Apogossypol derivative BI-97C1 (Sabutoclax) targeting Mcl-1 sensitizes prostate cancer cells to *mda*-7/IL-24–mediated toxicity

Rupesh Dash[a,1], Belal Azab[a,1], Bridget A. Quinn[a], Xuening Shen[a], Xiang-Yang Wang[a,c,d], Swadesh K. Das[a], Mohamed Rahmani[b], Jun Wei[e], Michael Hedvat[a,e], Paul Dent[c,d,f], Igor P. Dmitriev[g], David T. Curiel[g], Steven Grant[b,c,d], Bainan Wu[e], John L. Stebbins[e], Maurizio Pellecchia[e], John C. Reed[e], Devanand Sarkar[a,c,d], and Paul B. Fisher[a,c,d,2]

[a]Department of Human and Molecular Genetics, [b]Department of Medicine, [f]Department of Neurosurgery, [c]Virginia Commonwealth University Institute of Molecular Medicine, and [d]Virginia Commonwealth University Massey Cancer Center, School of Medicine, Virginia Commonwealth University, Richmond, VA 23298; [g]Department of Radiation Oncology, School of Medicine, Washington University, St. Louis, MO 63108; and [e]Sanford-Burnham Medical Research Institute, La Jolla, CA 92037

Edited* by Dennis A. Carson, University of California at San Diego, La Jolla, CA, and approved March 17, 2011 (received for review January 14, 2011)

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 prosurvival 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 unique 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 Ki-67 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.

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

Melanoma differentiation associated gene-7/interleukin-24 (*mda*-7/IL-24), a unique member of the IL-10-related cytokine gene family, exhibits nearly ubiquitous antitumor 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 adenovirus delivered *mda*-7/IL-24 (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 unique cytokine to inhibit angiogenesis and provoke a potent antitumor 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 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 antiapoptotic protein myeloid cell leukemia-1 (Mcl-1) (4, 7, 8).

In view of the essential roles of antiapoptotic 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 compared with Bcl-2 and Bcl-x_L (9), currently used inhibitors such as ABT-737 or its clinical counterpart, ABT-263, display limited affinity for Mcl-1 (10) (Figs. S1 and S2). Using NMR binding assays and computational docking studies, we have recently identified a series of unique 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-x_L, 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 of the properties of BI-79D10 along with superior in vitro and in vivo efficacy (11).

Because Mcl-1 is overexpressed in the majority of PCs (4), we hypothesized that suppressing Mcl-1 by treating human PC cells with BI-97C1 would sensitize them to *mda*-7/IL-24–mediated cytotoxicity. Our data suggest that treatment with the combination regimen of *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 *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.

Results

Mcl-1 Promotes Resistance to *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 *mda*-7/IL-24–mediated cytotoxicity. To facilitate delivery of *mda*-7/IL-24 to PC cells, which frequently contain reduced levels of Coxsackie Adenovirus Receptors, we used a tropism-modified chimeric 5/3 adenovirus, Ad.5/3-*mda*-7 (14). siRNA-mediated reduction of Mcl-1 significantly sensitized PC3 cells to Ad.5/3-*mda*-7–induced apoptosis even at low doses (50 pfu/cell) (Fig. 1*A*). On the other hand, treatment of PC3 cells with BI-79D10 or BI-97C1 resulted in reduced viability with an

Author contributions: X.-Y.W., D.T.C., S.G., M.P., J.C.R., and P.B.F. designed research; R.D., B.A., B.A.Q., X.S., S.K.D., M.R., J.W., M.H., I.P.D., B.W., J.L.S., and D.S. performed research; R.D., X.-Y.W., P.D., D.T.C., S.G., M.P., J.C.R., D.S., and P.B.F. analyzed data; and R.D., D.S., and P.B.F. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]R.D. and B.A. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: pbfisher@vcu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1100769108/-/DCSupplemental.

