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Approaches for Enhancing Therapeutic Efficacy of a Novel IL-10 Gene Family Member: MDA-7/IL-24

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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List of Abbreviations

Ad.  Adenovirus
Ad.5  Serotype 5 adenovirus
Ad.3  Serotype 3 adenovirus
Ad.5/3  tropism-modified chimeric 5/3 adenovirus
Ad.5-CTV  Serotype 5 CTV
Ad.5/3-CTV  tropism-modified chimeric 5/3 CTV
Ad.mda-7  Ad expressing mda-7/IL-24
Ad.5-mda-7  Ad.5 expressing mda-7/IL-24
Ad.5/3-mda-7  Ad.5/3 expressing mda-7/IL-24
Ad.5.vec  empty Ad.5
Ad.5/3.vec  empty Ad.5/3
Ad.5/3-Luc  Ad.5/3 expressing Luciferase
Ad.5-Luc  Ad.5 expressing Luciferase
Bad  BCL2-associated agonist of cell death
Bak  Bcl-2 homologous antagonist/killer
Bax  Bcl-2–associated X protein
Bcl-2  B-cell lymphoma 2
Bcl-xl  B-cell lymphoma-extra large
Bid  BH3 interacting domain death agonis
BiP/Grp78  Binding immunoglobulin protein/ 78 kDa glucose-regulated protein
CAR  Coxsackie Adenovirus Receptor
Caspase  Cysteine proteases with aspartate specificity
CD31  cluster of differentiation 31
CMV  
*Cytomegalovirus*

CRCAs  
Conditionally Replication-Competent Adenoviruses

CTV  
Cancer terminator Virus

DMEM  
Dulbecco's Modified Eagle Medium

DMSO  
Dimethyl sulfoxide

EDTA  
Ethylenediaminetetraacetic acid

ER  
Endoplasmic reticulum

EF1α  
elongation factor 1 alpha

eIF2α  
Phosphorylation of the eukaryotic initiation factor 2

ERK  
eextracellular-signal-regulated kinases

FBS  
Fetal bovine serum

FITC/PI  
Fluorescein isothiocyanate/Propidium iodide

GADD153  
Growth arrest and DNA damage protein 153

Grp94  
glucose-regulated protein 94

H & E  
hematoxylin and eosin stain

IC₅₀  
half maximal inhibitory concentration

IFN-β  
interferon β

IL-10  
interleukin-10

i.p.  
intraperitoneally

LC3  
Light Chain 3

Luc  
Luciferase

MB  
microbubble

Mcl-1  
myeloid cell leukemia sequence 1

*mda*-7/IL-24  
melanoma differentiation associated gene-7/interleukin-24

MEF  
mouse embryonic fibroblasts
MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide.
NMR NMR
NOXA Damage protein, a pro-apoptotic BH3-containing protein
Myc Myelocytomatosis oncogene cellular homolog
PARP Poly (ADP-ribose) polymerase
PBMC peripheral blood mononuclear cell
PBS Phosphate buffered saline
PC prostate cancer
PEG-3 Progression Elevated Gene-3
PERK PKR-like ER-localized eIF2α kinase
pfu Plaque Forming Unit
PIK3C3 Phosphatidylinositol 3-kinase catalytic subunit type 3
PKR dsRNA-dependent Ser/Thr protein kinase
RFP red fluorescent protein
s.c. subcutaneous
Smac/DIABLO Second mitochondria-derived activator of caspase/ Direct IAP binding protein with low pI
SV40 Simian vacuolating virus 40 or Simian virus 40
TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPR unfolded protein response
UTMD microbubble-targeted ultrasound destruction.
Z-VAD-FMK carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone
Abstract

Melanoma differentiation associated gene-7 (mda-7) was discovered in the Fisher laboratory by subtraction hybridization of temporally spaced subtracted cDNA libraries prepared from terminally differentiated human melanoma cells treated with human fibroblast interferon (IFN-β) and the protein kinase C activator mezerein (MEZ), an approach called ‘differentiation induction subtraction hybridization’ (DISH). mda-7 is located in human chromosome 1q32–33 and based on sequence homology, chromosomal localization, and its functional properties, the mda-7 gene is now classified as a member of the IL-10 family of cytokines and named IL-24. The mda-7/IL-24 cDNA encodes a protein of 206-amino acids with a predicted size of ~24-kDa, which contains an interleukin (IL)-10 signature motif at amino acids 101–121 (SDAESCYLVHTLLEFYLFVTF) shared by other members of the IL-10 family of cytokines. Sequence analysis revealed the presence of a 49-amino acid signal peptide suggesting that the molecule could be cleaved and secreted. Expression of MDA-7/IL-24 protein was detected in cells of the immune system (mainly by expression in tissues associated with the immune system, such as spleen, thymus and PBMC) and normal human melanocytes. Of interest, a progressive loss of MDA-7/IL-24 expression during melanoma progression suggests an inverse relationship between MDA-7/IL-24 expression and the evolution of melanocytes to various stages of melanoma. mda-7/IL-24 induces growth suppression in human melanoma and other cancer cells, without affecting normal cells. Subsequent studies provided consistent evidence that ectopic expression of mda-7/IL-24 employing a replication incompetent adenovirus (Ad.mda-7) resulted in apoptosis induction and cell death in a wide variety of solid tumors including melanoma, malignant glioma, carcinomas of the breast, kidney, cervix, colorectum, liver, lung, ovary and prostate sparing normal cellular counterparts, i.e., such as normal melanocytes, astrocytes,
fibroblasts, and mesothelial and epithelial cells. The *in vitro* antitumor activity of *mda*-7/IL-24 readily translated into the *in vivo* situation in animal models containing human breast, prostate, lung and colorectal carcinomas and in malignant glioma xenografts. Moreover, the ability of *mda*-7/IL-24 to induce a potent “bystander cancer-specific killing effect” provides an unprecedented opportunity to use this molecule to target for destruction not only primary tumors, but also metastases. Based on its profound cancer-selective tropism, substantiated by *in vivo* human xenograft studies in nude mice, *mda*-7/IL-24 (administered as Ad.*mda*-7) was evaluated in a Phase I clinical trial in patients with melanomas and solid cancers. These studies document that *mda*-7/IL-24 is well tolerated and demonstrates evidence of significant (44%) clinical activity. This review focuses on the recent enhancements in our understanding of the mode of action of *mda*-7/IL-24 and its potential applications as a unique and promising effective cytokine-based gene therapy for human cancers.

The first chapter explored the efficacy of a tropism-modified Ad-based cancer gene therapy approach for eradicating low CAR colorectal cancer cells. We show that in low CAR human colorectal cancer cells (RKO), a recombinant Ad.5/3 virus delivering *mda*-7/IL-24 (Ad.5/3-*mda*-7) is more efficient than Ad.5 delivering *mda*-7 (Ad.5-*mda*-7) in expressing MDA-7/IL-24 protein, inducing cancer-specific apoptosis and inhibiting *in vivo* tumor growth in a nude mouse xenograft model. Additionally, our *in vitro* and *in vivo* data confirms that BI-97C1 (Sabutoclax) profoundly sensitizes *mda*-7/IL-24 mediated toxicity in colorectal cancer. Thus, Ad.5/3-*mda*-7, alone and/or in combination with BI-97C1 (Sabutoclax), might represent an improved and more effective therapeutic approach for colorectal and other cancers.
In view of the essential roles of anti-apoptotic Bcl-2 family proteins in tumorigenesis and chemoresistance, efforts are focused on developing small molecule inhibitors of Bcl-2 family proteins as potential therapeutics for cancer. Unfortunately, due to the unique structure of Mcl-1 as compared with Bcl-2 and Bcl-xL, currently employed inhibitors, such as ABT-737 or its clinical counterpart, ABT-263, display limited affinity for Mcl-1. Using nuclear magnetic resonance (NMR) binding assays and computational docking studies, we have recently identified a series of new Apogossypol derivatives, compound 3 (BI-79D10) and compound 11 (BI-97C1), with pan-Bcl-2 inhibitory potency. BI-79D10 binds to Bcl- xL, Bcl-2, and Mcl-1 with IC_{50} values of 190, 360, and 520 nmol/L, respectively. BI-97C1 (Sabutoclax) is an optically pure individual Apogossypol derivative that retains all the properties of BI-79D10 along with superior \textit{in vitro} and \textit{in vivo} efficacy. Because Mcl-1 is over-expressed in the majority of PCs, we hypothesized that suppressing Mcl-1 by treating human PC cells with BI-97C1 (Sabutoclax) would sensitize them to \textit{mda}-7/IL-24-mediated cytotoxicity.

The second chapter study highlights the noteworthy potential of a combinatorial approach involving \textit{mda}-7/IL-24, a broad-acting anticancer gene, and BI-97C1 (Sabutoclax), which targets Mcl-1, to sensitize PC to \textit{mda}-7/IL-24-mediated cytotoxicity, thereby enhancing therapeutic efficacy. Our data suggests that treatment with the combination regimen of \textit{mda}-7/IL-24 and BI-97C1 (Sabutoclax) induces autophagy that facilitates apoptosis in association with up regulation of NOXA, accumulation of Bim, and activation of Bax and Bak. Treatment with \textit{mda}-7/IL-24 and BI-97C1 (Sabutoclax) inhibited the growth of PC xenografts and suppressed PC development in an immunocompetent transgenic mouse model of PC.
The third chapter study explored the efficacy of a tropism-modified CRCA cancer gene therapy approach for eradicating low CAR prostate cancer cells. We showed that in low CAR PC3 cells Ad.5/3-CTV is more efficient than Ad.5-CTV in delivering transgene (mda-7/IL-24), infecting tumor cells, expressing MDA-7/IL-24 protein, inducing cancer-specific apoptosis, inhibiting \textit{in vivo} tumor growth and exerting an antitumor ‘bystander’ effect in a nude mouse human prostate cancer xenograft and suppressed PC development in an immunocompetent transgenic mouse model of PC model.
Chapter 1

Enhanced delivery of mda-7/IL-24 using a serotype chimeric adenovirus (Ad.5/3) in combination with the Apogossypol derivative BI-97C1 (Sabutoclax) improves therapeutic efficacy in low CAR colorectal cancer cells
Abstract

Adenovirus (Ad)-based gene therapy represents a potentially viable strategy for treating colorectal cancer. The infectivity of serotype 5 adenovirus (Ad.5), routinely used as a transgene delivery vector, is dependent on Coxsackie adenovirus receptors (CAR). CAR expression is downregulated in many cancers thus preventing optimum therapeutic efficiency of Ad.5-based therapies. To overcome the low CAR problem, a serotype chimerism approach was used to generate a recombinant Ad (Ad.5/3) that is capable of infecting cancer cells via Ad.3 receptors in a CAR-independent manner. We evaluated the improved transgene delivery and efficacy of Ad.5/3 recombinant virus expressing melanoma differentiation associated gene-7/ interleukin-24 (mda-7/IL-24), an effective wide-spectrum cancer-selective therapeutic. In low CAR human colorectal cancer cells RKO, wild-type Ad.5 virus expressing mda-7/IL-24 (Ad.5-mda-7) failed to infect efficiently resulting in lack of expression of MDA-7/IL-24 or induction of apoptosis. However, a recombinant Ad.5/3 virus expressing mda-7/IL-24 (Ad.5/3-mda-7) efficiently infected RKO cells resulting in higher MDA-7/IL-24 expression and inhibition of cell growth both in vitro and in nude mice xenograft models. Addition of the novel Bcl-2 family pharmacological inhibitor Apogossypol derivative BI-97C1 (Sabutoclax) significantly augmented the efficacy of Ad.5/3-mda-7. A combination regimen of suboptimal doses of Ad.5/3-mda-7 and BI-97C1 profoundly enhanced cytotoxicity in RKO cells both in vitro and in vivo. Considering the fact that Ad.5-mda-7 has demonstrated significant objective responses in a Phase I clinical trial for advanced solid tumors, Ad.5/3-mda-7 alone or in combination with BI-97C1 would be predicted to exert significantly improved therapeutic efficacy in colorectal cancer patients.
Introduction

According to the American Cancer Society (www.cancer.gov), colorectal cancer is the third most common cancer and third leading cause of cancer-related deaths in the US. During 2010, 142,570 new cases of colorectal cancer were diagnosed and it was expected to cause about 51,370 deaths. Over 20% of patients present with metastatic (stage IV) colorectal cancer at the time of diagnosis, and up to 25% of this group will have isolated liver metastasis that is potentially resectable (1). The National Cancer Institute (www.cancer.gov) reported that the lifetime risk of being diagnosed with cancer of the colon or rectum is about 1 in 19 in males and about 1 in 20 in females in the US. While 20%-25% of colorectal cancer cases occur among individuals with a family history of colorectal cancer or a predisposing illness, about 75% of cases occur in people without these risk factors (2). In these contexts, there is need to develop novel, effective therapeutic approaches for treating colorectal cancer and genetic therapies represent promising therapeutic options for this neoplasm.

