Molecular Target-Based Therapy of Pancreatic Cancer

Irina V. Lebedeva, Devanand Sarkar, Zao-Zhong Su, Rahul V. Gopalkrishnan, Mohammad Athar, Aaron Randolph, Kristoffer Valerie, Paul Dent, and Paul B. Fisher

Departments of Pathology, Dermatology, Neurosurgery, and Urology, Herbert Irving Comprehensive Cancer Center, Columbia University, College of Physicians and Surgeons, New York, New York; Departments of Radiation Oncology and Biochemistry, Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia

Abstract
Pancreatic cancer is genetically complex, and without effective therapy. Mutations in the Kirsten-ras (K-ras) oncogene occur early and frequently (~90%) during pancreatic cancer development and progression. In this context, K-ras represents a potential molecular target for the therapy of this highly aggressive cancer. We now show that a bipartite adenovirus expressing a novel cancer-specific apoptosis-inducing cytokine gene, mda-7/interleukin-24 (II-24), and a K-ras AS gene, but not either gene alone, promotes growth suppression, induction of apoptosis, and suppression of tumor development mediated by K-ras mutant pancreatic cancer cells. Equally, the combination of an adenovirus expressing mda-7/II-24 and pharmacologic and genetic agents simultaneously blocking K-ras or downstream extracellular regulated kinase 1/2 signaling also promotes similar inhibitory effects on the growth and survival of K-ras mutant pancreatic carcinoma cells. This activity correlates with the reversal of a translational block in mda-7/II-24 mRNA in pancreatic cancer cells that limits message association with polysomes, thereby impeding translation into protein. Our study provides support for a “dual molecular targeted therapy” involving oncogene inhibition and selective cancer apoptosis-inducing gene expression with potential for effectively treating an invariably fatal cancer. (Cancer Res 2006; 66(4): 2403-13)

Introduction
Pancreatic cancer is the fourth leading cause of cancer deaths and it is estimated that >30,000 new cases will be diagnosed in the U.S. in 2005 and essentially all of these patients will die. In this context, long-term survival for individuals with organ-confined disease is only 20%, and in the majority of cases, in which the disease when diagnosed has already spread past the pancreas, survival is only 4% (1). Given its incidence and almost universal fatality, enhanced research efforts are mandated to comprehend, prevent, and effectively treat this devastating disease (1).

Although studied intensively, the crucial molecular determinants of pancreatic cancer and effective therapies for this disease remain elusive (1–3). Pancreatic cancer is a complex disorder in which multiple subsets of genes undergo genetic change, either activation or inactivation, during tumor development and progression (1–3). Common genetic modifications in pancreatic carcinomas include activation of the K-ras oncogene (85-95%), overexpression of specific growth factors and their associated receptors and inactivation of the p16/RB1 (>90%), p53 (75%), DPC4 (55%), and BRCA2 tumor suppressor genes (2, 3). These findings accentuate the complexity of this heterogeneous cancer and may underlie the aggressiveness and inherent resistance of this neoplasm to conventional therapies (1), including chemotherapy and radiation (2–4).

Defects in differentiation occur frequently in human cancers (5). By appropriate pharmacologic manipulation, it is possible to induce irreversible growth suppression and terminal differentiation in specific cancer subtypes (5, 6). Moreover, recent studies suggest that this approach, referred to as “differentiation therapy,” holds promise as a potentially less toxic form of cancer therapy (5, 6). In the case of human melanoma cells, exposure to recombinant human fibroblast IFN (IFN-α) and the protein kinase C–activating agent, mezerein, results in a rapid inhibition of growth and a loss of tumorigenic potential which correlates with the induction of terminal differentiation (5–7). Using this model system and subtraction hybridization, genes, displaying elevated expression as a result of cancer reversion and differentiation, have been identified and cloned, and were initially called melanoma differentiation associated (mda) genes (8). A novel gene identified using this scheme, mda-7 (9), displays wide spectrum antitumor activity resulting from induction of apoptosis selectively in cancer cells, both in vitro and in vivo in animal tumor models and more recently in a phase I clinical trial in patients with advanced carcinomas and melanomas (10–14). Based on its structure [including an interleukin (IL)-10 signature motif], properties (including secretion from cells) and chromosomal location (in an IL-10 cytokine cluster on chromosome 1q), mda-7 has now been classified as human IL-24 (11, 12, 14–18). Despite its diverse cancer-specific activity, injection of human pancreatic cancer cells with Ad.mda-7 does not significantly affect growth or induce apoptosis, whereas a dual combinatorial approach of infection with Ad.mda-7 and targeted inhibition of K-ras expression, using antisense phosphorothioate oligonucleotides, results in the induction of cell death uniquely in K-ras mutant pancreatic cancer cells (19). This strategy of modifying two distinct pathways in cancer cells, i.e., oncogene suppression and cancer suppressor gene replacement, represents a novel approach with potential for developing a rational therapy for this deadly disease (19).

In the present study, we define a means of improving the delivery of both gene-targeting vectors in pancreatic cancer cells and the mechanism underlying the synergy between mda-7/IL-24 and K-ras suppression in inducing apoptosis selectively in K-ras mutant pancreatic tumor cells. Inhibiting mutant K-ras directly or targeting one of its downstream activated pathways for inactivation, MEK1/2 or but not phosphatidylinositol-3-kinase (PI3K) or p38

Note: I.V. Lebedeva, D. Sarkar, and Z. Su contributed equally to this manuscript.