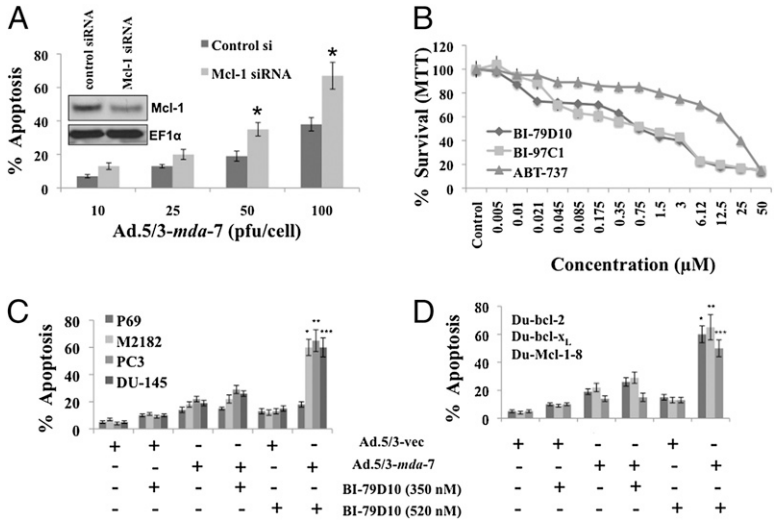

Fig. 1. Genetic or pharmacological knockdown of Mcl-1 sensitizes PC to *mda*-7/IL-24–mediated apoptosis. (*A*) PC3 cells were transiently transfected with indicated siRNA for 24 h (*Inset*) followed by infection with Ad.5/3-*mda*-7 at different pfu/cells for an additional 48 h, and the percentage of apoptotic cells was determined. SD (n = 3). *$P < 0.01$. (*B*) PC3 cells were exposed to the indicated concentrations of BI-79D10, BI-97C1, or ABT-737 for 48 h, after which the percentage of cell survival was evaluated. Bars, SD (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. SD (n = 3); *(M2182), **(PC3), and ***(DU-145) indicate $P < 0.01$ for the BI-97C1– and Ad.5/3-*mda*-7–treated groups. (*D*) *mda*-7/IL-24–resistant DU-145 cells stably expressing Bcl-x_L, Bcl-2, and Mcl-1 were treated as in *A* and apoptosis was evaluated. SD (n = 3); *(Du-bcl-2), **(Du-bcl-x_L), and ***(Du-Mcl-1-8) indicate $P < 0.01$ for the BI-97C1– and Ad.5/3-*mda*-7–treated groups.

IC_{50} value of 750 nM for both compounds (Fig. 1*B*), whereas that of ABT-737 was 12 μM.

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 sublethal 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-x_L) 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 (Fig. 1*C*). In contrast, the combination exerted little to no toxicity in normal P69 cells, indicating cancer-specific activity (Fig. 1*C*). Furthermore, the combination also showed a greater than additive induction of apoptosis in *mda*-7/IL-24–resistant DU-145 cells that stably overexpress Bcl-2, Bcl-x_L, or Mcl-1 (Du-bcl-2, Du-bcl-x_L, and Du-Mcl-1–8, respectively) (4, 15) (Fig. 1*D*). A similar enhanced induction of apoptosis was observed in these therapy-resistant PC cell lines after treatment with Ad.5/3-*mda*-7 and BI-97C1 (Fig. 2 *A* and *B*). 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 (Fig. 2*A*). Similarly, BI-97C1 potently induced apoptosis of PC3 cells with an IC_{50} of ~500 nM in combination with Ad.5/3-*mda*-7 (50 pfu/cell) (Fig. 2*B*). 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 (Fig. 2*C*). 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 Second mitochondrial-derived activator of caspase (Smac)/direct inhibitor of apoptosis protein-binding protein with low pI (DIABLO) as well as inactivation of cell proliferation signals, i.e., AKT and ERK (Fig. 2 *D–F*).

