Chemoprevention by perillyl alcohol coupled with viral gene therapy reduces pancreatic cancer pathogenesis

Irina V. Lebedeva,[1] Zhao-zhong Su,[3] Nicholaq Vozhilla,[3] Lejuan Chatman,[1] Devanand Sarkar,[3,5,6] Paul Dent,[4,5,6] Mohammad Athar,[2] and Paul B. Fisher[3,5,6]

Departments of [1]Urology and [2]Dermatology, Herbert Irving Comprehensive Cancer Center, Columbia University, College of Physicians and Surgeons, New York, New York; and Departments of [3]Human and Molecular Genetics and [4]Biochemistry and Molecular Biology, [5]VCU Institute of Molecular Medicine, and [6]Massey Cancer Center, Virginia Commonwealth University, School of Medicine, Richmond, Virginia

Abstract

Pancreatic cancer is one of the deadliest of cancers. Even with aggressive therapy, the 5-year survival rate is < 5%, mandating development of more effective treatments. Melanoma differentiation–associated gene-7/interleukin-24 (*mda*-7/IL-24) shows potent antitumor activity against most cancers displaying safety with significant clinical efficacy. However, pancreatic cancer cells display inherent resistance to *mda*-7/IL-24 that is the result of a ''protein translational block'' of *mda*-7/IL-24 mRNA in these tumor cells. We now show that a dietary supplement perillyl alcohol (POH) has significant chemopreventive effects for pancreatic cancer and, when coupled with adenovirus-mediated *mda*-7/IL-24 gene therapy (Ad.*mda*-7), effectively eliminates s.c. and i.p. xenografts of human pancreatic cancer cells in nude mice, promoting enhanced survival. The combination of POH and Ad.*mda*-7 efficiently abrogates the *mda*-7/IL-24 protein translational block, resulting in MDA-7/IL-24 protein production and growth suppression. Of direct translational relevance, clinically achievable concentrations of POH with Ad.*mda*-7, both of which have been found safe and without toxic effects in human trials, were used. This novel and innovative approach combining a dietary agent and a virally delivered therapeutic cytokine provides a means of both preventing and treating human pancreatic cancer with significant clinical translational potential. [Mol Cancer Ther 2008;7(7):2042–50]

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Requests for reprints: Paul B. Fisher, Department of Human and Molecular Genetics, Massey Cancer Center, Virginia Commonwealth University, School of Medicine, 1101 East Marshall Street, Sanger Hall Building, Room 11-015, Richmond, VA 23298-0033. Phone: 804-828-9632; Fax: 804-827-1124. E-mail: pbfisher@vcu.edu

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Introduction

Approximately 37,000 new cases of pancreatic ductal adenocarcinomas are diagnosed in the United States with virtually the same number of deaths annually, making it one of the most lethal cancers. In <20% of patients with early-stage detection, surgical resection allows 5-year survival (1–4). However, in the majority of patients, the overall 5-year survival is <5% attributed to a plethora of molecular changes causing resistance to chemotherapy and radiotherapy further compounded by lack of approaches targeting metastatic disease. Considering these appalling statistics, it is imperative to develop rational molecular target-based preventive and therapeutic strategies for this fatal disease.

Melanoma differentiation–associated gene-7/interleukin-24 (*mda*-7/IL-24; ref. 5) is a secreted cytokine showing broad-spectrum cancer-specific, apoptosis-inducing activities (6–11). It inhibits tumor angiogenesis, stimulates an antitumor immune response, sensitizes cancer cells to radiation, chemotherapy, small-molecule inhibitors, and therapeutic monoclonal antibodies, and manifests a potent ''bystander'' antitumor activity (6–11). The observation that *mda*-7/IL-24 does not harm normal cells prompted *in vivo* studies using several human tumor xenograft murine models that confirmed potent selective antitumor activity of this cytokine (6–11). A recent phase I clinical trial using a replication incompetent adenovirus expressing *mda*-7/IL-24 (Ad.*mda*-7; INGN 241) in patients with advanced carcinomas and melanoma was highly promising, confirming the retention of tumor-specific activity in patients (12–15). More impressively, Ad.*mda*-7 was well tolerated and showed no adverse effects in these patients. Investigations are now in progress to further evaluate the efficacy of *mda*-7/IL-24 for cancer gene therapy in phase II clinical trials.

Pancreatic cancer cells are unique, displaying inherent resistance to *mda*-7/IL-24–induced apoptosis induction (16–18). The underlying mechanism is a ''protein translational block'' causing limited conversion of *mda*-7/IL-24 mRNA into protein that could be reversed by inhibiting K-*ras* or its downstream signaling in mutant K-*ras* pancreatic cancer cells as well as by augmented generation of reactive oxygen species in pancreatic cancer cells

irrespective of their K-*ras* status (16–18). These combinatorial treatments that result in functional MDA-7/IL-24 protein production correlate with apoptosis induction.

The present studies were promulgated on the hypothesis that specific nontoxic dietary agent(s) with reactive oxygen species–inducing properties might cooperate with Ad.*mda*-7 and be safer for human use with potential to diminish the pathogenesis of pancreatic cancer. We chose perillyl alcohol (POH), a dietary monoterpene found in a variety of plants, including citrus plants, for evaluation. POH blocks several signaling pathways, such as *ras*, extracellular signal-regulated kinase, and nuclear factor-κB, and prevents the isoprenylation of the Ras family of small GTPase proteins (19–23). The pharmacokinetics of POH has been defined in both murine models and humans (24, 25) and phase I and II clinical trials have revealed that POH is well tolerated (23, 26). We now confirm for the first time that POH has significant chemopreventive effects for pancreatic cancer, and a chemoprevention plus gene therapy (CGT) protocol, involving a combination of POH with Ad.*mda*-7, efficiently eliminated both s.c. and i.p. xenografts of human pancreatic cancer cells in nude mouse models and significantly prolonged the disease-free survival of tumor-bearing animals. These exciting findings reveal a new CGT combinatorial therapeutic paradigm for both preventing and treating pancreatic cancer with potential to rapidly and effectively translate into the clinic.

