Cell Cycle Arrest by the Isoprenoids Perillyl Alcohol, Geraniol, and Farnesol Is Mediated by p21Cip1 and p27Kip1 in Human Pancreatic Adenocarcinoma Cells

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Received July 28, 2006; accepted November 28, 2006

ABSTRACT

Pancreatic cancer, the fourth leading cause of cancer-associated mortality in the United States, usually presents in an advanced stage and is generally refractory to chemotherapy. As such, there is a great need for novel therapies for this disease. The naturally derived isoprenoids perillyl alcohol, farnesol, and geraniol have chemotherapeutic potential in pancreatic and other tumor types. However, their mechanisms of action in these systems are not completely defined. In this study, we investigated isoprenoid effects on the cell cycle and observed a similar antiproliferative mechanism of action among the three compounds. First, when given in combination, the isoprenoids exhibited an additive antiproliferative effect against the three compounds. Furthermore, all three compounds induced a G0/G1 cell cycle arrest that coincided with an increase in the expression of the cyclin kinase inhibitor proteins p21Cip1 and p27Kip1 and a reduction in cyclin A, cyclin B1, and cyclin-dependent kinase (Cdk) 2 protein levels. Immunoprecipitation studies demonstrated increased association of both p21Cip1 and p27Kip1 with Cdk2 as well as diminished Cdk2 kinase activity after isoprenoid exposure, indicating a cell cycle-inhibitory role for p21Cip1 and p27Kip1 in pancreatic adenocarcinoma cells. When siRNA was used to inhibit expression of p21Cip1 and p27Kip1 proteins in MIA PaCa-2 cells, conditional resistance to all three isoprenoid compounds was evident. Given similar findings in this cell line and in BxPC-3 human pancreatic adenocarcinoma cells, we conclude that the chemotherapeutic isoprenoid compounds perillyl alcohol, farnesol, and geraniol invoke a p21Cip1- and p27Kip1-dependent antiproliferative mechanism in human pancreatic adenocarcinoma cells.

Perillyl alcohol (POH), geraniol (GOH), and farnesol (FOH) (Fig. 1) are plant-derived isoprenoid compounds (Elson and Yu, 1994; Craig, 1999). Dietary sources of perillyl alcohol include cherries, spearmint, sage, and celery seeds. Examples of geraniol dietary sources include carrot, lemon, lime, nutmeg, orange, blueberry, and blackberry. Farnesol is found in lemongrass and chamomile. Certain plants, such as lavender, lemongrass, and rosemary, are sources for more than one isoprenoid. Each isoprenoid has chemopreventive and therapeutic activity in a wide variety of in vitro and in vivo cancer models, including pancreatic cancer, for which there is little therapeutic success in the clinic (Crowell, 1999). Perillyl alcohol has undergone several phase I and II trials and shown therapeutic potential with relatively mild adverse effects (Hudes et al., 2000; Bailey et al., 2002; Azzoli et al., 2003). However, the specific mechanisms of action for perillyl alcohol, farnesol, and geraniol in pancreatic cancer cells are not fully characterized. The elucidation of their mechanisms of action could lead to the development of more potent analogs and novel therapeutic targets, resulting in new and improved therapeutic outcomes for pancreatic cancer.

To date, a number of molecular and cellular effects of all three isoprenoids have been reported in vitro and in vivo. In addition to early findings that isoprenoids may inhibit post-translational protein prenylation (Crowell et al., 1991; Ren et al., 1997), reported effects include induction of G0/G1 cell cycle arrest (Yu et al., 1995; Miquel et al., 1998; Clark et al., 2002; Elegbede et al., 2003), increased apoptosis (Haug et al., 1994; Mills et al., 1995; Reddy et al., 1997; Stayrook et al., 1997; Ariazi et al., 1999; Rioja et al., 2000; Burke et al., 2002; Clark et al., 2002; Elegbede et al., 2003; Xu et al., 2004), transient G0/M phase arrest (Shi and Gould, 2002; Rajesh and Howard, 2003), inhibition of cholesterol biosynthesis (Ren and Gould, 1994; Peffley and Gayen, 2003), inhibition of angiogenesis (Lourati et al., 2004), and increased sensitization to radiation and anticancer drugs (Samaila et al., 2004).