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-*mda*-7 (50 pfu/cell) and expression of Bak/Bax conformational changes (detected by immunoprecipitation using antibodies that specifically

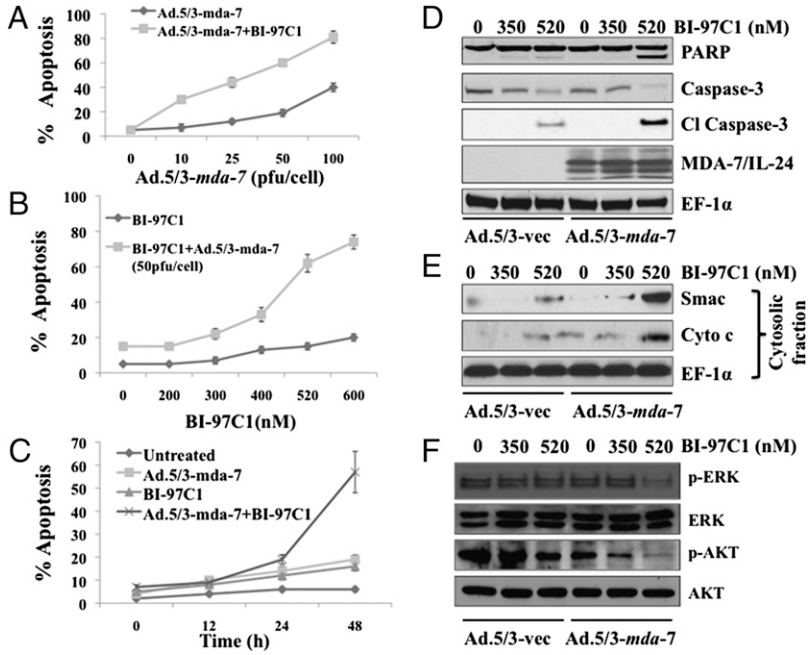

Fig. 2. BI-97C1 and *mda*-7/IL-24 cooperate to induce death in PC cells. (*A*) PC3 cells were infected with indicated doses of Ad.5/3-*mda*-7 for 6 h and then treated with DMSO or BI-97C1 for 48 h. (*B*) PC3 cells were infected with Ad.5/3-vec or Ad.5/3-*mda*-7 for 6 h and then treated with indicated 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*, the percentage of apoptosis was evaluated. Bars, SD (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 the 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.

recognize activated Bak or Bax) (16) and intracellular Bax localization were assessed. Treatment of PC3 with BI-97C1 and Ad.5/3-*mda*-7 moderately elevated expression of total Bax and Bak compared with either single agent alone (Fig. 3*A*). Treatment with the combination of BI-97C1 and Ad.5/3-*mda*-7 induced Bak or Bax conformational changes significantly greater than either agent alone (Fig. 3*B*). Similarly, combination treatment dramatically increased mitochondrial translocation of Bax (Fig. 3*B*) and Bak/Bax association (Fig. 3*C*). Transient shRNA-mediated knockdown of Bax and Bak promoted resistance to BI-97C1/*mda*-7/IL-24–mediated toxicity (Fig. 3*D*).

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 (Fig. 4*A*). When PC3 cells were transiently transfected with shRNA against NOXA or Bim, a significant degree of resistance toward BI-97C1/*mda*-7-IL-24–mediated apoptosis was observed (Fig. 4 *B* and *C*). Earlier, we reported that *mda*-7/IL-24 induces ER stress by activation of PKR-like ER-localized eIF2α kinase (PERK) (2). We observed that ER stress generated by *mda*-7/IL-24 was enhanced by combined treatment with BI-97C1 (Fig. 4*D*, *Upper*). 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 (Fig. 4*D*, *Lower*).

