A serotype 5/3 adenovirus expressing MDA-7/IL-24 infects renal carcinoma cells and promotes toxicity of agents that increase ROS and ceramide levels.


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Abbreviations: ERK: extracellular regulated kinase; MEK: mitogen activated extracellular regulated kinase; JNK: c-Jun NH2-terminal kinase; PI3K: phosphatidylinositol 3 kinase; MDA-7: melanoma differentiation associated gene-7; IL-24: interleukin-24; PERK: protein kinase R –like endoplasmic reticulum kinase; ER: endoplasmic reticulum; AR: anoikis resistant; MAPK: mitogen activated protein kinase; ca: constitutively active; dn: dominant negative; EGFR: epidermal growth factor receptor; PTEN: phosphatase and tensin homologue on chromosome ten; ROS: reactive oxygen species; 4HPR: N-(4-Hydroxyphenyl) retinamide (fenretinide); As2O3: arsenic trioxide; 17AAG: 17-N-allylamino-17-demethoxygeldanamycin; RCC: renal carcinoma cell; GST: glutathione-S-transferase; 5/3: serotype 5 / serotype 3 adenovirus; CMV: empty vector plasmid or virus; si: small interfering; SCR: scrambled; Calb.: calbindin; TRX: thioredoxin; IP: immunoprecipitation; MYR: myriocin; LASS: longevity assurance gene; Ad: adenovirus; DCF: dichloro-dihydrofluorescein; VHL: Von Hippel Lindau; CAR: coxsakie and adenovirus receptor; DISC: death-inducing signaling complex; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; VEH: vehicle; ATO: arsenic trioxide; GU: genito-urinary.
Abstract

It is recognized that agents which generate reactive oxygen species (ROS) enhance MDA-7/IL-24 lethality. The present studies focused on clarifying how such agents enhanced MDA-7/IL-24 toxicity in renal cell carcinoma cells (RCCs). Infection of RCCs with a tropism-modified serotype 5/3 adenovirus expressing MDA-7/IL-24, Ad.5/3-\textit{mda-7}, caused plasma membrane clustering of CD95 and CD95 association with pro-caspase 8, effects that were enhanced by combined exposure to 17AAG, As$_2$O$_3$ or 4HPR, and which correlated with enhanced cell killing. Knock down of CD95 or expression of c-FLIP-s blocked enhanced killing. Inhibition of ROS generation, elevated cytosolic Ca$^{2+}$, or \textit{de novo} ceramide synthesis blocked Ad.5/3-\textit{mda-7} +/- agent–induced CD95 activation and the enhancement of apoptosis. Ad.5/3-\textit{mda-7}– increased ceramide levels in a PERK-dependent fashion that were responsible for elevated cytosolic Ca$^{2+}$ levels that promoted ROS generation; 17AAG did not further enhance cytokine–induced ceramide generation. \textit{In vivo}, infection of RCC tumors with Ad.5/3-\textit{mda-7} suppressed the growth of infected tumors that was enhanced by exposure to 17AAG. Our data indicates that in RCCs Ad.5/3-\textit{mda-7}-induced ceramide generation plays a central role in tumor cell killing and inhibition of multiple signaling pathways may have utility in promoting MDA-7/IL-24 lethality in renal cancer.
Introduction

In the United States, renal cell carcinoma (RCC) is diagnosed in ~51,000 patients per annum. If the disease is detected at an early stage, in which a large portion or the entire kidney can be removed with the tumor, a high level of prolonged patient survival is noted (Gillett et al, 2005, and references therein). However, if the disease has spread beyond the capsule of the kidney into the adrenal gland or surrounding fascia with nodal involvement the prognosis is poor with rapid nadir. This occurs even under ideal circumstances where the disease is still only locally advanced and essentially all of the tumor can be surgically removed and the patients are maximally treated with palliative radiation and chemotherapy. In part, this resistance is due to the fact that RCC is characterized as frequently being highly refractory to multiple established cytotoxic chemotherapy regimens (Gillett et al, 2005).

The mda-7 gene (renamed Interleukin 24, IL-24) was isolated from human melanoma cells induced to terminally differentiate by treatment with interferon beta and mezerein (Jiang et al, 1995). The protein expression of MDA-7/IL-24 is decreased during melanoma progression, with nearly imperceptible levels in metastatic disease (Jiang et al, 1995; Ekmekcioglu et al, 2001; Ellerhorst et al, 2002). Based on an internal amino acid signature motif, MDA-7/IL-24 was classified as a member of the interleukin-10 (IL-10) gene family (Huang et al, 2001; Parrish-Novak et al, 2002; Caudell et al, 2002; Pestka et al, 2004; Gupta et al, 2006; Lebedeva et al, 2005; Fisher et al, 2003; Fisher, 2005). Enforced expression of MDA-7/IL-24, by use of a recombinant adenovirus Ad.5-mda-7, inhibits the growth and kills a broad spectrum of cancer cells, without exerting toxic effects in a wide assortment of non-transformed “normal” cell types (Gupta et al, 2006; Lebedeva et al, 2005; Fisher et al, 2003; Fisher, 2005; Su et al, 2001; Su et al, 1998). Mda-7/IL-24 was evaluated in a Phase I clinical trial in heavily pre-treated patients with advanced cancers and in this study Ad.5-mda-7 (INGN-241) injected intra-tumorally was safe and with repeated injections resulted in significant clinical activity in many patients (Lebedeva et al, 2005; Lebedeva et al, 2007; Fisher et al, 2003; Cunningham et al, 2005).
The apoptotic pathways by which MDA-7/IL-24 kills tumor cells are still not fully understood, however, current evidence suggests an inherent high degree of complexity and an involvement of proteins important for the onset of growth inhibition and apoptosis, including BCL-XL, BCL-2 and BAX (Gupta et al, 2006; Gupta et al, 2006a; Eulitt et al, 2010; Lebedeva et al, 2002; Fisher, 2005; Su et al, 2001; Su et al, 1998; Park et al, 2009). In prostate cancer cells, over-expression of either BCL-2 or BCL-XL protects cells from MDA-7/IL-24-induced toxicity in a cell type-dependent fashion (Su et al, 2006). In ovarian cancer, MDA-7/IL24 was reported to kill via the extrinsic apoptosis pathway (Gopalan et al, 2005) and we recently demonstrated that bacterially synthesized GST-MDA-7 killed multiple renal carcinoma cell lines also via activation of CD95 / FAS receptor (Park et al, 2009).