This work was supported by the Public Health Service, National Institutes of Health (Grant 5R01-CA10012-MA00) and by the American Institute for Cancer Research (Grant 99B047 to P.L.C.).

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.106.111666.

ABBREVIATIONS: POH, perillyl alcohol; GOH, geraniol; FOH, farnesol; Cdk, cyclin-dependent kinase; CKI, cyclin kinase inhibitor; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; NFDM, nonfat dry milk.
Perillyl alcohol has been associated with up-regulation of the mannose-6-phosphate/insulin-like growth factor type II receptor, transforming growth factor-β signaling pathways, and up-regulation of proapoptotic BCL-2-related proteins (Stayrook et al., 1997; Ariazi et al., 1999). Geraniol has been shown to disrupt membrane permeability and inhibit polyamine biosynthesis (Carnesecchi et al., 2001, 2002). In vivo, all three isoprenoids have shown anticancer as well as chemopreventive efficacy for pancreatic, skin, esophageal, and mammary epithelial carcinomas (Crowell, 1999).

Regulation of the mammalian cell cycle involves coordination of DNA replication with the physical act of mitosis. Essential cell cycle regulatory events involve a family of serine/threonine kinases, the cyclin-dependent kinases (Cdks), in association with their regulatory subunits (cyclins) and other activating and/or inhibiting cofactors (Meyerson et al., 1992). Mitogenic stimulation of a cell induces it to progress through the G1 phase through the kinase activities of Cdk4 and/or Cdk6 in association with the D-type cyclins (Sherr, 1994). The transition from G1 to S phase is mediated in part by the up-regulation and activation of Cdk2 initially associated with cyclin E and, later, the A-type cyclins. One well characterized target of cyclin E-Cdk2 kinase activity is the phosphorylation of histone H1 proteins (Arion et al., 1988), resulting in relaxation of the chromosome protein scaffolding structure and facilitating access to DNA for replication purposes. However, this process may be interrupted in apparent stoichiometric fashion by an increased level of cyclin kinase inhibitor (CKI) proteins. Cells with elevated CKI expression typically arrest in G1 phase and/or undergo apoptosis. The two canonical CKI protein families include the INK4 and the Cip/Kip families of proteins (Sherr and Roberts, 1999). Both families are classified as tumor suppressor families because inactivation of the corresponding genes is associated with increased risk of many forms of cancer and diminished prognosis during treatment (Balu proportions, 1998; Arcellana-Panlilio et al., 2002). Unlike the Cip/Kip family, however, members of the INK4 family, and especially that of p16INK4a, are typically not functional in pancreatic adenocarcinomas due to either inactivating mutation or deletion (Ghiorzo et al., 2004).

In this study, we demonstrate that perillyl alcohol, farnesol, and geraniol share a similar mechanism of action in human pancreatic adenocarcinoma cells. In keeping with their known cancer chemopreventive and chemotherapeutic activities, these isoprenoids cause arrest in G1/G0 phase of the cell cycle through induction of cyclin kinase inhibitors p21Cip1 and p27kip1, resulting in a reduction in Cdk2 activity and decreased expression of downstream cell cycle-related proteins.
Immunoprecipitates were recovered by centrifugation and washed twice with PBS + protease inhibitors and twice with kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, and Complete Mini protease inhibitors). After the final wash, pellets were resuspended in kinase buffer to a final volume of 30 µl. To this suspension, 5 µCi of [γ-³²P]ATP (GE Healthcare, Little Chalfont, Buckinghamshire, UK), 20 mM (final concentration) ice-cold ATP (Roche), and 2 µg of histone H1 protein (Roche Diagnostics) were added, and suspensions were incubated at 30°C for 30 min with agitation every 10 min. The kinase reaction was terminated by adding 5× sample buffer. Samples were then boiled for 5 min and resolved on a 12% SDS-polyacrylamide gel electrophoresis gel (Bio-Rad). Incorporation of ³²P onto histone H1 was quantified using a PhosphorImager with ImageQuant software (GE Healthcare). The data displayed are indicative of four independent experiments.

