preventive and therapeutic agents, but its effects on pancreatic cancer have not, to the best of our knowledge, been studied.

In the present study we examined the activity of a standardized, commercially available, and certified safe PE on the growth and viability of human pancreatic cancer cells *in vitro*. To ensure that our results were not cell line-dependent, two different human cancer cell lines were studied. We demonstrate that PE effectively inhibits the growth and viability of human pancreatic cancer cells by inducing cell cycle arrest, and reduces the tumor-initiating phenotype of the cancer cells. This is the first demonstration that PE may have efficacy against pancreatic cancer.

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Key Words: Pomegranate, pancreatic cancer cells, phytochemicals, cell cycle arrest.

Materials and Methods

Materials. POMELLA, a HPLC-standardized extract of pomegranate that retains the natural polyphenolic ratio of whole pomegranate fruit, was from Verdure Sciences (Noblesville, IN, USA). Fetal bovine serum, glutamine, RPMI-1640 medium, trypsin/EDTA and phosphate-buffered saline (PBS) were obtained from Hyclone (Logan, UT, USA). Dimethyl sulfoxide (DMSO), ribonuclease (RNase), Triton X-100, propidium iodide, sulforhodamineB (SRB),

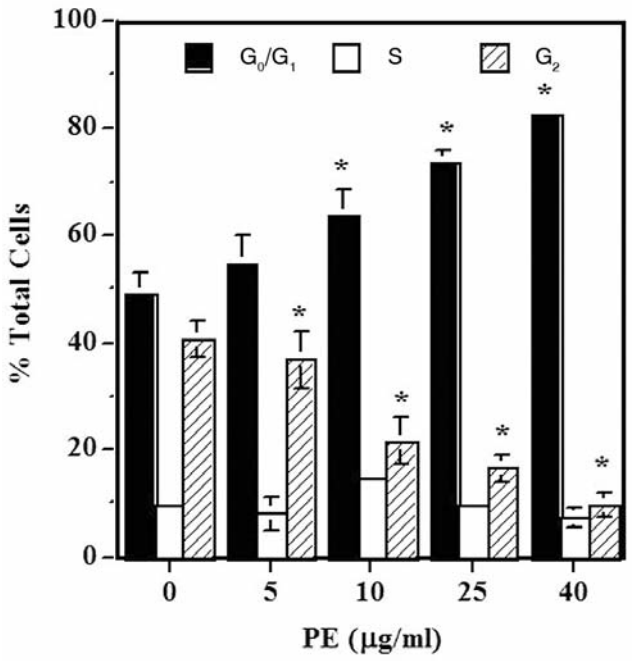
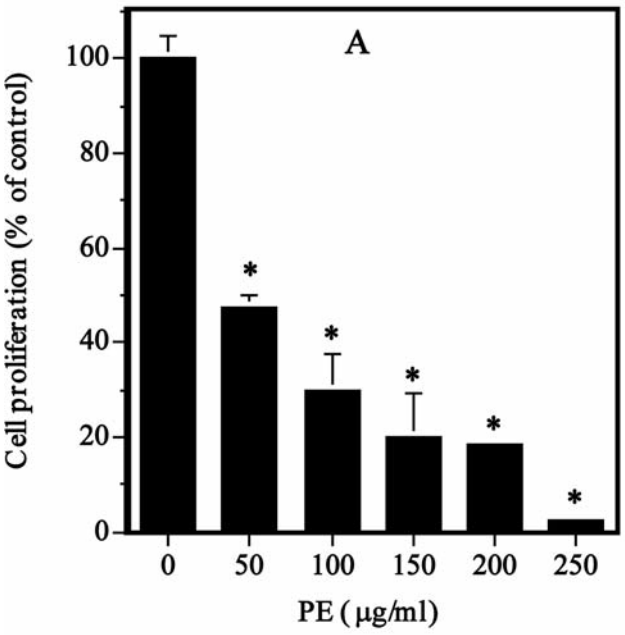


Figure 1. *Effect of PE on cell cycle progression. Cells were treated with the indicated concentrations of DMSO (control) or PE for 24 h. Cells were then trypsinized and analyzed for stage of cell cycle as described. n=4±s.e. *Indicates a significant difference from the control (p<0.05).*