MDA-7/IL-24 toxicity has also been linked to alterations in endoplasmic reticulum (ER) stress signaling (Yacoub et al, 2008; Yacoub et al, 2008a; Sarkar et al, 2002; Gupta et al, 2006a). In these studies, MDA-7/IL-24 physically associates with BiP/GRP78 and inactivates the protective actions of this ER-chaperone protein as judged by increased PKR-like endoplasmic reticulum kinase (PERK) auto-phosphorylation and increased phosphorylation of the downstream PERK target eIF2α. In addition to virus-administered mda-7/IL-24, delivery of this cytokine as a bacterially expressed GST fusion protein, GST-MDA-7, retains cancer-specific killing, selective ER localization and induces similar signal transduction changes in cancer cells (Sauane et al, 2004; Gupta et al, 2006a; Gupta et al, 2008; Yacoub et al, 2008; Yacoub et al, 2010). We have noted that high concentrations of GST-MDA-7 or infection with a serotype 5 virus to deliver the mda-7 transgene, Ad.5-mda-7, kill primary human glioma cells and do so in a PERK-dependent and ceramide-dependent fashion that requires mitochondrial dysfunction but not activation of the extrinsic pathway (Yacoub et al, 2008; Yacoub et al, 2010). Similar data were obtained in a genitor-urinary (GU) malignancy; prostate cancer. Thus, MDA-7/IL-24 lethality seems to occur by multiple distinct pathways in different tumor cell types, but in all of these studies, cell killing is reflected in a profound induction, downstream of primary effector molecules, in mitochondrial dysfunction.
Prior work with MDA-7/IL-24 in renal carcinoma cells and malignant gliomas, using bacterially synthesized GST-MDA-7 protein, demonstrated that in the low 0.25-2.0 nM concentration range GST-MDA-7 primarily causes growth arrest with little cell killing, whereas at ~20-fold greater concentrations, this cytokine causes profound growth arrest and tumor cell death (Yacoub et al, 2003; Park et al, 2009). Agents that are known to promote the generation of reactive oxygen species (ROS) in tumor cells, e.g., As$_2$O$_3$ or 4-HPR (Lebedeva et al, 2005a), promoted GST-MDA-7 toxicity that correlated with enhanced activation of p38 MAPK; p38 MAPK signaling being a key pro-apoptotic signal caused by MDA-7/IL-24 expression (Sarkar et al, 2002). The precise mechanisms by which ROS inducing agents interact with MDA-7/IL-24 to kill renal carcinoma cells, e.g. by altering MDA-7/IL-24 –induced activation of CD95, are unknown.

In the clinic, MDA-7/IL-24 has been examined in a phase I study using a gene therapy approach by means of a recombinant serotype 5 adenovirus to deliver the mda-7/IL-24 gene (INGN-241) (Fisher et al, 2003; Fisher, 2005; Lebedeva et al, 2007; Cunningham et al, 2005). Kidney cancer cells display low levels of the coxsakie and adenovirus receptor (CAR) resulting in many of the commercially available cell lines being relatively resistant to infection by a serotype 5 adenovirus. Utilizing established human RCC lines we have used a recently engineered novel chimeric serotype 5 / serotype 3 adenovirus to deliver the mda-7/IL-24 gene to RCCs; Ad.5/3-mda-7 (Dash et al, 2010; Eulitt et al, 2010; Hamed et al, 2010). Using Ad.5/3-mda-7 we determined the molecular mechanisms upstream and proximal to CD95 activation by which MDA-7/IL-24 and agents that elevate ROS levels (17AAG; As$_2$O$_3$; 4HPR) interact to kill RCCs.
Materials and Methods

Materials.

A plasmid expressing dominant negative PERK was kindly supplied by Dr. A. Diehl (University of Pennsylvania). Commercially available validated short hairpin RNA molecules to knock down RNA/protein levels were from Qiagen (Valencia, CA), as described in refs. (Gupta et al, 2006a; Yacoub et al, 2008; Yacoub et al, 2008a; Yacoub et al, 2010; Park et al, 2010). Antibody reagents, kinase and caspase inhibitors, cell culture reagents, and sources of non-commercial recombinant adenoviruses have been previously described (Gupta et al, 2006a; Yacoub et al, 2008; Yacoub et al, 2008a; Yacoub et al, 2010; Park et al, 2010; Hamed et al, 2010; Eulitt et al, 2010). Arsenic trioxide, 17AAG (17-N-Allylamino-17-demethoxygeldanamycin), and 4HPR ((4-hydroxy (phenyl) retinamide)) were obtained from EMD biosciences (Darmstadt, Germany).

Methods.

Generation of Ad.5.mda-7 and Ad.5/3.mda-7. Recombinant serotype 5 and serotype 5 / serotype 3 adenoviruses to express MDA-7/IL-24 (“Ad.mda-7”), control (“Ad.cmv” empty vector) were generated in the laboratory of Dr. Curiel as described in ref. (Su et al, 1998; Hamed et al, 2010; Dash et al, 2010).

Synthesis of GST-MDA-7. GST and GST-MDA-7 was generated in bacteria and purified by glutathione affinity chromatography as previously described (Sauane et al, 2004).

Cell culture and in vitro exposure of cells to GST-MDA-7 and drugs. All established RCC lines were cultured at 37 °C (5% (v/v CO₂) in vitro using RPMI medium supplemented with 5% (v/v) fetal calf serum and 10% (v/v) Non-essential amino acids. The 786-0 lines transfected with empty vector or to express VHL were originally generated and supplied by Dr. W. Kaelin (Harvard University, Boston, MA). For short-term cell killing assays and immunoblotting, cells were plated at a density of 3 x 10³ per cm² and 24h after plating were treated with GST-MDA-7 or infected with Ad.5/3.mda-7 and/or various drugs, as indicated. In vitro small molecule inhibitor treatments were from a 100 mM stock solution of each drug and the maximal
concentration of Vehicle (DMSO) in media was 0.02% (v/v). Cells were not cultured in reduced serum media during any study.

**Cell treatments, SDS-PAGE and Western blot analysis.** Cells were treated with various GST-MDA-7 concentrations or infected with Ad.5/3- mda-7 at various multiplicities of infection (moi) as indicated in the Figure legends. SDS PAGE and immunoblotting was performed as described using standard techniques. Briefly, At various time points after indicated treatment, hepatocytes were lysed in whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromphenol blue), and the samples were boiled for 30 min. The boiled samples were loaded onto 10-14% SDS-PAGE, and electrophoresis was run overnight. Proteins were electrophoretically transferred onto 0.22-μm nitrocellulose and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized using an Odyssey Infra red imager with associated software. For presentation, immunoblots were opened in Adobe PhotoShop CS2; the color was removed, and figures were generated in Microsoft PowerPoint (Park et al, 2010).

**Recombinant adenoviral vectors; infection in vitro.** We generated and purchased previously noted recombinant adenoviruses as per refs. (Su et al, 1998; Gupta et al, 2006a; Yacoub et al, 2008; Yacoub et al, 2008a; Yacoub et al, 2010). Cells were infected with these adenoviruses at an appropriate moi as indicated in the Figure / Legend. Cells were incubated for 24 h after infection to ensure adequate expression of transduced gene products prior to any subsequent drug exposures.

**Detection of cell death by Trypan Blue, Hoechst, TUNEL and flow cytometric assays.** Cells were harvested by trypsinization with Trypsin/EDTA for ~10 min at 37 °C. As some apoptotic cells detached from the culture substratum into the medium, these cells were also collected by centrifugation of the medium at 1,500 rpm for 5 min. The pooled cell pellets were resuspended. Trypan blue exclusion cell death assays were performed as individual assays in triplicate on each experimental occasion (Yacoub et al, 2010; Hamed et al, 2010).
**Plasmid transfection.** Plasmid DNA (0.5 μg / total plasmid transfected) was diluted into 50 μl of RPMI growth media that lacked supplementation with FBS or with penicillin-streptomycin. Lipofectamine 2000 reagent (1 μl) (Invitrogen, Carlsbad, CA) was diluted into 50 μl growth media that lacked supplementation with FBS or with penicillin-streptomycin. The two solutions were then mixed together and incubated at room temperature for 30 min. The total mix was added to each well (4-well glass slide or 12-well plate) containing 200 μl growth media that lacked supplementation with FBS or with penicillin-streptomycin. The cells were incubated for 4 h at 37°C, after which time the media was replaced with RPMI growth media containing 5% (v/v) FBS and 1x Pen-Strep.

