Reversing Translational Suppression and Induction of Toxicity in Pancreatic Cancer Cells Using a Chemoprevention Gene Therapy Approach

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ABSTRACT

Pancreatic cancer is an aggressive disease with limited therapeu tic options. Melanoma differentiation–associated gene-7/interleukin-24 (mda-7/IL-24), a potent antitumor cytokine, shows cancer-specific toxicity in a vast array of human cancers, inducing endoplasmic reticulum stress and apoptosis, toxic autophagy, an antitumor immune response, an antiangiogenic effect, and a significant “bystander” anticancer effect that leads to enhanced production of this cytokine through autocrine and paracrine loops. Unfortunately, mda-7/IL-24 application in pancreatic cancer has been restricted because of a “translational block” occurring after Ad.5-mda-7 gene delivery. Our previous research focused on developing approaches to overcome this block and increase the translation of the MDA-7/IL-24 protein, thereby promoting its subsequent toxic effects in pancreatic cancer cells. We demonstrated that inducing reactive oxygen species (ROS) after adenoviral infection of mda-7/IL-24 leads to greater translation into MDA-7/IL-24 protein and results in toxicity in pancreatic cancer cells. In this study we demonstrate that a novel chimeric serotype adenovirus, Ad.5/3-mda-7, displays greater efficacy in delivering mda-7/IL-24 compared with Ad.5-mda-7, although overall translation of the protein still remains low. We additionally show that d-limonene, a dietary monoterpene known to induce ROS, is capable of overcoming the translational block when used in combination with adenoviral gene delivery. This novel combination results in increased polysome association of mda-7/IL-24 mRNA, activation of the preinitiation complex of the translational machinery in pancreatic cancer cells, and culminates in mda-7/IL-24–mediated toxicity.

Introduction

Pancreatic cancer is a deadly disease with a 5-year survival of less than 5%. The lack of early stage symptoms often leads to advanced disease at the time of diagnosis. Additionally, it exhibits intrinsic resistance to chemotherapy and radiation (Hezel et al., 2006). Consequently, there is a dire need for the development of novel therapies for this devastating disease. Melanoma differentiation–associated gene-7/interleukin-24 (mda-7/IL-24) is an anticancer cytokine originally cloned in our laboratory during studies of terminal differentiation of human melanoma cells (Jiang and Fisher, 1993; Jiang et al., 1995, 1996). This gene is a member of the interleukin-10 family of cytokines and functions as a tumor suppressor (Dash et al., 2010a). Reintroduction of this cytokine into cancer cells leads to significant endoplasmic reticulum (ER) stress, apoptosis, and anticancer immune effects, but shows no toxicity in normal cells (Jiang et al., 1996; Su et al., 1998; Dash et al., 2010a). Additionally, mda-7/IL-24 exhibits a potent “bystander effect,” wherein cells that express mda-7/IL-24 can also secrete it and further activate its expression through autocrine and paracrine loops in both normal and cancer cells (Dash et al., 2010a). Clinically, this suggests that mda-7/IL-24 has the capability of traveling from the original source of treatment to affect distant tumors and metastases (Sauane et al., 2003, 2008; Dash et al., 2010a).

Gene therapy studies using replication-incompetent adenovirus containing the full-length mda-7 gene (Ad.mda-7) have been shown to be extremely effective in multiple cancer types in both laboratory and clinical settings (Fisher, 2003; 2005, 2007; Tong et al., 2005; Cunningham et al., 2005; Eager et al., 2008). Although mda-7/IL-24 has ubiquitous activity in multiple cancer types, it is difficult to fully exploit these in pancreatic cancer owing to a block in mda-7/IL-24 translation after delivery into these cells. Infected cells accumulate mda-7/IL-24 mRNA, and translation into protein is not observed.

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ABBREVIATIONS: Ad, adenovirus; Ad.5, serotype 5 adenovirus; Ad.5/3, serotype 5 and serotype 3 chimeric adenovirus; Ad.mda-7, replication-incompetent adenovirus containing the full-length mda-7 gene; CAR, Coxsackie and adenovirus receptors; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl cysteine; PCR, polymerase chain reaction; POH, perillyl alcohol; ROS, reactive oxygen species.
mRNA, which exhibits a reduced ability to interact with polyomaviruses and create MDA-7/IL-24 protein (Su et al., 2001; Lebedeva et al., 2005, 2006, 2007, 2008a,b; Sarkar et al., 2013).