Adenoviruses represent a new therapeutic modality that can be designed to deliver therapeutic genes and to specifically replicate in and kill cancer cells. Clinical studies proved that these viruses can safely be administered locally, regionally and systemically (3) and that these agents have efficacy in gastrointestinal cancer (4). Recombinant forms of the type 5 adenovirus (Ad.5) are the most frequently used serotype of Ad for gene therapy. However, to be able to infect the cells, Ad.5 requires the Coxsackie Adenovirus Receptor (CAR) (5, 6). In many tumor types, such as malignant glioma, ovarian, renal, bladder, prostate, colorectal cancers, and particularly when examined in primary tumor specimens, the expression of CAR is reduced or absent in tumor cells compared to surrounding non-tumor tissue (5, 7-10). Established cell lines
express higher levels of CAR compared to that observed in primary tumors or in patients. Reduced CAR expression limits the efficiency of transduction of cancer cells by Ad.5 (11, 12). This finding may in part explain why gene therapy approaches using Ad.5 have not been as robust or successful as the studies performed in vitro using established cell lines. An approach to circumventing the low efficiency of Ad.5 infection of tumor cells involves ‘tropism modification’ in which the virus capsid proteins that normally associate with CAR are modified, permitting CAR-independent infectivity of tumor cells. The infective type 3 Ad sequence within the Ad type 5 virus knob (Ad.5/3 recombinant virus) allows viral infectivity regardless of the CAR expression status of tumor cells thereby permitting efficient viral infectivity of low and high CAR expressing tumor cells (13, 14).

Melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24) was cloned in our laboratory by subtraction hybridization technique by inducing human melanoma cells to terminal differentiation upon treatment with fibroblast interferon and mezerein (15, 16). This novel tumor suppressor cytokine is a member of the interleukin-10 (IL-10) gene family (17-22). Ectopic expression of mda-7/IL-24, using Ad.5 (Ad.5-mda-7) inhibits growth and induces cell death in many types of cancer cell lines in vitro, without harming comparable normal human cells, including epithelial cells, fibroblasts, melanocytes or astrocytes (21-26). Considering its robust cancer-specific apoptosis-inducing ability (by inducing ER stress) and tumor growth-suppressing properties in nude mice xenograft models, Ad.mda-7 (INGN 241) was evaluated in a phase I clinical trial in patients with advanced cancers. The results from this trial demonstrated safety and clinical efficacy (27). In addition to its direct apoptosis inducing properties, Ad.mda-7 also shows antiangiogenic, immunostimulatory, and potent ‘‘bystander’’ antitumor activities (28, 29). These exciting results provide direct support for using mda-7/IL-24 in developing an
effective gene-based therapy for cancer. Recently, we reported that in prostate and ovarian cancers and in malignant gliomas, mda-7/IL-24 induced an ER stress response and subsequent apoptosis by suppressing expression of the anti-apoptotic Bcl-2 family members thus demonstrating the crucial role of Bcl-2 family proteins in regulating mda-7/IL-24 function (30-32). In this context antagonists of anti-apoptotic Bcl-2 family members might sensitize cancer cells to mda-7/IL-24-mediated cytotoxicity. The novel Apogossypol derivative BI-97C1 (Sabutoclax) suppresses the pro-survival Bcl-2 family members within a clinically achievable dose range (33). We recently demonstrated that BI-97C1 (Sabutoclax) sensitizes prostate cancer cells to mda-7/IL-24-mediated toxicity (34).

The present study explored the efficacy of a tropism-modified Ad-based cancer gene therapy approach for eradicating low CAR colorectal cancer cells. We show that in low CAR human colorectal cancer cells (RKO), a recombinant Ad.5/3 virus delivering mda-7/IL-24 (Ad.5/3-mda-7) is more efficient than Ad.5 delivering mda-7/IL-24 (Ad.5-mda-7) in expressing MDA-7/IL-24 protein, inducing cancer-specific apoptosis and inhibiting in vivo tumor growth in a nude mouse xenograft model. Further, our in vitro and in vivo data shows BI-97C1 (Sabutoclax) profoundly sensitizes mda-7/IL-24 mediated toxicity in colorectal cancer. Thus, Ad.5/3-mda-7, alone and/or in combination with BI-97C1 (Sabutoclax), might represent an improved and more effective therapeutic approach for colorectal and other cancers.
Results

*Enhanced infectivity of tropism-modified adenovirus (Ad.5/3) in RKO low CAR colorectal cancer cells.* Experiments were designed to compare the infectivity of Ad.5/3 chimeric viruses (expressing luciferase or mda-7/IL-24) and Ad.5 viruses (expressing luciferase or mda-7/IL-24) in low and high CAR colorectal cancer cells. The Kolmogorov-Smirnov two-sample test was used to analyze flow cytometric histograms to determine the cumulative distribution function of fluorescence intensity for each sample and the difference between the cumulative distributions, with the $D$ value indicating the greatest difference between the two curves. RKO cells have a reduced level of CAR (D value 0.03), whereas HCT116 cells have a high level of CAR (D value 0.82) (Fig. 1A). Luciferase (Luc) activity was evaluated following infection with Ad.5/3-Luc and Ad.5-Luc in RKO and HCT116 cells (Fig. 1B). The infection of RKO cells with Ad.5/3-Luc showed a dramatic increase in luciferase activity when compared to Ad.5-Luc. On the other hand the relative luciferase activity of Ad.5/3-Luc compared to that of Ad.5-Luc in HCT116 was much lower than that observed in RKO cells. These findings indicate that transduction efficiency in a low CAR tumor cell background was significantly enhanced by the Ad.5/3 modification. Next we compared Ad.5/3 and Ad.5 expressing mda-7/IL-24 (Ad.5/3-mda-7 and Ad.5-mda-7, respectively) by evaluating cell viability using standard MTT assay (Fig. 1C). Ad.5-vec (empty Ad.5 virus) and Ad.5/3-vec (empty Ad.5/3 virus) were used as controls. In RKO cells, Ad.5/3-mda-7 infected group had a significant reduction in cell viability whereas Ad.5-mda-7 treatment did not affect the cell viability. In HCT116 cells, both Ad.5-mda-7 and Ad.5/3-mda-7 inhibited growth with comparable efficiency (Fig. 1C).
**Ad.5/3-mda-7** induces MDA-7 expression and enhances killing in low CAR expressing RKO cells. We compared the ability of Ad.5/3-mda-7 and Ad.5-mda-7 (50 or 100 pfu/cell) in expressing mda-7/IL-24 in RKO and HCT116 cells (Fig. 2A and B). In RKO cells, Ad.5/3-mda-7 generated MDA-7/IL-24 at both high and low titer whereas Ad.5-mda-7 infected groups did not generate MDA-7/IL-24 protein (Fig. 2A). HCT116 cancer cells generated comparable amounts of MDA-7/IL-24 protein upon infection with both Ad.5/3-mda-7 and Ad.5-mda-7 (Fig. 2B). As a corollary, Ad.5/3-mda-7, but not Ad.5-mda-7, efficiently induced apoptosis in RKO cells, while both Ads were equally effective in inducing apoptosis in HCT116 cells as determined by PARP cleavage analysis (Fig. 2A and B). Similar profiles of induction of cell death were also observed using the trypan blue-dye exclusion test (Fig. 2C and D). Ad.5/3-mda-7, but not Ad.5-mda-7, induced cell killing (Fig. 2C) activity in RKO cells, which correlated with the expression level of MDA-7/IL-24 protein. As anticipated, both Ad.5-mda-7 and Ad.5/3-mda-7 induced cell death in HCT116 cells with comparable efficiency (Fig. 2D).

Annexin V staining followed by flow cytometry further quantified induction of apoptosis. In RKO cells, infection of Ad.5/3-mda-7 at 100 pfu/cell resulted in ~70% apoptotic cells, while no apoptosis was observed with Ad.5-mda-7 (Fig. 3A and B). As a corollary, infection of RKO cells with Ad.5/3-mda-7, and not with Ad.5-mda-7, induced an ER stress response as evidenced by increased expression of ER stress markers BiP/GRP78 and GRP94 and downregulation of anti-apoptotic protein Bcl-2 (Fig. 3C) These findings confirm that Ad.5/3 chimerism maintains the bona fide downstream effects exerted by mda-7/IL-24.

**Ad.5/3-mda-7 exerts anti-tumor effects in RKO xenografts in nude mice.** To test if the in vitro superiority of Ad.5/3-mda-7 over Ad.5-mda-7 in low CAR colorectal cancer cells translates into
the in vivo situation, we established subcutaneous xenografts of RKO cells in athymic nude mice. After palpable tumors of ~100 mm³ developed, the animals received 9 intratumoral injections over a 4-week period with 4 X 10⁸ pfu of Ad.5-vec, Ad.5/3-vec, Ad.5-mda-7 or Ad.5/3-mda-7 (Fig. 4). RKO cells formed large, aggressive and actively growing tumors in Ad.5-vec, Ad.5/3-vec and Ad.5-mda-7 treated animals, while Ad.5/3-mda-7 significantly inhibited the growth of the RKO tumors, which was evident at weeks 4 after initiating the therapeutic treatment protocol (Fig. 4A and B). The reduction in tumor growth by Ad.5/3-mda-7 was associated with increased expression of MDA-7/IL-24 and reduced expression of CD31, a marker for microvessels, in the tumor sections (Fig. 5A and B). H & E sections of the tumors also showed decreased cell density in Ad.5/3-mda-7-treated group compared to the other groups (Fig. 5C). These findings indicate that in low CAR colorectal cancer cells, Ad.5/3-mda-7 can efficiently generate MDA-7/IL-24 protein leading to inhibition of cell proliferation and angiogenesis.

**Suboptimal doses of Ad.5/3-mda-7 and BI-97C1 (Sabutoclax) promote synergistic anti-tumor effect in vitro and in vivo in RKO colorectal cancer cells.** We determined if suboptimal doses of the combination regimen of Ad.5/3-mda-7 and BI-97C1 (Sabutoclax) are capable of inhibiting growth of RKO cells. We first evaluated the dose and time kinetics of BI-97C1 (Sabutoclax) alone on RKO cells viability in vitro. A significant inhibition of cell viability was observed with 3 µM BI-97C1 on day 3 and day 6 (Fig. 6A). A combination of suboptimal doses of BI-97C1 (2µM) and Ad.5/3-mda-7 (50 pfu/cell) resulted in significant inhibition of viability of RKO cells compared to either agent alone (Fig. 6B). The combinatorial treatment resulted in downregulation of prosurvival proteins Bcl-2 and Mcl-1 and induction of apoptosis as revealed by PARP cleavage (Fig. 6C).
To evaluate the effect of combinatorial treatment *in vivo*, nude mice bearing subcutaneous RKO xenografts received nine intratumoral injections of a sub-optimal dose of $1 \times 10^8$ pfu per injection of Ad.5/3-vec or Ad.5/3-mda-7 and 12 intraperitoneal injections of 3 mg/kg of BI-97C1 (Sabutoclax) over a 4-week period (33, 34). The treatment was started when tumor size was ~100 mm$^3$. Mice were divided into four groups; Ad.5/3-vec; Ad.5/3-mda-7; Ad.5/3-vec plus BI-97C1 (Sabutoclax); and Ad.5/3-mda-7 plus BI-97C1 (Sabutoclax). Tumor growth and weight were markedly inhibited by the combination treatment of Ad.5/3-mda-7 and BI-97C1 (Sabutoclax) compared to the other groups at the end of the treatment period (Fig. 7). These findings showed that BI-97C1 (Sabutoclax) is efficient in sensitizing low CAR colorectal cancer cells to Ad.5/3-mda-7 treatment. BI-97C1 (Sabutoclax) was non-toxic to athymic nude mice (NCRnu/nu) when injected intraperitonially (up to 10 mg/kg) (34).