**Mass spectrometric determination of ceramide and dihydroceramide lipid levels.** A498 cells were scraped into PBS 6 h after exposure and isolated by centrifugation followed by freezing at -80°C. Lipids were isolated from the cells and ceramide isoforms analyzed by liquid chromatography, electrospray ionization-tandem mass spectrometry with a Shimadzu (Columbia, MD, USA) LC-10 binary pump system coupled to a PerkinElmer Series 200 auto-injector and an Applied Biosystems 4000 QTRAP operating in a triple quadrupole mode, as described previously (Hait et al, 2009).

**Animal studies.** Athymic Nu/Nu mice (8 week old, female) were obtained from the NCI. Mice were irradiated (1.5 Gy) 48 h prior to injection of animals with 1.0 x 10^7 A498 cells further suppressing animal immune systems thereby improving take rate. Tumors of ~150 mm^3 grew over the following 29 days. Animals were segregated into tumor volumes of approximate equivalent mean tumor size and standard errors were determined. For studies in Figure 6A, the tumor was injected with 2 μl (1 x 10^8 infectious particles) of either Ad.5/3-cmv or Ad.5/3-mda-7 suspended in 2 μl of PBS were delivered by slow infusion over a 6 min period. Two days after the first virus infection, tumors were again infected in an identical fashion with adenovirus. The mean volumes of the tumors on each flank are presented as Fold-increase over the pre-infected volume (defined as 1.00) (n = 3, +/- SEM; 9-10 mice per group total). For the studies in
Figures 6B and 6C adenoviral vectors, Ad.5/3-\textit{mda-7} or Ad.5/3-\textit{cmv} were administered 29 days after tumor cell implantation into animals. Viral vectors (Ad.5/3-\textit{mda-7} or Ad.\textit{cmv}; \textit{nota bene}, 0.5 x 10^7 p.f.u.) suspended in 2 \(\mu\)l of PBS were delivered by slow infusion over a 6 min period. This infection procedure was repeated one week later. Animals were treated 24 h after each virus infection with either vehicle or 4HPR (100 mg/kg) or 17AAG (10 mg/kg) every day for three days. Animals then received a 2 day treatment break. Tumor volumes were measured every two-three days, as indicated.

\textit{Immunohistochemistry and staining of fixed tumor sections} Post sacrifice, tumors were fixed in OCT compound (Tissue Tek); cryostat sectioned (Leica) as 12 \(\mu\)m sections. Nonspecific binding was blocked with a 2 \%(v/v) Rat Sera, 1 \%(v/v)Bovine Sera, 0.1\%(v/v) Triton X100, 0.05\%(v/v) Tween-20 solution then sections were stained for apoptosis and growth markers: Cleaved Caspase 3 (rabbit IgG, 1:100; Cell Signaling); Ki67 (mouse IgG, 1:100; Santa Cruz). For staining of sectioned tumors, primary antibodies were applied overnight, sections washed with phosphate buffer solution, and fluorescein-tagged secondary antibodies applied for detection (as indicated in the Figure): goat antirat Alexa 488/647 (1:500; Invitrogen); goat anti-mouse Alexa 488/647 (1:500; Invitrogen) secondary antibody as per the primary antibody used. Sections were then de-hydrated, cleared and mounted with cover-slips using DAPI mounting media (Vectastain). Apoptotic cells with double stranded DNA breaks were detected using the Upstate TUNEL Apoptotic Detection Kit according to the manufacturer’s instructions. Slides were applied to high powered light/confocal microscopes (Zeiss LSM 510 Meta-confocal scanning microscope; Zeiss HBO 100 microscope with Axio Cam MRm camera). The proliferation zone which included both tumor and normal tissue was usually selected as the site of interest, within 2mm of, or juxtaposed to leading edge of the tumor.

\textit{Analysis of DISC formation.} Cells were lysed as described in ref. (Park et al, 2009; Park et al, 2010). Portions of lysate (5\%) were used to determine total protein levels. The remaining portion of lysate was subjected to immunoprecipitation for CD95. The amount of co-immunoprecipitating caspase 8 was determined after SDS PAGE and anti-caspase 8 blotting.
Analysis of reactive oxygen species (ROS) and cytosolic Ca^{2+} levels. ROS levels were determined in a Vector 3 plate reader as described previously (Park et al., 2009; Yacoub et al., 2010). Briefly, cancer cells were plated in 96-well plates. Dichloro-dihydrofluorescein (DCFH2CA), which is non-fluorescent in its dihydro form but becomes highly fluorescent upon reaction with reactive oxygen species (ROS) to DCF, was used to monitor production of cellular ROS. Cells were pre-incubated with dihydro-DCF (Molecular Probes Eugene, OR, 5 mmol/dm^{3} for 30 min). Fluorescence measurements were obtained 0 to 30 minutes after drug addition with a Vector 3 plate reader. Data are presented corrected for basal fluorescence of vehicle-treated cells at each time point and expressed as a fold increase in reactive oxygen species (ROS) levels. For cytosolic Ca^{2+} levels carcinoma cells, seeded in 96-well plates, with fura-2 acetoxymethylester as an indicator. The ratio of fura-2 acetoxy-methylester emissions, when excited at the wavelengths of 340 and 380 nm, was recorded, and analysis software supplied with the plate reader were used to process and statistically analyze data.

Data analysis. Comparison of the effects of various treatments was performed using one way analysis of variance and a two tailed Student’s t-test. Differences with a p-value of < 0.05 were considered statistically significant. Statistical examination of in vivo animal survival data utilized log rank statistical analyses between the different treatment groups. Experiments shown are the means of multiple individual independent points (at least 3 per experiment) from multiple experiments (at least 2 experiments; minimum independent points per value = 6) (± SEM).
Results

MDA-7 interacts with ROS-producing agents in a greater than additive fashion to cause RCC death; killing is suppressed by re-expression of VHL. Initial experiments focused on defining the impact of GST-MDA-7 in combination with agents that generate intracellular ROS. In UOK121LN and A498 renal carcinoma cells the lethality of GST-MDA-7 was enhanced in a greater than additive fashion by As$_2$O$_3$, 17AAG and 4HPR (Figures 1A-1C). The majority of RCCs in patients have lost expression of the Von Hippel Lindau (VHL) protein, an E3 ligase, resulting in an increased protein stress load in tumor cells (Turcotte et al, 2008). Re-expression of the VHL protein in 786-0 RCCs suppressed GST-MDA-7 toxicity and the interaction between GST-MDA-7 and 17AAG or As$_2$O$_3$ (Figure 1D).

To achieve metastatic spread from the primary tumor site through the blood, tumor cells need to acquire some degree of resistance to undergoing anoikis–induced apoptosis. We generated anoikis resistant A498 cells and determined the impact of this survival process on GST-MDA-7 lethality. Anoikis resistant kidney cancer cells were more sensitive to MDA-7/IL-24 lethality than tumor cells that grow attached to a substratum (Figures 1E and 1F). Killing of anoikis and parental cells was blocked by inhibition of caspase 8.