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 (Fig. 5*A*). Because *mda*-7/IL-24 selectively targets transformed cells, we transfected MEFs with a human pcDNA3.1H-ras construct. Human H-ras transfected $Bax^{-/-}Bak^{-/-}$ MEFs were resistant to *mda*-7/IL-24 + BI-97C1-induced cytotoxicity compared with wild-type MEFs, indicating that cell death induced by the combination is due to apoptosis, not autophagy (Fig. 5*B*). On the other hand, we also detected a slight induction of autophagy in $Bax^{-/-}Bak^{-/-}$ MEFs compared with wild-type MEFs following combination treatment (Fig. S3*C*). 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 (Fig. 5 *C* and *G*). 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 (Fig. 5*D*). This finding was supported by the observation that 24 h after combination treatment, the interaction between Mcl-1 and Beclin-1 was disrupted (Fig. 5*E*). 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 (Fig. 5*F*). When caspase-3 activity was inhibited using a pharmacological inhibitor, PC3 cells developed resistance toward the cytotoxicity induced by the combination of Ad.5/3-*mda*-7 and BI-97C1 (Fig. 5*F*).

Combination Treatment with *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 in vivo, we performed studies that used two animal models, one using immune deficient athymic nude mice xenografted with human prostate tumor cells (M2182) (11) and one using a spontaneous immunocompetent transgenic mouse model of PC (the Hi-Myc mouse) (19). Initial studies were performed in nude mice bearing

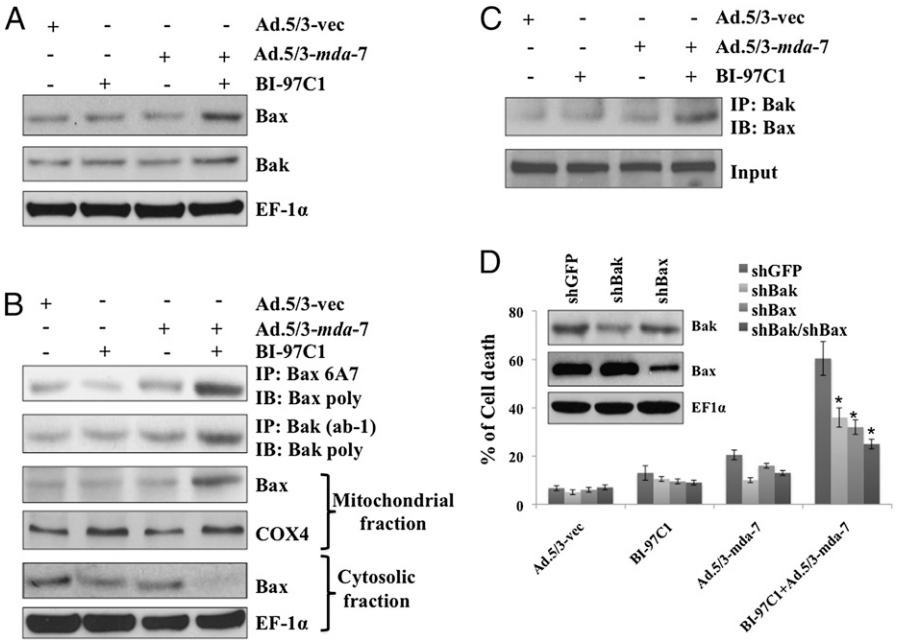

Fig. 3. Combination of *mda*-7/IL-24 and BI-97C1 results in Bak and Bax conformational change, whereas knockdown of these molecules markedly attenuates apoptosis. (*A*) PC3 cells were infected with 50 pfu/cell of Ad.5/3-vec or Ad.5/3-*mda*-7 for 6 h and then exposed to DMSO or BI-97C1 for 48 h. Protein 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 *Materials and Methods* (*Upper*). Alternatively, cytosolic and mitochondrial fractions were separated and subjected to Western blotting analysis (*Lower*). (*C*) PC3 cells were treated as mentioned in *B* and subjected to immunoprecipitation using anti-Bak antibodies. (*D*) PC3 cells transiently transfected with shGFP, shBax, or shBak (*Inset*) for 24 h as described in *Materials and Methods* and were treated as described in *A*. Cell death was determined using Trypan blue dye exclusion. SD ($n = 3$), $^*P < 0.01$.