**Discussion**

HCT116 cells have high CAR and therefore are efficiently infected with Ad.5. On the other hand RKO cells have low CAR, which makes it relatively resistant to Ad.5 infection. Our analysis reveals a variable level of CAR expression in different colorectal cancer cell lines (Fig. 1A) indicating that for Ad delivery to be effective a strategy facilitating efficient infection of all types of colorectal cancer cells needs to be utilized. To overcome the low infectivity limitation of Ad.5, multiple groups are trying to solve the relative inefficiency of Ad.5 infection by modifying specific sequences within the virus capsid proteins that directly associate with CAR; i.e., targeting strategies are being developed to enhance viral infectivity via CAR-independent pathways. Several laboratories have modified the infective viral capsid “knob” to bind to surface
integrin proteins enhancing transformation (RGD modification) (6, 11-14). Additionally, insertion into the knob of multiple lysine residues (pK7) increases viral interaction with cells by electrostatic effects; and including portions of type 3 Ad in the viral capsid knob has also enhanced infectivity via CAR-independent pathways (6, 11-14). In the present study, the potent tumor suppressor gene mda-7/IL-24 driven by a CMV promoter has been delivered by a CAR-independent Ad containing a chimeric fiber with the knob domain of human Ad.3 in the Ad.5 capsid (Ad.5/3), which showed more efficient transduction of prostate, renal cancer and malignant glioma cells having low CAR compared with wild-type Ad.5 (35-38). This concept is supported by our observation that Ad.5/3-Luc showed superior luciferase transgene expression when compared to Ad.5-Luc in low CAR colorectal cancer cells. Similarly, Ad.5/3-Luc also showed enhanced infectivity in ovarian cancer cells OV-4 and SKOV3 which display moderate or low levels of CAR (39), as well as in renal cancer cells (40), melanoma cells (41) and prostate cancer (35). We presently extend these observations by demonstrating that Ad.5/3 can effectively deliver the potent anti-cancer therapeutics, mda-7/IL-24, in low CAR colorectal cancer cells; and it has similar transduction efficiency as the wild-type Ad.5 in high CAR colorectal cancer cells. More importantly the 5/3 modification did not interfere with the downstream killing pathway of mda-7/IL-24 as evidenced by induction of ER stress response markers such as BiP/GRP78 and GRP94; downregulation of pro-apoptotic Bcl-2 family members such as Bcl-2 and induction of apoptosis upon Ad.5/3-mda-7 infection of RKO cells. Therefore, Ad.5/3 might be an effective tool for therapeutic delivery of mda-7/IL-24 into genetically diverse colorectal tumors in vivo.

mda-7/IL-24 induces apoptosis by downregulating the proapoptotic Bcl-2 family proteins (33, 42) and ectopic expression of anti-apoptotic Bcl-2 family proteins, including Mcl-1, interferes with the anti-tumor activity of mda-7/IL-24. Apogossypol derivatives, such as BI-97C1
(Sabutoclax), function as broad spectrum antagonists of anti-apoptotic Bcl-2 proteins. These compounds bind to pockets formed by the BH domains of the anti-apoptotic Bcl-2 proteins and inhibit their ability to bind their targets (33). BI-97C1 (Sabutoclax) that also targets Mcl-1 profoundly sensitizes prostate cancer cells to mda-7/IL-24-mediated cell toxicity (34). Similarly, a combination of suboptimal doses of BI-97C1 (Sabutoclax) and Ad.5/3-mda-7 profoundly inhibited cell viability and promoted cell death in colorectal cancer cells and also inhibited Colo tumor xenografts in vivo. In our studies, BI-97C1 (Sabutoclax) was found to be non-toxic even at high doses and although clinical trials with BI-97C1 (Sabutoclax) remain to be initiated it is assumed that a suboptimal dose of BI-97C1 might not be toxic and will be well tolerated in human subjects as well. Based on the safety profile of the individual agents, and the fact that mda-7/IL-24 is now in the clinic and has been shown to be safe and efficacious, this novel combinatorial approach may be translatable into the clinic for the treatment of advanced colorectal cancers in the near future.

Materials and Methods

Cell lines, culture conditions, and viability assays. Human HCT116 colorectal carcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Dr. Jeffrey Schlom kindly provided human RKO colorectal carcinoma cell line from the National Center Institute (NIH, Bethesda, MD, USA). The cells were cultured in DMEM High Glucose (GIBCO, Carlsbad, California), supplemented with 10% FBS and 1% penicillin and streptomycin (Life technologies Inc. Carlsbad, California) at 37°C in a 5% CO2 95% air
humidified incubator. Cell viability was determined by standard 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assays (43).

**Generation of Ad.5.mda-7 and Ad.5/3-mda-7.** Recombinant serotype 5 and serotype 5 / serotype 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 (25, 35).

**Trypan blue dye exclusion and Annexin V binding assays.** Cells were harvested by treatment with 0.05% trypsin/0.53 mM EDTA. Cells were centrifuged at 200 x g for 5 min to remove trypsin/EDTA. After the cell pellets were resuspended in 100 µl of PBS, 100 µl of 0.4% trypan blue solution were added and mixed. Trypan blue cell suspension was left at room temperature for 10 min before the cells were counted with a hemocytometer. Cell death was defined as the percentage of the stained dead cells in the whole-cell population (43). Annexin V binding assay was performed as previously described (42).

**Detection of Coxsackie-adenovirus receptors (CARs) on the cell surface, and preparation of whole-cell lysates and Western blotting analyses.** Quantification of surface expression of CAR was performed as described previously (35, 42). Preparation of whole-cell lysates and Western blotting analyses were performed as described (44). The primary antibodies used were anti-MDA-7/IL-24 (Gen Hunter Corporation ), anti-EF1α (1:1,000; mouse monoclonal; , Millipore), anti-Mcl-1 (1:500; mouse monoclonal; Santa Cruz), anti-BiP/GRP78 (1:500; rabbit monoclonal; Santa Cruz), anti-GRP94 (1:1000; rabbit monoclonal; Sigma), anti-Bcl-2 (1:1000; mouse monoclonal; BD Biosciences) and anti-PARP (1:1000; rabbit monoclonal; Cell Signaling).
Human colorectal cancer xenografts in athymic nude mice and treatment with Ad.5/3-mda-7 and BI-97C1 (Sabutoclax). RKO cells (2 × 10^6) were injected s.c. in 100 µL in male athymic nude mice (NCRnu/nu, 4 weeks old, ~20 g body weight) (44, 45). After establishment of visible tumors of ~100-mm³, requiring ~8-10 days, different adenoviruses were injected intratumorally. The injections were given 3X the first week and then 2X a week thereafter for a total of nine injections. BI-97C1 (Sabutoclax) was injected intraperitoneally (i.p.) at a sub-toxic level (33) at 3 mg/kg 3X a week throughout the study (total 12 injections). Compound was dissolved in 500 µL of solvent (ethanol/Cremophor EL/saline = 10:10:80). A minimum of five animals was used per experimental point. Tumor volume was measured with a caliper and calculated using the formula: π/6 × larger diameter × (smaller diameter)². At the end of the experiment, the animals were sacrificed and the tumors were harvested and preserved in formalin before embedding in paraffin for immunohistochemical analysis.

**Immunohistochemical staining.** Immunohistochemical staining was performed as described (35) with anti-MDA-7/IL-24 (1:200) and monoclonal anti-CD31 (1:200) (Dako Corporation, Carpenteria, California, USA).

**Statistical analysis.** Statistical analysis was done using the Student t-test. P < 0.05 was considered significant.

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References


Figure Legends

Figure 1. Enhanced infectivity of tropism-modified adenovirus (Ad.5/3) in RKO low CAR colorectal cancer cells. A) Coxsackie-adenovirus receptors (CARs) expression on the surface of different colorectal cancer cell lines, the K-S value of RKO is only 3%, whereas it reaches 83% for HCT116. B) Cells were infected with Ad.5-Luc and Ad.5/3-Luc at the indicated doses; and luciferase activity was measured after 48 hr. Data represent luciferase activity of Ad.5/3-Luc divided by that of Ad.5-Luc. C) Cells were infected with the indicated doses of virus and cell viability was quantified using MTT assay after 3 and 6 days of infection.

Figure 2. Ad.5/3-mda-7 induces MDA-7/IL-24 expression and enhances killing in low CAR expressing RKO cancer cells. A and B. HCT116 and RKO cells were infected with the indicated doses of Ad.5-mda-7 or Ad.5/3-mda-7 and respective controls. After 48 hr of infection total proteins were isolated. The expression of MDA-7/IL-24, activation of PARP (as a marker for apoptosis) and expression of EF1α (as a loading control) were analyzed by Western blotting analyses in RKO (A) and HCT 116 (B) cells. C and D. Percentage of dead cells detected by Trypan blue dye exclusion staining for RKO (C) and HCT 116 (D) cells. Results are mean ± S.D. (n=3).

Figure 3. Ad.5/3-mda-7 induces killing, apoptosis and ER stress in RKO low CAR colorectal cancer cells.
RKO Cells were treated with the indicated Ad for two days. A) Histogram of different groups assayed by flow cytometry following Annexin V-FITC/PI staining. B) Graph showing percentage of Annexin V- positive cells. Results are the mean ± S.D. (n=3). C) Changes in GRP94, BiP/GRP78, Bcl-2, MDA-7/IL-24 and EF1α were evaluated by Western blotting analysis.

**Figure 4. Anti-tumor effect of Ad.5-mda-7 and Ad.5/3-mda-7 in RKO xenografts in nude mice.** Subcutaneous tumor xenografts from RKO cells were established in athymic nude mice; tumors were injected with the indicated Ads over a 4-week period (total of nine injections). A) Measurements of RKO xenograft tumor volumes; points, average (with a minimum of five mice in each group); bars, ± S.D. B) A representative photomicrographs of tumor and wights at the end of the study; bars, ± S.D.

**Figure 5. Ad.5/3-mda-7 shows enhanced MDA-7/IL-24 production and inhibition of proliferation and angiogenesis in RKO low CAR colorectal cancer cells.** Subcutaneous tumor xenografts from RKO cells were established in athymic nude mice; the tumors were injected with the indicated Ads over a 4-week period (total of nine injections). At the end of the experiment the tumors were harvested and formalin-fixed, paraffin-embedded, sectioned and were subjected to further analysis A) Immunohistochemical analysis for MDA-7/IL-24 expression. B) Immunohistochemical analysis for CD31 expression. C) Monitoring of H&E stained sections for cellular density.
Figure 6. Suboptimal doses of tropism-modified adenoviruses (Ad.5/3) and BI-97C1 (Sabutoclax) synergize resulting in decreased viability of RKO low CAR colorectal cancer cells. A) RKO cells were treated with the indicated concentrations of BI-97C1 (Sabutoclax). After 3 and 6 days, cell viability was evaluated by MTT assays. Bars, ± S.D. (n = 3). B) RKO cells were infected with 50 pfu/cell of Ad.5/3-vec or Ad.5/3-mda-7 for 24 hr after which cells were treated with indicated concentrations BI-79C1 (Sabutoclax) and MTT assay was performed at 3 and 6 days. C) Analysis of expression of the indicated proteins by Western blotting.

Figure 7. The combination of Ad.5/3-mda-7 and BI-97C1 (Sabutoclax) additively inhibit the growth of low-CAR colorectal cancer xenografts in vivo. Subcutaneous tumor xenografts from RKO cells were established in athymic nude mice; the tumors were injected with the indicated Ads with and without BI-97C1 (Sabutoclax) over a 4-week period (total of nine injections each). A) Measurements of RKO xenograft tumor volumes; points, average (five mice in each group); bars, ± S.D. B) Left: Measurement of tumor weight at the end of the study; Right: Images of the tumors at the end of the study; columns, mean; bars, ± S.D.
Figure 1
Figure 2
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An Apogossypol derivative BI-97C1 (Sabutoclax) targeting Mcl-1 sensitizes prostate cancer cells to *mda-7/IL-24*-mediated toxicity
Abstract

Limited options are available for treating patients with advanced prostate cancer (PC). Melanoma differentiation-associated gene-7/interleukin-24 (mda-7/IL-24), an IL-10 family cytokine, exhibits pleiotropic anticancer activities without adversely affecting normal cells. We previously demonstrated that suppression of the pro-survival Bcl-2 family member, myeloid cell leukemia-1 (Mcl-1), is required for mda-7/IL-24-mediated apoptosis of prostate carcinomas. Here we demonstrate that pharmacological inhibition of Mcl-1 expression with the novel Apogossypol derivative BI-97C1, also called Sabutoclax, is sufficient to sensitize prostate tumors to mda-7/IL-24-induced apoptosis, whereas ABT-737, which lacks efficacy in inhibiting Mcl-1 does not sensitize mda-7/IL-24-mediated cytotoxicity. A combination regimen of tropism-modified adenovirus delivered mda-7/IL-24 (Ad.5/3-mda-7) and BI-97C1 enhances cytotoxicity in human PC cells including those resistant to mda-7/IL-24 or BI-97C1 alone. The combination regimen causes autophagy that facilitates NOXA- and Bim-induced, and Bak/Bax-mediated mitochondrial apoptosis. Treatment with Ad.5/3-mda-7 and BI-97C1 significantly inhibits the growth of human PC xenografts in nude mice and spontaneously induced PC in Hi-myc transgenic mice. Tumor growth inhibition correlated with increased TUNEL staining and decreased Ki67 expression in both PC xenografts and prostates of Hi-myc mice. These findings demonstrate that pharmacological inhibition of Mcl-1 with the Apogossypol derivative, BI-97C1, sensitizes human PCs to mda-7/IL-24-mediated cytotoxicity, thus potentially augmenting the therapeutic benefit of this combinatorial approach toward PC.
Introduction

Despite improvements in detection and treatment, PC remains the most common cancer and the second leading cause of cancer-related death in men in the USA (1). Because PC is a relatively slow-growing disease, repeated systemic gene therapy applications alone or in combination with anti-tumor chemotherapeutic agents over the life span of the patient may be required (1).

Melanoma differentiation associated gene-7/Interleukin-24 (mda-7/IL-24), a novel member of the IL-10-related cytokine gene family, exhibits nearly ubiquitous anti-tumor properties *in vitro* and *in vivo* (2-4), which culminated in its successful entry into the clinic where safety and clinical efficacy was achieved (5). Unique therapeutic aspects of Ad.mda-7 lie in its selective induction of apoptosis in cancer cells, profound antitumor ‘bystander’ effect exerted by the secreted MDA-7/IL-24 protein, and the ability of this novel cytokine to inhibit angiogenesis and provoke a potent anti-tumor immune response (2). Upon ectopic expression of *mda-7/IL-24* by an adenovirus, MDA-7/IL-24 interacts with the endoplasmic reticulum (ER) chaperone protein BiP/GRP78 and initiates an “unfolded protein response (UPR)” in tumor cells that promotes apoptosis (6). Recently, we reported that in PC, ovarian and malignant gliomas, *mda-7/IL-24*-induced ER stress response produced apoptosis by suppressing expression of the anti-apoptotic protein Mcl-1 (4, 7, 8).