A chimeric serotype 5 / serotype 3 recombinant adenovirus expressing MDA-7/IL-24 (Ad.5/3- mda-7) infects and kills renal carcinoma cells and secretes MDA-7/IL-24 into the growth media where it has a toxic bystander effect on uninfected RCCs. RCCs are resistant to infection by serotype 5 adenoviruses, e.g., Ad.5- mda-7, due to the fact that they do not express the coxsakie and adenovirus receptor (CAR) (Yacoub et al, 2003). To circumvent this problem, we developed a chimeric serotype 5/serotype 3 modified knob-serotype adenovirus to deliver mda-7/IL-24 to RCCs: Ad.5/3- mda-7 (Dash et al, 2010; Eulitt et al, 2010; Hamed et al, 2010). Infection of primary renal epithelial cells that express CARs with Ad.5- mda-7 produced expression of MDA-7/IL-24 without causing significant toxicity to these non-transformed cells (Eulitt et al, 2010, data not shown). Infection of RCCs with Ad.5/3- mda-7 caused cell death and this toxicity
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was enhanced in a greater than additive fashion by As$_2$O$_3$, 17AAG and by 4HPR (Figures 2A-2C). Prior studies have shown that bacterially synthesized GST-MDA-7 kills RCCs by activating the death receptor CD95 / FAS- receptor (Park et al, 2009).

MDA-7/IL-24 is a secreted cytokine and we next determined whether direct infection of RCCs with Ad.5/3- mda-7, that contains the mda-7 cDNA to express a non-tagged form of the protein which in mammalian cells is glycosylated and also a dimer, permitted MDA-7/IL-24 to be secreted from an infected cell into the culture media and whether this secreted MDA-7/IL-24 could then act to kill uninfected RCCs and by what mechanism this killed uninfected RCCs. Transfer of media containing MDA-7/IL-24 onto uninfected RCCs promoted cell killing, and the transfer of conditioned media containing the cytokine enhanced 17AAG and As$_2$O$_3$ toxicity, and did so in a CD95 and MDA-7/IL-24–dependent fashion within the cell treated with conditioned media (Figure 2D). Similar data to those in Figure 2D were obtained when non-transformed primary human renal epithelial cells were infected with Ad.5/3-mda-7 and conditioned media containing MDA-7/IL-24 transferred on to uninfected kidney cancer cells, resulting in tumor cell apoptosis (Figures 2E and 2F). A mutant form of MDA-7/IL-24 that lacked the secretion peptide signal did not display a bystander effect (data not shown) (Sauane et al, 2004; Sauane et al, 2004a; Sauane et al, 2008; Sauane et al, 2010; Eulitt et al, 2010).

As$_2$O$_3$ and 17AAG enhance Ad.5/3-mda-7–induced activation of CD95 in an ROS-dependent manner. As MDA-7/IL-24, regardless of synthetic origin or in combination with ROS-inducing agents, was killing RCCs via increased CD95/caspase 8 signaling, we determined whether these ROS–inducing agents were truly altering the levels of CD95 activation. Treatment of Ad.5/3-mda-7-infected cells with 17AAG or As$_2$O$_3$ enhanced CD95 surface localization and promoted caspase 8 association with CD95 (DISC formation) (Figure 3A). Similar data were obtained with 4HPR (data not shown). In agreement with prior studies and data in Figure 1E and Figure 2D showing death receptor dependence for cell killing, over-expression of the caspase 8 inhibitor c-FLIP-s blocked the enhancement of Ad.5/3-mda-7 lethality.
by 17AAG (Figure 3B) (Park et al, 2009). Ad.5/3- mda-7 and 17AAG or 4HPR interacted to cause a dramatic greater than additive increase in the levels of ROS in RCCs, an effect that was blocked by over-expression of thioredoxin; by quenching of cytosolic Ca^{2+} using calbindin; by knock down of ceramide synthase 6 (LASS6) expression; or by expression of dominant negative PERK (Figures 3C and 3D, data not shown). Of note was that although thioredoxin reduced As2O3 –induced ROS production neither calbindin nor knock down of LASS6 expression, in contrast to data using 17AAG or 4HPR, suppressed the As2O3 –induced ROS levels. (Figure 3D).

**Ad.5/3- mda-7 increases CD95 surface levels, an effect that is enhanced by 17AAG or As2O3, and that is blocked by quenching of ROS or Ca^{2+} or inhibition of de novo ceramide synthesis.** The increase in cell surface CD95 levels caused by Ad.5/3- mda-7 and 17AAG treatment was blocked by the quenching of ROS; the quenching of Ca^{2+} or knockdown of LASS6 (Table 1). Quenching of ROS using thioredoxin also suppressed enhanced levels of induced CD95 tyrosine phosphorylation, DISC formation and the ability of 17AAG and As2O3 to kill RCCs (Figures 3E and 3F).

**Geldanamycin (17AAG) and fenretinide (4HPR) enhance Ad.5/3- mda-7–induced cytosolic Ca^{2+} levels, an effect blocked by molecular quenching of de novo ceramide synthesis and by expression of dominant negative PERK; As2O3-induced cytosolic Ca^{2+} levels are ROS-dependent.** Treatment of Ad.5/3- mda-7-infected cells with As2O3, 17AAG or 4HPR enhanced cytosolic Ca^{2+} levels (Figure 4A). Of note, quenching of ROS did not block either 17AAG or 4HPR–induced Ca^{2+} levels whereas quenching of ROS blocked As2O3 -induced Ca^{2+} levels. Quenching of Ca^{2+} significantly reduced the increase in CD95 plasma membrane levels and reduced DISC formation (Table 1 and Figure 4B). Quenching of Ca^{2+} also suppressed the ability of As2O3, 17AAG or 4HPR to kill RCCs (Figure 4C, data not shown). Based on these findings, and our prior analyses in glioblastoma and prostate cancer cells showing that MDA-7/IL-24 expression can lead to increased ceramide levels, we determined whether ceramide generation was involved in the interaction between Ad.5/3- mda-7 and As2O3 / 17AAG / 4HPR (Park et al, 2009; Sauane et al, 2010).
Ad.5/3-mda-7-induced CD95 activation is dependent on the de novo ceramide synthesis pathway.

Ad.5/3-mda-7-induced CD95 plasma membrane localization was blocked by inhibition of the de novo ceramide synthesis pathway (Table 1 and Figure 5A, lower graph). The abilities of 4HPR, 17AAG and As$_2$O$_3$ to stimulate CD95 surface localization and DISC formation were also blocked by inhibition of the de novo ceramide synthesis pathway (Table 1 and Figure 5A, lower graph; Figure 5A, upper blot). Ad.5/3-mda-7–induced tumor cell killing, and the abilities of 4HPR and 17AAG to further stimulate tumor cell death in the presence of MDA-7/IL-24 were also blocked by inhibition of the de novo ceramide synthesis pathway or knockdown of LASS6 expression (Figures 5B and 5C). Ad.5/3-mda-7 increased the levels of C16 ceramide and C16 dihydro-ceramide in RCCs in a PERK and LASS6–dependent fashion (Figure 5D, data not shown). Combined exposure of cells to Ad.5/3-mda-7 and to 17AAG did not further enhance ceramide generation; however the combination of agents did permit ceramide generation when dominant negative PERK was expressed.

Ad.5/3-mda-7 infection of RCC tumors suppresses RCC tumor growth that is enhanced by 17AAG.