Requests for reprints: Paul B. Fisher, Department of Pathology, College of Physicians and Surgeons of Columbia University Medical Center, 30 West 168th Street, New York, NY 10032. Phone: 212-305-3642; Fax: 212-305-8177; E-mail: pbf1@columbia.edu.

doi:10.1158/0008-5472.CAN-05-3510

©2006 American Association for Cancer Research.

mitogen-activated protein kinase (MAPK), results in enhanced translation of mda-7/IL-24 mRNA into protein and consequently apoptosis in mutant K-ras pancreatic carcinoma cells in vitro and tumor growth suppression in vivo in nude mice. This process correlates with enhanced association of mda-7/IL-24 mRNA with polysomes, thereby facilitating the production of protein. Our studies provide support for a “dual molecular target–based therapeutic approach” for inducing programmed cell death in pancreatic cancer cells. Moreover, these findings provide a framework for a reasoned method for effectively treating one of the most aggressive cancers, pancreatic cancer, which may also have potential applications for other neoplastic diseases. In addition, it may be possible to combine this model system with mda-7/IL-24 and combinatorial and natural product chemistry approaches to identify new agents that can directly or indirectly inhibit ras and ras-signaling pathways permitting induction of apoptosis by mda-7/IL-24. With appropriate follow-up studies, this new strategy may pave the way for successfully translating these provocative laboratory findings into the clinic.

Materials and Methods

Cell lines, culture conditions, and growth assays. AsPC-1, Mia PaCa-2, Panc-1, and BaPC-3 pancreatic carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). An SV40 T-antigen-immortalized human melanocyte cell line, FMS16-S (9), and an H-TERT-immortalized normal human fetal astrocyte cell line, PHFA-IM (20), were described previously. All cells were cultured as described (19). Cell counting assays were done over a 7-day period with a media change at day 4 using a hemocytometer and trypan blue dye exclusion assay to determine viable cell counts (21). 3,4-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done as described in ref. (21). Statistical analyses of the results were done using the Analysis ToolPack provided by Microsoft Excel. A Student’s two-sample t test, assuming unequal variances, was used to determine the equality of the means of two samples. The confidence level α was 0.05.

Virus construction, purification, and infectivity assays. The recombinant replication-defective Ad.mda-7 virus was created in two steps as described previously (22), and plaque-purified by standard procedures. Ad.K-ras AS virus was produced by recombination as described and constructed by the Massey Cancer Center Virus Vector Shared Resource (23). The recombinant replication-defective Ad.m7/KAS bipartite virus was constructed using AdenoQuick Cloning System (O.D. 260 Inc., Boise, ID) as previously described for construction of Ad.PEG-E1A-IFN-γ (24). Cells were infected with 100 plaque-forming units (pfu)/cell of different adenoviruses or their combination (50 pfu/cell of each virus) and analyzed as described (21, 22).

RNA interference. Small interfering RNAs (siRNA) for MEK1 and MEK2 RNA were generated using Silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer’s protocol. The sequences of oligonucleotides were AAGAAGGAGAGCCTCACAGCA (for MEK2; ref. 25). The nonspecific random sequence, AAGGGTTGCATATAGGATGTGCAT, was used for control transfections. siRNA duplexes (100 nmol/L) were transfected into cells using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Suppression of MEK1 and MEK2 protein was verified by Western blotting with MEK1- and MEK2-specific antibodies (Cell Signaling Technology, Beverly, MA).

Apoptosis assays. Annexin V binding assays were used to determine apoptosis induction under various treatment conditions. Briefly, 24 hours after treatment, cells (5 × 10^6 cells per sample) were trypsinized, washed with complete medium and PBS, resuspended in 500 μL of binding buffer containing 2.5 mM L-CaCl_2 and stained with FITC-labeled Annexin V (BD Biosciences, Palo Alto, CA) and propidium iodide for 15 minutes at room temperature. Flow cytometry was done immediately after staining (26).

Western blotting. Protein lysates were prepared on ice and protein concentrations were determined as described (21). Equal aliquots of proteins were evaluated for MDA-7/IL-24, p21 K-Ras, and EF-1α protein levels by Western blotting as previously described (19, 20, 26). To determine the activities of extracellular regulated kinase 1/2 (ERK1/2), phosphoinos- munoobluting was done with ERK phosphospecific and total antibodies obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; ref. 27).

Purification of polysomes, RNA extraction, and Northern blot analysis. Polysomes were purified essentially as described (28). Cells (2 × 10^6) were infected with adenovirus and 48 hours later, the cells were harvested in 500 μL of buffer A [200 mMol/L Tris-HCl (pH 8.5), 50 mMol/L KCl, 25 mMol/L MgCl_2, 2 mMol/L EGTA, 100 μg/mL heparin, 2% polyoxyethylene 10-tridecyl ether, and 1% sodium deoxycholate supplemented with Complete Mini protease inhibitor cocktail and RNase inhibitor] and centrifuged at 12,000 rpm for 10 minutes at 4°C to clear cell debris. The supernatant was loaded on top of a 10% to 50% sucrose gradient prepared in buffer B (50 mMol/L Tris-HCl 25 mMol/L KCl, and 10 mMol/L MgCl_2) and centrifuged at 40,000 rpm for 1 hour at 4°C. Each fraction was taken at 500 μL monitoring the absorbance at 260 nm, and polysome fractions were identified (typically fractions 10–20). RNA was extracted from each fraction with QIAGEN RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol, and Northern blotting was done as described using radiolabeled mda-7 cDNA probe.