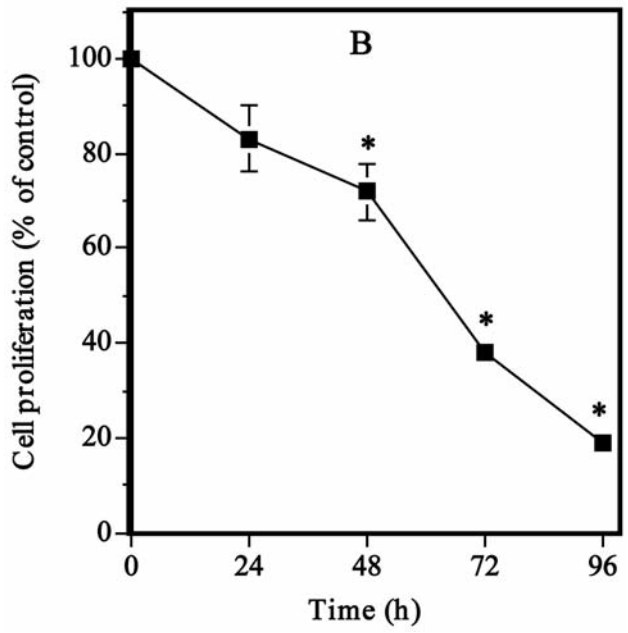


Figure 2. *Effect of PE on PANC-1 cell proliferation. A: Subconfluent PANC-1 cells were treated with DMSO (control) or PE at the indicated concentrations and their proliferation was measured 96 h later. B: Subconfluent PANC-1 cells were treated with 100 μg/ml PE and their proliferation was measured at the indicated times. For both A and B, n=4±s.e. *Indicates a significant difference from the control (p<0.05).*

tricholoroacetic acid and Tris-base buffer were purchased from Sigma Chemical (St. Louis, MO, USA). CD44 and CD24 antibodies were bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The annexin V kit was from Clontech (Mountain View, CA, USA). The EnzChek Caspase-3 assay kit was from Invitrogen (Carlsbad, CA, USA).

Cell culture. PANC-1 and Aspc-1 human pancreatic cancer cellswere obtained from ATCC (Manassas, VA, USA). Both cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine and maintained in monolayer culture at 37˚C and 5% CO_2 in a humidified incubator. Cells were passed twice weekly using 0.05% trypsin.

Proliferation and cytotoxicity assays. To measure proliferation, pancreatic cancer cells were passed into 24-well plates at 5,000 (PANC-1) or 2,000 (AsPC-1) cells per well. After 24 h the cells were treated with DMSO (control) or PE and the viable cell population was determined using SRB as described elsewhere (9). For the cytotoxicity assay, cells were passed 20,000 (PANC-1) or 5,000 (AsPC-1) cells per well and grown to >80% confluence before adding treatment with DMSO or PE as indicated and the viable cells were determined 48 h later by the SRB assay. SRB has been validated to be an accurate measure of the viable cell population. DMSO at the concentrations used had no effect on cell viability.

Cell cycle analysis. To determine the cell cycle distribution, cells were passed into 6-well plates and treated with DMSO (control) or PE for 24 h. Cells were trypsinized, washed with PBS, and fixed in 1% methanol-free formaldehyde for 20 minutes on ice. The cell suspension was added to 70% ice-cold ethanol overnight. Cells were pelleted and stained with 50 μg/mL propidium iodide, 0.1 mg/mL RNase and 0.05% TritonX-100 for 45 minutes at 37˚C. Cell cycle analysis of the stained