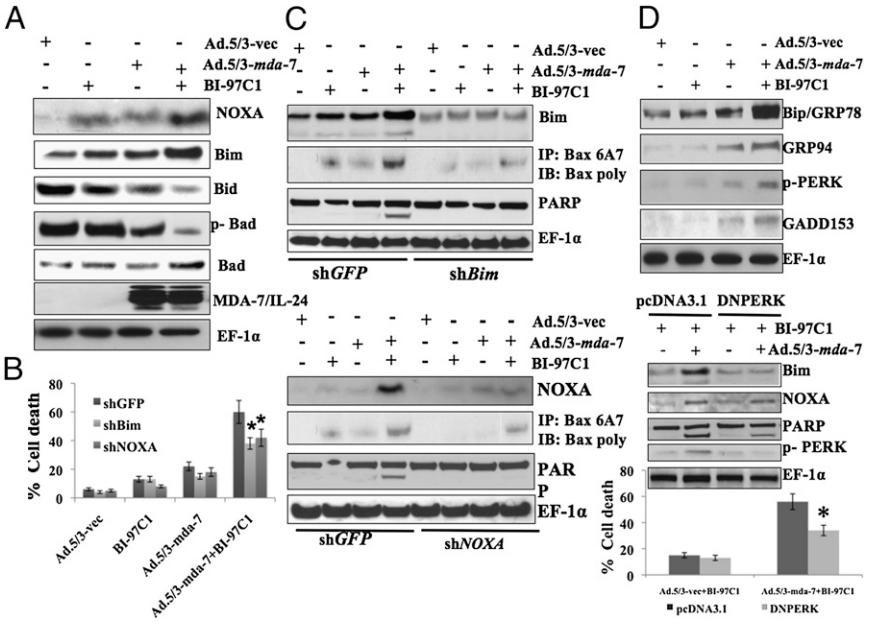

Fig. 4. Bim and NOXA play critical roles in Bax- and Bak-mediated cytotoxicity induced by *mda*-7/IL-24 and BI-97C1. (*A*) PC3 cells were infected with Ad.5/3-vec or Ad.5/3-*mda*-7 for 6 h and then treated with indicated concentrations of BI-97C1 for 48 h and Western blotting was performed. (*B*) PC3 cells transiently transfected with shGFP, shBim, or shNOXA (*Insert*) and treated as described in *A*. Cell death was determined using Trypan blue dye exclusion. SD ($n = 3$). *$P < 0.01$. (*C*) *Left* and *Right*: PC3 cells were treated as mentioned in *B*, after which cell lysates were subjected to determine Bax conformation analysis as indicated in *Materials and Methods* (*D*) *Left*: PC3 were treated as mentioned in *A* and Western blotting was performed. *Right*: PC3 cells transiently transfected with pcDNA3.1 or pcDNA3.1DNPERK (*Inset*) as described in *Materials and Methods* were treated as mentioned in *A* and cell death was determined using Trypan blue dye exclusion assay (*Lower*) and Western blotting was performed. SD ($n = 3$). *Significantly lower vs. pcDNA3.1 transfected cells ($P < 0.01$).

s.c. M2182 human prostate tumors established on both flanks that stably express luciferase (M2182-Luc). Mice were treated when tumor size was ~100 mm^3, using a suboptimal dose of 0.5×10^8 pfu/injection of Ad.5/3-vec or Ad.5/3-*mda*-7 (seven injections). BI-97C1 was injected i.p. (11) either at 1 mg/kg or 3 mg/kg 3 times per week for the 3-wk duration of the study (nine injections). Tumor growth was markedly inhibited by combination treatment compared with treatment with either agent alone (Fig. 6*A*). Combined treatment of Ad.5/3-*mda*-7 and BI-97C1 (3 mg/kg) significantly reduced Ki-67 expression and increased TUNEL signal compared with treatment with either agent alone (Fig. 6*B*). BI-97C1 was observed to be nontoxic to athymic nude mice (NCRnu/nu) when injected i.p. (up to 10 mg/kg) or when injected i.v. (25 mg/kg) (Fig. S4 and Tables S1 and S2).