In view of the essential roles of anti-apoptotic Bcl-2 family proteins in tumorigenesis and chemoresistance, efforts are focused on developing small molecule inhibitors of Bcl-2 family proteins as potential therapeutics for cancer (9). Unfortunately, due to the unique structure of Mcl-1 as compared with Bcl-2 and Bcl-xL (9), currently employed inhibitors, such as ABT-737 or its clinical counterpart, ABT-263, display limited affinity for Mcl-1 (10) (Supplemental Figure 1). Using nuclear magnetic resonance (NMR) binding assays and computational docking studies, we have recently identified a series of new Apogossypol derivatives, compound 3 (BI-79D10) and
compound 11 (BI-97C1; Sabutoclax), with pan-Bcl-2-inhibitory potency (11, 12). BI-79D10 binds to Bcl-xL, Bcl-2, and Mcl-1 with IC_{50} values of 190, 360, and 520 nmol/L, respectively (13). BI-97C1 is an optically pure individual Apogossypol derivative that retains all the properties of BI-79D10 along with superior \textit{in vitro} and \textit{in vivo} efficacy (11).

Because Mcl-1 is over-expressed in the majority of PCs (4), we hypothesized that suppressing Mcl-1 by treating human PC cells with BI-97C1 would sensitize them to \textit{mda}-7/IL-24-mediated cytotoxicity. Our data suggests that treatment with the combination regimen of \textit{mda}-7/IL-24 and BI-97C1 induces autophagy that facilitates apoptosis in association with up regulation of NOXA, accumulation of Bim, and activation of Bax and Bak. Treatment with \textit{mda}-7/IL-24 and BI-97C1 inhibited the growth of PC xenografts and suppressed PC development in an immunocompetent transgenic mouse model of PC.

\textbf{Results}

\textit{Mcl-1 promotes resistance to \textit{mda}-7/IL-24-mediated apoptosis of human PC cells.} Genetic and pharmacological inhibitors of Mcl-1 were used to determine if suppression of Mcl-1 expression sensitizes PC cells to \textit{mda}-7/IL-24-mediated cytotoxicity. To facilitate delivery of \textit{mda}-7/IL-24 to PC cells, which frequently contain reduced levels of Coxsackie Adenovirus Receptors (CAR), we employed a tropism-modified chimeric 5/3 adenovirus, Ad.5/3-\textit{mda}-7 (14). siRNA-mediated reduction of Mcl-1 significantly sensitized PC3 cells to Ad.5/3-\textit{mda}-7-induced apoptosis even at low doses (50 pfu/cell) (Figure 1A). On the other hand, treatment of PC3 cells with BI-79D10 or BI-97C1 resulted in reduced viability with an IC_{50} value of 750 nM for both compounds (Figure 1B) while that of ABT-737 was 12 \mu M.
To evaluate the combinatorial effect of Mcl-1 inhibition and mda-7/IL-24 we treated PC3, DU-145, M2182 and normal, SV40 T-antigen-immortalized prostate epithelial cells (P69) with a sub-lethal dose of Ad.5/3-mda-7 (50 pfu/cell) and BI-79D10 at either 350 nM (at which concentration it inhibits Bcl-2 and Bcl- xL) or 520 nM (at which level it inhibits Mcl-1) (13). Treatment of PC3, DU-145 or M2182 cells with 350 nM of BI-79D10 in combination with 50 pfu/cell of Ad.5/3-mda-7 had a minimal effect on promoting apoptosis, whereas infection with Ad.5/3-mda-7 (50 pfu/cell) and treatment with 520 nM of BI-79D10 profoundly promoted apoptosis (Figure 1C). In contrast, the combination exerted little to no toxicity in normal P69 cells, indicating cancer-specific activity (Figure 1C). Furthermore, the combination also showed a greater than additive induction of apoptosis in mda-7/IL-24-resistant DU-145 cells that stably over-express Bcl-2, Bcl-xL or Mcl-1 (Du-bcl-2, Du-bcl-xL and Du-Mcl-1-8, respectively) (4, 15) (Figure 1D). A similar enhanced induction of apoptosis was observed in these therapy-resistant prostate cancer cell lines by treatment with Ad.5/3-mda7 and BI-97C1 (Figure 2A and 2B). BI-97C1 is an optically purified small molecule suitable for clinical use (11). Induction of apoptosis was observed with as low as 10 pfu/cell of Ad.5/3-mda-7 in combination with BI-97C1 (Figure 2A). Similarly, BI-97C1 potently induced apoptosis of PC3 cells with an IC50 of approximately 500 nM in combination with Ad.5/3-mda-7 (50 pfu/cell) (Figure 2B). A time course analysis revealed that 48 h was required for the combination of Ad.5/3-mda-7 and BI-97C1 to significantly promote apoptosis of human PC cells (Figure 2C). Treatment of human PC cells with Ad.5/3-mda-7 and BI-97C1 resulted in PARP cleavage, activation of Caspase-3, cytosolic release of cytochrome c and Smac/DIABLO, as well as inactivation of cell proliferation signals, i.e., AKT and ERK (Figure 2D to 2F).

**Bim and NOXA activity are required for BI-97C1/mda-7-IL-24-mediated Bak- and Bax-dependent apoptosis.** To analyze the involvement of mitochondrial pathway of apoptosis (16), PC3
cells were treated with BI-97C1 (520 nM) and Ad.5/3-\textit{mda}-7 (50 pfu/cell) and expression of Bak/Bax conformational changes (detected by immunoprecipitation using antibodies that specifically recognize activated Bak or Bax) (16) and intracellular Bax localization were assessed. Treatment of PC3 with BI-97C1 and Ad.5/3-\textit{mda}-7 moderately elevated expression of total Bax and Bak compared to either single agent alone (Figure 3A). Treatment with the combination of BI-97C1 and Ad.5/3-\textit{mda}-7 induced Bak or Bax conformational changes significantly greater than either agent alone (Figure 3B). Similarly, combination treatment dramatically increased mitochondrial translocation of Bax (Figure 3B) and Bak/Bax association (Figure 3C). Transient shRNA-mediated knockdown of Bax and Bak promoted resistance to BI-97C1/\textit{mda}-7/IL-24-mediated toxicity (Figure 3D).

Considering the potential roles that the BH3-only proteins, NOXA and Bid play in apoptosis induced by various stimuli (17), expression and/or phosphorylation of these proteins were examined following combination treatment in PC3 cells. After 48 h of combined treatment, we observed a significant increase in NOXA expression, Bim accumulation, and suppression of Bid and p-Bad expression compared with treatment with either agent alone (Figure 4A). When PC3 cells were transiently transfected with shRNA against NOXA or Bim, a significant degree of resistance towards BI-97C1/\textit{mda}-7-IL-24-mediated apoptosis was observed (Figure 4B and 4C). Earlier we reported that \textit{mda}-7/IL-24 induces ER stress by activation of PERK (PKR-like ER-localized eIF2\textalpha kinase) (2). We observed that ER stress generated by \textit{mda}-7/IL-24 was enhanced by combined treatment with BI-97C1 (Figure 4D: Left panel). Interestingly, inhibition of PERK by a dominant negative construct in PC3 cells protected them from cytotoxicity of the combination treatment accompanied by a reduction in Bim accumulation and PARP cleavage, but with minimal effects on elevated NOXA expression (Figure 4D).
**Induction of Autophagy by the combination of mda-7/IL-24 and BI-97C1 precedes apoptosis.**

Autophagy is usually initiated in response to increased metabolic requirements during cellular stress induced by therapeutic treatments (18). For that reason, we evaluated autophagy in response to treatment with mda-7/IL-24 and BI-97C1. Induction of LC3 in autophagic vacuoles was determined by transient transfection of PC3 cells with a baculovirus-expressing RFP fused with LC3. Treatment with BI-97C1 or mda-7/IL-24 alone resulted in a significant increase in the LC3-RFP signal and LC3-II expression that was further augmented by combination treatment (Figure 5A). Because mda-7/IL-24 selectively targets transformed cells, we transfected MEFs with a human pcDNA3.1-H-ras construct. Human H-ras transfected Bax-/-Bak-/- MEFs were resistant to mda-7/IL-24 + BI-97C1-induced cytotoxicity compared to wild type MEFs, indicating that cell death induced by the combination is due to apoptosis, not autophagy (Figure 5B). On the other hand, we also detected a slight induction of autophagy in Bax-/-Bak-/- MEFs as compared to wild type MEFs following combination treatment (Supplementary Figure 3C). These results suggest that autophagy induced by the combination treatment might protect cells from initial stress followed by a switch to apoptosis at a later time point. Furthermore, the increase in LC3-RFP signal and expression of LC3-II was maximum at 24 h and decreased by 48 h of post treatment, whereas apoptosis, detected by TUNEL assay and PARP cleavage, was evident at 48 h, but not at 24 h post-treatment (Figure 5C and 5G). Notably, we observed that down-regulation of the essential autophagy genes Beclin-1 or ATG5 (to a lesser extent) (but not ATG7) by siRNA reduced the cytotoxicity of the combination effect (Figure 5D). This finding was supported by the observation that 24 h after combination treatment, the interaction between Mcl-1 and Beclin-1 was disrupted (Figure 5E). The switch between autophagy and apoptosis is a complex process that is currently poorly understood (18).
Previously, it was found that in a cellular model system where autophagy precedes apoptosis, IL-3 depletion of Ba/F3 cells caused caspase-mediated cleavage of Beclin-1 and PI3KC3 (18). Interestingly, cleavage of Beclin-1 during apoptosis not only inactivates its autophagic function, but also generates a Beclin-1-derived fragment that translocated to the mitochondria and enhanced the release of cytochrome c and hence augmented apoptosis (18). We also observed that 48 h after combined treatment, activation of caspase-3 was associated with cleavage of Beclin-1 (Figure 5F). When Caspase-3 activity was inhibited using a pharmacological inhibitor, PC3 cells developed resistance towards the cytotoxicity induced by the combination of Ad.5/3-\textit{mda}-7 and BI-97C1 (Figure 5F).

**Combination treatment with \textit{mda}-7/IL-24 and BI-97C1 potentiates inhibition of human prostate tumor growth in vivo in immune deficient and immune competent animals.** To test efficacy \textit{in vivo}, we performed studies that utilized two animal models, one employing immune deficient athymic nude mice xenografted with human prostate tumor cells (M2182) (11) and one using a spontaneous immunocompetent transgenic mouse model of prostate cancer (the Hi-Myc mouse) (19). Initial studies were performed in nude mice bearing subcutaneous M2182 human prostate tumors established on both flanks that stably express luciferase (M2182-Luc). Mice were treated, when tumor size was \~100 mm\textsuperscript{3}, using a sub-optimal dose of 0.5 X 10\textsuperscript{8} pfu per injection of Ad.5/3-vect or Ad.5/3-\textit{mda}-7 (7 injections). BI-97C1 was injected intraperitoneally (i.p.) (11) either at 1mg/kg or 3 mg/kg 3X a week for the 3-week duration of the study (9 injections). Tumor growth was markedly inhibited by combination treatment compared to treatment with either agent alone (Figure 6A). Combined treatment of Ad.5/3-\textit{mda}-7 and BI-97C1 (3 mg/kg) significantly reduced Ki-67 expression and increased TUNEL signal compared to treatment with either agent alone (Figure 6B). BI-97C1 was observed to be non-toxic to athymic nude mice (NCRnu/nu) when
injected intraperitonially (up to 10 mg/kg) or when injected intravenously (25 mg/kg) (Supplemental Figure 4 and Supplemental Tables 1 and 2).

Next, we tested the efficacy of Ad.5/3- mda-7 and BI-97C1 in a spontaneous model of prostate cancer, the Hi-Myc mouse (19). For optimal therapeutic outcome for adenocarcinoma, we began treatment at 22 weeks of age. The ability to deliver adenoviruses systemically is limited by sequestering of the virus in the liver and clearance of the virus by the immune system. We have developed a novel strategy to circumvent both of these problems that employs microbubbles treated with complement and targeted release of the therapeutic virus at the tumor site using ultrasound, an approach referred to as microbubble-targeted ultrasound destruction (UTMD) (20). In this context, systemic and targeted delivery of mda-7/IL-24 to the prostate of Hi-Myc mice was achieved by intravenous injection of complement-treated microbubble (MB) encapsulated Ad.5/3- mda-7 followed by sonoporation in the prostatic area, UTMD (20). A total of 9 tail vein injections of MB/Ad.5/3- mda-7 were administered over a 4-week period. BI-97C1 was administered intraperitoneally (i.p.) at 3 mg/kg 3X a week throughout the study. The size of the prostates of Hi-Myc mice treated with the combination of Ad.5/3- mda-7 and BI-97C1 were significantly reduced compared to treatment with either agent alone or the vehicle (and Ad.5/3- vec) (Figure 6C). Decreased Ki67 and increased TUNEL expression accompanied tumor growth inhibition was observed in sections of the prostates of Hi-myc mice with Ad.5/3- mda-7 and BI-97C1 as compared to each single agent alone (Figures 6C and 6D).