Our previous work has demonstrated that the “translational block” seen after Ad.5-mda-7 treatment can be abrogated by two strategies: inhibition of onecogene Kirsten-ras (K-ras) or in combination with a reactive oxygen species (ROS)-inducing agent (Su et al., 2001; Lebedeva et al., 2005, 2006, 2007, 2008a,b). We have shown that agents such as arsenic trioxide or perillyl alcohol (POH) are able to induce MDA-7/IL-24 protein translation and subsequent toxic effects in pancreatic cancer cells. The creation of a clinically useful drug that specifically targets K-ras has been a long-standing and complex problem that still remains unresolved. Additionally, targeting K-ras only enhances MDA-7/IL-24 expression in cells that express mutated forms of K-ras. Because of this, we have focused on identifying novel ROS-inducing agents that could translate easily into a clinical setting.

Furthermore, the use of adenoviruses (Ad) in pancreatic cancer is often hampered by poor infectivity rates. Serotype 5 Ad (Ad.5) viruses use Coxackie and adenovirus receptors (CAR) to infect cells. Unfortunately, cancer cells, including pancreatic cancer cells, often downregulate these receptors, which leads to inefficient viral infectivity (Pearson et al., 1999). Using alternate viral serotypes to create chimeric viruses can help with this issue (Dash et al., 2010b, 2011; Hamed et al., 2010; Azab et al., 2012, 2014). The Ad.3 serotype relies on expression of CD46 and desmoglein for viral entry into cells (Wang et al., 2011). The creation of Ad.5/3 chimeric Ad combines both of these serotypes to create a virus that is capable of using CAR, CD46, and/or desmoglein for infection. We demonstrated that Ad.5/3-mda-7 is able to infect prostate, renal, glioblastoma multiforme, and colorectal cancer cells with greater efficiency compared with Ad.5-mda-7 and that this translates into greater toxicity (Dash et al., 2010b, 2011; Eulitt et al., 2010; Hamed et al., 2010; Azab et al., 2012).

In this study, we determined if Ad.5/3-mda-7 would improve the transduction efficiency in pancreatic cancer cells compared with Ad.5-mda-7. We also evaluated the ability of n-limonene, a dietary monoterpene, to increase the translation of mda-7/IL-24 mRNA and lead to toxicity in combination with Ad.mda-7. Limonene is a major component of citrus oil, shows minimal toxicity in low doses, and, like POH, is also able to induce ROS (Rahi and Bishayee, 2009). We hypothesized that it would function just as well as would increasing mda-7/IL-24 mRNA translation following Ad.mda-7 infection.

Materials and Methods

Cell Lines and Generating Stable Clones. AsPC-1 and BxPC-3 cell lines were obtained from the American Type Culture Collection, maintained in RPMI-1640 media (Gibco; Invitrogen, Auckland, New Zealand) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO); MIA PaCa-2 and PANC-1, also obtained from the American Type Culture Collection, were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% FBS. Immortalized normal pancreatic mesenchymal cell line L72 was obtained from EMD Millipore (Billerica, MA) and maintained per Millipore instructions. All cell lines were cultured at 37°C in a 5% CO2 and 95% air-humidified atmosphere. To obtain mda-7/IL-24-overexpressing clones, MIA PaCa-2 and PANC-1 cells were transfected with pcDNA3.1-mda-7. Individual clones were selected after ~3–4 weeks of continuous maintenance in culture medium containing hygromycin (Lebedeva et al., 2003), and were further characterized for the presence/expression of the inserted plasmid by real-time polymerase chain reaction (PCR).

Generation of Ad.5.mda-7 and Ad.5/3-mda-7. Recombinant serotype 5 and serotype 5/3 chimeric adenoviruses expressing mda-7/IL-24 (Ad.5-mda-7 and Ad.5/3-mda-7) and control empty adenovirus (Ad.5.vec and Ad.5/3.vec) were generated as described (Su et al., 1998; Dash et al., 2010b).

Adenoviral Infection. Pancreatic cancer cell lines were infected with Ad.mda-7 or Ad.5/3-mda-7 in medium lacking FBS for 3 hours followed by treatment with limonene (200 μM) in complete medium (10% FBS) for time points as described for particular assays.