Tumorigenesis assays. Tumorigenic potential of in vitro–treated pancreatic cancer cells subsequently injected into animals were done as described (19, 22, 24, 29). Briefly, Panc-1 or AsPC-1 cells were untreated or treated with PD98059 (50 μmol/L) and infected with 100 pfu/cell of Ad. vec, Ad.mda-7, Ad.K-ras AS, Ad.m7/KAS alone, or with the combination of Ad.mda-7 with PD98059 or Ad.K-ras AS, and 24 hours later, 2 × 10^6 cells were mixed with Matrigel and injected s.c. into athymic nude mice. Animals were monitored for tumor formation, and tumor volume was determined. Statistical significance was evaluated with Student’s t test using the computer program SIGMA STAT (Jandel, San Rafael, CA; refs. 22, 29). Therapeutic studies were done on established tumors as described previously (24, 29). AsPC-1 xenografts were established in athymic nude mice by injecting 2 × 10^6 tumor cells suspended in 100 μL sterile PBS s.c. into animals. When the tumor reached a size of ~125 to 150 mm^3, the animals were randomized into groups (n = 5 animals per group) and treatment was initiated. For the treatment, 100 μL of sterile PBS or sterile PBS containing 5 mg/mL of PD98059 or 10^6 pfu of Ad. vec, Ad.mda-7, Ad.K-ras AS, Ad.m7/KAS, or a combination of Ad.mda-7 and Ad.K-ras AS (total 10^6 pfu) or Ad.mda-7 and PD98059 were injected intratumorally. The injection protocol was as follows: thrice a week for the first week, then twice a week for 3 weeks. Tumor measurements were recorded twice a week, and tumor volumes were calculated by the formula V (mm^3) = A × B^2 / 2, where A is the largest dimension and B is the perpendicular diameter (24, 29). Antitumor efficacy data are presented as average tumor volumes and tumor weights for all animals in each group.

Statistical analysis. All of the experiments were done at least thrice. Results are expressed as the mean ± SE. Statistical comparisons were made using an unpaired two-tailed Student’s t test. A P < 0.001 was considered significant. The statistical significance of the animal experimental results was calculated using Student’s t test for tumor measurements, and Kaplan-Meier survival test for animal survival.

Results

Dual molecular targeting of pancreatic carcinoma cells for apoptosis. Despite profound proapoptotic and antiplayin tumor activity in a broad spectrum of human cancers, both in vitro and in vivo in animal models and phase I clinical trials (reviewed in refs. 10–14, 17, 18), Ad.mda-7 infection or transfection with an mda-7/IL-24 cDNA expression vector does not induce growth suppression or apoptosis in pancreatic cancer cells (19). In contrast, when mutant K-ras expression is ablated in mutant K-ras pancreatic cancer cells using antisense phosphorothioate oligonucleotides targeting K-ras to identify new agents that can directly or indirectly inhibit ras and ras-signaling pathways permitting induction of apoptosis by mda-7/IL-24.
or by means of an antisense K-ras expression vector, growth is profoundly inhibited, cells become apoptotic and tumor formation in vivo in nude mice is prevented (19). To enhance the clinical utility of this dual targeting approach, we created a replication-defective adenovirus expressing a K-ras AS gene (Ad.K-ras AS) to be used together with Ad.mda-7 and a bipartite replication incompetent adenovirus that simultaneously expresses both mda-7/IL-24 and K-ras AS (Ad.m7/KAS). The K-ras AS gene construct used to produce Ad.K-ras AS and Ad.m7/KAS is targeted to the initiation codon of this gene, resulting in a decrease in both mutant and wild-type K-ras proteins as shown by Western blotting in PANC-1, AsPC-1, and MIA PaCa-2 (mutant K-ras) and BxPC-3 (wild-type K-ras) cells (Fig. 1A). Infection of mutant K-ras pancreatic cancer cells (PANC-1, MIA PaCa-2, or AsPC-1) with Ad.mda-7 + Ad.K-ras or Ad.m7/KAS resulted in a temporal decrease (evident by day 3) in cell growth and survival (Fig. 1B). In contrast, similar treatment of wild-type K-ras (BxPC-3) cells or normal telomerase immortalized primary human fetal astrocytes (PHFA-1M) or SV40 T-antigen immortalized normal human melanocytes (FM516-SV) did not significantly inhibit growth or decrease viability (Fig. 1B). The mechanism by which simultaneous suppression of K-ras and expression of mda-7/IL-24 decreased viability in mutant K-ras pancreatic carcinoma cells was previously shown to involve induction of apoptosis (19). Similarly, infection of mutant K-ras (data shown for PANC-1), but not wild-type K-ras (BxPC-3), cells with Ad.mda-7 + Ad.K-ras AS, but not either gene alone, or Ad.m7/KAS induced apoptosis as documented by nucleosomal DNA ladder formation, induction of hypodiploidy (A2 DNA content), Annexin V staining, and the TUNEL reaction (Fig. 1C and data not shown). These findings provide support for a molecular target-based strategy for inducing pancreatic cancer cell death, involving the inhibition of mutant oncogene (K-ras) expression and simultaneously expressing the cancer-specific apoptosis-inducing cytokine gene, mda-7/IL-24.