Materials and Methods

Cell Lines and Virus Infection

AsPC-1, MIA PaCa-2, PANC-1, and BxPC-3 pancreatic carcinoma cells and immortalized human melanocyte FM516-SV, human prostate epithelial cell P69, human fetal astrocyte IM-PHFA, and human pancreatic mesenchymal cell LT2 were cultured as described (16–18, 27, 28). Stable clones of PANC-1, AsPC-1, and MIA PaCa-2 cells expressing *mda*-7/IL-24 mRNA were established as described (17). Ad.*mda*-7 was created and plaque purified as described (29). Cells were infected with 100 plaque-forming units per cell of Ad.*vec* or Ad.*mda*-7 (50 plaque-forming units per cell of each virus) and analyzed as described (16).

RNA Isolation and Northern Blot Assays

Total RNA was extracted from cells using the Qiagen RNeasy Mini kit (Qiagen) according to the manufacturer's protocol and used for Northern blotting using a radiolabeled *mda*-7/IL-24 cDNA probe as described (18).

Cell Viability Assays

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described (16).

Preparation of Cell Extracts and Western Blotting Analysis

Cell extraction, preparation, and Western blot analysis were done as described (16). For all Western blots, equal loading of protein was verified by reblotting of membranes for EF-1α protein (16).

Tumorigenesis Assays

Athymic nude mice were injected s.c. in both flanks with pancreatic cancer cells (2×10^6). The mice were injected by the i.p. route daily with vehicle or POH (75 mg/kg/5 mL in tricaprylin).

For therapy studies, athymic nude mice were separated into three groups, receiving the vehicle (tricaprylin) or POH starting at different times as described in Results. Human pancreatic cancer xenografts were established in mice by injecting 2×10^6 tumor cells s.c. When the tumor reached a size of ~ 100 mm^3, in each group, the animals were randomized into subgroups ($n = 5$ animals per subgroup) and gene therapy treatment was initiated. For the treatment, 100 μL of sterile PBS or sterile PBS containing 10^{10} virus particles of Ad.*vec* or Ad.*mda*-7 were injected intratumorally. The injection protocol was as follows: thrice weekly for the first week and then twice weekly for 2 wk. Animals were monitored for tumor progression, and tumor volume was determined. Statistical significance was evaluated with the Student's t test using the computer program GraphPad Prism (GraphPad Software, Inc.). Tumor measurements were recorded weekly, and tumor volumes were calculated by the formula V (mm^3) = $\pi \times A \times B^2/6$, where A is the largest dimension and B is the perpendicular diameter (29). At indicated times, some animals were opened and tumors were excised, weighed, and fixed for histologic evaluation. Antitumor efficacy data are presented as average tumor volumes and tumor weights for all animals in each group.

For the i.p. model, athymic nude mice were injected i.p. with 1×10^7 AsPC-1 cells. At indicated time points, some animals were terminated, the abdominal regions were opened, and visible tumors were excised, weighed, and fixed for histologic evaluation. Antitumor efficacy data are presented as the average tumor volumes and tumor weights for all animals in each group.

Histopathology

Tissue samples were fixed in 10% formalin (24 h), embedded in paraffin, sectioned (5 μm), and stained with H&E to analyze tissue morphology. Alternatively, samples were blocked with 10% normal goat serum in 1% bovine serum albumin-PBS before staining with anti-CD31/PECAM-1 (1:100; BD Biosciences-PharMingen). Apoptosis in paraffin-embedded sections was revealed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining using an In Situ Cell Detection kit (Roche Diagnostics) according to the manufacturer's instructions.

Statistical Analysis

All of the experiments were done at least thrice. Results are expressed as mean ± SE. Statistical comparisons were made using an unpaired two-tailed Student's t test. A P value of <0.05 was considered significant.

Results and Discussion

Combinatorial Treatment with Ad.*mda*-7 and POH Induces Growth Inhibition in Pancreatic Cancer Cell Lines *In vitro*

In this study, we selected various aggressive well-characterized and established pancreatic cancer cell lines

Table 1. Sensitivity of various human normal immortalized and pancreatic carcinoma cell lines to POH

	FM-516SV	IM-PHFA	P69	LT2	PANC-1	MIA PaCa-2	AsPC-1	BxPC-3
IC_{50}	750	600	>800	>800	350	350	300	550

NOTE: Cells were treated with different concentrations of POH, and their viability was assessed by MTT assays 72 h after treatment, as described in Materials and Methods. Based on these data, concentrations of POH (in μmol/L) that cause ~50% inhibition of cell growth (IC_{50}) were determined for each cell line. FM516-SV, SV40 T antigen–immortalized normal human melanocytes; IM-PHFA, H-TERT–immortalized primary human fetal astrocytes; P69, SV40-immortalized normal human prostate epithelial cells; LT2, immortalized human pancreatic mesenchymal cells; PANC-1, MIA PaCa-2, AsPC-1, and BxPC-3, human pancreatic adenocarcinoma cell lines.