Cell Proliferation Assays. Pancreatic cancer cell lines were infected with Ad.mda-7 or Ad.5/3-mda-7 in medium lacking FBS for 3 hours followed by treatment with n-limonene in complete medium (10% FBS) for 72 hours, and cell proliferation assays were performed at optical density 560 nm after adding 100 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye (1 mg/ml; Sigma-Aldrich). To further confirm cell proliferation, a chemiluminescent-based BrdU Cell Proliferation Assay kit (Cell Signaling Technology, Danvers, MA) was used.

Apoptotic Assays. Apoptotic assays were performed using an FITC Annexin V Apoptosis Detection Kit I (Pharmingen; BD Biosciences, San Diego, CA) according to the manufacturer’s instructions. Flow cytometry assays were performed immediately after staining using FACS Canto (BD Biosciences). Data were analyzed using FACS DIVA software (BD Biosciences).

ROS Measurements. To determine ROS production, cells were stained with 100 μM of 10 μM carboxy-2’,7’-dichlorodihydrofluorescein diacetate (Molecular Probes; Life Technologies, Grand Island, NY) in phosphate-buffered saline for 30 minutes followed by treatment with limonene, and fluorescence was measured with a fluorometer using a green filter at the indicated time points.

Preparation of Whole-Cell Lysates and Western Blotting Analyses. Cells were treated for 48 hours, lysed using cell lysis buffer (Cell Signaling Technology) supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) with protease inhibitor cocktail, phosphatase inhibitor (Roche, Indianapolis, IN). Whole-cell lysates were collected after centrifugation at 12,000 rpm at 4°C. For Western blotting analyses, the primary antibodies used were mouse monoclonal anti-MDA-7/IL-24 (1:2000; Gen Hunter Corporation, Nashville, TN), anti-EF1α (1:5000; EMD Millipore), mouse monoclonal anti-human K-Ras (1:1000; Bio-Rad Laboratories, Raleigh, NC), rabbit monoclonal anti-Bcl-xL, anti-PARP, anti-Mcl-1, anti-phospho-p70S6K (Thr-389), anti-phospho-ERK14 (Ser202/204), anti-phospho-AKT (Thr-37/44), anti-Cell Signaling Technology, rabbit polyclonal anti-BiP/GRP-78 (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX). The secondary antibodies used were polyclonal goat anti-mouse IgG (1:1000; Dako, Carpinteria, CA) and polyclonal swine anti-rabbit IgG (1:3000; Dako).

Polysome Studies. Cells (2 × 106) were infected with Ad.mda-7 and 48 hours later harvested in 500 μl buffer A (200 mM Tris-HCl pH 8.5, 50 mM KCl, 25 mM MgCl₂, 2 mM EGTA, 100 mg/ml heparin, 100 mg/ml cycloheximide, 2% polyoxylethylene 10-tridecyl ether, and 1% sodium deoxycholate supplemented with complete Mini protease inhibitor cocktail and RNase inhibitor (Roche). Cells were centrifuged at 12,000 rpm for 10 minutes at 4°C to clear cell debris. The supernatant was loaded on top of a 10–50% sucrose gradient prepared in buffer B (50 mM Tris-HCl, 25 mM KCl, and 10 mM MgCl₂) and centrifuged at 40,000 rpm for 1 hour at 4°C. Fractions of 500 μl were collected, the optical density at 260 nm was monitored, and polysome fractions were identified (typically fractions 10–20). The fractions were pooled into two separate tubes: fraction I (1–10 fraction number) and fraction II (polysome-enriched fraction, 11–20 fraction number). RNA was extracted by adding equal volume of 8 M guanidine hydrochloride, and precipitated by adding 2 volumes of ethanol. RNA was extracted from each fraction with a Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA was prepared and reverse transcription PCR was performed using a probe.
for mda-7/IL-24 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for relative quantification of mRNA.

**Statistical Analyses.** Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Student’s t test or 1-way analysis of variance was used as indicated, to study the level of significance (P < 0.05).