Finally, as our medium-term aim is to translate MDA-7/IL-24 as a kidney cancer therapy into the clinic we wished to determine whether MDA-7/IL-24 exhibited a bystander effect in vivo, prolonged animal survival and could exhibit any interaction between 4HPR or 17AAG. To address the first issue, we established tumors on both flanks of an athymic mouse and injected one tumor with either an empty vector virus or a virus to express MDA-7/IL-24. Both the injected and non-injected contra-lateral tumor in Ad.5/3-mda-7-infected animals displayed a reduced growth rate that correlated with increased animal survival: 50% of the Ad.5/3-cmv infected animals had to be sacrificed 10 days after virus infection compared to a 50% survival at 29 days* after Ad.5/3-mda-7 infection (data not shown, * $p < 0.05$ survival greater than Ad.5/3-cmv).

To determine whether MDA-7/IL-24 toxicity could be enhanced in vivo we titrated downwards and used 20-fold less Ad.5/3-mda-7 than in our prior studies (Eulitt et al, 2010; Hamed et al, 2010). A498 RCC tumors were established on one flank of an athymic mouse after ~29 days and the tumor was injected with
Ad.5/3-cmv or with Ad.5/3-mdx-7 (total 2 injections) and animals were treated with 4HPR or 17AAG subsequently for the following 3 days. Infection of tumors with a 20-fold lower dose of Ad.5/3-mdx-7 did not significantly reduce the growth rate of tumors (Figures 6A and 6B). Surprisingly based on our in vitro interactions, 4HPR (fenretinide) did not significantly enhance Ad.5/3-mdx-7 toxicity (at least at the dose and administration schedule tested herein) whereas 17AAG did further reduce tumor growth (Figures 6A and 6B) Reduced growth in Ad.5/3-mdx-7 + 17AAG-treated tumors correlated with tumor cyto-architecture disruption, decreased Ki67 staining and increased caspase 3 cleavage and TUNEL staining (Figure 6C).
Discussion.

Previous studies from our laboratories have demonstrated that bacterial synthesized GST-MDA-7 reduces proliferation and causes tumor cell- and transformed cell-specific killing and radiosensitization in glioma, and breast, prostate and renal cancer cells. We noted that GST-MDA-7 killed RCCs by promoting activation of the death receptor CD95 and that single agent killing also required ROS and ceramide production. The studies in this manuscript were designed to determine the mechanisms by which clinically relevant agents that generate ROS promote mammalian cell synthesized MDA-7/IL-24 toxicity in RCCs.

RCCs are relatively resistant to infection by serotype 5 adenoviruses due to low CAR levels (e.g., Yacoub et al, 2003). Considering this problem in using serotype 5 adenoviruses for gene transduction in RCCs, we used a novel chimeric serotype 5 / serotype 3 adenovirus, Ad.5/3-mdad7 (Dash et al, 2010; Eulitt et al, 2010; Hamed et al, 2010), which infects tumor cells using the CD46/80/86 receptors. The Ad.5/3-mdad7 virus readily infected multiple RCC cell lines with a subsequent robust expression of MDA-7/IL-24 protein. Over-expression of c-FLIP-s or the knockdown of CD95 expression reduced Ad.5/3-mdad7 toxicity in RCCs.

In our initial report examining MDA-7/IL-24 toxicity in RCCs, detailing the interactions between GST-MDA-7 and 4HPR / As2O3; we demonstrated that agents which generate ROS enhance MDA-7/IL-24 lethality (Yacoub et al, 2003). Similar combination effects between mda-7/IL-24 and ROS inducing agents have also been observed in prostate and pancreatic cancer (Lebedeva et al, 2005a; Lebedeva et al, 2007). We subsequently noted that unlike GBM and prostate cancer cells, MDA-7/IL-24 (GST-MDA-7) killed RCCs by causing activation of the CD95 death receptor and activation of the extrinsic apoptosis pathway (Park et al, 2009). Our present findings have now determined the molecular mechanisms by which MDA-7/IL-24 –induced cell killing is enhanced by ROS inducing agents in RCCs, using glycosylated untagged dimeric MDA-7/IL-24, by employing a novel chimeric serotype modified adenovirus to deliver the mda-7/IL-24 transgene.
A priori we would have predicted that: As$_2$O$_3$ would enhance MDA-7/IL-24 toxicity via its degeneration causing additional generation of ROS and mitochondrial dysfunction and not by causing further activation of CD95; 4HPR would kill by promoting additional ceramide generation that would increase MDA-7/IL-24–induced CD95 activation by increasing ceramide levels in lipid rafts; 17AAG would enhance MDA-7/IL-24 toxicity by causing additional generation of ROS and loss of protective signaling pathway activities and not by causing further activation of CD95. We determined that As$_2$O$_3$, 4HPR and 17AAG all enhanced MDA-7/IL-24–induced activation of CD95 in RCCs as judged both by plasma membrane localization; by increased CD95 tyrosine phosphorylation; and by enhanced DISC formation / caspase 8 association. The enhanced amount of CD95 activation was dependent on the enhanced levels of ROS, Ca$^{2+}$ and ceramide generated in cells treated with Ad.$5/3$-$mda$-$7$ and with MDA-7/IL-24 combined with the agents 4HPR or 17AAG. In this regard, it is well known that 4HPR can increase ceramide levels in cells and this effect has also been linked to the ability of this agent to elevate ROS production (Lai et al, 2008). The HSP90 antagonist 17AAG has been shown by our group and by others to increase ROS levels, in part based on the chemical structure of this drug but also potentially by inhibiting expression of ROS detoxification enzymes (Park et al, 2008; Mitchell et al, 2007; Azad et al, 2009). Nonetheless, without CD95 signaling, 17AAG was not effective at promoting MDA-7/IL-24 lethality.

We noted that 4HPR or 17AAG promoted Ad.$5/3$-$mda$-$7$–induced ROS and Ca$^{2+}$ levels. For both 4HPR and 17AAG the enhanced generation of ROS was Ca$^{2+}$-dependent. Inhibition of the de novo ceramide synthesis pathway, or specifically knockdown of LASS6, suppressed the abilities of Ad.$5/3$-$mda$-$7$ as well as 4HPR or 17AAG to promote CD95 activation and also suppressed the induction of cytosolic Ca$^{2+}$ and ROS by either 17AAG or 4HPR. Surprisingly, however, our data demonstrated that 17AAG did not significantly enhance bulk MDA-7/IL-24–induced ceramide or dihydro-ceramide levels. But, of note, when ER stress signaling was compromised by expression of dominant negative PERK that blocked the MDA-7/IL-24–induced increase in ceramide levels; 17AAG was able to partially overcome the block to ER stress signaling and facilitate MDA-7/IL-24–induced ceramide generation. Clearly; a more detailed time course analysis of ceramide generation and degradation pathways will be required to fully understand the interaction of 17AAG and MDA-7/IL-24.
with respect to lipid signaling and how ceramide levels are regulated. Thus: our data argues that ceramide generation or alterations in ceramide metabolism caused by expression of MDA-7/IL-24 is essential for Ad.5/3-mda-7 lethality and for the ability of agents that generate ROS, such as 17AAG or 4HPR, to activate CD95; and to ultimately cause tumor cell killing (Figure 7).