carrying both wild-type and mutant K-*ras* to investigate whether a combinatorial treatment with Ad.*mda*-7 and POH induces growth inhibition in these cells in culture. We also included several immortalized cells of diverse origins in addition to those of pancreas to test whether this treatment differentially affects normal versus cancer cells. First, we tested the concentration-dependent effects of POH on the viability of pancreatic carcinoma cells as well as immortalized normal cells using MTT assays. These data reveal that pancreatic carcinoma cells are significantly more sensitive to POH than the immortalized normal cells irrespective of their origin. The IC_{50} values for pancreatic cancer cells ranged between 300 and 500 μmol/L, whereas these were found to be >600 μmol/L for immortalized normal cells (Table 1). Based on these data, we chose a concentration of 200 μmol/L for all further experiments as this concentrations was well tolerated by these cells and did not manifest any apparent toxicity. Importantly, it has been shown that in clinical trials, humans receiving low, nontoxic doses of POH (1.6–2.1 g/m^2) have similar serum concentration of perillic acid, a major metabolite of POH. The effects of treatment of POH and Ad.*mda*-7 alone or in combination on pancreatic carcinoma cells or immortalized normal cells were studied up to 6 days following their administration. In this study, there was no change in the growth of normal immortalized cells in any of the treatment groups. Treatment with POH or Ad.*mda*-7 alone did not affect the viability of pancreatic cancer cells; however, the viability of carcinoma cells was significantly reduced in the treatment groups receiving a combination of Ad.*mda*-7 and POH. The cell death recorded by day 6 was between 50% and 75% and was independent of K-*ras* mutation status of the cell line (Fig. 1A).

MDA-7/IL-24 protein expression was detected in pancreatic cancer cells receiving a combinatorial treatment of POH and Ad.*mda*-7, but not either agent alone, indicating that POH treatment overrides the inherent ''translational block of *mda*-7/IL-24 mRNA'' observed in pancreatic cancer cells (Fig. 1B). These results were similar in pancreatic carcinoma cells irrespective of carrying wild-type or mutant K-*ras* and our treatment protocol did not alter the expression level of p21 K-RAS (data not shown).

Evaluating Chemoprevention and Therapeutic Effects of POH Alone or in Combination with Ad.*mda*-7 in Murine Models

Our initial *in vitro* findings prompted us to assess potential *in vivo* efficacy of an Ad.*mda*-7 and POH combinatorial approach to block the growth of human pancreatic carcinoma cells. We used two different xenograft models in athymic nude mice: s.c. and a quasi-orthotopic i.p. model.

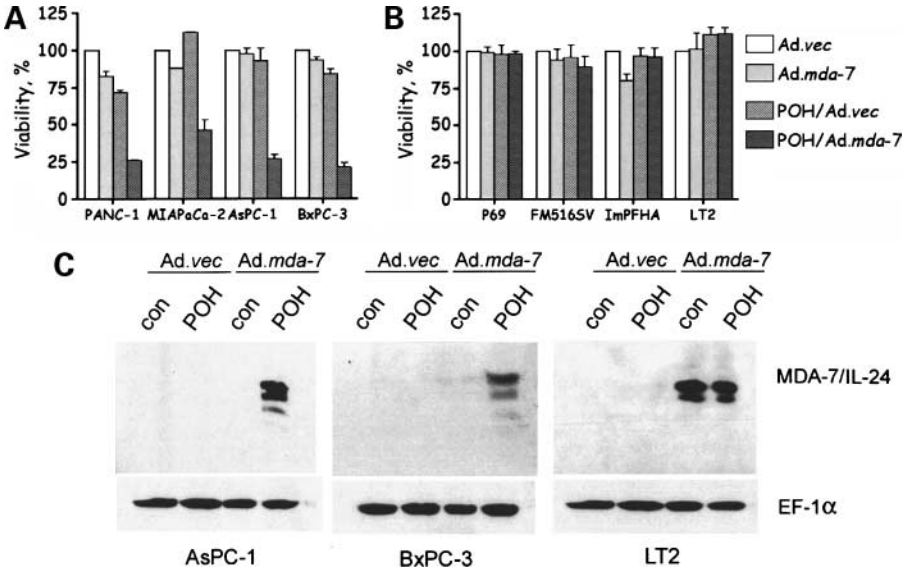

Figure 1. Combination of Ad.*mda*-7/IL-24 with POH efficiently inhibits *in vitro* growth of pancreatic cancer cells irrespective of their K-*ras* genotype without affecting immortal normal human cells. Cells were seeded as described in Materials and Methods and the next day infected with Ad.*vec* or Ad.*mda*-7 at 50 plaque-forming units per cell. Three hours later, POH or vehicle (DMSO) was added. **A,** viability of pancreatic cancer cells was measured by MTT assays 6 d after treatment. **B,** viability of normal immortal human cell lines was measured by MTT assays 6 d after treatment. **C,** MDA-7/IL-24 protein production was analyzed in AsPC-1 cells by Western blot analysis. Protein samples were collected in radioimmunoprecipitation assay buffer 48 h after treatment.