**Results**

**Enhanced Delivery of mda-7/IL-24 Using Serotype Ad.5/3-mda-7 in Pancreatic Cancer Cells.** Initial studies with mda-7/IL-24 focused on gene delivery using the Ad.5 serotype, which utilizes CAR for infection. Pancreatic cancer cells express relatively low levels of CAR, making infection with Ad.5 inefficient. To circumvent this problem, a serotype chimeric recombinant adenovirus, Ad.5/3, was generated. Ad.5/3, while able to use CAR receptors, is also reliant on expression of CD46 and desmoglein for infection. Pancreatic cancer cell lines AsPC-1, PANC-1, MIA PaCa-2, and BxPC-3, as well as an immortal pancreas fibroblast cell line, LT2, all express CD46 protein at much higher levels compared with CAR (Fig. 1A). AsPC-1 and BxPC-3 cells also express high levels of desmoglein (Fig. 1B). These results were also confirmed by measuring mRNA transcript levels (Fig. 1C). To compare Ad.5/3 infection to that of Ad.5, we used viruses expressing a luciferase reporter gene. In all pancreatic cell lines, higher luciferase levels were found using the Ad.5/3 serotype compared with the Ad.5 serotype (Fig. 1D). Pretreatment of cells with blocking antibodies to CAR, CD46, desmoglein, or a combination of the three confirmed the importance of all three receptors in determining infectivity of pancreatic cancer cells, with infection being inhibited the most when antibodies to all three receptors were used in combination (Fig. 1E). These results support the use of the Ad.5/3 virus as a means of effectively delivering mda-7/IL-24 to pancreatic cancer cells.

**D-Limonene Inhibits Growth of Pancreatic Cancer Cells and Induces ROS.** D-Limonene is able to inhibit the proliferation of multiple pancreatic cancer cells but does not affect growth of an immortalized pancreatic mesenchymal cell line, LT2 (Fig. 2A). D-Limonene is a monoterpene and capable of inhibiting the prenylation of GTPases, such as K-ras (Chen et al., 1999). However, the doses of d-limonene we used in our study did not affect the normal prenylation of K-ras (Fig. 2B). We next evaluated the ability of d-limonene to induce ROS and found that even at nontoxic or sublethal doses d-limonene promoted ROS induction, specifically peroxides, and this increase was abrogated with N-acetyl cysteine (NAC) pretreatment (Fig. 2C).

**D-Limonene Enhances Translation of mda-7/IL-24 mRNA and Promotes Toxicity of Ad.mda-7.** Previous studies indicate that the translational block seen with mda-7/IL-24 mRNA in pancreatic cancer cells can be overcome with ROS induction (Lebedeva et al., 2008a,b). Because d-limonene showed significant ROS induction, we infected cells with Ad.5-mda-7 or Ad.5/3-mda-7 in the presence or absence of d-limonene to determine if this compound could enhance mda-7/IL-24 mRNA translation and promote toxicity. In LT2 cells, we found no effect on growth (Fig. 3A). However, in pancreatic cancer cell lines PANC-1 (Fig. 3B) and MIA PaCa-2 (Fig. 3C), the addition of d-limonene to Ad.mda-7-infected cells was able to significantly enhance growth inhibition. Additionally, in agreement with prior results comparing Ad.5 versus Ad.5/3, Ad.5/3-mda-7 alone was able to induce a greater growth inhibition compared with Ad.5-mda-7 (Dash Fig. 1.)
et al., 2010b; Azab et al., 2012, 2014). Western blotting showed similar results, with Ad.5/3-mda-7 inducing greater MDA-7/IL-24 protein expression, both with and without the addition of D-limonene compared with Ad.5-mda-7 (Fig. 3, D and E). This correlated with an increase in cell death, as indicated by increased poly(ADP-ribose) polymerase cleavage in these cells. This effect correlated with decreases in the expression of the Bcl-2 anti-apoptotic proteins Mcl-1 and Bcl-xL.

Effects of D-Limonene in Inducing Cancer-Specific Cell Death Are Dependent on ROS Induction. To evaluate whether cell death induction and growth inhibition following treatment with D-limonene are related to its ability to induce ROS, we performed MTT assays and Western blotting to assess the extent of ROS production and the expression of markers of apoptosis. These experiments showed that D-limonene treatment led to a significant increase in ROS levels, as well as a decrease in the expression of the anti-apoptotic proteins Mcl-1 and Bcl-xL.