Arsenic trioxide (As$_2$O$_3$; also called ATO) is a therapeutic agent whose actions in leukemic cells have largely been linked to the generation of ROS and the induction of tumor cell differentiation (Zhou et al, 2007). Unlike either 4HPR or 17AAG, As$_2$O$_3$ can degenerate to release arsenic as well as O$^-$ and hence it’s ability enhance MDA-7/IL-24 toxicity would be expected to have some differences to those of 4HPR or 17AAG. Indeed, unlike 4HPR or 17AAG, quenching of cytosolic Ca$^{2+}$ or inhibition of de novo ceramide synthesis pathways did not prevent As$_2$O$_3$ from interacting with MDA-7/IL-24 to profoundly increase ROS levels. And, the reverse of our data with 4HPR or 17AAG with respect to changes in cytosolic Ca$^{2+}$, was observed using As$_2$O$_3$; the As$_2$O$_3$-induced increase of Ca$^{2+}$ was ROS dependent. However, despite all of these differences and in a manner similar to 4HPR or 17AAG, and despite all of the additional ROS generation caused by As$_2$O$_3$ + Ad.5/3-mda-7 treatment, As$_2$O$_3$ still required CD95 expression / functionality and LASS6 function to enhance MDA-7/IL-24 lethality. Thus as with MDA-7/IL-24 itself which can kill tumor cells through multiple convergent apoptotic mechanisms, agents that generate ROS can facilitate MDA-7/IL-24 lethality through multiple mechanisms that converge on enhanced death receptor signaling.

MDA-7/IL-24 is a secreted cytokine and has been shown in several studies to have a “toxic bystander” effect on distant tumor cells (Sauane et al, 2008; Su et al, 2005; Sarkar et al, 2002; Emdad et al, 2009, Eulitt et al, 2010 and references therein). We discovered that secreted MDA-7/IL-24 produces a conditioned media that when placed onto uninfected RCCs suppresses the growth of the uninfected cells and elevates apoptosis levels in a CD95–dependent fashion; this lethality is further enhanced by As$_2$O$_3$, 4HPR and by 17AAG. Based on simple mass action effects, it is not possible to infect every tumor cell within a tumor using an adenovirus, and this has been one possible reason why so many gene therapy approaches have failed in the clinic. As MDA-7/IL-24 is
secreted, our findings argue that this cytokine could have therapeutic utility in chemotherapy resistant metastatic renal carcinoma. That a 20-fold lower Ad.5/3-\textit{mda}-7 virus dose resulted in no significant alteration in tumor growth argues that for translation into the clinic a virus with serotype modification plus tumor conditional replication in addition to MDA-7/IL-24 production and an approach to target virus delivery using systemic administration may be required to achieve optimal results i.e. Ad.5/3-\textit{CTV} (Greco et al, 2010).

One characteristic hallmark of RCC is loss of VHL protein expression (Ishizawa et al, 2004). We found that re-expression of VHL in VHL \textit{-/-} RCCs suppressed MDA-7/IL-24 toxicity, in agreement with data arguing that VHL expressing RCCs are less tumorigenic and that MDA-7/IL-24 toxicity is reduced in non-transformed cells. MDA-7/IL-24 kills tumor cells, in part, by causing a toxic form of ER stress, and one plausible mechanism for the disparity in MDA-7/IL-24 toxicity in transformed versus non-transformed cells is due to the greater levels of protein expression and unfolded proteins in transformed cells. As VHL is an E3 ligase whose actions will tend to reduce the toxic protein load in a tumor cell, our findings are also compatible with VHL expressing cells having a lower load of unfolded (toxic) proteins.

Prior studies from our laboratory have noted that MDA-7/IL-24 can suppress the growth of RCC tumors; has a bystander effect; an effect that can be enhanced by the multi-kinase inhibitor sorafenib (Eulitt et al, 2010). Based on the findings in the present manuscript we attempted to determine whether agents that generate ROS could enhance MDA-7/IL-24 toxicity in RCC tumors. To our surprise 4HPR did not appear to interact \textit{in vivo} with MDA-7/IL-24 expression to suppress tumor growth rates. Clearly, further studies are required using higher doses of 4HPR and altered administration schedules to confirm this lack of in vivo effect. In contrast, 17AAG significantly reduced growth in tumors infected with Ad.5/3-\textit{mda}-7. It is possible that the effect of 17AAG was due to generation of both ROS and to inhibition of protective signaling pathways in the tumors. Further studies, including the clinical translation of viruses to express MDA-7/IL-24 into patients, will be required to define potential therapeutic efficacy of MDA-7/IL-24 in kidney cancer.
Acknowledgements.

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Authorship Contribution

PD designed the studies and directed MAP, HAH, CM, NC, RD, JA whom performed the studies. AY and GT assisted PD in bench-side supervision of MAP, HAH, CM, NC, RD. SS assisted PD in bench-side supervision of JA. IPD and DTC developed and provided recombinant adenoviruses for these studies. BO provided ceramide manipulation agents. PBF, SG assisted PD with the writing and proofing of the manuscript.


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Figure 1. GST-MDA-7 interacts with ROS-producing agents in a greater than additive fashion to cause RCC death; killing is suppressed by re-expression of VHL. Panels A-C. UOK121LN and A498 cells were treated 24 h after plating with GST or GST-MDA-7 (1 nM) and in parallel with either vehicle (DMSO) or 17AAG (100 nM); As$_2$O$_3$ (0.5, 1.0 μM); 4HPR (0.5, 1.0 μM). Forty eight h after GST-MDA-7 treatment, cell viability was determined using trypan blue exclusion (n = 3, +/- SEM; * p < 0.05 greater than corresponding treatment with GST). Panel D. Parental Von Hippel Lindau (VHL) null 786-0 cells transfected with empty vector or 786-0 cells stably transfected to express VHL were treated 24 h after plating with GST or GST-MDA-7 (1 nM) and in parallel with either vehicle (DMSO), 17AAG (100 nM) or As$_2$O$_3$ (0.5, 1.0 μM). Forty eight h after GST-MDA-7 treatment, cell viability was determined using trypan blue dye exclusion (n = 3, +/- SEM; # p < 0.05 less than corresponding values in with vector cells). Panel E. A498 cells (parental and anoikis resistant) were treated with GST or GST-MDA-7 (20 nM) in the presence or absence of vehicle (DMSO) or the caspase 8 inhibitor IETD (50 μM). Cell viability was determined by trypan blue dye exclusion assay after 48h (n = 3, +/- SEM; * p < 0.05 greater than corresponding treatment with GST; # p < 0.05 less than corresponding value in anoikis resistant cells). Panel F. A498 cells (parental and anoikis resistant) were infected with Ad.5-cmv; Ad.5/3-cmv; Ad.5-mda-7; Ad.5/3-mda-7 (20 moi) and 48 h later viability was determined by trypan blue dye exclusion assay (n = 3, +/- SEM). Upper inset: the levels of cell surface CAR were determined using an anti-CAR antibody labeled with FITC via flow cytometry (n = 3, +/- SEM; * p < 0.05 greater than corresponding treatment with Ad.5/3-cmv).