**Inhibiting a downstream target of K-ras signaling, MAPK, sensitizes mutant K-ras pancreatic carcinoma cells to Ad.mda-7-induced growth suppression and apoptosis.** To define the mechanism by which inhibiting mutant K-ras permits mda-7/IL-24 to induce growth suppression and apoptosis, we focused on potential downstream targets that are activated by this oncogene (1, 30). The K-ras gene signals through MAPK pathways including ERK1/2, JNK1/2, p38MAPK, and the PI3K pathways (30). Based on this consideration, the effect of selectively blocking these downstream ras-activated pathways, using pharmacologic inhibitors, on Ad.mda-7 effects on mutant and wild-type K-ras pancreatic carcinoma cells was evaluated. Inhibition of ERK1/2 function using the MEK1/2-specific inhibitors PD98059 (50 μmol/L) or U0126 (2 μmol/L), but not the p38MAPK inhibitor SB203580 (2 μmol/L), substitutes for targeting K-ras expression in selectively inducing apoptosis in Ad.mda-7-infected mutant K-ras, but not wild-type K-ras, pancreatic cancer cells (Figs. 2 and 3). In contrast, blocking the ras-activated PI3K pathway using wortmannin (200 nmol/L) or LY294002 (5 μmol/L) does not facilitate apoptosis induction by mda-7/IL-24 (Fig. 3). As predicted based on its proposed mode of action, the ability of PD98059 and U0126 to sensitize mutant K-ras to Ad.mda-7-induced apoptosis occurs without altering the level of K-ras protein in treated cells (Fig. 2B). Moreover, because both PD98059 and U0126 inhibitors can also block MEK5 signaling (31, 32), additional studies were done with the PD184352 inhibitor that is reported to be highly specific toward MEK1/2 kinases (31, 33). PD184352 in combination with Ad.mda-7 resulted in significant growth inhibition, cell death, and apoptosis induction in pancreatic cancer cells with mutant K-ras (Fig. 3B and data not shown).

Furthermore, to confirm that ablation of MEK1 and MEK2 would sensitize pancreatic cancer cells to mda-7/IL-24-induced killing, MEK1 and MEK2 RNAs were inhibited using a siRNA approach and the efficacy of these siRNAs were evaluated by Western blotting using appropriate antibodies (Fig. 4A). Pancreatic cancer cells were transfected with control, MEK1, or MEK2 siRNA oligoduplexes, and the next day, cells were infected with Ad.mda-7. Following infection, cell viability was assessed by MTT and colony formation assays. Apoptosis induction was monitored by Annexin V binding assay and by staining with propidium iodide for the A0 cell population. As shown by MTT and colony formation assays, MEK1/2 knockdown in combination with Ad.mda-7 infection resulted in decreased survival of PANC-1 and AsPC-1 cells that possess mutant K-ras (Fig. 4B). However, this treatment did not affect the viability of BxPC-3 cells (wild-type K-ras). Both Annexin V binding and propidium iodide staining assays corroborated an apoptotic mode of cell death induced by MEK1/2 knockdown and Ad.mda-7 infection in mutant K-ras cell lines (Fig. 4C). MDA-7/IL-24 protein expression was assayed by Western blot (Fig. 4A). As anticipated, MDA-7/IL-24 protein expression was only apparent with a combination of MEK1/2 siRNA + Ad.mda-7. The levels of induction of MDA-7/IL-24 protein in PANC-1 and AsPC-1 cells using these combinations were modest in comparison with infection with Ad.mda-7 + Ad.K-ras AS or infection with Ad.m7/KAS. MDA-7/IL-24 protein expression correlated with inhibition of cell growth and apoptosis induced by the combination of MEK1/2 siRNA and Ad.mda-7, suggesting that a threshold level of MDA-7/IL-24 protein is required to induce growth suppression and apoptosis in K-ras mutant pancreatic tumor cells (Fig. 4). In contrast, a single treatment with MEK1 or MEK2 siRNA, or a control siRNA sequence, alone or in combination with Ad.mda-7 did not produce MDA-7/IL-24 protein and therefore did not affect cell growth or viability (Fig. 4). MDA-7/IL-24 protein expression, as well as the deleterious effects of this combination treatment, was not observed in wild-type K-ras BxPC-3 cells.

The present results support the importance of ERK1/2 signaling in mutant K-ras pancreatic cancer cells in inhibiting the ability of mda-7/IL-24 to induce growth suppression and apoptosis. Moreover, based on the intrinsic resistance of pancreatic cancer cells to Ad.mda-7-induced therapy, this cell culture system represents a valuable model for identifying pharmaceutical compounds and/or small-molecule drugs that can cooperate with mda-7/IL-24 in inducing pancreatic cancer cell death (27).