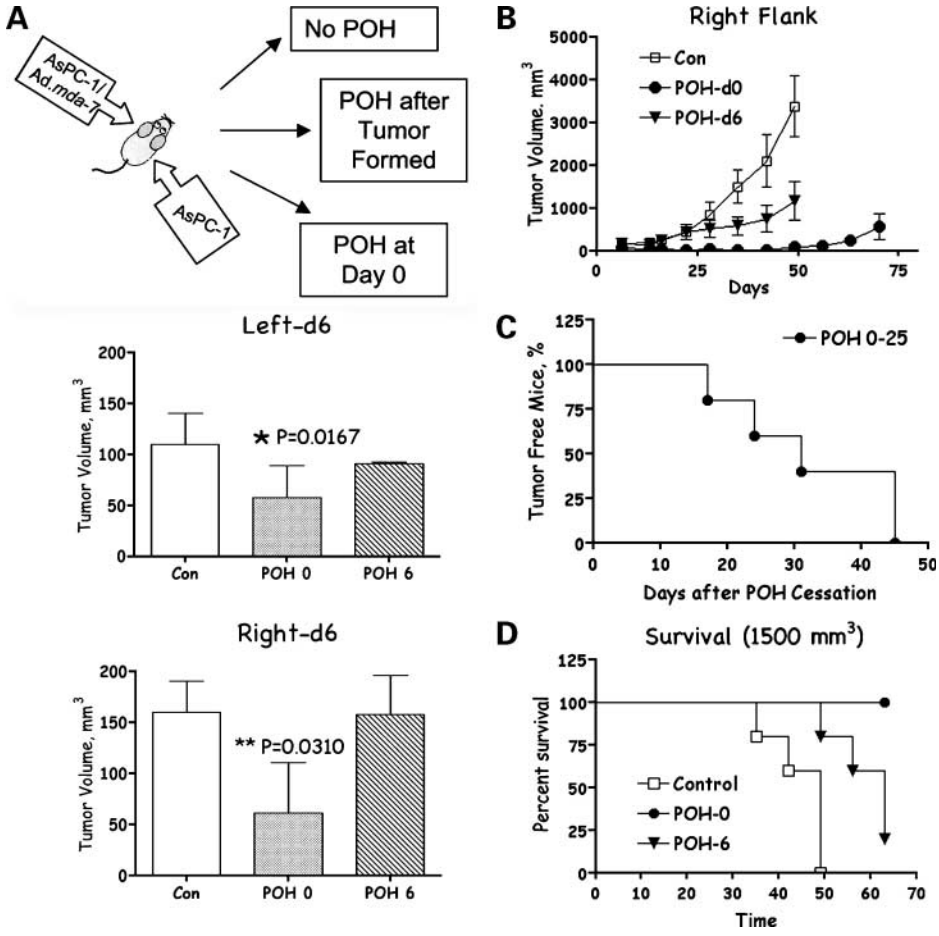

Figure 2. Chemopreventive and therapeutic effects of POH in athymic nude mice injected with AsPC-1 cells infected with Ad.*mda*-7/IL-24 (AsPC-1/Ad.*mda*-7, left flank) and uninfected AsPC-1 cells (right flank). **A,** experimental design (*top*) and volume of the tumors on the left side and on the right side of the animals at day 6 of the experiment (*bottom*). **B,** tumor growth on the right flank of mice. **C,** tumor regrowth in mice after POH administration was terminated. **D,** prolonged survival in AsPC-1/Ad.*mda*-7 and AsPC-1 pancreatic cancer tumor-bearing mice treated with POH. The animals were sacrificed when tumor size reached 1,500 mm^3 and survival was recorded. Animal survival was estimated using the Kaplan-Meier survival test.

To minimize experimental bias, we developed tumor xenografts using s.c. injections of AsPC-1 cells in the right flank and AsPC-1 cells *ex vivo* infected with Ad.*mda*-7 (50 plaque-forming units per cell) in the left flank in athymic nude mice (Fig. 2A, *top*). This led to the establishment of tumor xenografts in both left and right flanks that temporally attained similar sizes and did not show a significant effect of Ad.*mda*-7 *ex vivo* infection on tumor growth (Fig. 2A, *bottom*). We expected the infected AsPC-1 cells to produce *mda*-7/IL-24 mRNA for at least 3 weeks, as we showed earlier for pancreatic cancer cells (18). These mice were divided into three groups: (*a*) received vehicle (tricaprylin) only (control), (*b*) received POH (daily i.p. injections for 25 days) beginning from day 0 when these mice were injected with tumor cells (chemoprevention protocol), and (*c*) received POH beginning when xenografts achieved a size of ~100 mm^3 (day 6, therapeutic intervention protocol). The animals were sacrificed when the tumor size reached 1,500 mm^3 and survival was recorded. Animals receiving POH developed significantly smaller tumors in their left flank (AsPC-1 infected with Ad.*mda*-7) compared with right flank [AsPC-1 alone; Fig. 2A (*bottom*) and B]. However, in both flanks, POH-treated tumors were smaller in size compared with animals that received only vehicle (Fig. 2B). Furthermore, in both chemoprevention and therapeutic protocols, 25 days of continuous treatment with POH resulted in the appearance of tumor-free left flanks in all animals. Additionally, left flanks in the animals remained tumor-free until the termination of the experiment (70 days). The growth inhibition of the xenograft developed by AsPC-1 cells on the right flank by POH was treatment dependent [Fig. 2A (*bottom*) and B]. Both protocols provided significant growth suppression of the right flank tumors. A chemoprevention protocol treatment for 25 days also resulted in appearance of tumor-free right flanks. With the cessation of POH treatment, the right flank tumors started proliferating (Fig. 2C). In both treatment protocols, POH treatment led to a significant increase in the survival of the animals compared with the control group ($P = 0.0029$ for chemoprevention protocol and $P = 0.0077$ for therapeutic protocol; Fig. 2*D*). Interestingly, all of these animals were more active and healthy looking compared with vehicle-treated controls, which manifested high morbidity. These results correspond with our *in vitro* findings that *mda*-7/IL-24 in combination with POH is effective in abrogating the growth of human pancreatic carcinoma cells *in vivo* and this combination is more effective than either agent alone.