**Discussion**

Even though mda-7/IL-24 can induce apoptosis in diverse human cancers, it is apparent that some subsets of cancer cells are relatively resistant to this therapy (2). One mode of resistance to mda-
7/IL-24-based therapies occurs through elevated expression of either Mcl-1 or Bcl-xL, which promotes resistance to mda-7/IL-24-induced apoptosis (4, 15). Exposure of PC cells to mda-7/IL-24 moderately down-regulates Bcl-xL, whereas this treatment completely extinguishes Mcl-1 expression, suggesting that the Mcl-1 gene may be more relevant in conferring resistance to mda-7/IL-24-mediated cytotoxicity and may provide a more appropriate target for enhancing sensitivity to this therapeutic cytokine. To test this hypothesis, we inhibited Mcl-1 expression by siRNA in Du-Bcl-xL, which are resistant to Ad.mda-7-mediated apoptosis (15). Inhibiting Mcl-1 expression was able to overcome resistance of Du-Bcl-xL cells to Ad.mda-7-induced apoptosis. In contrast, siRNA-mediated reduction of Bcl-xL in mda-7/IL-24 resistant Du-Mcl-1-8 cells (4) did not sensitize them to Ad.mda-7-induced apoptosis (Supplemental Figures 2A and 2B). These findings indicate that Mcl-1, rather than Bcl-xL, plays a more definitive role in generating resistance to mda-7/IL-24-induced apoptosis. Treatment with novel BI-97C1 in nanomolar range is sufficient to sensitize mda-7/IL-24-resistant cells, which over-express Bcl-2, Bcl-xL or Mcl-1, to mda-7/IL-24-dependent apoptosis. Because the Bcl-2/Bcl-xL inhibitor ABT-737/ABT-263 does not inhibit Mcl-1, treatment with ABT-737 failed to sensitize PC cells to mda-7/IL-24-induced apoptosis (Supplemental Figure 3A and 3B).

Based on the present data, a hypothetical model is proposed by which mda-7/IL-24 and BI-97C1 cooperate to induce Bim- and NOXA-dependent intrinsic apoptosis (Figure 6E). Although the mechanism by which Bim promotes apoptosis remains unclear, it has been shown that up-regulation of Bim results in the release of pro-apoptotic multidomain Bcl-2 family members Bak and Bax. Once activated Bax and Bak permeabilize the mitochondrial membrane, thereby facilitating the release of apoptotic inducing factors such as cytochrome c, AIF, and Smac/DIABLO (16). Evidence for direct activation of Bax by Bim also exists (21). Similarly, inhibition of Mcl-1 by treatment with
BI-97C1 results in up regulation of NOXA, which may lead to not only activation of Bak (16), but also the ability of Mcl-1 to efficiently sequester Bim (22). Alternatively, activation of Bak/Bax by BI-97C1 and mda-7/IL-24 may inhibit Bcl-2 and Bcl-xL. It is important to note that in the present combination studies, the concentration of BI-97C1 employed was 520 nM, which also inhibits Bcl-2 and Bcl-xL. Our data also suggest that at early times, autophagy induction by the combination may provide an initial cytoprotective response, based on evidence that knocking down Beclin1 or ATG5 expression protected cells from mda-7/IL-24/BI-97C1 toxicity. On the other hand, at later times (e.g., 48 h) caspase-mediated cleavage of Beclin-1 may instead augment apoptosis rendered by the combination regimen (Figure 6E).

In summary, the present study highlights the noteworthy potential of a combinatorial approach involving mda-7/IL-24, a broad-acting anticancer gene, and BI-97C1, which targets Mcl-1, to sensitize PC to mda-7/IL-24-mediated cytotoxicity, thereby enhancing therapeutic efficacy. Based on the safety profile of the individual agents, and the fact that mda-7/IL-24 is now in the clinic and has been shown to be safe and efficacious, this novel combinatorial approach may be translatable into the clinic for the treatment of advanced PC in the near future.

**Materials and Methods**

**Cells, stable clones, culture conditions and adenoviral constructs.** P69 and Human PC cells M2182, DU-145 and PC3, Wild type (wt), and Bax<sup>−/−</sup>/Bak<sup>−/−</sup> MEF were cultured as described (15). DU-145 clones stably overexpressing Bcl-2, Bcl-x<sub>L</sub>, Mcl-1 and M2182-Luc were generated as described (4, 15).

**Assessment of cell viability, cell death and apoptosis.** Apoptotic cells were identified and quantified by Annexin V–fluorescein isothiocyanate staining and DeadEnd Colorimetric TUNEL
Assay kit (Promega, Madison, WI) (15), and cell death was quantified by trypan blue dye exclusion assays (4) and cell viability was measured by MTT assays (14).

**Transient transfections.** Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol with different plasmids for 24 h before treatment, 100 nM SMARTpool Mcl-1, Bcl-xL siRNAs or control siRNA (siControl) (Dharmacon, Inc.), siATG5, siBeclin-1, siATG-7 (Santa Cruz Biotechnology Inc) (23, 24).

**Immunoprecipitation and immunoblotting.** Immunoprecipitation and immunoblotting were performed as previously described (4). The primary antibodies used in this study were as follows: Mcl-1, BiP/GRP78, Bak, Phosho-PERK, Smac/DIABLO, GADD153, cytochrome c, NOXA (Santa Cruz Biotechnology Inc); PARP, Bcl-xL, total and p-ERK1/2 (Thr-202/Tyr-204), total and p-Akt (Thr-183/Tyr-185), Bid, p-Bad (Cell Signaling Technology, Danvers, MA); MDA-7/IL-24 (GenHunter Corporation, Nashville, TN); anti-EF1α (Upstate Biotechnology, Lake Placid, N.Y); and GRP94 (Sigma). Bax, Bim (BD Pharmigen). LC3, ATG-5, ATG-7, Beclin-1 and Caspase-3 (Novus Biologicals). zVAD-FMK (Enzyme Systems Products). Cisplatin (Bristol-Meyer Squibb Oncology NY, USA). ABT-737 (SYNthesis Med Chem, China). Luciferin (Roche Applied Science).

**Detection of Bak and Bax activation.** Detection of Bax and Bak activation was performed by flow cytometry as described (16). For Bax translocation studies, mitochondrial and cytosolic fractions were prepared by using the mitochondria fractionation kit (Active Motif) according to the manufacturer’s protocol.

**Human prostate cancer xenografts.** M2182-Luc (1 X 10^6) cells were injected s.c. in the left and right flank of male athymic nude mice as described (11). After establishing visible tumors of ~100-mm^3, intratumoral injections of Ad.5/3-vec or Ad.5/3-*mda*-7 were given to the tumors on the left
flank at a dose of 0.5 X 10^8 pfu in 100 µl. The injections were given 3X the first week and then 2X a week for two more weeks for a total of seven injections. BI-97C1 (dissolved in ethanol/Cremophor EL/saline = 10:10:80) was injected i.p. at a sub-toxic level (11) either at 1 mg/kg or 3 mg/kg 3X a week throughout the study (total 9 injections).

*Preparation of microbubbles (MBs), ultrasound (US) platform and ultrasound-targeted microbubble destruction (UTMD).* Hi-Myc (19) mice were obtained from the Mouse Repository at NCI Frederick, MD, USA and genotyping was done based on the primer information available in NCI. Preparation of MBs followed by UTMD for delivery of mda-7/IL-24 expressing Ads has been described (20). The mice were divided into four groups: I) Ad.5/3-vec, II) Ad.5/3-vec + BI-97C1, III) Ad.5/3-mda-7, and IV) Ad-5/3-mda-7 + BI-97C1 group. Ultrasound (sonoporation) was performed (20) in the ventral side of mice in the prostatic area. BI-97C1 was administered intraperitoneally at a dose level of 3 mg/kg 3X a week for the duration of the study (total 9 injections).

*Immunohistochemical staining.* For immunohistochemical analysis, formalin-fixed and paraffin-embedded specimens of 3–4-µm thickness were sectioned and stained with ant-MDA-7/IL-24 and Ki-67 as described (4).

*Monitoring autophagy.* For detection of autophagy, PC3 cells were infected with BacMam LC3B-RFP baculovirus reagent (PremoTM Autophagy Sensor Kit, Invitrogen). For staining cytoskeleton, MAP4-GFP (Cellular Lights MAP4-GFP, Invitrogen) transduction reagents were used and after treatment samples were analyzed by confocal laser-scanning microscope (Zeiss 510 Meta confocal imaging system).

*Molecular Modeling and NMR studies.* A ^15^N-labeled Mcl-1 sample was prepared and NMR measurements of Mcl-1 and compound interactions were carried out using a series of ^1^H-^15^N HSQC
spectra as described (9). Molecular modeling studies were conducted on a Linux workstation and a 64 3.2-GHz CPUs Linux cluster. Docking studies were performed using the crystal structure of Mcl-1 in complex with a BH3 mimetic ligand (Protein Data Bank code 2NL9).

**Statistical analysis.** Data are represented as the mean ± S.D. and analyzed for statistical significance using one-way ANOVA followed by Newman-Keuls test as a post hoc test. P value of <0.05 was considered significant.

**Acknowledgements**

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References


Figure Legends

Figure 1: Genetic or pharmacological knockdown of Mcl-1 sensitizes PC to mda-7/IL-24-mediated apoptosis:  A) PC3 cells were transiently transfected with indicated siRNA for 24 h (inset) followed by infection with Ad.5/3-mda-7 at different pfu/cells for an additional 48 h, and % of apoptotic cells was determined. S.D. (n = 3). * indicates P <.01. B) PC3 cells were exposed to the indicated concentrations of BI-79D10, BI-97C1 or ABT-737 for 48 h after which % of cell survival was evaluated. Bars, S.D. (n = 3). C) DU-145, PC3, M2182 and P69 cells were infected with 50 pfu/cell of Ad.5/3-vec or Ad.5/3-mda-7 for 6 h and then treated with indicated concentrations BI-79D10 for 48 h and apoptosis was monitored. S.D. (n = 3), *(M2182), ** (PC3) and *** (DU-145) indicates P <.01 for BI-97C1 and Ad.5/3-mda-7 treated group. D) mda-7/IL-24-resistant DU-145 cells stably expressing Bcl-xL, Bcl-2 and Mcl-1 were treated as in A and apoptosis was evaluated. S.D. (n = 3) * (Du-bcl-2), ** (Du-bcl-xL) *** (Du-Mcl-1-8) indicates P <.01 for BI-97C1 and Ad.5/3-mda-7 treated group.

Figure 2: BI-97C1 and mda-7/IL-24 cooperate to induce death in prostate cancer cells:  A) PC3 cells were infected with indicated doses of Ad.5/3-mda-7 for 6 h and then treated with DMSO or BI-97C1 for 48 h. B) PC3 cells were infected with Ad.5/3-vec or Ad.5/3-mda-7 for 6 h and then treated with indicated concentrations of BI-97C1 for 48 h. C) PC3 cells were infected with Ad.5/3-vec or Ad.5/3-mda-7 for 6 h and then treated with DMSO or 520 nM of BI-97C1 for the indicated time. In A, B and C, % of apoptosis was evaluated. Bars represent S.D. (n = 3). D) PC3 cells were infected with Ad.5/3-vec or Ad.5/3-mda-7 for 6 h and then exposed indicated concentrations of BI-
97C1 for 48 h after and Western blotting was performed with indicated antibodies. E) PC3 cells were treated as mentioned in D and cytosolic protein fractions were isolated and Western blotting was performed. F) PC3 cells were treated as mentioned in D and Western blotting was performed.

**Figure 3:** Combination of *mda-7/IL-24* and BI-97C1 results in Bak and Bax conformational change, whereas knockdown of these molecules markedly attenuates apoptosis. A) PC3 cells were infected with 50 pfu/cell of Ad.5/3-vec or Ad.5/3-*mda-7* for 6 h and then exposed to DMSO or BI-97C1, for 48 h. Protein lysates were collected and Western blotting was performed. B) PC3 Cells were treated as mentioned in A and then activated Bak and Bax proteins were monitored as described in the Material and Methods section (top panel). Alternatively, cytosolic and mitochondrial fractions were separated and subjected to Western blotting analysis (bottom panels). C) PC3 cells were treated as mentioned in panel B and subjected to immunoprecipitation using anti-Bak antibodies. D) PC3 cells transiently transfected with shGFP, shBax or shBak (insert) for 24 h as described in Materials and Methods section and were treated as described in A. Cell death was determined using trypan blue dye exclusion. S.D. (n=3), * indicates P <.01.

**Figure 4:** Bim and NOXA play critical roles in Bax- and Bak-mediated cytotoxicity induced by *mda-7/IL-24* and BI-97C1. A) PC3 cells were infected with Ad.5/3-vec or Ad.5/3-*mda-7* for 6 h and then treated with indicated concentrations of BI-97C1 for 48 h and Western blotting was performed. B) PC3 cells transiently transfected with shGFP, shBim or shNOXA (insert) and treated as described in A. Cell death was determined using trypan blue dye exclusion. S.D. (n=3). * indicates P <.01. C) Left and Right panel: PC3 cells were treated as mentioned in panel B after
which cell lysates were subjected to determine Bax conformation analysis as indicated in Materials and Methods. 

D) Left Panel: PC3 were treated as mentioned in A and Western blotting was performed. Right Panel: PC3 cells transiently transfected with pcDNA3.1 or pcDNA3.1DNPERK (insert) as described in Materials and Methods were treated as mentioned in A and cell death was determined using trypan blue dye exclusion assay (lower panel) and Western blotting was performed. S.D. (n=3). * Significantly lower vs pcDNA3.1 transfected cells (P <0.01).