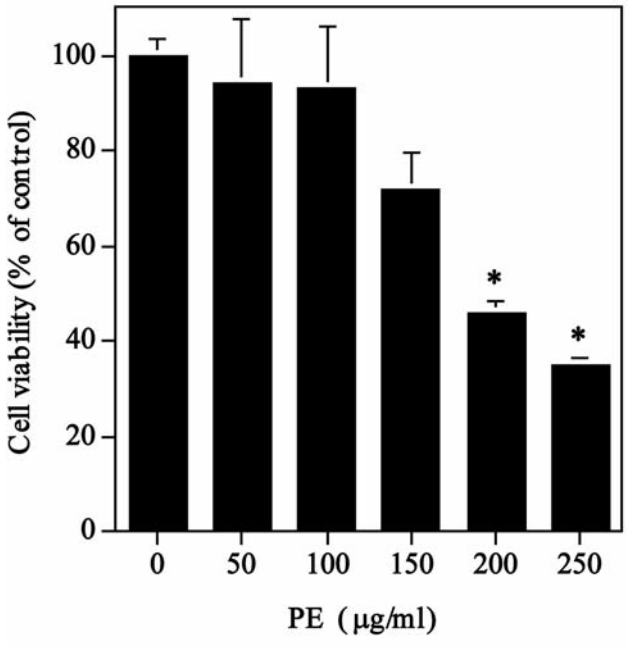


Figure 3. *Effect of PE on PANC-1 viability. PANC-1 cells were allowed to grow until just confluent and then treated with DMSO (control) or PE at the indicated concentrations. Cell viability was assessed 48 h later. n=4±s.e. *Indicates a significant difference from the control (p<0.05).*

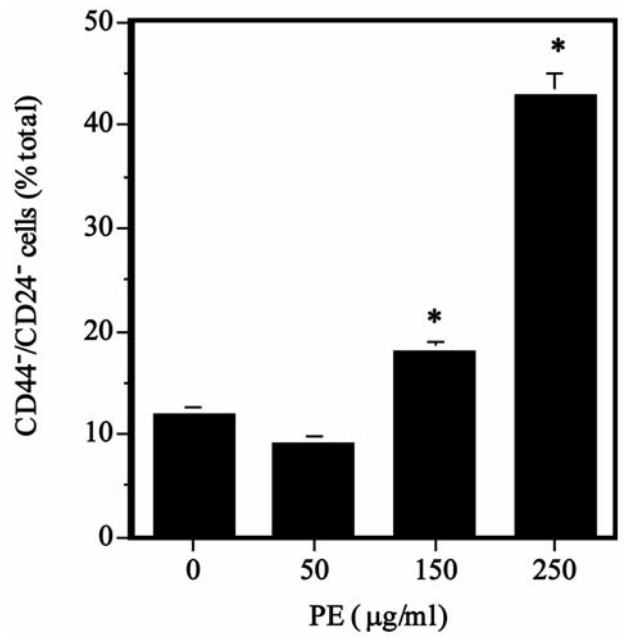


Figure 4. *Effect of PE on CD44 and CD24 expression. PANC-1 cells were treated with the indicated concentrations of DMSO (control) or PE for 24 h and the expression of CD44 and CD24 were analyzed by flow cytometry as described. n=3±s.e. *Indicates a significant difference from the control (p<0.05).*

cells was performed immediately with FACS-Calibur (BD, Franklin Lakes, NJ, USA). A minimum of 10,000 cells per sample were used for the assay and analysis was performed with FlowJo software.

Apoptosis assays. The cells were grown to confluence in 6-well plates and treated with DMSO (control) or serial concentrations of PE for 24 h. Each sample of 1×10^6 cells was trypsinized and washed with PBS twice. Each sample was stained with Annexin V (FITC) (10 μL in 100 μL of buffer) for 15 min and counterstained with 7-AAD (10 μL in 390 μL of buffer). The stained cells were analyzed immediately for apoptosis with FACS-Calibur. Caspase-3 activity was assayed by a kit as per the manufacturer's instructions.

CD24/CD44 assay. Cells were passed into 6-well plates and treated with DMSO (control) or PE for 24 h. Each sample of 1×10^6 cells was trypsinized and washed with PBS twice. Each sample was stained with 5 μL of anti-CD44 antibody and 20 μL of anti-CD24 antibody in 100 μL of BSA and placed on ice for 60 min. The stained cells were washed, suspended and dissolved in PBS and analyzed with FACS-Calibur.

Statistics. Data is presented as the mean of triplicate or quadruplicate determinants with standard error (s.e.). Assays were repeated at least two times. Statistical analysis was performed to assess the difference between the means of the untreated and treated samples using two-tailed Student's *t*-test with SPSS statistical software and GraphPad Prism software. A *p*-value of <0.05 was considered statistically significant.