Next, we established the growth-inhibitory efficacy of a combination of POH and direct intratumoral injection of

Ad.*mda*-7 in established xenografts. S.c. AsPC-1 xenografts were established both on the right and left flanks of nude mice (Fig. 3A). These mice were divided into three groups: (*a*) received vehicle (control), (*b*) received POH beginning from day 0 when these mice were injected with tumor cells (chemoprevention protocol), and (*c*) received POH beginning when xenografts achieved a size of ~100 mm^3 (day 7, therapeutic intervention protocol). On day 7, each group was then divided into three subgroups, which received, respectively, intratumoral injections of (*a*) PBS, (*b*) Ad.*vec*,

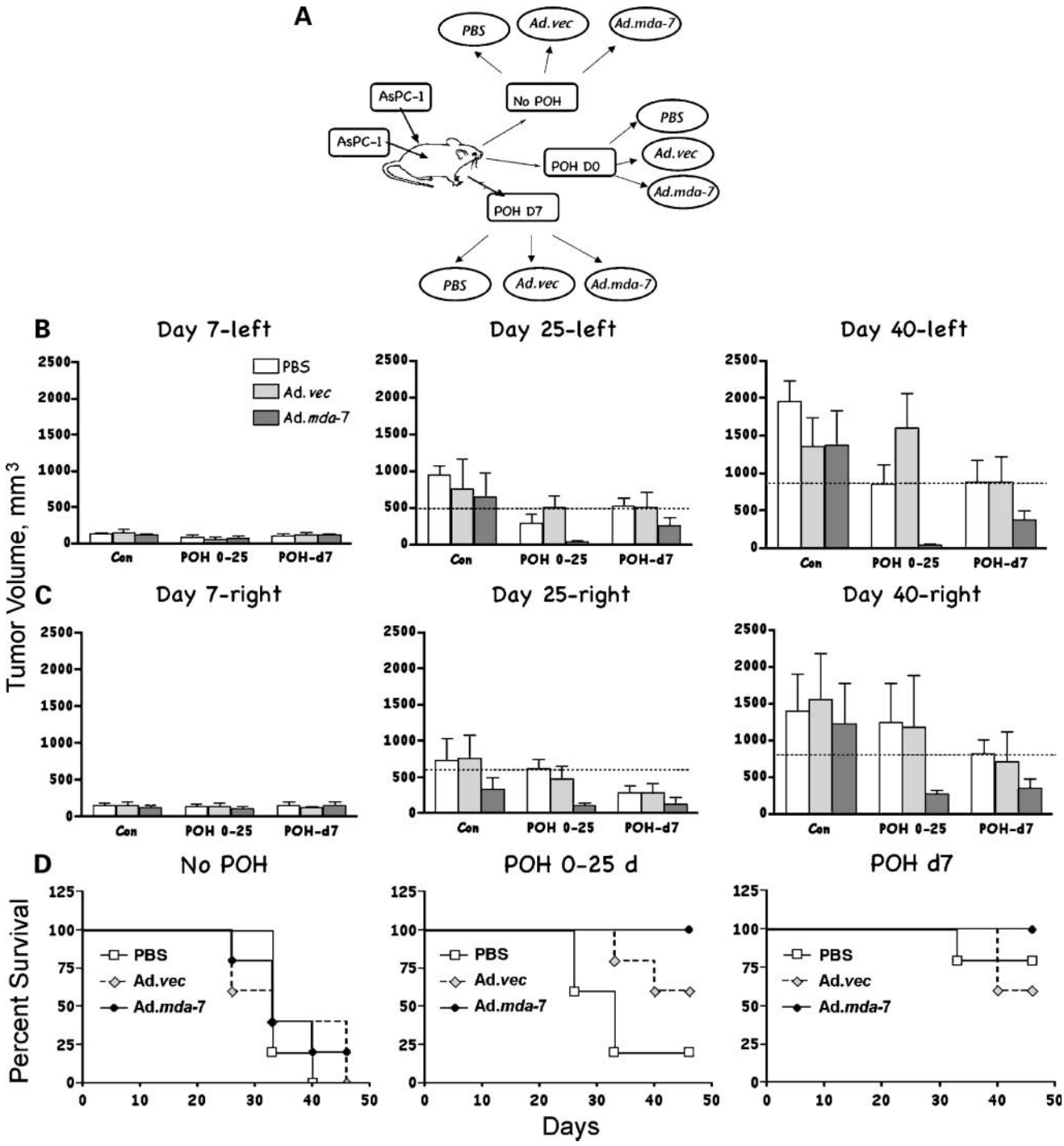

Figure 3. Chemopreventive and therapeutic effects of POH or a combination of Ad.*mda*-7 + POH in athymic nude mice injected with AsPC-1 cells on both flanks. **A,** experimental design. **B** and **C,** volumes of tumors on days 7, 25, and 40. **D,** prolonged survival in AsPC-1 pancreatic cancer tumor-bearing mice treated with POH or combination of Ad.*mda*-7/IL-24 and POH. Animal survival was estimated using the Kaplan-Meier survival test.