Fig. 2. D-Limonene (D-Lim) inhibits cellular proliferation by inducing ROS. (A) Pancreatic cells were treated with increasing doses of D-Lim, and MTT assays were performed after 72 hours to measure cell proliferation. (B) Western blotting analysis of K-ras prenylation status after treatment with D-Lim. (C) MIA PaCa-2 cells were treated with D-Lim with or without NAC pretreatment and stained with dihydrodichlorofluorescein to detect ROS. ROS induction was quantified using fluorescence measurements. A.U., arbitrary units; Conc., concentration.

Fig. 3. D-Limonene (D-Lim) synergizes with mda-7/IL-24, in inducing cancer-specific apoptosis in pancreatic cancer cells. Pancreatic cells were infected with increasing m.o.i (multiplicity of infection; pfu/cell) of Ad.5-mda-7 and its recombinant chimeric Ad.5/3-mda-7, followed by 100 μM D-Lim treatment. MTT assays were performed in (A) LT2 cells, (B) PANC-1 and (C) MIA PaCa-2 cells 72 hours after treatment. (D and E) Western blotting of MIA PaCa-2 lysates for MDA-7 and markers of apoptosis. O.D., optical density.
ROS, we pretreated cells with NAC before Ad infection and d-limonene treatment. As expected, this addition did not show any change in proliferation in LT2 cells (Fig. 4A). However, in both mutant K-ras–bearing MIA PaCa-2 (Fig. 4B) and wild type K-ras–bearing BxPC-3 cells (Fig. 4C) pretreatment with NAC significantly rescued cells from the growth-inhibitory effects of Ad.5/3-mda-7 plus d-limonene. To further confirm that these changes were reflective of alterations in proliferation, bromo-deoxyuridine (5-bromo-2'-deoxyuridine) incorporation assays were done in both LT2 and MIA PaCa-2 cells. These studies confirmed the observations seen by MTT, showing very similar results (Supplemental Fig. 1). The observation of ROS levels (specifically peroxide) confirmed that NAC pretreatment prevented d-limonene–induced ROS generation (Fig. 4D). Pyruvate, another ROS scavenger, was also shown to inhibit this increase in ROS (Fig. 4D). Similar results were obtained when monitoring apoptosis induction, with LT2 cells showing no increase in apoptosis, but MIA PaCa-2 and BxPC-3 cells infected with Ad.5/3-mda-7 and treated with d-limonene showing increases in Annexin V staining, cleaved poly(ADP-ribose) polymerase, and cleaved caspase-3 (Fig. 5). Furthermore, these cells also showed increased mda-7/IL-24 protein expression as well as an increase in an ER stress marker, BiP/GRP-78, which is induced by mda-7/IL-24. These effects were also prevented by pretreatment with NAC (Fig. 5).

**Fig. 4.** d-Limonene enhances mda-7/IL-24–mediated inhibition of cell growth by inducing ROS (specifically peroxide). Pancreatic cells were pretreated with 10 mM NAC prior to treatment with Ad.5/3-mda-7 (25 pfu/cell) and d-Lim. MTT assays were performed on (A) LT2 cells, (B) MIA PaCa-2, and (C) BxPC-3 cells 72 hours after treatment. (D) MIA PaCa-2 cells were pretreated with either NAC or peroxide scavenger, pyruvate, and then stained with DCF to detect ROS induction (specifically peroxide). A.U., arbitrary units; O.D., optical density.

**ROS Induction by d-Limonene Activates Translational Machinery in Cells.** The effects of d-limonene when used in conjunction with Ad.mda-7 infection are similar to what we have observed previously with other ROS-inducing agents (Lebedeva, et al., 2005, 2008a,b). In an effort to enhance our understanding of the mechanism behind these observations, we evaluated the effects of d-limonene further. We found that d-limonene–induced ROS generation occurs in both a dose- and time-dependent manner (Fig. 6A). We know that without this compound, mda-7/IL-24 mRNA is very weakly translated when introduced into pancreatic cancer cells via Ad infection and that the addition of d-limonene results in greater translation of mda-7/IL-24 mRNA into protein. To further confirm that d-limonene is able to increase the translation of this mRNA into protein, we looked at the expression of the preinitiation complex proteins after d-limonene treatment. In accordance with our observed time-dependent ROS induction, we found that d-limonene is able to activate the transient translational machinery within a few hours of treatment. Increases in phosphorylated eIF4E were found to peak at around 3 hours post-treatment. Translational repressor 4EBP-1, which inhibits eIF4E in its unphosphorylated form, also demonstrated an increase in phosphorylation that peaked at 3 hours after d-limonene treatment. We also saw activation of upstream kinase P70S6K in this same time frame (Fig. 6, B and C). The dose of...
D-Limonene used in these experiments showed potent induction of ROS at 3 hours after treatment (Fig. 6A), supporting a potential role of ROS in the activation of the translational machinery. To confirm that this activation of the preinitiation complex was related to the induction of ROS, we pretreated cells with NAC and also included the potent ROS-inducer arsenic trioxide as a positive control. D-Limonene was able to induce the phosphorylation of P70S6K, eIF4E, and 4EBP-1 to the same extent as arsenic trioxide. Importantly, these increases in phosphorylation were completely abrogated if cells were pretreated with NAC (Fig. 6D).