To determine protein-protein interactions, samples were immunoprecipitated as above. Samples were washed four times in lysis buffer. Samples were then resuspended in lysis buffer and 5× sample buffer to a final volume of 35 µl and boiled for 5 min. Samples were analyzed for proteins of interest using Western blot analysis as described above.

**RNA Interference of p21Cip1 and p27Kip1.** MIA PaCa-2 cells were plated in 24-well plates at a density of 1×10⁵ cells/ml and incubated for 24 h in 0.5 ml of antibiotic-free DMEM. Media were then removed and exchanged for 100 µl of serum-free, antibiotic-free DMEM with either Lipofectamine 2000 (LF2000) alone or LF2000 in combination with 5 nM RNAi SmartPool (Dharmacon, Lafayette, CO) against p21Cip1, p27Kip1, a combination (5 nM total) of p21Cip1 and p27Kip1, or a mixture (5 nM total) of scrambled RNAi sequences matched for nucleotide content to the proprietary sequences of each SmartPool. After overnight incubation, LF2000 media were exchanged for antibiotic-free DMEM, and cells were allowed to incubate for an additional 24 h. At this time, three random samples of each 24-well plate were trypsinized and counted to determine cell population at beginning of therapy time course (time = 0 h). Remaining wells were randomly assigned to either control (normal DMEM)
or isoprenoid (300 and 600 μM POH, 200 and 400 μM GOH, and 60 and 90 μM FOH) groups (n = 3). After 24 h of incubation, all samples were trypsinized, washed in PBS, counted, and analyzed by Western analysis for expression of p21Cip1 p27Kip1, and cyclin A as described previously.

Results
Perillyl Alcohol, Farnesol, and Geraniol Inhibit Pancreatic Adenocarcinoma Cell Growth. Subconfluent MIA PaCa-2 and BxPC-3 human pancreatic adenocarcinoma cells were exposed to various concentrations of perillyl alcohol, farnesol, and geraniol for 24 h. Cell proliferation was inhibited in a dose-dependent manner, and statistically significant (P ≤ 0.05) inhibition was achieved at all concentrations higher than 300 μM perillyl alcohol, 20 μM farnesol, and 200 μM geraniol, respectively, in MIA PaCa-2 cells (Fig. 2A), and 500 μM perillyl alcohol, 60 μM farnesol, and 400 μM geraniol, respectively, in BxPC-3 cells (Fig. 2B).

Simultaneous Combination of Isoprenoids Results in an Additive Antiproliferative Effect. To begin to test whether the isoprenoids had similar or different mechanisms of action, we performed cell proliferation assays with isoprenoid drug combinations, assuming that combinations of compounds with similar mechanisms would be additive in nature. With all isoprenoid combinations, exposure to two drugs at low concentration resulted in an antiproliferative effect equal to that of the single-agent in higher concentration (Fig. 2C), suggesting that the isoprenoid combinatorial effects were additive in nature.

Perillyl Alcohol, Farnesol, and Geraniol Arrest Pancreatic Cancer Cells in G1 Phase of the Cell Cycle. Based on our prior observation that perillyl alcohol and farnesol decrease DNA replication in pancreatic epithelial cells (Burke et al., 2002), we tested the hypotheses that isoprenoid-induced inhibition of pancreatic cancer proliferation would inhibit G1 to S phase progression through the cell cycle. Therefore, we analyzed MIA PaCa-2 cell-cycle phase distribution using propidium iodide staining and flow-cytometric analysis after a 24-h exposure to varying doses of each isoprenoid. Isoprenoid-treated pancreatic cancer cells exhibited dose-dependent increases in the percentage of cells in the G0/G1 phase of the cell cycle and decreases in the percentage of cells present in either S or G2/M phases (Figs. 3, A and B).