Figure 5: Autophagy induced by the combination of mda-7/IL-24 and BI-97C1 progresses to apoptosis by caspase-dependent cleavage of Beclin-1: A) Upper panel: PC3 cells were infected with LC3-RFP and MAP-4-GFP. After incubation 12 h, the cells were infected with 50 pfu/cell of Ad.5/3-vec or Ad.5/3-mda-7 for 6 h and then exposed to BI-97C1. Localization of LC3 (red) and the cytoskeleton (green) were examined by confocal laser microscopy (magnification x 100). Lower panel: To quantitate endogenous LC3II the indicated cells were treated as described above in upper panel and LC3 expression was analyzed by Western blotting. B) Wild type and Bax-/-Bak-/- double knockout MEFs were transiently transfected with pcDNA3.1H-ras vector. After 24 h cells were treated as in A and cell death was measured by Trypan blue dye exclusion assay. S.D. (n = 3). C) Localization of LC3 (red) and cytoskeleton (green) were examined as described in A after PC3 cells were infected with 50 pfu/cell of Ad.5/3-vec or Ad.5/3-mda-7 for 6 h and then exposed to 520 nM of BI-97C1 for indicated times. Apoptosis was determined by TUNEL staining. D) PC3 cells were transfected with the indicated siRNAs. Following 24 h incubation, cells were treated as described in A. Cell death (right panel) was determined by trypan blue dye exclusion. S.D. (n=3). Western blotting was performed with indicated antibodies (left panel). E) PC3 cells were treated as described in A for 24 h. Cell lysates were immunoprecipitated using anti-Beclin-1 antibody and then immunoblotted with anti-
Mcl-1 antibody or vice versa. **F)** Left panel: PC3 cells were treated as described in **A** and Western blotting was performed. Right panel: PC3 cells were treated as described in **A** in the presence or absence of 25 µM of z-VAD-FMK. Cell death was measured by trypan blue dye exclusion. S.D. (n=3). **G)** Temporal induction of PARP cleavage and LC3-II expression by Western blotting following infection with 50 pfu/cell of Ad.5/3- md7 and BI-97C1.

**Figure 6:** The combination regimen of *mda-7/IL-24* and BI-97C1 additively inhibits prostate tumor growth in athymic and immunocompetent mice, and a hypothetical model of the combination regimen:  
**A)** Athymic nude mice-bearing M2812 xenografts stably expressing luciferase in both right and left flanks were treated as indicated in the Materials and Method section. Tumor growth was visualized by bioluminescence measured by the Xenogen imaging system. **B)** At the end of the study in panel **A**, tumors were harvested and stained for Ki-67, MDA-7/IL-24 and TUNEL. **C)** Left Panel: The prostatic region of male Hi-Myc mice were sonoporated for 10 min following tail-vein injection of the indicated complement-treated microbubble/Ad complexes and treated as described in the Materials and Method section. At the end of the experiment the mice were sacrificed and the prostates were harvested, weighed and photographed. Right panel: Hi-Myc mice were treated as described above and proliferative index was measured by Ki-67 staining of paraffin-embedded sections of prostate from treated mice as described in panel **D**, S.D. (n=7) **D)** Hi-Myc mice were treated as described in panel **C**, paraffin-embedded sections obtained from the prostate were analyzed by immunohistochemistry using ant-Ki-67 and anti-MDA-7/IL-24 antibodies. Apoptosis in the prostate sections was detected by TUNEL assay. **E)** Hypothetical model of action for the combination regimen of *mda-7/IL-24* and BI-97C1 (Sabutoclax) in prostate cancer cells.
Figure 1
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Fig. S1. Binding studies to Mcl-1. (A) Chemical structure of BI-97C1 (also called Sabutoclax), BI-79D10, and ABT-737. Compounds BI-79D10 and BI-97C1 were synthesized as described previously by Wei et al. (1) and Wei et al. (2), respectively. Compound purity for BI-97C1 (99%) was verified by HPLC. ABT-737 was purchased from SYNthesis Med Chem. (B) Docked structure of BI-97C1 into the BH3 binding groove of Mcl-1. The structure of BI-97C1 is represented in ball and stick and colored in atom types. The surface of Mcl-1 (PDB code: 2NL9) is calculated via MOLCAD (Tripos) and colored according to cavity depth: blue, shallow; yellow, deep. (C) Superimposition of HSQC spectra of Mcl-1 (0.1 mM) recorded in the absence (red) and presence (blue) of ABT737 (0.2 mM). The spectra are nearly identical, indicating no significant binding to Mcl-1 by ABT-737 under these experimental conditions. (D) Superimposition of HSQC spectra of
Mcl-1 (0.1 mM) in the absence (red) and presence (green) of BI-97C1 (0.2 mM). Several changes can be observed that can be mapped to the BH3 binding groove of Mcl-1.


Fig. S2. Mcl-1 but not Bcl-xL plays a critical role in mda-7/IL-24 mediated cytotoxicity in PC.

(A) Left: A stable clone (Du-Bcl-xL) of DU-145 cells ectopically expressing Bcl-xL (Inset) cells were transfected with control or Mcl-1 siRNA and 48 h later whole cell lysates were subjected to Western blotting analysis to quantify Mcl-1 expression. Right: After transfection with control siRNA or Mcl-1 siRNA, cells were infected with 100 pfu/cell of Ad.vec or Ad.mda-7 for 48 h, after which the percentage of apoptosis was determined by flow cytometry after AnnexinV-FITC/
PI staining. Results are the mean + SD (n = 3) from three independent studies. (B) Left: Stable clone of DU-145 cells ectopically expressing Mcl-1 (Du-Mcl-1-8) (Inset) were transfected with the control or Bcl-xL siRNA and 48 h later whole cell lysates were subjected to Western blotting analysis to quantify Bcl-xL expression. Right: 48 h after transfection with control siRNA or Bcl-xL siRNA, cells were infected with the 100 pfu/cell of Ad.vec or Ad.mda-7 for an additional 48 h, and the percentage of apoptosis was determined by flow cytometry after AnnexinV-FITC/PI staining. Results are the mean + SD (n = 3) from three independent studies.
Fig. S3. BI-97C1 but not ABT-737 cooperates with mda-7/IL-24 to show enhanced cytotoxic effect in PC. (A) M2182 cells were infected with 50 pfu/cell of Ad.5/3-vec or Ad.5/3-mda-7 for 6h, after which the cells were exposed to 1 µM of ABT-737 or BI-97C1 (also called Sabutoclax) and cell viability was measured by MTT assay as described in Materials and Methods. Bar, SD (n = 3). (B) M2182 cells were treated as mentioned in A and the lysates were collected to perform Western blotting with indicated antibodies. (C) Wild-type and Bax/Bak-negative MEF (DKO) cells were infected with Ad.5-vec or Ad.5-mda-7 for 6 h and then treated with indicated concentrations of BI-97C1 for 48 h. Western blotting was performed with the indicated antibodies.
Fig. S4. Tolerance level of BI-97C1 (also called Sabutoclax) in athymic nude mice (NCRnu/nu). Six- to 8-wk-old female athymic nude mice were acquired from Harlan Laboratories. Groups of three female mice were treated on d 0, 2, 6, 8, 10, and 13 as indicated by arrows. i.v. or i.p. doses of vehicle (CT) or BI-97C1 were administered at concentrations shown. Vehicle consisted of 84% PBS/8% Tween-20/8% DMSO. Data reflects survival.
### Table S1. Blood chemistry data after i.p. doses of vehicle (CT) or Bi-97C1 administered at the concentrations shown

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Vehicle consisted of 81% PBS/19% Tween-20/8% DMSO. n = number of injections after which samples analyzed. Samples were analyzed according to manufacturer specifications using the VetScan Comprehensive Diagnostic Profile on a VetScan HMII (Abaxis). ALT, albumin; ALA, alanine aminotransferase; ALKP, alkaline phosphatase; AMYL, amylase; BUN, urea nitrogen; CA, calcium; CREA, creatinine; GLOB, globulin; GLU, glucose; PHOS, phosphorus; TBIL, total bilirubin; TP, total protein.

### Table S2. Blood chemistry data after i.v. doses of vehicle (CT) or Bi-97C1 administered at the concentrations shown

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Vehicle consisted of 84% PBS/18% Tween-20/8% DMSO. All samples analyzed after 14 and 26 doses. Samples were analyzed according to manufacturer specifications using the VetScan Comprehensive Diagnostic Profile on a VetScan HMII (Abaxis). ALT, albumin; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AMYL, amylase; BUN, urea nitrogen; CA, calcium; CREA, creatinine; GLOB, globulin; GLU, glucose; PHOS, phosphorus; TBIL, total bilirubin; TP, total protein.
Chapter 3

Using a new serotype chimera Cancer Terminator Virus (Ad.5/3-CTV) to improve the efficiency and specificity of prostate cancer gene transfer and therapy
Abstract

Gene therapy using Conditionally Replication-Competent Adenoviruses (CRCAs) is being examined as a potential strategy for treating prostate cancer (PC). We have earlier successfully constructed and evaluated a CRCA, termed Cancer terminator Virus (CTV), in which the adenoviral replication is controlled by the cancer selective promoter of the Progression Elevated Gene-3 (PEG-3) and which simultaneously expresses melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24) from the E3 region of the adenovirus. The CTV was constructed on serotype 5 background (Ad.5-CTV) and thus its infectivity is dependent on Coxsackie-adenovirus Receptors (CARs). Many tumor types including PC show a downregulation in CAR, thereby limiting therapeutic gene transduction. Using serotype chimerism, we generated a new CTV (Ad.5/3-CTV) in which the Ad.5 fiber knob is replaced by Ad.3 fiber knob thus facilitating infection via Ad.3 receptors in a CAR-independent manner. The current study evaluated Ad.5/3-CTV, in comparison to Ad.5-CTV, in low CAR human PC, demonstrating higher efficacy in inhibiting cell viability in vitro as well as inhibiting tumor growth in nude mice xenograft model and spontaneously induced PC in Hi-myc transgenic mice in vivo. Ad.5/3-CTV exerted a marked anti-tumor ‘bystander’ effect in vivo thus establishing its therapeutic utility. Considering the fact that non-replicating Ad.5-mda-7 showed significant responses in a Phase I clinical trial for solid tumors, Ad.5/3-CTV might exert significantly enhanced therapeutic benefit and clinical efficacy.
Introduction

Prostate Cancer (PC) is the most frequently diagnosed cancer and is the second leading cause of cancer death among men in the US (1). It is estimated that 217,730 new PC cases were diagnosed in 2010 alone. Patients with localized disease may be treated with surgery or radiation, whereas the treatment for patients with metastatic disease is purely palliative. Current therapies include hormonal therapy, radiotherapy and cytotoxic chemotherapeutic agents (2). These therapies are inefficient on the overall survival of patients. Therefore a novel, more efficient and innovative treatment is needed, and genetic therapies represent promising approaches for the treatment of this neoplasm.

Using subtraction hybridization combined with induction of cancer cell terminal differentiation, our laboratory cloned melanoma differentiation-associated gene-7/interleukin-24 (mda-7/IL-24) (3), a novel member of the IL-10–related cytokine gene family (4). Subsequent studies documented that mda-7/IL-24 has almost ubiquitous antitumor properties in vitro and in vivo, leading to its entry into the clinic, where its safety and clinical efficacy, when administered by adenovirus (Ad.mda-7; INGN 241), was observed in a phase I clinical trial in humans with advanced carcinomas and melanomas (5, 6). Its mode of action involves preferential induction of apoptosis in cancer cells while exerting no discernible toxic effects toward normal cells (7, 8) by eliciting potent “antitumor bystander activity” as a consequence of autocrine and paracrine secretion (8-10).

Since PC is commonly a relatively slow-growing disease, it may require repeated gene therapy treatments, with single or multiple genes, over the lifespan of the patient. Conditionally replication-competent adenoviruses (CRCAs) provide a valuable potential tool for gene therapy. Using subtraction hybridization we cloned a novel rodent gene, PEG-3, in the context of tumor
progression in transformed rat embryo cells (11). PEG-3: (i) displays elevated expression as a function of oncogenic transformation (by diverse oncogenes) (11, 12); (ii) induces an aggressive cancer phenotype without promoting transformation when expressed in normal cells (13, 14); and (iii) gene promoter (PEG-Prom) has been isolated and shown to display elevated expression in both rodent and human tumors (including PC), with negligible expression in normal cells (including human prostate epithelium) (15-17). Considering the cancer-specific expression aspects of the PEG-Prom, we constructed a bipartite serotype 5 CRCA (CRCA, called a cancer terminator virus – Ad.5-CTV) in which the expression of E1A and E1B genes of Ad, necessary for replication, is regulated by the PEG-Prom (18). This novel Ad.5-CTV also expresses mda-7/IL-24 from the E3 region (Ad.PEG-E1A-mda-7). The ability of Ad.5-CTV to infect and express MDA-7/IL-24 in prostate cancer cells depends on the presence of Coxsackie-Adenovirus Receptors (CAR) on their surface. Ad.5-CTV is capable of efficiently infecting high CAR receptor cells, such as DU-145, whereas it fails to efficiently infect and consequently efficiently express MDA-7/IL-24 in low CAR receptor cells, such as PC-3. An approach to circumvent the low efficiency of Ad.5 infection of tumor cells involves ‘tropism modification’ in which virus capsid proteins that normally associate with CAR are modified, permitting CAR-independent infectivity of tumor cells. Studies using various tumor cell types have shown that inclusion of the infective type 3 Ad sequence within the Ad type 5 virus knob (Ad.5/3 recombinant virus) promotes viral infectivity in tumor cells showing reduced or no CAR expression (19-21). It should be noted that Ad.5/3 also infects high CAR-expressing tumor cells with equal efficacy when compared with Ad.5, thereby providing an expanded scope of utility for Ad.5/3, in both low and high CAR-expressing tumor cells. This study explored the efficiency of Ad.5/3-CTV infection of low CAR tumor cells by using a recombinant Cancer Terminator Virus Ad.5/3-CTV
in which the virus capsid proteins that normally associate with CAR are modified, permitting CAR-independent infectivity of tumor cells. This study explored the efficacy of a tropism-modified CRCA cancer gene therapy approach for eradicating low CAR prostate cancer cells. We showed that in low CAR PC3 cells Ad.5/3-CTV is more efficient than Ad.5-CTV in delivering transgene (mda-7/IL-24), infecting tumor cells, expressing MDA-7/IL-24 protein, inducing cancer-specific apoptosis, inhibiting in vivo tumor growth and exerting an antitumor ‘bystander’ effect in a nude mouse human prostate cancer xenograft and suppressed PC development in an immunocompetent transgenic mouse model of PC model.