Figure 2. A serotype 5/serotype 3 recombinant adenovirus to express MDA-7/IL-24 (Ad.5/3-mda-7) infects and kills renal carcinoma cells. Panels A. and B. UOK121LN and A498 cells were infected 24 h after plating with Ad.5/3-cmv; Ad.5/3-mda-7 (20 moi) and 12 h after infection treated with either vehicle (DMSO) or 17AAG (100 nM); As$_2$O$_3$ (0.5, 1.0 μM). Forty eight h after infection, cell viability was determined using trypan blue dye exclusion (n = 3, +/- SEM; * p < 0.05 greater than corresponding
Panel C. Parental VHL null 786-0 cells transfected with empty vector or 786-0 cells stably transfected to express VHL were infected 24 h after plating with Ad.5/3-cmv; Ad.5/3-mda-7 (20 moi) and 12 h after infection treated with either vehicle (DMSO) or 17AAG (100 nM); As2O3 (0.5, 1.0 μM). Forty eight h after infection, cell viability was determined using trypan blue dye exclusion (n = 3, +/- SEM; # p < 0.05 less than corresponding treatment in CMV vector cells). Panel D. UOK121LN cells were transfected with either scrambled siRNA molecules (siSCR) or molecules to knock down expression of CD95 (siCD95) or MDA-7/IL-24 (siMDA-7) as indicated. In parallel, other portions of UOK121LN cells were infected with Ad.5/3-cmv; Ad.5/3-mda-7 (50 moi) and the growth media isolated 48 h after infection. Transfected UOK121LN cells were treated with conditioned media in parallel with vehicle (DMSO), 17AAG (100 nM) or As2O3 (0.5 μM). Cell viability in the transfected cells was determined 48 h after treatment by trypan blue exclusion (n = 3, +/- SEM; # p < 0.05 less than corresponding treatment with in siSCR cells). Panel E. Conditioned media from infected primary renal epithelial cells was placed onto UOK121LN cells and cells were in parallel treated with either vehicle (DMSO), 4HPR (0.5 μM); As2O3 (0.5 μM). Cells were isolated 48h after treatment with conditioned media and viability determined by trypan blue exclusion (n = 3, +/- SEM; * p < 0.05 greater than corresponding treatment with media from Ad.5/3-cmv infected cells). Panel F. Conditioned media from infected primary renal epithelial cells was placed onto UOK121LN cells and cells were in parallel treated with either vehicle (DMSO), 17AAG (0.1 μM); 17DMAG (0.1 μM). Cells were isolated 48 h after treatment with conditioned media and viability determined by trypan blue dye exclusion (n = 3, +/- SEM; * p < 0.05 greater than corresponding treatment with media from Ad.5/3-cmv infected cells).

Figure 3. As2O3 and 17AAG enhance Ad.5/3-mda-7–induced activation of CD95 in an ROS-dependent manner. Panel A. UOK121LN cells, in 4-well chambered slides or 60-mm dishes, were infected with Ad.5/3-cmv; Ad.5/3-mda-7 (50 moi). Twenty four h after infection cells were treated with vehicle (DMSO), 17AAG (100 nM) or As2O3 (0.5 μM), and 6 h later cells were fixed for determination of CD95 plasma
membrane levels or were lysed for immunoprecipitation of CD95 for determination of caspase 8 – CD95
association (DISC complex formation). IHC data from 40 cells per experiment (n = 3, +/- SEM; * p < 0.05
greater than corresponding treatment with media from Ad.5/3-cmv infected cells). DISC immunoblotting
data, to the right, is from a representative of 3 independent studies. **Panel B.** UOK121LN cells were
transfected with either empty vector control plasmid (CMV) or a plasmid to express c-FLIP-s. Twelve h
after transfection cells were infected with Ad.5/3-cmv; Ad.5/3-mda-7 (50 moi). Twenty-four h after
infection cells were treated with vehicle (DMSO), 17AAG (100 nM) or As2O3 (0.5 μM). Forty-eight h after
infection cells were isolated and viability determined by trypan blue dye exclusion assay (n = 3, +/- SEM; #
p < 0.05 less than corresponding treatment in CMV vector cells). **Panels C. and D.** UOK121LN cells in
sextuplicate were transfected with empty vector plasmid (CMV), and plasmids to express thioredoxin
(TRX); calbindin D28 (Calb.) or to knockdown ceramide synthase 6 expression (siLASS6). Twelve h after
transfection cells were infected with Ad.5/3-cmv or Ad.5/3-mda-7 (50 moi). Twenty four h after infection
cells were loaded with DCFH2CA and then treated with vehicle (DMSO); 4HPR (0.5 μM), As2O3 (0.5 μM)
or 17AAG (100 nM). The levels of ROS in cells were examined 1 h (17AAG, As2O3) or 2 h (4HPR) after
drug addition (n = 2, 12 data points, +/-SEM; # p < 0.05 less than corresponding treatment in CMV vector
cells). **Panel E.** UOK121LN cells were transfected with either empty vector control plasmid (CMV) or a
plasmid to express thioredoxin (TRX). Twelve h after transfection cells were infected with Ad.5/3-cmv;
Ad.5/3-mda-7 (50 moi). Twenty-four h after infection cells were treated with vehicle (DMSO), 17AAG
(100 nM) or As2O3 (0.5 μM). Six h after drug treatment cells were isolated and CD95 immunoprecipitated
to determine caspase 8 association and CD95 tyrosine phosphorylation. Representative studies are shown (n
= 3). **Panel F.** UOK121LN and A498 cells were transfected with either empty vector control plasmid
(CMV) or a plasmid to express thioredoxin (TRX). Twelve h after transfection cells were infected with
Ad.5/3-cmv; Ad.5/3-mda-7 (50 moi). Twenty-four h after infection cells were treated with vehicle (DMSO),
17AAG (100 nM) or As2O3 (0.5 μM). Twenty-four h after drug treatment cells were isolated and viability
determined by trypan blue dye exclusion assay (n = 3, +/- SEM; # p < 0.05 less than corresponding treatment in CMV vector cells lacking TRX).