Isoprenoid Effects on Cyclin, Cdk, and Cdk Inhibitor Proteins in MIA PaCa-2 Cells. From our observations that perillyl alcohol, farnesol, and geraniol induce G1 arrest in human pancreatic cancer cells, we hypothesized that they would either reduce the level of G1 cyclins D and/or E or would increase the expression of G1 CKIs, such as p21Cip1, p27Kip1, or p57Kip2. To test this hypothesis, we compared treated and untreated subconfluent populations of MIA PaCa-2 cells exposed for 24 h to each isoprenoid and measured cyclin and CKI protein expression relative to β-actin protein level by Western blot analysis. Treatment of human pancreatic adenocarcinoma cells with perillyl alcohol, farnesol, or geraniol resulted in significant increases in the expression of the cyclin-dependent kinase inhibitor proteins p21Cip1 and p27Kip1 as well as reductions in the levels of cyclin A, cyclin B1, and cdk2 (Fig. 4). In addition, farnesol and geraniol reduced cyclin D1 protein levels in MIA PaCa-2 cells but not

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Fig. 3. Isoprenoid exposure causes a G0/G1 phase cell cycle arrest in MIA PaCa-2 cells. MIA PaCa-2 cells were exposed for 24 h to media containing perillyl alcohol (0–800 μM), farnesol (0–90 μM), or geraniol (0–800 μM). Representative histograms for each experimental group are displayed in A, and the resulting cell cycle distribution percentages are given in B. Error bars, mean ± S.E.M. * P ≤ 0.05 versus untreated controls (n ≥ 5).

Increased Association of Cyclin-Dependent Kinase Inhibitor Proteins p21Cip1 and p27Kip1 with Cdk2 following Isoprenoid Treatment. Because isoprenoids increased the expression of p21Cip1 and p27Kip1 CKI proteins, we hypothesized that the increases in CKI protein concentration would result in greater CKI association with cell cycle-promoting kinases such as Cdk2. Indeed, we found that, in the presence of perillyl alcohol, farnesol, or geraniol, significantly greater amounts of p21Cip1 and p27Kip1 coimmunoprecipitated with Cdk2 protein in situ (Fig. 5).

Inhibition of Cdk2 Kinase Activity by Isoprenoid Treatment in MIA PaCa-2 Cells. Based on the isoprenoid-induced elevation in the levels of p21Cip1 and p27Kip1 and the increased association of these CKIs with Cdk2, we hypothesized that Cdk2 activity would be reduced in response to isoprenoid treatment. Cdk2 exhibited a significant decrease in enzymatic activity in pancreatic adenocarcinoma cells
treated with isoprenoids at concentrations consistent with those that caused increases in p21Cip1 and p27Kip1 protein levels and increased association with Cdk2 (Fig. 6).

RNA Interference of p21Cip1 and p27Kip1 Causes Conditional Resistance to Isoprenoids in MIA PaCa-2 Cells. Given the previous data, we hypothesized that human pancreatic cancer cells could be rescued from the antiproliferative effects of perillyl alcohol, farnesol, and geraniol through reduction of intracellular protein concentrations of p21Cip1 and p27Kip1. Using commercially available small interfering RNA “pools,” significant reductions in both proteins were achieved. However, it was important during this study not to completely eliminate the expression of either protein from the cells because it has been shown that basal levels of p21Cip1 and p27Kip1 are required for proper assembly of promitogenic cyclin-cdk complexes (Cheng et al., 1999; Sherr and Roberts, 1999). Therefore, we used RNAi concentrations that significantly reduced but did not entirely eliminate p21Cip1 and p27Kip1 protein expression (Fig. 7). In the presence of perillyl alcohol, farnesol, or geraniol, simultaneous RNA interference of p21Cip1 and p27Kip1 CKI proteins resulted in significant protection from the isoprenoid antiproliferative effect (Fig. 7), whereas no such protection occurred in untransfected, single-gene, or scrambled RNAi-treated cells. Thus, p21Cip1 and p27Kip1 serve complimentary roles as inhibitors of cell-cycle progression in pancreatic cancer cells treated with the isoprenoids perillyl alcohol, farnesol, or geraniol.