**Molecular basis of the synergy between mda-7/IL-24 and ablation of K-ras expression in inducing apoptosis selectively in mutant K-ras pancreatic carcinoma cells.** Previous studies confirmed that infection of both mutant and wild-type K-ras pancreatic cancer cells with Ad.mda-7 results in abundant mda-7/IL-24 mRNA expression, but negligible protein (19). However, when mutant K-ras pancreatic carcinoma cells were simultaneously exposed to an AS K-ras phosphorothioate oligonucleotide, MDA-7/IL-24 protein was produced, which coincided with the induction of growth suppression and apoptosis (19). In a similar manner, infection of mutant K-ras pancreatic cancer cells, but not wild-type K-ras, with Ad.mda-7 + Ad.K-ras AS or Ad.m7/KAS results in the production and secretion of MDA-7/IL-24 protein (Figs. 1 and 2C; data not shown). Blocking K-ras signaling with PD98059 or U0126
(MEK1/2 inhibitors) or, alternatively, with MEK1/2 siRNA, results in MDA-7/IL-24 protein in the cell lysates and supernatants of mutant K-ras pancreatic carcinoma cells infected with Ad.m7/7 (Figs. 2C and 4; data not shown), without altering the levels of K-Ras p21 protein (Fig. 2B). In contrast, the p38MAPK inhibitor SB203580 and the PI3K inhibitors wortmannin or LY294002 did not relieve the mda-7/IL-24 mRNA "translational block" in Ad.mda-7-infected mutant K-ras pancreatic cancer cells (Fig. 2C). Similarly, infection

**Figure 1.** Selective growth inhibition and induction of apoptosis in mutant K-ras pancreatic carcinoma cells following infection with Ad.mda-7 + Ad.K-ras AS or Ad.m7/KAS. **A,** combination of Ad.K-ras AS + Ad.mda-7 or Ad.m7/KAS results in decreased K-Ras p21 protein in mutant and wild-type K-ras pancreatic tumor cells and production of MDA-7/IL-24 protein in mutant, but not in wild-type K-ras pancreatic tumor cells. Mutant K-ras (PANC-1, AsPC-1, and MIA PaCa-2) and wild-type K-ras (BxPC-3) cells were infected with 100 pfu/cell of Ad.vec, Ad.mda-7, Ad.K-ras AS, Ad.m7/KAS or a combination of Ad.mda-7 and Ad.K-ras AS viruses (total 100 pfu/cell), and 48 hours later, protein lysates were harvested and analyzed by Western blotting for MDA-7/IL-24 and p21 K-Ras proteins as described in Materials and Methods. Blots were immunostained for EF-1α protein to confirm equal loading. **B,** effect of different viruses on growth and viability of pancreatic carcinoma and normal immortal cells. The indicated cell type was infected with 100 pfu/cell of the indicated virus (in the case of Ad.mda-7 + Ad.K-ras AS infection, 50 pfu/cell of each virus) and viable cell counts were determined after 1, 3, 5, and 7 days. Medium with no virus added was replaced at day 4. Average of triplicate plates that varied by ±10%. Qualitatively similar results were obtained in two additional repeat experiments. **C,** effect of different viruses on apoptosis induction in PANC-1 and BxPC-3 cells. The indicated cell type was infected with Ad.vec (control) or the different test viruses and Annexin V staining was determined at 24 hours as described in Materials and Methods. Results are the average of three independent experiments ± SE. Annexin V staining was also increased under similar experimental conditions, i.e., infection with Ad.K-ras + Ad.mda-7 or Ad.m7/KAS, in MIA PaCa-2 and AsPC-1 cells (data not shown).
Figure 2. Effect of pharmacologic inhibitors with and without infection with Ad.vec or Ad.mda-7 on ERK1/2 phosphorylation, p21 K-Ras protein and MDA-7/IL-24 protein in pancreatic tumor cells. A, treatment with MEK1/2 inhibitors alone or in combination with Ad.mda-7 infection promotes down-regulation of ERK1/2 activity. The indicated pancreatic carcinoma cell type was untreated or treated with different MAPK inhibitors and infected with 100 pfu/cell of Ad.vec or Ad.mda-7. Doses of inhibitors used were 50 μmol/L of PD98059, 20 nmol/L of U0126, 10 μmol/L of LY294002, and 2 μmol/L of SB203580. Twenty-four hours after the addition of inhibitors, cells were lysed, protein lysates were harvested and subjected to SDS-PAGE and immunoblotting to determine the phosphorylation (activity) of ERK1/2, as described in Materials and Methods.

B, treatment of PANC-1 or BxPC-3 with MAPK inhibitors alone or in combination with Ad.vec or Ad.mda-7 infection does not cause down-regulation of p21 K-Ras protein levels. The indicated pancreatic carcinoma cell types were untreated or treated with different MAPK inhibitors (the same doses as in A) and infected with 100 pfu/cell of Ad.vec or Ad.mda-7. As a positive control for down-regulation of K-Ras protein, infection with Ad.K-ras AS virus was used. After 24 hours, cells were harvested, protein lysates were prepared and analyzed by Western blotting as described in Materials and Methods. Blots were immunostained for EF-1α protein to confirm equal loading. A similar lack of effect on K-Ras p21 protein occurred when MIA PaCa-2 or AsPC-1 cells were infected with the viruses and treated with the various pharmacologic inhibitors of MAPK. C, induction of MDA-7/IL-24 protein in mutant K-ras pancreatic cancer cells by ablation of K-ras expression or treatment with specific downstream inhibitors of MAPK signaling. To determine the levels of MDA-7/IL-24 protein, protein lysates and supernatants (data not shown) from the treated cells were prepared 24 hours posttreatment and Western blotting was done as described in Materials and Methods. Equal protein loading for protein lysates was confirmed by Western blotting with EF-1α antibody. Data for wild-type K-ras cells (BxPC-3) are not shown because there is no protein expression under any of the indicated conditions.
with Ad.mda-7 + Ad.K-ras AS, Ad.m7/KAS, or Ad.mda-7 + treatment with PD98059 or U0126 did not result in the conversion of mda-7/IL-24 mRNA into protein in wild-type K-ras BxPC-3 cells (data not shown). These results confirm a direct relationship between the conversion of mda-7/IL-24 mRNA into protein and induction of growth suppression and apoptosis in mutant K-ras pancreatic carcinoma cells.