Results

Ad.5/3-CTV enhanced mda-7/IL-24 expression and inhibition of cell viability in low CAR prostate cancer cells. The schema for construction of Ad.5/3-CTV, in which viral replication is controlled by the PEG-Prom and which also expresses mda-7/IL-24 in Ad.5/3 background, is depicted in Fig. 1 and described in details in Materials & Methods. As a control we used Ad.5-vec (replication-incompetent empty Ad.5), Ad.5/3-vec (replication-incompetent empty Ad.5/3), Ad.5-PEG-E1A, in which viral replication is controlled by the PEG-Prom in Ad.5 background, and Ad.5/3-PEG-E1A, in which viral replication is controlled by the PEG-Prom in Ad.5/3 background. We compared MDA-7/IL-24 expression upon infection of Ad.5/3-CTV and Ad.5-CTV in prostate carcinoma cells that contain low and high CAR on their surface. For this purpose, we used PC-3 cells, which have a reduced level of CAR (D value 0.32) in comparison with high CAR prostate carcinoma cells DU-145 (D value 0.92) (19). In PC-3 cells MDA-7/IL-24 expression was significantly higher upon infection with Ad.5/3-CTV, as compared to Ad.5-
CTV, whereas infection with both Ad.5/3-CTV and Ad.5-CTV in DU-145 cells resulted in the expression of similar amount of MDA-7/IL24 (Fig. 2A and 2B). Infection with none of the control Ad resulted in MDA-7/IL-24 expression. These findings indicate that infection with Ad.5/3-CTV resulted in enhanced transgene delivery in low CAR containing prostate cancer cells compared to Ad.5-CTV, and both CTVs are similarly efficient in transgene delivery in high CAR prostate cancer cells. A similar pattern was also observed when Ad replication was analyzed by E1A expression in PC3 and DU-145 cells. In DU-145 cells replicating viruses from both Ad.5 and Ad.5/3 background showed similar replication pattern, while in PC-3 cells replication of Ad.5/3 was significantly higher than that of Ad.5 (Fig. 2A and 2B). The efficacy of Ad.5/3-CTV and Ad.5-CTV in reducing the cell viability of prostate cancer cells was evaluated by MTT assay. Ad.5/3-CTV infection resulted in significantly augmented reduction in the viability of PC-3 cells compared to Ad.5-CTV infection at m.o.i. of 500 and 1000VP/ cell on day 3 and day 6 post infection (Fig. 2C). In DU-145 cells both Ad.5/3-CTV and Ad.5-CTV showed comparable efficiency in reducing viability at similar doses and time points (Fig. 2D). It should be noted that Ad.5-PEG-E1A and Ad.5/3.-PEG-E1A was as effective as Ad.5-CTV and Ad.5/3-CTV in reducing cell viability in both cell lines indicating that the profound effect of Ad replication in inhibiting cell viability might mask the in vitro growth inhibitory effects of mda-7/IL-24. In these contexts, in vivo evaluation of CTV is mandatory to confirm the ‘by-stander’ effect exerted by mda-7/IL-24.

**Ad.5/3-CTV, but not Ad.5-CTV, induces ER stress and apoptosis, and overcomes the therapy resistant PC-3-Bcl-2 tumor cells.** We next analyzed the modulation of mda-7/IL-24 downstream genes and signals that confer its tumor suppressor properties upon infection with Ad.5/3-CTV and Ad.5-CTV in PC-3 cells. Ad.5/3-CTV induces an ER stress response (unfolded protein
response) and we therefore checked the expression levels of ER-stress markers. In PC-3 cells, the levels of BiP/GRP78 and GRP94 were significantly higher upon infection with Ad.5/3-CTV when compared with Ad.5-CTV. Ad.5/3-CTV also efficiently induced apoptosis as evidenced by increased cleavage of PARP (Fig. 3A). These findings further confirm that Ad.5/3 chimerism maintains the bona fide downstream effects exerted by mda-7/IL-24.

Bcl-2 gene family plays a central role in PC and over expression of Bcl-2 gene family confers resistance to cancer therapeutics (22). In this context, we evaluated the efficacy of Ad.5/3-CTV vs Ad.5-CTV in the PC-3-Bcl-2 cells (PC3 cells that stably over express Bcl-2) which are resistant to mda-7/IL-24-mediated killing (22). As a control we used PC-3-Neo cells that are stably transfected with the empty vector. In MTT assays Ad.5-CTV did not significantly affect the cell viability of the PC-3-Bcl-2 cells, although it significantly inhibited the viability of PC-3-Neo cells (Fig. 3). However, Ad.5/3-CTV markedly inhibited viability of both PC-3-Neo and PC-3-Bcl-2 cells. These findings indicate the potential therapeutic application of Ad.5/3-CTV in prostate cancer patients frequently showing Bcl-2 over expression.

*Ad.5/3-CTV eradicates primary and inhibits distant PC-3-Bcl-2 xenografts in nude mice.* To validate if the in vitro enhanced efficacy of Ad.5/3-CTV compared to Ad.5-CTV is also translated to the in vivo situation in low CAR PC cells, subcutaneous PC-3-Bcl-2 tumor xenografts were established in the right and left flanks of athymic nude mice. After ~7 to 10 days palpable tumors of ~100 mm$^3$ developed and the mice received 9 intratumoral injections only in the left flank tumors over a 4-week period with $1 \times 10^{10}$ viral particles per 100 µL. The Ad used were Ad.5-vec, Ad.5/3-vec, Ad.5-PEG-E1A, Ad.5/3-PEG-E1A, Ad.5-CTV and Ad.5/3-CTV. No injections were administrated to right flank tumors. PC-3-Bcl-2 cells formed large, aggressive and actively proliferating tumors on both flanks in Ad.5-vec, Ad.5/3-vec, Ad.5-PEG-E1A and Ad.5-CTV
treated groups. Although Ad.5/3-PEG-E1A inhibited the growth of tumors on the left flank, it had no effect on the distant tumor on the right flank (Figure 4). In contrast, Ad.5/3-CTV produced a dramatic effect in eradicating the tumor on the left flank and profoundly inhibiting tumor growth on the right flank compared to the rest of the groups. These results show definitive evidence for enhanced therapeutic efficacy of Ad.5/3-CTV as compared to Ad.5-CTV in prostate tumor cells with reduced CAR, and highlight the potent ‘by-stander’ anticancer activity of mda-7/IL-24 that causes growth inhibition of right-sided non-injected tumors. We detected the efficacy of Ad.5/3-CTV and Ad.5-CTV in expressing mda-7/IL-24 in vivo by western blot analysis of the total protein extracts from the harvested tumors. Ad.5/3-CTV generated a stronger MDA-7/IL-24 protein in both flanks, validating the previously reported “bystander” effect of MDA-7/IL-24 (9, 10, 23), when compared to the weak expression of Ad.5-CTV on the left flank and no expression on the right flank (Figure 4E). These findings support that in low CAR prostate cancer cells, Ad.5/3-CTV can generate robust expression of MDA-7/IL-24 protein that is sufficient for inhibition of cell proliferation and angiogenesis, and exerting the ‘by-stander’ activity of MDA-7/IL-24 in the distant tumor.

**Combination treatment of Ad.5/3-CTV and BI-97C1 (Sabutoclax) potentiates inhibition of prostate tumor growth in vivo in immune competent animals.** Because PC is a relatively slow-growing disease, repeated systemic gene therapy applications in combination with anti-tumor chemotherapeutic agents over the life span of the patient may be appropriate (24). For chemotherapeutic agent we used BI-97C1 (Sabutoclax), which is a pure optical derivative of the Apogossypol (25). Apogossypol derivatives antagonize the antiapoptotic Bcl-2 family members including Bcl-2 and Mcl-1 (26). MDA-7/IL-24 induces cancer-specific apoptosis through the translational inhibition of Mcl-1(27). BI-97C1 targeting sensitizes prostate cancer cells to mda-
7/IL-24-mediated toxicity *in vitro* (28). Experiments were performed to determine if Ad.5/3-CTV in combination with BI-97C1 could inhibit prostate tumor growth *in vivo*. The used animal model in this study is the spontaneous immunocompetent transgenic mouse model of prostate cancer (the Hi-Myc mouse), in which the prostate-specific expression of human c-Myc is driven by the rat probasin promoter with two androgen response elements (ARR2/probasin promoter). Hi-Myc mouse develop prostatic intraepithelial neoplasia (mPIN) as early as 2 to 4 weeks of age and invasive adenocarcinoma of the prostate at 6 months (29). We started the treatment at 22 weeks of age. The ability to deliver Adenoviruses systemically is limited by sequestering of the virus in the liver and clearance of the virus by the immune system (30-32). To overcome those problems we employed microbubble-targeted ultrasound destruction (UTMD) (33, 34) approach in which microbubbles incorporating Adenoviruses are targeted to release the therapeutic virus at the tumor site using ultrasound. Using the ultrasound to sonicate the microbubbles creates transient nonlethal perforations in cells and other membranes (35). In this context, systemic and targeted delivery of *mda*-7/IL-24 to the prostate of Hi-Myc mouse was performed by tail vein injection of microbubble (MB) incorporating Ad.5/3-vec, Ad.5/3-PEG-E1A or Ad.5/3-CTV followed by sonoporation in the prostatic area (33). A total of 8 tail vein injections of each group were administered over a 4-week period. BI-97C1 was administered intraperitoneally (i.p.) in each group at 3mg/kg thrice a week throughout the study. The prostates of Hi-myc mice were sectioned and stained for MDA-7/IL-24 and Ki-67, proliferation marker, and apoptosis induction was analyzed by TUNEL assay. MDA-7/IL-24 expression was accompanied by increased TUNEL positive cells and decreased Ki-67 positive cells in the Ad.5/3-CTV and BI-97C1 treated group compared to the control groups (Figure 5). Although Ad.5/3-CTV alone induced significant apoptosis it was markedly augmented in combination with BI-97C1.
Discussion

Since prostate cancer is commonly a relatively slow-growing disease, it may be necessary to use repeated gene therapy approaches, with single or multiple genes, over the lifespan of the patient (36). In these contexts, the use of conditionally replicating adenoviruses to administer therapeutic gene or gene products in prostate tumor cells represents a viable treatment option (37, 38). A major challenge for effective gene therapy using conditionally replicating Ads is the ability to specifically deliver nucleic acids directly into diseased tissue. Progress in gene therapy has been hampered by concerns over the safety and practicality of viral vectors, particularly for intravenous delivery, and the inefficiency of currently available non-viral transfection techniques (36). Recombinant Ads are one of the most common gene transfer vectors utilized in human clinical trials, but systemic administration of this virus is thwarted by host innate and adaptive antiviral immune responses which can limit and/or preclude repetitive treatment regiments (39). The advantage and superiority of our gene therapy approach is because the used therapeutic gene is a novel IL-10-family member cytokine; mda-7/IL-24. Early phase clinical studies suggest that mda-7/IL-24 may be an ideal agent for gene therapy of primary and metastatic melanoma (6). It is capable of selectively targeting cancer cells in vitro and in vivo for apoptosis or toxic autophagy, whereas in diverse normal cells it does not show cell toxicity (5-8, 40, 41). Additionally, the secreted MDA-7/IL-24 exhibits a potent “bystander” anti-tumor effect on cancer (9, 10, 23, 42).

The infectivity of Adenoviruses and their limitation of infecting low CAR on different prostate cancer cells have been previously analyzed (43, 44). DU-145 has high CAR, while PC-3 has lower cell surface CAR, which makes it relatively resistant to adenovirus infection. To overcome
the viral resistance of PC-3, it has been genetically engineered to increase 35% of CAR-positive cells to 86% CAR-positive cells (44). Consequently, infection with a recombinant Ad.5 CMV-p21 virus showed relatively higher levels of p21 protein in virus-infected cells resulting in apoptosis (44). These studies highlight the importance of relative levels of CAR as a major determinant of Ad-based gene therapy approaches. Further, in our study, we constructed a bipartite Ad where the selective tumor cytotoxic gene mda-7/IL-24 driven by a CMV promoter and E1A is driven by a minimal active region of the promoter of progression elevated gene-3 (PEG-3), that restricts viral replication to cancer cells with limited activity in normal cells (18, 45). Consequently, the PEG-Promoter may provide a generic tool for ensuring cancer selectivity. Ad.5/3-CTV contains a chimeric fiber with the knob domain of human Ad.3 in the Ad.5 capsid (Ad.5/3), which allows viral infectivity regardless of the CAR expression status of tumor cells thus permitting efficient viral infectivity of low and high CAR expressing tumor cells (46, 47) , The finding that Ad.5/3-CTV eradicated not only primary injected tumors, but also distant non-injected tumors derived from a resistant prostate cancer cell line in a nude mouse xenograft model support the anticancer potency of this cancer therapeutic virus. The Ad.5/3-CTV is capable of infecting cancer cells regardless of their cell surface CAR status, which makes it more useful and efficient than Ad.5-CTV that fails to efficiently deliver the therapeutic gene in low CAR receptor prostate cancer cells.