Discussion

We demonstrate with these data that the chemically related isoprenoid compounds perillyl alcohol, farnesol, and geraniol act though a similar antiproliferative mechanism to cause G1 arrest in human pancreatic adenocarcinoma cells. All three isoprenoids cause cell cycle arrest in a dose-dependent manner at concentrations that are pharmacologically relevant in humans (Hudes et al., 2000; Bailey et al., 2002; Azzoli et al., 2003). The pharmacological effect of these antitumorigenic isoprenoids on the cell cycle entails increases in the expression of the cyclin kinase inhibitor proteins p21Cip1 and p27Kip1 and enhancement of their asso-

Fig. 4. Isoprenoid-induced changes in cell cycle-associated protein expression. MIA PaCa-2 (A–D) and BxPC-3 (E and F) human pancreatic adenocarcinoma cells were treated for 24 h with perillyl alcohol (0–800 μM), farnesol (0–90 μM), or geraniol (0–800 μM). After treatment, protein lysates were analyzed by immunoblotting. A, average expression (n ≥ 5) of each protein relative to the untreated control in MIA PaCa-2 cells, normalized to 100%. Statistically significant differences are indicated in boldface type. Representative immunoblots are shown for MIA PaCa-2 cells treated with perillyl alcohol (B), farnesol (C), and geraniol (D), and BxPC-3 cells exposed to all three isoprenoids (F).
interactions with cyclin-E/Cdk2 protein complexes. These interactions, in turn, seem to be responsible for the isoprenoid-induced, dose-dependent decrease in Cdk2 activity we observed (Fig. 6). Furthermore, these isoprenoids, when administered in simultaneous combination, exhibit additive rather than synergistic or antagonistic effects. All of these findings argue that perillyl alcohol, farnesol, and geraniol act through a similar mechanism of action in pancreatic cancer cells. Furthermore, this mechanism seems to be K-ras mutation status-independent because similar cell-cycle effects were observed in MIA PaCa2 cells, which harbor a constitutively active, oncogenic K-ras mutation typical of the majority of human pancreatic adenocarcinomas, and in BxPc-3 cells, which have wild-type K-ras genes.