**Figure 4.** 17AAG and 4-HPR enhance Ad.5/3-*mda*-7–induced Ca\(^{2+}\) levels, an effect blocked by molecular quenching of *de novo* ceramide synthesis and by expression of dominant negative PERK; As\(_2\)O\(_3\)-induced Ca\(^{2+}\) levels are ROS-dependent. Panel A. UOK121LN cells in sextuplicate were transfected with empty vector plasmid (CMV), and plasmids to express dominant negative PERK, thioredoxin (TRX); calbindin D28 (Calb.) or to knock down ceramide synthase 6 expression (siLASS6). Twelve h after transfection cells were infected with Ad.5/3-*cmv* or Ad.5/3-*mda*-7 (50 moi). Twenty four h after infection cells were loaded with Fura-2 and then treated with vehicle (DMSO); 4-HPR (0.5 μM) or 17AAG (100 nM). The levels of Ca\(^{2+}\) in cells were examined 1h (17AAG) or 2h (4HPR) after drug addition (n = 2, 12 data points, +/-SEM; # p < 0.05 less than corresponding treatment in CMV vector cells). Panel B. UOK121LN cells were transfected with empty vector plasmid or a plasmid to express Calbindin D28. Twelve h after transfection, cells were infected with Ad.5/3-*cmv* or Ad.5/3-*mda*-7 (50 moi). Twenty-four h after infection cells were treated with vehicle (DMSO) or 17AAG (100 nM). Six h after drug exposure cells were isolated and CD95 immuno-precipitated. The amount of caspase 8 associated with CD95 was determined after PAGE / blotting (n = 3 independent studies). Panel C. A498 and UOK121LN cells were transfected independently in triplicate with empty vector plasmid or a plasmid to express Calbindin D28. Twelve h after transfection cells were infected with Ad.5/3-*cmv* or Ad.5/3-*mda*-7 (50 moi). Twenty-four h after infection cells are treated with vehicle (DMSO) or 17AAG (100 nM). Cells were isolated 24 h after drug exposure and viability determined by trypan blue dye exclusion (n = 2, +/- SEM; # p < 0.05 less than corresponding treatment in CMV vector cells).
Figure 5. Ad.5/3-mda-7–induced CD95 activation is dependent on the de novo ceramide synthesis pathway. Panel A. A498 cells in 4-well chambered slides or 60-mm dishes were transfected with empty vector plasmid to express a scrambled siRNA or a plasmid to knockdown ceramide synthase 6 (siLASS6). Twelve h after transfection cells were infected with Ad.5/3-cmv or Ad.5/3-mda-7 (50 moi) and treated with either vehicle (DMSO) or myriocin (1 μM). Twenty-four h after infection cells were treated as indicated with vehicle (DMSO), 4HPR (0.5 μM) or 17AAG (100 nM), as indicated. Six h after drug exposure cells were either fixed for IHC to determine the levels of plasma membrane CD95 (lower graph) or lysed followed by CD95 immunoprecipitation to determine caspase 8 association (DISC) complex formation (upper immunoblot) (n = 3, +/-SEM; # p < 0.05 less than corresponding treatment in siSCR cells). Panels B and C. A498 and UOK121LN cells were infected independently in triplicate with Ad.5/3-cmv or Ad.5/3-mda-7 (50 moi) and treated with either vehicle (DMSO) or myriocin (1 μM). Twenty-four h after infection cells were treated as indicated with vehicle (DMSO), 4HPR (0.5 μM), or 17AAG (100 nM). Cells were isolated 24 h after drug-exposure and viability determined by trypan blue dye exclusion (n = 2, +/- SEM; # p < 0.05 less than corresponding treatment in VEH cells). Panels D and E. A498 and UOK121LN cells were transfected independently in triplicate with empty vector plasmid to express a scrambled siRNA or a plasmid to knockdown ceramide synthase 6 (siLASS6). Twelve h after transfection cells were infected with Ad.5/3-cmv or Ad.5/3-mda-7 (50 moi). Twenty-four h after infection cells are treated as indicated with vehicle (DMSO), 4HPR (0.5 μM) or 17AAG (100 nM). Cells were isolated 24 h after drug exposure and viability was determined by trypan blue dye exclusion (n = 2, +/- SEM; # p < 0.05 less than corresponding treatment in VEH cells). Panel D. A498 cells were transfected independently in triplicate with empty vector plasmid, or a plasmid to express dominant negative PERK. Twelve h after transfection cells were infected with Ad.5/3-cmv or Ad.5/3-mda-7 (50 moi). Twenty-four h after infection, cells were treated as indicated with vehicle (DMSO) or 17AAG (100 nM). Cells were isolated 6 h after drug-exposure and the levels of ceramide determined by mass spectrometry (n = 2, +/- SEM; * p < 0.05 greater than corresponding Ad.5/3-cmv control).
Figure 6. Ad.5/3-md-a-7 infection of RCC tumors suppresses RCC tumor growth that is enhanced by 17AAG. Panels A and B. A498 cells were injected into the rear right flanks of athymic mice. Tumors were grown over the following 29 days. Animals were segregated into tumor volumes of approximate equivalent mean tumor size and standard error; the tumor was injected with either Ad.5/3-cmv or Ad.5/3-md-a-7. Animals were treated 24 h later with either vehicle or 4HPR (100 mg/kg) (Panel A) or 17AAG (100 mg/kg) (Panel B) every day for three days. One week after the first virus infection, tumors were again infected in an identical manner with adenovirus. Tumor volumes were measured every two-three days. The mean volume of the tumor is presented as a Fold-increase over the pre-infected volume (defined as 1.00) (n = 2, +/- SEM; 9-10 mice per group total over 2 studies; # p < 0.05 less than vehicle or individual treatments). Panel C. A498 tumors were isolated 2 days after the second virus infection. Sections (10 microns) were taken and stained for H&E (morphology); TUNEL (apoptosis); Cleaved caspase 3; Ki67 and MDA-7 / IL-24 expression. Data are from representative images from multiple tumors shown for H&E at X20 magnification and for other slides using a confocal microscope at X100 magnification.

Figure 7. Mechanisms by which agents that generate ROS interact with MDA-7/IL-24 to kill kidney cancer cells. As a single agent MDA-7 / IL-24 kills tumor cells by generating ceramide that in turn promotes elevation in cytosolic Ca^{2+} levels that in turn promotes increased ROS levels. Increased ceramide (via lipid rafts); Ca^{2+} and ROS by altering CD95 phosphorylation and through unknown mechanisms all also independently facilitate activation of CD95 (extrinsic pathway). Activation of the extrinsic pathway through caspase 8 / BID cleavage causes kidney cancer cell killing by promoting mitochondrial dysfunction. The drugs 17AAG and 4HPR both enhance signaling proximal to MDA-7 / IL-24 to “boost” the established MDA-7 / IL-24 pro-apoptotic pathway. The drug As_{2}O_{3} (heavier arrows) acts distally to ceramide resulting in increased ROS levels that increases Ca^{2+} levels; again with MDA-7 / IL-24 –induced ceramide combining with elevated ROS and Ca^{2+} levels to activate CD95 and cause tumor cell killing.
Table 1. Ad.5/3-\textit{mda}-7 increases CD95 surface levels, an effect that is enhanced by 17AAG or As$_2$O$_3$, and that is blocked by quenching of ROS or Ca$^{2+}$ or inhibition of \textit{de novo} ceramide synthesis.

UOK121LN cells in 4 chambered glass slides were transfected with vector control plasmid (CMV or siSCR) or with plasmids to quench ROS (thioredoxin, TRX), quench Ca$^{2+}$ (Calbindin D28, Calb.) or to knock down ceramide synthase 6 expression (siLASS6). Twelve h after transfection, cells are infected with Ad.5/3-\textit{cmv} or Ad.5/3-\textit{mda}-7. Twenty four h after infection cells are treated with vehicle or MnTBAP (10 $\mu$M) as indicated and then with vehicle, 17AAG (100 nM) or As$_2$O$_3$ (0.5 $\mu$M). Cells were fixed 6h after 17AAG / As$_2$O$_3$ treatment. Cell surface levels of CD95 were determined by IHC as described in the Methods with the cell surface density of CD95 determined in 40 cells per experiment per condition (n = 3, +/- SEM). * p < 0.05 greater than corresponding Ad.5/3-\textit{cmv} infected value; # p < 0.05 less than Ad.5/3-\textit{mda}-7 + VEH value.

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Figure 6

6A

![Graph A](image1)

6B

![Graph B](image2)

6C

![Image C](image3)

**6A**

- **Ad.5/3-cmv + VEH**
- **Ad.5/3-cmv + 4HPR**
- **Ad.5/3-mda-7 + VEH**
- **Ad.5/3-mda-7 + 4HPR**

**6B**

- **Ad.5/3-cmv + VEH**
- **Ad.5/3-cmv + 17AAG**
- **Ad.5/3-mda-7 + VEH**
- **Ad.5/3-mda-7 + 17AAG**

**6C**

- **H&E**
- **Cleaved Caspase 3**
- **TUNEL**
- **Ki67**
- **MDA-7/IL-24**
Figure 7

- MDA-7/IL-24
- 4HPR
- 17AAG
- As$_2$O$_3$
- ROS
- Ca$^{2+}$
- CD95
- mitochondrial dysfunction
- cell death

Pathway:
- MDA-7/IL-24 → ceramide
- 4HPR
- 17AAG
- As$_2$O$_3$ → ROS
- Ca$^{2+}$
- CD95 activation
- mitochondrial dysfunction
- cell death