Recent studies highlight the importance of translational regulation in determining mRNA association with polysomes, and consequently, the levels of specific proteins in the context of ras and Akt signaling (34–36). These experiments illustrate an interesting relationship between suppression of the ras pathway and the enhanced ability of specific mRNAs to associate with polysomes and to be translated into protein (34–36). In this context, abrogating K-ras expression, and consequently, its downstream MEK1/2 signaling pathway might result in the conversion of mda-7/IL-24 mRNA into protein by inducing translational enhancement of this mRNA, thereby facilitating its recruitment into polysomes, and consequently, translation into protein. We have now tested this hypothesis and confirm that coinfection of mutant K-ras (data

Figure 3. Combination treatment with Ad.mda-7 and MEK1/2 inhibitors decreases viability and induces apoptosis in mutant K-ras pancreatic cancer cells. A, inhibition of MEK1/2 sensitizes mutant, but not wild-type, K-ras pancreatic cancer cells to lose viability after infection with Ad.mda-7. The indicated cell types were treated with the pharmacologic agents and infected with 100 pfu/cell of Ad.vec or Ad.mda-7. MTT assays were done after 5 days as described in Materials and Methods, and cell viability was determined as the ratio to untreated cells. Results are the average from at least three experiments ± SE. B, inhibition of MEK1/2 sensitizes PANC-1 cells to undergo apoptosis following infection with Ad.mda-7. Treatment with PD98059 (50 μmol/L), U0126 (2 μmol/L), or PD184352 (10 μmol/L) and infection with Ad.mda-7 (100 pfu/cell) results in enhanced Annexin V binding 24 hours posttreatment. The results are average of three independent experiments ± SE. Induction of Annexin V staining is also evident in MIA PaCa-2 or AsPC-1 cells treated with these agents and infected with Ad.mda-7, but not in wild-type K-ras BxPC-3 cells (data not shown).
shown for AsPC-1), but not wild-type K-ras (data shown for BxPC-3), pancreatic carcinoma cells with a combination of Ad.mda-7 + Ad.K-ras AS enhances the proportion of mRNA in infected cells that associates with polysomes (Fig. 5). These results support a model in which ablating K-ras expression (and consequently its signaling pathways) allows Ad.mda-7 to kill mutant K-ras pancreatic cancer cells by relieving the translational block of mda-7/IL-24 mRNA mediated by the K-ras oncogene.

Figure 4. MEK1 and MEK2 knockdown by siRNA results in sensitization of mutant K-ras pancreatic cancer cells to Ad.mda-7-induced cell death and apoptosis. PANC-1, AsPC-1, and BxPC-3 cells were transiently transfected with the indicated siRNAs (100 nmol/L final concentration) and infected the next day with Ad.vec or Ad.mda-7 (50 pfu/cell). A, MEK1/2, MDA-7/IL-24, and EF-1α protein levels were determined by Western blot analysis with corresponding antibodies 48 hours postinfection. B, cell viability was assessed by MTT assay on day 6 after infection as described in Materials and Methods. Colony formation assays were done as described in Materials and Methods. C, apoptosis changes measured by Annexin V and propidium iodide staining assays were done 24 and 48 hours postinfection, respectively. Cell growth was strongly inhibited and apoptosis was induced in mutant K-ras pancreatic carcinoma cells on combination treatment with MEK1/2 siRNA and Ad.mda-7. *, nonspecific hybridization signal (A); **, P < 0.0001, statistically significant difference between Ad.vec and Ad.mda-7 samples (B and C).
Dual molecular targeting approach inhibits tumorigenesis induced by mutant K-ras pancreatic cancer cells. Transfection of MIA PaCa-2 with an AS K-ras expression plasmid in combination with Ad.mda-7 infection ex vivo suppressed tumor development when these cells were injected into athymic nude mice (19). We have now endeavored to further test the efficacy of our "dual molecular targeting approach" for suppressing tumor growth of mutant K-ras pancreatic carcinoma cells using both ex vivo (PANC-1 and AsPC-1) and in vivo (AsPC-1) therapy protocols (Fig. 6). Infection of PANC-1 or AsPC-1 with Ad.mda-7 + Ad.K-ras AS or with the bipartite Ad.m7/KAS virus prior to injection into nude mice inhibited tumor development (Fig. 6A and B). Similarly, infection with Ad.mda-7 + treatment with PD98059 (50 μmol/L) significantly inhibited tumor growth, whereas treatment with Ad.mda-7, Ad.vec, Ad.K-ras AS or PD98059 alone either had no effect or only modestly inhibited tumor growth (Fig. 6A and B). The effect of different treatments of AsPC-1 prior to injection into animals on tumor development in representative nude mice is shown in Fig. 6B. To define direct antitumor activity in established tumors, AsPC-1 cells were injected into nude mice and when tumors reached ~125 mm³ in size (Fig. 6C), animals were either untreated or injected (twice a week, for a total of six injections) with 1 × 10⁸ pfu of Ad.vec, Ad.mda-7, Ad.K-ras AS, Ad.mda-7 + Ad.K-ras AS, or Ad.m7/KAS and tumor volume (Fig. 6C), tumor weight (Fig. 6C), and survival (Fig. 6D) were determined at day 55. These studies documented reduced tumor volume / weight and increased survival in AsPC-1 xenografted nude mice injected with Ad.mda-7 + Ad.K-ras AS or Ad.m7/KAS. These studies confirm that the combinatorial approach described here could be extrapolated to antitumor activity in vivo in nude mice containing human mutant K-ras pancreatic tumors.