Results

Effect of PE on cell cycle progression in PANC-1 cells. As seen in Figure 1, a low concentration (≤40 μg/ml) of PE caused the percentage of PANC-1 cells in the G_0/G_1 phase of the cell cycle to profoundly and significantly increase in a concentration-dependent manner, with an accompanying significant decrease in the percentage of cells in G_2. This indicates that PE caused a cell cycle arrest in PANC-1 cells.

Effect of PE on the proliferation and viability of PANC-1 cells. As shown in Figure 2, PE caused a concentration- (Figure 2A) and time- (Figure 2B) dependent decrease in the proliferation of PANC-1 cells with a half-maximal inhibitory concentration (IC_{50}) of approximately 50 μg/ml. In addition to inhibiting proliferation of subconfluent cells, PE was also cytotoxic to quiescent cells (Figure 3). However, much higher PE concentrations were required to achieve a significant reduction in cell viability than was necessary to inhibit proliferation. Even a high concentration of PE was unable to induce apoptosis, as assessed by Annexin V staining and caspase-3 activity (data not shown).

Effect of PE on PANC-1 cell phenotype. We examined the effect of PE on the expression of CD44 and CD24 cell

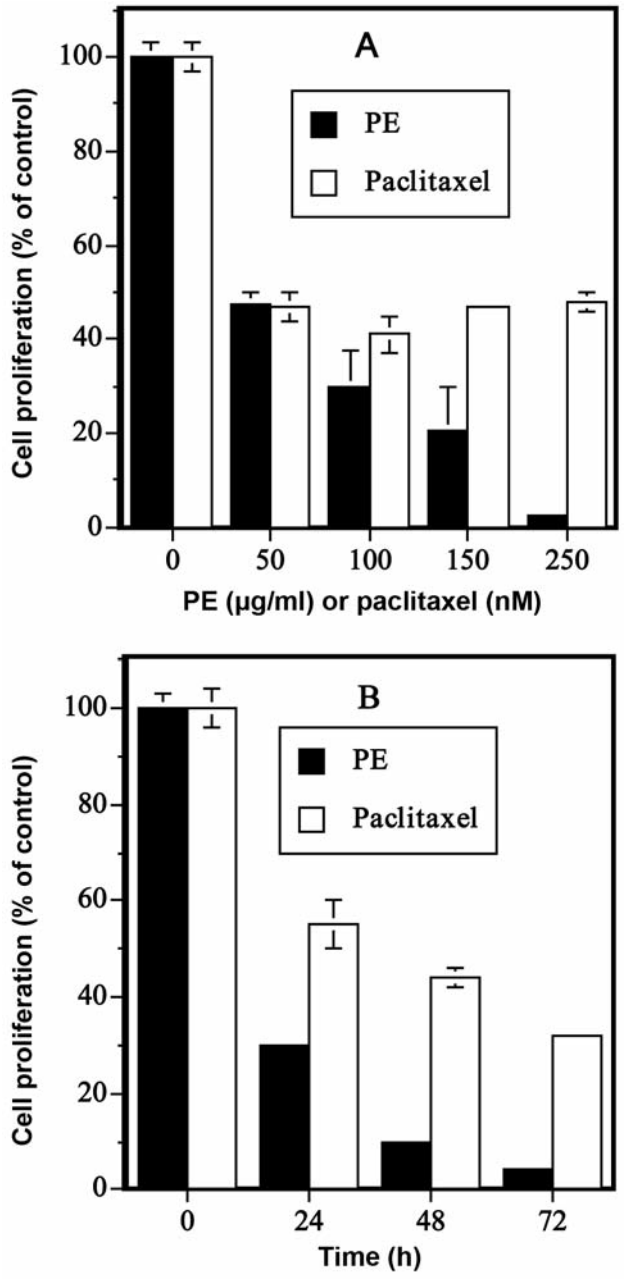


Figure 5. *Comparison of PE with paclitaxel in inhibiting cell proliferation. A: Subconfluent PANC-1 cells were treated for 72 h with the indicated concentrations of DMSO (control), PE or paclitaxel. B: Subconfluent cells were treated with DMSO (control), PE (100 μg/ml), or paclitaxel (100 nM) for the times indicated. For both A and B, n=4±s.e. There was a significant reduction in proliferation at all concentrations of PE and paclitaxel tested.*

surface glycoproteins. As shown in Figure 4, PE treatment caused a concentration-dependent increase in the proportion of cells lacking expression of CD44 and CD24.