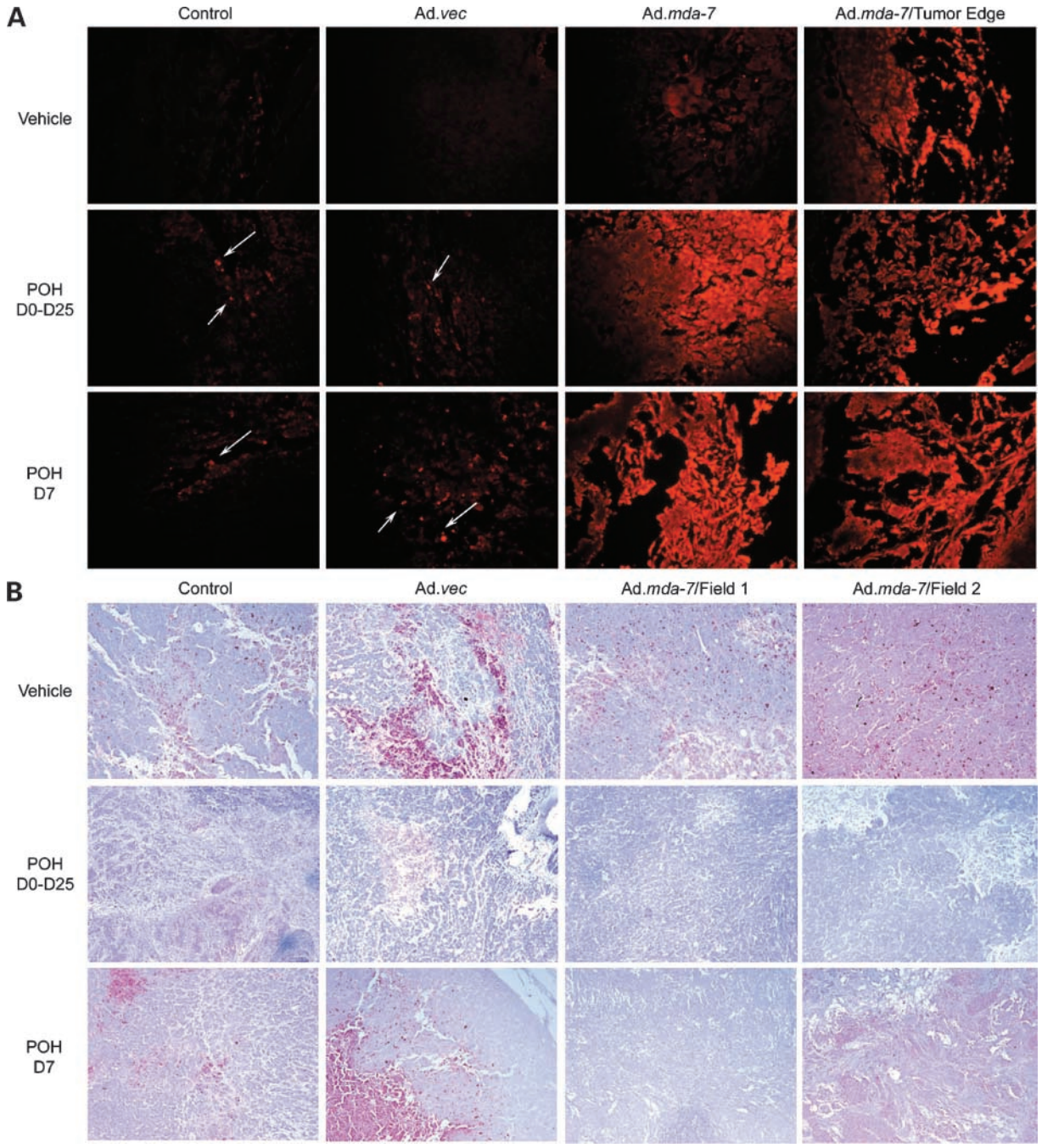

Figure 4. Combination of Ad.*mda*-7 and POH induces apoptosis (**A**) and suppresses angiogenesis (**B**) in *in vivo* xenograft mouse model of human pancreatic cancer. AsPC-1 pancreatic cancer xenografts were established in nude mice by s.c. injection of 2×10^6 cells and treated as described in Results. Fifty days after treatments started, representative tumors were excised and formalin-fixed, paraffin-embedded sections were prepared and stained for apoptosis (**A**) using In Situ Cell Death Detection kit (Roche Diagnostics). *Arrows,* single apoptotic cells. **B,** blood vessel formation was evaluated using CD31 antibody staining followed by hematoxylin counterstaining as described in Materials and Methods.

or (*c*) Ad.*mda*-7 only in the left flank tumors. The right flank tumors did not receive any injection. Animals receiving intratumoral injection of PBS or Ad.*vec* served as negative controls. As expected, the size of both left and right flank tumors in animals receiving a combinatorial treatment of POH and Ad.*mda*-7 (groups IIc and IIIc) was significantly smaller when compared with tumors developed in vehicle-treated mice (groups Ia, Ib, and Ic) as well as POH-treated animals receiving PBS or Ad.*vec* injection (groups IIa, IIb, IIIa, and IIIb; Fig. 3B and C). In