Discussion
Pancreatic cancer presents as one of the most aggressive malignancies with an extremely poor prognosis (2–4). Despite efforts on multiple fronts, there has been little success in significantly ameliorating disease progression that translates into enhanced disease-free survival of patients. Of the plethora of changes occurring in pancreatic cells as they evolve into pancreatic carcinomas, mutations in the K-ras oncogene represent one of the earliest and most prevalent alterations, occurring in 85% to 95% of tumors (1–4). Based on the confirmed activity of the K-ras gene in promoting cell transformation (1–4), strategies have been developed to nullify K-ras expression, using antisense, and more recently, siRNA approaches, or to target its downstream signaling pathways, including MAPK and PI3K (37–40). These approaches have had limited success in preclinical models, and few if any have now emerged as mainstream therapeutic options for treating this horrific disease. We now show that a "dual molecular target–based therapy" combining targeting of K-ras itself or a downstream pathway, MEK1/2, with expression of a cancer-specific apoptosis-inducing gene mda-7/IL-24, represents a rational and successful approach for selectively inducing apoptosis in mutant K-ras pancreatic tumor cells. The advantages of the current protocol include, specificity of action (10–14, 17, 21, 22, 26, 41–45), the potent capacity to enhance radiosensitivity of human cancers (20, 46–50), and an ability to inhibit tumor angiogenesis (51, 52). In this context, the present approach offers significant promise for ultimately developing an effective and enduring therapy for pancreatic cancer.

A significant component of our combinatorial approach for treating pancreatic cancer is the mda-7/IL-24 gene (19). This gene was identified as part of a molecular screen designed to detect cDNAs that display reactivation (or enhanced expression) as a function of reversion of the cancer phenotype of malignant human melanoma cells to that of a more differentiated melanocyte-like state by treatment with human fibroblast IFN + mezerein (5–9). This differentiation induction subtraction hybridization approach (8) has provided insight into the processes of cancer growth control and terminal differentiation resulting in the cloning of novel genes involved in cell cycle control, cancer aggressiveness, cellular senescence, and cytokine response (5–9, 11). When expressed at elevated levels, either by transfection or using a replication incompetent adenovirus, mda-7/IL-24 inhibits growth and induces apoptosis selectively in a diverse group of human cancer subtypes, without inducing harmful effects in normal cells (11, 12, 14, 20, 21, 26, 42–45, 53). In contrast to its activity in most cancer contexts, human pancreatic cancer cells were found to be...
refractive to the effects of this gene (19). This prompted us to investigate the potential mechanism underlying this resistance, leading to the observation that K-ras activation was a primary contributor to the inability of mda-7/IL-24 to induce apoptosis in pancreatic cancer cells (19). By nullifying K-ras, using antisense approaches (expression vector or phosphorothioate oligonucleotides), we showed sensitization of mutant K-ras pancreatic cancer cells to induction of apoptosis by mda-7/IL-24 (19). These observations have now been extended indicating that not only blocking K-ras, but inhibiting one of its specific activated

Figure 6. Therapeutic effect of treatment with a bipartite virus Ad.m7/KAS or combination treatment with Ad.mda-7 and Ad.K-ras AS or PD98059 on the growth of pancreatic cancer cells in nude mice. A, nude athymic mice were injected with PANC-1 or AsPC-1 cells either untreated or treated as indicated and described in Materials and Methods. Tumor measurements were done 3 weeks after injection. Presented data are an average from two independent experiments ± SE. Statistical significance was determined by a Student’s t test (*, P < 0.05). B, animals receiving AsPC-1 cells pretreated with bipartite virus Ad.m7/KAS or combination treatment with Ad.mda-7 + Ad. K-ras AS or PD98059 show significant inhibition of tumor growth. C, therapeutic model of pancreatic cancer. AsPC-1 tumors were established in mice as described in Materials and Methods and mice were divided into six groups (n = 5 animals per group) and treated as indicated. Tumors were measured using calipers, and the statistical significance of tumor volume changes was calculated using the Student’s t test (C, left). Experiments were terminated when tumors started to show signs of necrosis or when the tumors exceeded 2,000 mm³, according to NIH guidelines. Tumor volume at the beginning and at the end of the experiment (C, left). Columns, mean tumor volume for each animal group; bars, ± SD. D, prolonged survival in AsPC-1 pancreatic cancer tumor-bearing mice treated with bipartite virus Ad.m7/KAS or combination of Ad.mda-7 and Ad.K-ras AS. Animal survival was estimated by using the Kaplan-Meier survival test.
pathways, MEK1/2, allows mda-7/IL-24 to kill mutant pancreatic cancer cells (Figs. 3 and 4). These findings provide further support for targeting K-ras expression (either directly or its downstream signaling pathways) as a means of converting mda-7/IL-24-resistant pancreatic tumor cells to permissive cells, thereby decreasing tumor cell survival. Contrary to our findings, a recent report describes sensitivity of pancreatic cancer cells to apoptosis by Ad.mda-7 alone that might be explained by high titer of the virus used, the specific virus construct employed or culture conditions (54).