D-Limonene Increases mda-7/IL-24 mRNA Polysome Association. These experiments provide a novel role for D-limonene, showing that this compound induces ROS that can subsequently activate the translational machinery in pancreatic cancer cells. We have also shown that the overall effect of this phenomenon is increased mda-7/IL-24 translation, thereby resulting in cancer-specific toxicity. We finally wanted to determine if the activation of the translational machinery actually resulted in increased association of mda-7/IL-24 mRNA with the polysomes. Although we have demonstrated that MDA-7/IL-24 protein levels increase, showing increased polysome association confirmed our hypothesis that the ROS generated by D-limonene not only activated the preinitiation complex of the translational machinery but also subsequently facilitated the association of mda-7/IL-24 mRNA with polysomes. As a proof-of-principle, MIA PaCa-2 cells were infected with either Ad.5/3-mda-7 or Ad.5/3-mda-7, with or without subsequent D-limonene treatment. Polysomal fractions from these cells were separated using sucrose gradient gels (Fig. 7A) and then assayed for the presence of mda-7/IL-24 mRNA using real-time PCR. We found that with both Ad serotypes, the addition of D-limonene resulted in a significantly increased polysome association of mda-7/IL-24 mRNA as observed by a decreased Ct value (Fig. 7B). There was no apparent change in the association of the housekeeping gene GAPDH mRNA with polysomes (Fig. 7, B and C), indicating that D-limonene induces the translation of weakly translated mRNAs such as mda-7/IL-24. In order to see the fold-change in polysomal-associated mda-7/IL-24 mRNA with respect to polysomal-associated GAPDH mRNA following treatment with Ad.mda-7/IL-24 and/or D-limonene, the relative quotient was calculated. It was found that D-limonene significantly increased the association of weakly translated mda-7/IL-24 mRNA with polysomes when MIA PaCa-2 cells were treated with mda-7/IL-24 using either serotype of Ad (Fig. 7C). Furthermore, pretreating cells with NAC prevented increased polysome association, supporting our conclusion that it is indeed the induction of ROS that ultimately leads to increased mda-7/IL-24 translation. Western blots confirmed that this combination resulted in increased MDA-7/IL-24 protein expression following phosphorylation of translation machinery proteins P70S6K and eIF4E, which leads to the formation of the preinitiation complex. All of these changes were inhibited with NAC pretreatment (Fig. 7D).

D-Limonene Induces mda-7/IL-24 Expression and Toxicity in Cell Lines That Stably Overexpress mda-7/IL-24 mRNA. To ensure that the effects of D-limonene on mda-7/IL-24 mRNA translation were not dependent on the adenoviral delivery of mda-7/IL-24, we evaluated this compound in pancreatic cancer cell lines that stably overexpress mda-7/IL-24 mRNA, without detectable protein. PANC-1 (Fig. 8A) and MIA PaCa-2
Fig. 8B stable mda-7/IL-24 mRNA–expressing clones were treated with increasing doses of D-limonene for 3 days and evaluated for effects on proliferation by MTT assays. We found that compared with parental cell lines containing an empty vector, the mda-7/IL-24–overexpressing clones exhibited greater growth inhibition by D-limonene, with left-shifting dose curves and lower IC₅₀ values. The IC₅₀ values of PANC-1 and MIA PaCa-2 treated with D-limonene were 413.15 and 385.12 µM, respectively. The IC₅₀ values were decreased to 337.12 and 195.11 µM, respectively, for PANC-1 overexpressing mda-7/IL-24 (PANC-1-m7) and MIA PaCa-2 overexpressing mda-7/IL-24 (MIA PaCa-2-m7), respectively. Western blotting results confirmed that both D-limonene and POH were able to induce MDA-7/IL-24 protein expression in pancreatic cancer cells overexpressing mda-7/IL-24, and this expression resulted in cell death as demonstrated by poly(ADP-ribose) polymerase cleavage (Fig. 8C).