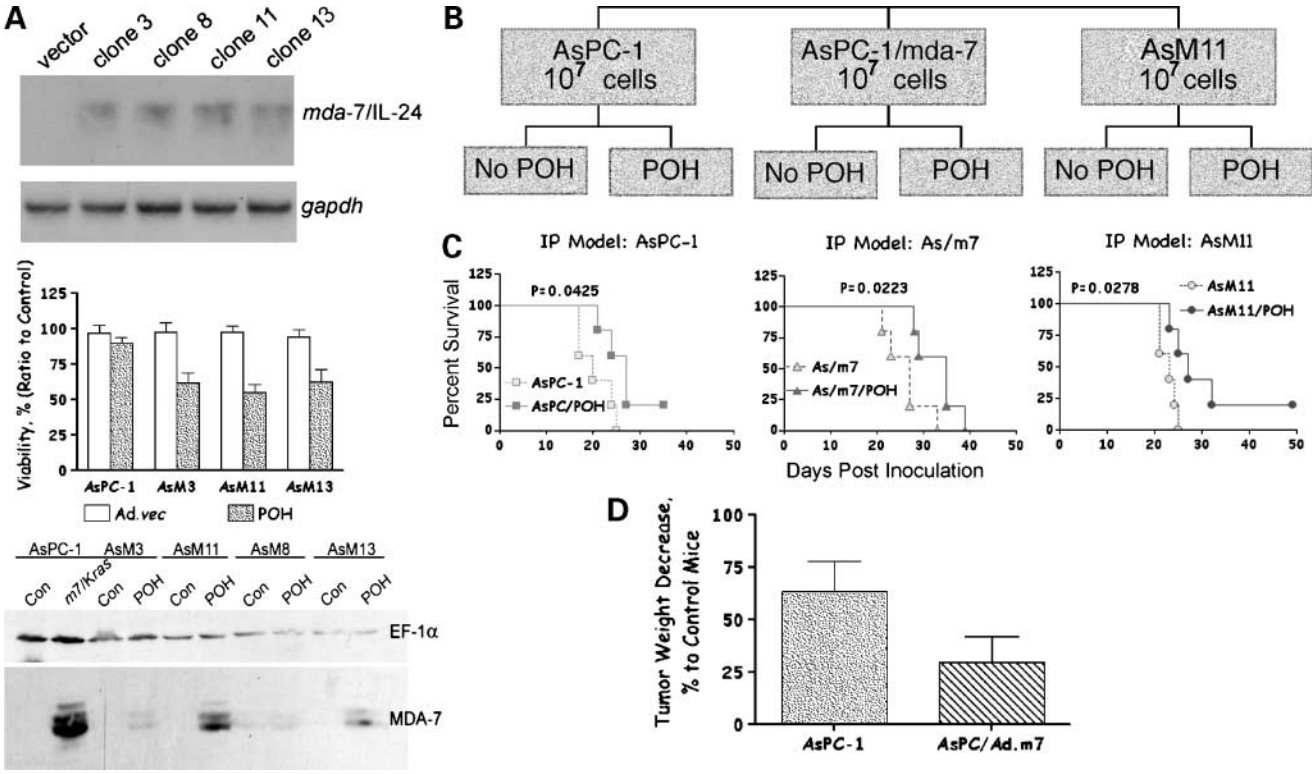

Figure 5. Chemopreventive and therapeutic effects of POH or a combination of Ad.*mda*-7 + POH in athymic nude mice injected i.p. with 1×10^7 AsPC-1 parental cells, AsPC-1 cells infected with 50 plaque-forming units per cell of Ad.*mda*-7/IL-24 (AsPC/mda-7), or a stable clone of AsPC-1 cells expressing *mda*-7/IL-24 mRNA (AsM11). **A,** characterization of stable clones of AsPC-1 cells stably expressing *mda*-7/IL-24 mRNA. Expression of *mda*-7/IL-24 mRNA and protein detected by Northern (*top*) and Western (*bottom*) blots, respectively. *Middle,* effect of single POH treatment on the viability of different stable clones of AsPC-1 cells overexpressing *mda*-7/IL-24 mRNA (MTT assay, day 6). **B,** experimental design. **C,** POH treatment prolongs median survival of the animals. Animal survival was estimated using the Kaplan-Meier survival test. **D,** POH treatment significantly decreases the weight of the tumors compared with the vehicle-treated animals. On day 21, the animals were euthanized and tumors were excised and weighed.

POH-treated and Ad.*mda*-7–treated animals, tumor growth inhibition was sustained even after cessation of POH treatment on day 25 until the end of the experiment at day 45. The growth inhibition was more profound in the chemoprevention protocol (group IIc) when compared with the therapeutic intervention protocol (group IIIc). Although POH treatment alone inhibited tumor growth when compared with vehicle treatment, following cessation of POH treatment on day 25, tumors started growing in mice receiving PBS or Ad.*vec* injections (groups IIa, IIb, IIIa, and IIIb). These findings indicate that a combination of POH and Ad.*mda*-7 is required to ensure a sustained growth-inhibitory effect. In this experiment, mice receiving combination treatment also survived longer than mice receiving only POH ($P = 0.0102$) or Ad.*mda*-7 ($P = 0.0382$; Fig. 3D).

At the end of the experiment, tumors were excised in each group, and formalin-fixed, paraffin-embedded tissue sections were prepared. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining and CD31 staining were done to evaluate the effect of the treatments on apoptosis induction and angiogenesis (Fig. 4A and B). Very few apoptotic cells were detected in control, Ad.*vec*-treated, and Ad.*mda*-7–treated cells (Fig. 4A, *top*). Interestingly, apoptosis could be detected at the borders but not in the middle of the tumors treated with Ad.*mda*-7 alone (Fig. 4A, *top*). These results tempt us to speculate that this may be the result of ''a bystander'' antitumor effect of MDA-7/IL-24 that we observed in our previous investigations (18). Most likely, Ad.*mda*-7 infection of normal mouse cells surrounding the xenograft resulted in MDA-7/IL-24 protein production and secretion of MDA-7/IL-24 protein that promoted apoptosis in xenografted pancreatic carcinoma cells. This hypothesis needs to be confirmed experimentally. POH treatment alone induced significant apoptosis when compared with vehicle treatment (Fig. 4A, *middle* and *bottom*), indicating the chemopreventive effect of POH. As expected, a combination of Ad.*mda*-7 and POH induced significant apoptosis both in the middle and at the edges of the xenografts (Fig. 4A, *middle* and *bottom*). Furthermore, a combination of Ad.*mda*-7 and POH decreased angiogenesis in the xenografts as evidenced by CD31 staining (Fig. 4*B*). POH treatment alone had little effect on angiogenesis (purple staining for CD31). Ad.*mda*-7 treatment reduced the number of big blood vessels. The combination of

Ad.*mda*-7 and POH almost completely eliminated blood vessel development in tumors. These findings confirm that a combination of Ad.*mda*-7 and POH exerts a profound antitumor effect against pancreatic cancer cells by inducing apoptosis, inhibiting angiogenesis, and generating a ''bystander'' antitumor effect.