Infection of pancreatic cancer cells with Ad.mda-7 was previously shown to result in high-levels of mda-7/IL-24 mRNA without significant production of protein (19). In contrast, when K-ras expression was extinguished using AS K-ras phosphorothioate oligonucleotides, mda-7/IL-24 mRNA was converted into protein and cell death occurred, uniquely in the context of mutant K-ras pancreatic tumor cells (19). In this context, the translational block in the conversion of mda-7/IL-24 mRNA into protein seems to be the predominant impediment to antitumor activity of this gene in mutant K-ras pancreatic tumor cells. Similarly, we now find that blocking K-ras expression using a viral approach, Ad.K-ras AS or Ad.m7/KAS, or blocking K-ras-activated MEK1/2 activity using pharmacologic agents, PD98059 or U1026, eliminates the mda-7/IL-24 mRNA translational block in mutant K-ras pancreatic cancer cells resulting in protein production and apoptosis. This raises the intriguing question as to how blocking K-ras expression results in the conversion of mda-7/IL-24 mRNA into protein in pancreatic tumor cells? Possible insights into this mechanism come from a recent study by Rajasekhar et al. (36) documenting that ras and Akt signaling pathways exert profound effects in defining which subsets of mRNAs associate with polysomes and become translated into protein. Moreover, a surprising observation was that blocking ras or Akt signaling only modestly affected mRNA levels of genes when monitoring total cellular RNA, whereas the levels of polysome-associated mRNAs were greatly altered following ras or Akt blockade (37). In this context, a simple hypothesis, which we have tested and confirmed, is that inhibiting K-ras signaling enhances the proportion of mda-7/IL-24 mRNA associated with polysomes (Fig. 5), thereby resulting in production of MDA-7/IL-24 protein and apoptosis in mutant, but not in wild-type (Figs. 2 and 3), K-ras pancreatic cancer cells. These results highlight an additional significant question, what is the mechanism by which mutant K-ras signaling in pancreatic cancer cells prevents mda-7/IL-24 mRNA translation? Although the mechanism is not known, it is possible that K-ras signaling inhibits the phosphorylation of eukaryotic initiation factor 4E (eIF4) and eIF4E binding protein-1 that are required for the initiation of translation from most mRNAs (34, 35, 55). The specificity of inhibition of specific mRNAs might relate to their structure, which could include long 5′-untranslated regions and upstream AUGs present in specific subsets of mRNAs encoding important regulatory factors that affect cell growth and proliferation (34–36, 55).

In summary, the present studies provide proof-of-principle for a dual molecular target–based therapy with significant promise for improving pancreatic cancer therapy. This approach exploits a molecular defect found in the vast majority (82–95%) of pancreatic cancers, i.e., mutations in K-ras, in combination with a nontoxic cancer-specific apoptosis-inducing gene, mda-7/IL-24 (19). This unique combinatorial approach can be applied using a viral vector that contains both the mda-7/IL-24 gene and the K-ras AS gene in a single bipartite virus or by using Ad.mda-7, which is now showing promise for gene therapy in the clinic (10–14), in combination with pharmacologic agents (including PD184352 and CI-1040) that specifically target downstream K-ras signaling pathways. Moreover, these MAPK K-ras/2 inhibitors are now being tested clinically (33) and studies employing Ad.mda-7 in combination with these agents could be evaluated for safety in phase I clinical trials and ultimately for clinical efficacy. Based on the provocative results obtained thus far, and the profound bystander antitumor effect of this combinatorial approach (10–14, 19, 44), this strategy merits further evaluation as a mainstream therapy for use in patients with pancreatic carcinoma.

Acknowledgments

Received 9/29/2005; revised 11/18/2005; accepted 12/19/2005.

Grant support: Supported in part by NIH/National Cancer Institute grants CA907318, CA908172, and PO1 CA104177; the Lustgarten Foundation for Pancreatic Cancer Research; the Samuel Waxman Cancer Research Foundation; and Chernow Endowment. P.B. Fisher is a Michael and Stella Chernow Urological Cancer Research Scientist and a Samuel Waxman Cancer Research Foundation Investigator.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Nicholaq Vozhilla and Lejuan Chatman for assistance with the tumorigenesis assays.

References

16. Su ZZ, Lebedeva IV, Gopalakrishnan RV, et al. A
Molecular Target-Based Therapy of Pancreatic Cancer


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/4/2403

Cited articles
This article cites 55 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/4/2403.full#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/66/4/2403.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/66/4/2403.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.