Systematic delivery of adenoviruses is challenged by sequestering of adenoviruses in the liver limiting virus delivery to disseminated tumors (32) and neutralization of viruses by the immune system (32). To prevent trapping of CTV in the liver and elimination of viruses by the immune system of the Hi-Myc PC transgenic mouse we have developed a novel approach in which
tropism modified \textit{CTV} is incorporated in a perfluorocarbon microbubble that is administered systemically to be released in the prostate area through ultrasound, a UTMD approach (33).

In summary, gene therapy for prostate cancer is a promising field; the prostate gland is not vital for survival and is accessible by ultrasound; Ad.5/3-\textit{CTV} can be either injected directly into the primary tumor or delivered through UTMD of the microbubbles incorporated viruses and therefore express MDA-7/IL-24; the replication of this virus and the expression of the therapeutic genes can be specified in cancer cells but not normal cells through its cancer-specific and tissue-specific PEG promoter; and disease progression can be monitored by measuring prostate-specific antigen (PSA) (48, 49). In these contexts, the use of Ad.5/3-\textit{CTV} to administer the therapeutic and cytotoxic \textit{mda}-7/IL24 selectively induce cytolysis in prostate tumor cells represent viable treatment options (38, 50).

\textbf{Materials and Methods}

\textit{Cell lines, culture conditions, and viability assays}. DU-145 and PC-3 prostate cancer cells were obtained from the American Type Culture Collection and cultured as described (22). The cell viability was determined by standard 3-(4, 5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assays (22).

\textit{Construction of Ad.5/3-\textit{CTV}}. The genome of Ad5/3.PEG-E1.\textit{mda}-7 was generated in three consecutive steps (Fig. 1). 1) Homologous recombination of pAd5/3 genomic plasmid with pShuttleE3 plasmid containing the \textit{mda}-7/IL-24 expression cassette and kanamycin selection results in pAd5/3.E3-\textit{mda}-7 genome. 2) pAd5/3.E3-\textit{mda}-7 is cut with \textit{Swa} I to excise the
kanamycin resistance gene. 3) The resultant pAd5/3.E3-mda-7 plasmid is recombined with pShuttleE1 plasmid containing E1A-1B genes under control of PEG3 promoter resulting in Ad5/3.PEG-E1.mda-7 genomic plasmid. This plasmid is digested with Pac I to release viral ITRs and transfected in A549 cells to rescue the CRCA Ad.5/3-CTV.

**Preparation of whole-cell lysates and Western blot analyses.** Preparation of whole-cell lysates and Western blot analyses were performed as previously described (18, 33). The primary antibodies used were anti-MDA-7/IL-24 (1:1,000; mouse monoclonal; Gen Hunter Corporation), anti-EF1α (1:1,000; mouse monoclonal; Millipore), anti-Mcl-1 (1:500; mouse monoclonal; santa cruz), anti-BiP/Grp78 (1:500; rabbit monoclonal; santa cruz), anti-GRP94 (1:1000; rabbit monoclonal; sigma), anti-PARP (1:1000; rabbit monoclonal; cell signalling).

**Human prostate cancer xenografts in athymic nude mice.** PC-3-Bcl-2 cells (2× 10^6) were injected s.c. in 100 µL of 1:1 PBS and DBmatrigel in left and right flanks of male athymic nude mice (NCRnu/nu, 6-8 weeks old, ~20 g body weight) (18, 19). After establishment of visible tumors of ~100-mm³, requiring ~7-10 days, intratumoral injections of different adenoviruses were given only to the tumors on the left flank at a dose of 1× 10^{10} viral particles in 100 µl. The injections were given twice a week for four weeks. A minimum of five animals was used per experimental point. Tumor volume was calculated using the formula: \( \pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2 \). At the end of the experiment, the animals were sacrificed, and the tumors were removed and weighed.

**Hi-Myc mice and animal husbandry protocol.** The VCU Institutional Animal Care and Use Committee approved the experimental protocol used in this study and the animals were cared for in accordance with institutional guidelines. Hi-Myc transgenic mice, in which the prostate-
specific expression of human c-Myc is driven by the rat probasin promoter with two androgen response elements (ARR2/probasin promoter) were used in this study (29). Mice were obtained from the Mouse Repository of the National Cancer Institute Mouse Models of Human Cancer Consortium at NCI Frederick, MD, USA. Mouse-tail DNA was isolated using the DNeasy Blood & Tissue Kit from QIAGEN (Valencia, CA) and subjected to a PCR-based screening assay for genotyping. For genotyping Hi-MYC mice, the upstream primer (located within the ARR2-PB promoter), 5’-AAACATGATGACTACCAAGCTTG GC-3’ and the downstream primer (within the MYC cDNA sequence) 5’-ATGATAGCATCTTGTTCTAGTCTTTTTCTTAATAGGG-3’ were used to generate a PCR product of 177 base pairs.

Preparation of microbubbles (MBs), ultrasound (US) platform, ultrasound-targeted microbubble destruction (UTMD) and BI-97C1. Preparation of MBs followed by UTMD for delivery of mda-7/IL-24 expressing Ads has been described previously (33). Targeson (Targeson) custom synthesis US contrast agent (perfluorocarbon MBs, encapsulated by a lipid monolayer and poly(ethyleneglycol) stabilizer) were obtained. MBs were reconstituted in the presence or absence of 1 ml of $1 \times 10^{11}$ viral particles of indicated Ads and unenclosed surface-associated Ads were treated with complement as previously described (34). For in vivo experiments US exposure was achieved with a Micro-Maxx SonoSite (SonoSite) US machine equipped with the transducer L25 set at 0.7 Mechanical Index, 1.8 MPa for 10 minutes. Mice were sedated in an IMPAQ6 anesthesia apparatus (VetEquip, Pleasanton, CA) that was saturated with 3–5% isofluorane and 10–15% oxygen with the aid of a precision vaporizer to deliver the appropriate amount of anesthetic and to induce anesthesia. For microbubble/Adenovirus injection a 27-gauge needle with a heparin lock was placed within a lateral tail vein for administration of contrast material. The mice received injections of 100 µl of MBs with Ads
through the tail vein 8 times in the span of 4 weeks. Ultrasound (sonoporation) was performed with a SonoSite scanner (SonoSite) equipped with the transducer L25 set at 0.7 Mechanical Index, 1.8 MPa for 10 min in the ventral side of mice in the prostatic area. BI-97C1 was administered intraperitoneally at a dose level of 3 mg/kg thrice a week for the duration of the study (total 12 injections). Compounds dissolved in 500 µL of solvent (ethanol/Cremophor EL/saline = 10:10:80) were injected intraperitoneally. At the end of the experiment, the Hi-Myc mice were sacrificed and the prostate was dissected. The harvested prostate was preserved in neutral buffered formalin at 4°C before embedding in paraffin for immunohistochemical analysis.

**Immunohistochemical staining.** For immunohistochemical (IHC) analysis, formalin-fixed and paraffin-embedded specimens were sectioned 3–4-µm thick. Sections were deparaffinized, re-hydrated and then quenched in 3% H₂O₂ for 20 min. Sections were washed with PBS and blocked in PBS containing 1% BSA for 20 min at 37°C. Monoclonal anti-MDA-7/IL-24 (1:200) was incubated for 3 hr at room temperature and then washed 3X in PBS. Sections were incubated with an avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) and then washed 2X in PBS. The immunoreactivity was determined using diaminobenzidine (DAB) as the final chromogen. Finally, sections were counterstained with Meyer’s Hematoxylin, dehydrated through a sequence of increasing concentration of alcohol, cleared in xylene and mounted with epoxydic medium. Sections were also processed for hematoxylin and eosin (H&E) staining.

**Determination of apoptotic cells by TUNEL assay.** For TUNEL assays, we used the DeadEnd Colorimetric TUNEL Assay kit (Promega, Madison, WI) performed according to the
manufacturer’s instructions. Briefly, paraffin-embedded slides were deparaffinized and rehydrated. Pre-equilibrated slides were labeled with a labeling DNA-strand break solution containing a biotinylated nucleotide mix (60 min at 37°C). After several washes in 2X SSC and PBS, slides were blocked with hydrogen peroxide (3–5 min at room temperature). After several washes in PBS, the slides were mounted with mounting solution with DAPI. Apoptotic cells on the slides were observed under an Olympus epifluorescence microscope (X10 magnification; Olympus, Center Valley, PA) in randomly chosen fields. For detection of apoptosis in a time-dependent manner in vitro, PC3 cells were grown in microscopic slide culture chambers (BD Bioscience) and cells were treated with Ad.5/3-mda-7 and BI-97C1 after which the cells were fixed with 4% formaldehyde at the indicated time and TUNEL assays were performed as per the manufacturer’s instruction using an Olympus epifluorescence microscope (X10 magnification; Olympus, Center Valley, PA)

Statistical analysis. Statistical analysis was done using student t test. P < 0.05 was considered significant.

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References


Figure Legends:

Figure 1. Generation of tropism-modified cancer terminator virus (Ad.5/3-CTV). Schematic representation showing the construction of tropism modified cancer terminator virus for delivery of mda-7/IL-24. The detailed procedure has been described in material and methods.

Figure 2. Ad.5/3-CTV enhanced mda-7/IL-24 expression and inhibition of cell viability in low CAR prostate cancer cells. a) DU-145 and PC-3 cells were infected with the indicated vp/cell of Ad.5-vec, Ad.5-PEG-E1A, Ad.5-CTV, Ad.5/3-vec, Ad.5/3-PEG-E1A, and Ad.5/3-CTV for 48 hr and total proteins were isolated. The expression of MDA-7/IL-24, E1A and EF-1α (as a loading control) proteins were analyzed by Western blot analyses. b) Cell viability using the MTT assay was quantified after 3 days and 6 days with the indicated doses of vp/cell of Ad.5-CTV, Ad.5/3-CTV and their respective controls. Results are the mean ± S.D (n=3).

Figure 3. Ad.5/3-CTV, but not Ad.5-CTV, induces ER stress and apoptosis, and overcomes the therapy resistant PC-3-Bcl-2 tumor cells. a) Changes in BiP/GRP78, GRP94 and activation of PARP were detected by Western blot analysis after 2 days of treating PC-3 cells with the indicated Adenoviruses. b+c) Cell viability using the MTT assay was quantified after 3 days and 6 days with the indicated doses of vp/cell of Ad.5-CTV, Ad.5/3-CTV and their respective controls. Results are the mean ± S.D (n=3).
Figure 4. Ad.5/3-CTV eradicates primary and inhibits distant PC-3-Bcl-2 xenografts in nude mice. Tumor xenografts from PC-3-Bcl-2 cells were established in athymic nude mice in both right and left flanks; and only tumors on the left side were injected with the indicated adenoviruses over a 4 week period (total of nine injections). Measurements of PC-3 xenograft tumor volumes of a) left and b) right flanks; points, average (with a minimum of five mice in each group); bars, ± S.D. Inset contains a photograph of the animals of each representative group. c) Photograph of the PC-3-Bcl-2 xenograft tumor at the end of the study. D) Measurement of tumor weight at the end of the study; columns, mean (with at least five mice in each group); bars, ± S.D. E) Western blot analysis of protein extracts from representative PC-3-Bcl-2 tumor samples treated Ad.5-vec, Ad.5/3-vec, Ad.5-PEG-E1A, Ad.5/3-PEG-E1A, Ad.5-CTV, Ad.5/3-CTV. The immunoblot was reacted with anti-MDA-7/IL-24.

Figure 5: Combination treatment of Ad.5/3-CTV and BI-97C1 potentiates inhibition of prostate tumor growth in vivo in immune competent animals. Hi-Myc mice were treated as described in panel C and at the end of the experiment the mice were sacrificed and the prostates were collected. The paraffin-embedded sections obtained from the prostate and immunohistochemistry was performed to measure systemic transgene delivery by staining with anti-MDA-7/IL-24. Apoptosis in the prostate section was detected by TUNEL assay. Nuclei were visualized with DAPI. Wild type mice served as a control for these experiments.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Vita

Belal Mohammed Azab was born in Timisoara, Romania on March 25, 1981. He attended elementary school in Salt/ Jordan, middle and high school in Amman/ Jordan and graduated high school in 1999. Belal attended the Jordan University of Science and Technology in Irbid, Jordan, where his father worked, and he completed his undergraduate degree in Biotechnology and Genetic Engineering in 2003. Since 2006, Belal has been at Virginia Commonwealth University, Richmond, Virginia, pursuing his graduate degree in Human and Molecular Genetics. His future plans include applying for a clinical molecular genetics fellowship program.