In this study, we observed that perillyl alcohol, farnesol, and geraniol caused dose-dependent decreases in cyclins A and B1, and Cdk2 protein levels in pancreatic adenocarcinoma cells, consistent with their ability to arrest the cell cycle in the G1 phase. After mitogenic induction, cells arise from a quiescent G0 phase, entering G1 phase typically with an increase in protein levels of the D-type cyclins, Cdk4 and Cdk6. It is believed that the resulting Cdk4-cyclin D and Cdk6-cyclin D holoenzyme complexes assemble and function to promote cell-cycle progression. These complexes translocate to the nucleus, are phosphorylated by a CDK-activating kinase, and become catalytically active, phosphorylating serine and threonine residues on target proteins, including the transcriptional repressor and tumor suppressor protein Rb (Chen et al., 1989). Once phosphorylated, Rb disassociates from its binding partners, including the E2F transcription factor, which is now free to activate transcription of genes necessary for DNA synthesis. The genes for cyclins E and A are both believed to be regulated by E2F (Nevins et al., 1991). A second proposed function of the cyclin D-Cdk4/6 complexes is the sequestration of CKIs, including gp21Cip1 and p27Kip1, from binding to and inhibiting cyclin E-Cdk2 and cyclin A-Cdk4 complexes (Sherr and Roberts, 1999). Furthermore, it is proposed that these CKIs act as a “biological rheostat” in that any signal that causes a net protein concentration increase of p21Cip1 and p27Kip1 allows them to overwhelm the sequestration function of cyclin D-Cdk4/6; thus, unsequestered p21Cip1 and p27Kip1 are free to inhibit the Cdk2 complexes, ultimately halting the progression of the cell cycle. Significant effects on the protein level, CKI association, and kinase activity of Cdk2 were observed after isoprenoid treatment, whereas preliminary experiments indicated that the isoprenoids did not affect Cdk4 and Cdk6. The isoprenoid-induced increases in p21Cip1 and p27Kip1 proteins associating with Cdk2, the decreased kinase activity of Cdk2, and the attenuation of the isoprenoid antiproliferative effects in the presence of p21Cip1 and p27Kip1 siRNAs argue for a p21Cip1- and p27Kip1-dependent isoprenoid mechanism in pancreatic adenocarcinoma cells. In addition to the data presented here, similar G1 arrest has been noted in colorectal and mammary
carcinoma cell lines treated with perillyl alcohol (Karlson et al., 1996; Reddy et al., 1997; Bardon et al., 1998; Ariazi et al., 1999; Shi and Gould, 2002). As such, it is increasingly clear that one of the primary in vitro mechanisms of action of perillyl alcohol, farnesol, and geraniol is inducing cell-cycle arrest in the G0/G1 phase.

The mechanisms by which perillyl alcohol, farnesol, and geraniol induce p21Cip1 and p27Kip1 protein expression in pancreatic cancer cells is not currently known and will be the subject of future investigation. Also not known are the reasons behind the differences in potency of these three compounds. As such, several possibilities exist, including differences in binding efficiency to an as-of-yet unidentified isoprenoid receptor or that perhaps farnesol can interact with a greater number of receptors than do geraniol or perillyl alcohol. Farnesoid receptors, of which farnesol is one identified ligand, have been identified and characterized (Kozak et al., 1996), but it is not known whether geraniol and/or perillyl alcohol are ligands for this receptor. Our finding that perillyl alcohol was the only isoprenoid of the three we studied that did not down-regulate cyclin D1 expression in MIA PaCa-2 cells argues for the possibility that differing abilities to modulate cell cycle-associated proteins as an explanation. However, in BxPC-3 cells, both perillyl alcohol and geraniol exposure caused increased cyclin D1 protein expression, with no apparent difference in antiproliferative potency relative to MIA PaCa-2 cells.

In summary, the data presented here strongly suggest that isoprenoids, as well as other pharmacological agents that induce p21Cip1 and p27Kip1 expression, may have chemother-

Fig. 7. RNAi-mediated down-regulation of p21Cip1 and p27Kip1 attenuates isoprenoid effects on pancreatic cancer cell proliferation. MIA PaCa-2 cells were treated with 0- to 5-nm RNAi pools of either p21Cip1 or p27Kip1, and protein extracts were immunoblotted. The RNAi effect was concentration dependent (A), and down-regulation of both CKI proteins was shown to be independent of isoprenoid exposure (B and C). Cell proliferation assays after RNAi treatment with either single-gene pools or in combination (for 5 nM total RNAi concentration) showed resistance for all three compounds (D–F) relative to control cells treated only with Lipofectamine (LF2000). As a control for potential RNAi-mediated toxicity, pools of RNAi oligomers with equal nucleotide content for both p21Cip1 and p27Kip1 pools were included (Scramble). Error bars, ± SEM. *, P < 0.05 versus LF2000 controls (n ≥ 4).
apeutic activity toward pancreatic cancer. This CKI induc-
tion could be used in the screening and development of more
potent isoprenoid or other compounds for the chemotherapy
of pancreatic cancer, which remains recalcitrant to available
systemic therapies.

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Received for publication June 28, 2004; accepted for publication August 19, 2004.

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