Discussion

Pancreatic cancer is an invariably fatal disease that currently lacks effective therapeutic options for patients (Hezel et al., 2006). The development of novel ways to combat this cancer is essential. mda-7/IL-24 has shown great promise as a cancer therapeutic (Fisher et al., 2003, 2005; Cunningham et al., 2005; Tong et al., 2005; Eager et al., 2008; Dash et al., 2010a). This tumor suppressor, when introduced back into cancer cells, is capable of inducing ER stress and apoptosis, initiating an autocrine and paracrine feedback-loop to increase mda-7/IL-24 expression, and fostering an anticancer immune response (Fisher, 2005; Dash et al., 2010a). Furthermore, this cytokine shows a bystander effect as a result of its feedback loop, whereby MDA-7/IL-24 protein secretion from cells is able to bind to receptors on neighboring normal and cancer cells and induce mda-7/IL-24 expression in those cells (Su et al., 2005; Sauane et al., 2008). This loop intensifies the overall anticancer response and contributes significantly to its efficacy as a cancer therapeutic.

Unfortunately, the use of mda-7/IL-24 in pancreatic cancer has been restricted, owing to decreased translation of its encoded mRNA into protein (Su et al., 2001; Lebedeva et al., 2008a,b; Dash et al., 2014). Adenoviral delivery of this cytokine results in robust mda-7/IL-24 mRNA expression with only low or limited levels of expressed MDA-7/IL-24 protein, and consequently minimal changes in cellular phenotype. We have studied strategies to overcome this block in protein translation of the mda-7/IL-24 mRNA (Su et al., 2001; Lebedeva et al., 2005, 2006, 2007, 2008a,b; Sarkar et al., 2013; Dash et al., 2014), and thereby increase the utility of mda-7/IL-24 for the therapy of pancreatic cancer. Our previous work has shown
that inhibiting oncogenic K-ras is one mechanism of increasing the translation of mda-7/IL-24 mRNA. The mechanism through which K-ras influences MDA-7/IL-24 protein expression is currently unknown. K-ras signaling involves a multitude of downstream target proteins and it is possible that one of these K-ras effector proteins is involved in protein translation. While this is a useful strategy in vitro, it is difficult to translate this into the clinic, as inhibiting K-ras in patients is problematic because of the lack of success in the development of drugs specifically targeting this oncogene (Maurer et al., 2012; Sun et al., 2012; Hocker et al., 2013). We also found that the induction of ROS was able to increase expression of MDA-7/IL-24 protein and produce a subsequent therapeutic effect. Potent ROS inducers such as arsenic trioxide show great success when used in combination with mda-7/IL-24 in experimental settings (Lebedeva et al., 2005).

We then turned our studies to more natural compounds capable of inducing ROS. Drug toxicity is a major problem in the treatment of cancer and this only intensifies as patients are treated with increasing numbers of drugs in combination. We evaluated POH, a dietary agent known to induce ROS at nontoxic doses. It was demonstrated that the combination of mda-7/IL-24 and POH also induced MDA-7/IL-24 expression and potent cell death in pancreatic cancer cells (Lebedeva et al., 2008b).

While we had made advances in increasing the utilization of mda-7/IL-24 as a therapy for pancreatic cancer, a few outstanding questions needed to be addressed. The goal of this study was to examine some of those questions. Could we improve our viral delivery of mda-7/IL-24 as a means of increasing therapeutic efficacy? Do dietary agents other than POH also function to increase mda-7/IL-24 mRNA translation and promote subsequent phenotypic effects?