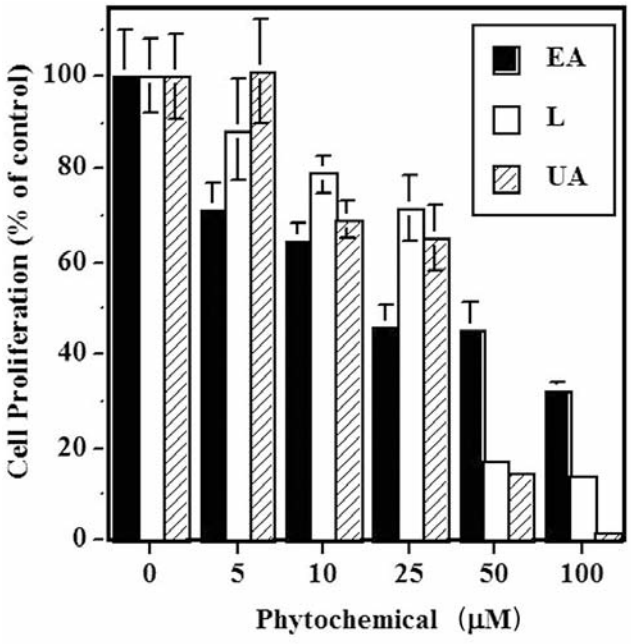


Figure 6. *Effect of individual pomegranate phytochemicals on cell proliferation. Subconfluent cells were treated with the indicated doses of individual phytochemicals for 72 h. n=4±s.e. There was a significant reduction in proliferation compared to the control at ≥25 μM of ellagic acid (EA), and ≥50 μM of luteolin (L) and ursolic acid (UA) ($p<0.05$).*

Effect of PE in comparison to paclitaxel on cell proliferation. We compared the ability of PE and the chemotherapeutic drug paclitaxel to inhibit cell growth. As seen in Figure 5A, compared to paclitaxel, PE had a more profound impact on cell growth. PE resulted in an almost complete inhibition of cell growth, while even high concentrations of paclitaxel did not completely prevent proliferation. Furthermore, the inhibitory effect of PE was more rapid than that of paclitaxel (Figure 5B).

Effect of pomegranate phytochemicals on cell proliferation and cytotoxicity. The effects of the predominant phytochemicals present in PE on PANC-1 proliferation were examined. Ellagic acid, luteolin, and ursolic acid caused a concentration-dependent decrease in PANC-1 cell proliferation (Figure 6). Combinations of individual phytochemicals were no more effective than equivalent concentrations of a single agent (data not shown).

Discussion

Patients with pancreatic cancer face a grim prognosis, as it is highly aggressive and refractory to chemotherapeutics. New treatments are therefore needed. Dietary fruits and vegetables are proving to be an ample source of alternative agents for

the treatment of many types of cancer. Phytochemicals, the naturally occurring chemicals in fruits and vegetables, have been shown to be effective anticancer agents in laboratory studies and are well tolerated (10). Pomegranate, being rich in phytochemicals, has been of great interest for the treatment of cancer, and many studies have demonstrated its anticancer effects in both *in vitro* and *in vivo* experiments on various cancer types, particularly of the breast, prostate, and lung (4-8). However, there have been no published studies on pomegranate and pancreatic cancer, and only a few studies on the effect of phytochemicals on pancreatic cancer (11-14). Therefore, in the present study, we examined the effect of PE on human pancreatic cancer cells *in vitro*. PE is a standardized extract of pomegranate that is commercially available as a dietary supplement and is certified "generally regarded as safe" by the Food and Drug Administration (USA). The extract is standardized by HPLC to contain no less than 70% total polyphenolic compounds, with a composition similar to whole pomegranate, and these polyphenolic compounds were shown to be bioavailable in humans following ingestion (15). The main cell line used in our experiments was the human PANC-1 pancreatic cancer cell line. This cell line was derived from a primary pancreatic ductal carcinoma from a 56-year-old male Caucasian patient and has been widely used as an *in vitro* model of pancreatic cancer (11-13).