POH in Combination with Ad.*mda*-7 Diminishes Growth of Pancreatic Cancer in a Quasi-Orthotopic Murine Model of Human Pancreatic Cancer

Schwarz et al. (30) developed a quasi-orthotopic tumor model of human pancreatic cancer by i.p. injection of PANC-1 cells into severe combined immunodeficient mice. These animals developed pancreatic cancer that shared many characteristics of the human disease (30). Similarly, we observed that i.p. injection of AsPC-1 cells into nude mice (1×10^7 per animal) leads to the development of tumor nodules in the pancreas and peripancreatic area and metastases to the lungs, liver, and diaphragm (data not shown). The animals showed obvious signs of cachexia and died within 2 to 3 weeks after i.p. injections of AsPC-1 cells. We used this murine model to investigate the effects of administration of POH alone or in combination with Ad.*mda*-7 on the survival of pancreatic cancer bearing animals by generating cumulative survival statistics using the Kaplan-Meier test. Athymic nude mice were divided into three groups receiving i.p. injections of (*a*) AsPC-1 cells, (*b*) AsPC-1 cells infected with Ad.*mda*-7, and (*c*) AsM11 clone of AsPC-1 cells stably expressing *mda*-7/IL-24 mRNA (clone AsM11). We isolated several clones of AsPC-1 cells expressing *mda*-7/IL-24 mRNA (Fig. 5A, *top*). These clones did not express MDA-7/IL-24 protein that was detected at variable levels only after POH treatment (Fig. 5A, *bottom*). As a corollary, although POH alone had little effect on parental AsPC-1 cells, it significantly reduced the viability of clones of AsPC-1 cells expressing *mda*-7/IL-24 mRNA (Fig. 5A, *middle*). The AsM11 clone was chosen for *in vivo* studies because it showed maximum expression of MDA-7/IL-24 protein production following POH treatment. Each group of animals was divided into two subgroups receiving (*a*) vehicle (tricaprylin) or (*b*) POH the day before i.p. injection of pancreatic cancer cells (Fig. 5B). All the animals receiving POH showed a prolonged survival compared with those receiving vehicle only (Fig. 5C). However, mice receiving a combination of Ad.*mda*-7 and POH (groups IIb and IIIb) manifested significantly improved survival compared with the animals receiving POH alone ($P = 0.0223$ and 0.0278, respectively; Fig. 5C; Table 2). In addition, these animals showed much less cachexia and looked much healthier compared with vehicle-treated controls.

In an additional experiment, on day 15 after the i.p. injection of pancreatic cancer cells, the animals were sacrificed and the tumors were excised and weighed (Fig. 5D). POH treatment resulted in an ~40% decrease in tumor weight compared with the vehicle-treated animals. As expected, the combination of Ad.*mda*-7 and POH treatment further decreased tumor weight by ~75% compared with the untreated animals. In these animals, the tumors were much smaller in size and were confined to the pancreas and peripancreatic areas (data not shown). However, at day 15, we did not observe any metastatic lesions in the liver or lungs.

This study shows that a combination of agents with complementary mechanisms of action prove more efficacious than administering a single agent in the therapy of pancreatic cancer. Earlier studies by several groups showed chemotherapeutic effects of POH in animal models (rat and hamster) of liver and pancreatic cancer (31, 32). In the majority of these experiments, animals were subjected to POH treatment after tumors developed. However, in this investigation, we show for the first time that POH exhibits significant chemopreventive effects in murine models that use human pancreatic cancer cells. In all of our experiments, the tumor growth in POH-treated animals was significantly delayed and survival of animals was prolonged [Figs. 2A (*bottom*) and E, 3B and D, and 5C and D]. In our study, the effect of POH alone was much smaller than the chemoprevention effect of POH reported in other animal models of cancer (33, 34). However, our studies used low doses of POH because high doses of POH that were effective in animal models induced extreme gastroenterotoxicity when applied in humans (23). Moreover, POH treatment even at low doses significantly augmented gene therapy by making inherently resistant pancreatic carcinoma cells sensitive to Ad.*mda*-7/IL-24 therapy [Figs. 2A (*bottom*), 3B and D, and 5C and D].

An obvious question is how POH relieves the protein translational block of *mda*-7/IL-24 in pancreatic cancer cells infected with Ad.*mda*-7. Ongoing experiments are elucidating the mechanism underlying this profound synergy between POH and *mda*-7/IL-24 in pancreatic carcinoma cells. These studies indicate that the generation of reactive oxygen species mediated by xanthine oxidase, a major source of superoxide radical production, by POH in combination with Ad.*mda*-7 is a significant contributory factor in this cancer-specific toxicity (35). A combinatorial treatment of pancreatic carcinoma cells with POH and Ad.*mda*-7 results in the association of *mda*-7/IL-24 mRNA with polysomes and concomitant translation of this message into functional protein. Additional studies are planned to evaluate the effect of this combination in the context of transgenic mice displaying a phenotype that recapitulates pancreatic cancer development and progression in humans (36). These investigations will

Table 2. Median survival time (days) of nude mice injected i.p. with various pancreatic cancer cell lines

	AsPC-1	AsM11	AsPC/*mda*-7
Vehicle	20.6 ± 3.4	22.8 ± 1.6	26.2 ± 4.2
POH	26.8 ± 4.7	31.2 ± 9.4	33.2 ± 4.1

NOTE: Nude mice were injected i.p. with 1×10^7 cells per animal with the indicated pancreatic carcinoma cell line and then were injected thrice weekly with vehicle (tricaprylin) or with POH. Survival was recorded and analyzed using the Kaplan-Meier method.

provide additional insights into the role of an intact immune system in chemoprevention and chemotherapeutic activity of this combinatorial CGT treatment protocol.

POH is a naturally occurring nonnutritive dietary monoterpene and has also been tested in humans (24). The detailed pharmacokinetics and toxicity of this agent is already known in both animals and humans (23, 24). POH is currently being tested in phase I and II clinical trials in patients with refractory solid malignancies (19, 26). In this investigation, we used a modest dose regimen, which is feasible in humans. Additionally, Ad.*mda*-7 has been evaluated in a phase I clinical trial in patients with melanomas and advanced carcinomas, not including pancreatic cancers, and has been found to be safe with significant clinical activity (8, 9, 12, 13, 15). These findings support the application of a novel dual CGT approach for both the prevention and therapy of pancreatic cancer. Our data also indicate that POH may be a useful adjunct to conventional anticancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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