We began this study by evaluating our delivery system of mda-7/IL-24. Adenoviral delivery mechanisms can be a successful means of gene therapy but are reliant on certain factors for success. Adenoviral serotypes use different cellular...
receptors as a means of introducing their genetic material into target cells (Bergelson et al., 1998; Pearson et al., 1999; Dash et al., 2010b; Wang et al., 2011; Azab et al., 2012, 2014). Ad.5-mda-7, the traditional type 5 adenovirus serotype used in our prior work, is dependent on the expression of Coxsackie and adenovirus receptors (CAR) for efficacy. Our initial experiments showed that, as a whole, pancreatic cancer cells expressed relatively low levels of CAR, a factor that was most likely preventing us from successfully delivering optimum levels of mda-7/IL-24 to these cells. In contrast, the Ad.5/3 serotype uses desmoglein and CD46, as well as CAR, receptors for entry into cells. We show that pancreatic cancer cells express higher levels of these receptors, both on an mRNA and protein level and that expression of all three receptors yielded the highest rate of success in gene delivery (Fig. 1E).

However, despite being able to increase the infection efficacy of Ad, we still only observed minimal MDA-7/IL-24 protein expression. Our prior studies have shown that POH, a dietary monoterpen, is able to relieve this translational block when combined with Ad.mda-7 infection and enhance MDA-7/IL-24 protein production and subsequent expression through the induction of ROS (Lebedeva et al., 2008a,b). These observations supported the hypothesis that other monoterpenes that are capable of inducing ROS might also have similar effects on MDA-7/IL-24 protein expression. In this study, we evaluated D-limonene, also a dietary monoterpen known to induce ROS. This compound has a low toxicity profile and has been shown to be safe when used in humans. Adverse effects reported include skin irritation when D-limonene was used in cosmetic products (Kim et al., 2013). As a systemic therapy, this compound has been used in humans in clinical trials at up to 15 gm/day without major toxicities being observed. Some mild side effects, such as nausea or fatigue, were seen at higher doses (Sun, 2007). Given its safe drug profile, D-limonene is a viable candidate for inclusion as adjuvant treatment in patients.

We found that, similar to POH, nontoxic doses of D-limonene are able to enhance MDA-7/IL-24 protein production in cells infected with Ad.mda-7, which translates into mda-7/IL-24–induced toxicity. Our experiments demonstrated that this ROS induction was necessary for MDA-7/IL-24 protein production and that it enhanced translation through activation of the translation machinery and hence increased polysome association of mda-7/IL-24 mRNA and subsequent protein expression. These observations correlate with studies in the literature demonstrating that ROS generated via extracellular hydrogen peroxide is capable of inducing activation of p70S6K and its downstream target 4EBP-1 (Bae et al., 1999). We observed that ROS generated through D-limonene was also able to activate these members of the preinitiation complex and that led to subsequent activation of translation. Furthermore, we showed that this strategy was successful using both an adenoviral delivery strategy as well as stable transfection of mda-7/IL-24, resulting in mRNA expression with little protein production, in pancreatic cancer cells.

As a whole, our data supports the efficacy of mda-7/IL-24 gene therapy in pancreatic cancer. The use of mda-7/IL-24 as a cancer therapeutic is exciting and promising in many cancer types, but progress in pancreatic cancer has been slow. We
have made major strides in developing strategies to use this cancer-specific cytokine in this disease but continue to work on this issue. The presented data help to answer some of our outstanding questions and represent novel ways of increasing the efficacy and therapeutic use of mda-7/IL-24 in this devastating disease.

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Authorship Contributions

Participated in research design: Fisher, D. Sarkar, Das, Emdad, Conducted experiments: S. Sarkar, Quinn, Shen, Contributed new reagents or analytic tools: Dent, Performed data analysis: S. Sarkar, Quinn, Das, Emdad, Fisher, Wrote or contributed to the writing of the manuscript: S. Sarkar, Quinn, Emdad, Das, D. Sarkar, Dent, Fisher.

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Reversing translational suppression and induction of toxicity in pancreatic cancer cells using a chemoprevention gene therapy (CGT) approach

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Supplementary Figure 1

**Figure 1:** D-Lim enhances mda-7/IL-24-mediated inhibition of cell growth by inducing ROS. Pancreatic cells were pretreated with 10 mM NAC prior to treatment with Ad.5-mda-7 (25 pfu/cell) and D-Lim. BrdU incorporation assays were performed on (A) LT2 cells and (B) MIA PaCa-2 cells.