PE caused a profound cell cycle arrest and prevented the proliferation of pancreatic cancer cells (Figures 1 and 2). PE was only mildly cytotoxic to quiescent cells; high concentrations were needed to achieve a significant reduction in viability (Figure 3) and PE did not induce apoptosis (data not shown). These data demonstrate that PE acts primarily by inhibiting cell cycle progression, and not through the induction of apoptosis. To ensure that these results were not cell type-specific, we repeated most experiments using another human pancreatic cancer cell line, the AsPC-1 line. These cells were derived from an ascites metastasis of a primary pancreatic adenocarcinoma from a female. AsPC-1 cells were equally sensitive to PE, with approximately the same IC_{50} for both proliferation and cytotoxicity. These results indicate that the potentially efficacious activities of PE against pancreatic cancer are not cell-type specific.

The stem cell hypothesis of cancer is that there is a population of cells in tumors that are long-lived, highly resistant to chemotherapeutics, and possess the capacity of self-renewal. First isolated in leukemia and later in solid tumors such as breast cancer, cancer stem cells are thought to also underlie pancreatic cancer (16). Research has determined that side populations of the PANC-1 cell line exhibit the attributes of stem cells, and this side population can be identified by the expression of the cell surface markers CD44 and CD24 (17-19). PANC-1 cells that express CD44 and CD24 are highly tumorigenic in nude mice, a hallmark of stemness. Indeed, $CD44^-/CD24^-$ PANC-1 cells fail to cause tumors unless a very high number of cells are injected (17, 18). Thus, the $CD44^+/CD24^+$ pancreatic cancer cell is a critical target for the treatment of pancreatic cancer. Figure 4 shows that following treatment with PE, the proportion of PANC-1 cells lacking expression of these stem cell markers was greatly increased. These results demonstrate that PE preferentially targets the most tumorigenic population of cells in the PANC-1 cell line and suggests that PE would reduce the tumorigenicity of PANC-1 cells. The mechanism underlying this effect is currently unknown. This result extends our recent finding that PE targets mouse mammary cancer stem cells (20).

Chemotherapeutic drugs are largely ineffective against pancreatic cancer. The reasons for this are not understood, but may result from the presence of multidrug-resistant cancer stem cells which are readily able to repopulate tumors once more differentiated cancer cells are eradicated by the drug. We decided to compare the efficacy of PE to that of paclitaxel, a mitotic inhibitor that has recently been studied for efficacy against pancreatic cancer (21, 22). Compared to a clinical achievable concentration of paclitaxel (23), PE caused a more profound decrease in cell proliferation: >90% inhibition compared to a maximal inhibition of 60% for paclitaxel, and this inhibition occurred more quickly than that with paclitaxel (Figure 5 A and B).

The anticancer effects of pomegranate have been ascribed to ellagic acid, a metabolite of the ellagitanins, an abundant class of phytochemicals in pomegranate. Other active polyphenolic constituents that have been shown to possess anticancer activities include ursolic acid (24) and luteolin (25). We therefore examined the effect of these phytochemicals alone and in combination on proliferation of PANC-1 cells. All three of these phytochemicals inhibited the proliferation of PANC-1 cells (Figure 6). However, the concentrations required for significant inhibition were in excess of their concentration in PE (15). This suggests that combinations of pomegranate phytochemicals are responsible for inhibition; however, we observed no greater inhibitory activity when we tested various combinations of these three phytochemicals (data not shown). Thus, the component of PE that is active against pancreatic cancer cells is not known, but is probably not any of the identified constituents that are commonly studied for their anticancer activity.

To summarize, our present study establishes PE, for the first time, as a potent inhibitor of the growth of human pancreatic epithelial cancer *in vitro* with a greater efficacy than current chemotherapeutics. Our data indicates that PE acts through mechanisms that target cell cycle progression. Furthermore, PE targets those subpopulations of cells in heterogeneous tumors most likely to cause a recurrence of the tumor, the pancreatic cancer stem cell.

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