Next, we tested the efficacy of Ad.5/3-*mda*-7 and BI-97C1 in a spontaneous model of PC, the Hi-Myc mouse (19). For optimal therapeutic outcome for adenocarcinoma, we began treatment at 22 wk of age. The ability to systemically deliver adenoviruses is

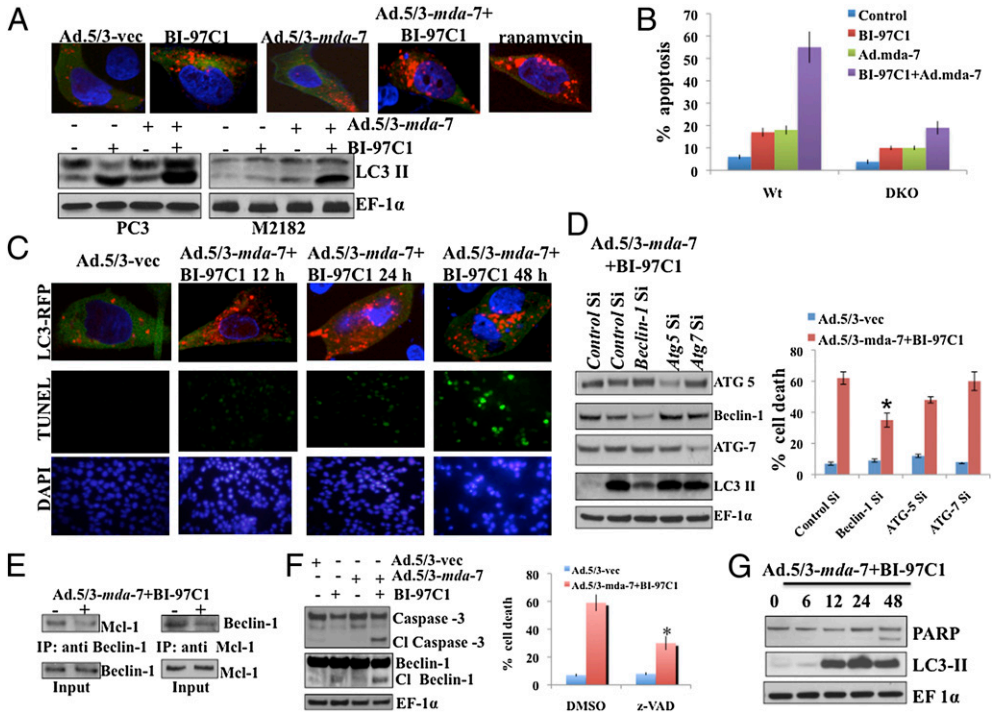

Fig. 5. Autophagy induced by the combination of *mda*-7/IL-24 and BI-97C1 progresses to apoptosis by caspase-dependent cleavage of Beclin-1. (*A*) *Top*: PC3 cells were infected with Baculovirus expressing LC3-RFP and MAP-4-GFP. After incubation 12 h, the cells were infected with 50 pfu/cell of Ad.5/3-vec 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 × 100). *Lower*: To quantitate endogenous LC3II, the indicated cells were treated as described above in the top 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. SD ($n = 3$). (*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*) was determined by Trypan blue dye exclusion. SD ($n = 3$). Western blotting was performed with indicated antibodies (*Left*). (*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*: PC3 cells were treated as described in *A* and Western blotting was performed. *Right*: 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. SD ($n = 3$). (*G*) Temporal induction of PARP cleavage and LC3-II expression by Western blotting following infection with 50 pfu/cell of Ad.5/3-*mda*-7 and BI-97C1.

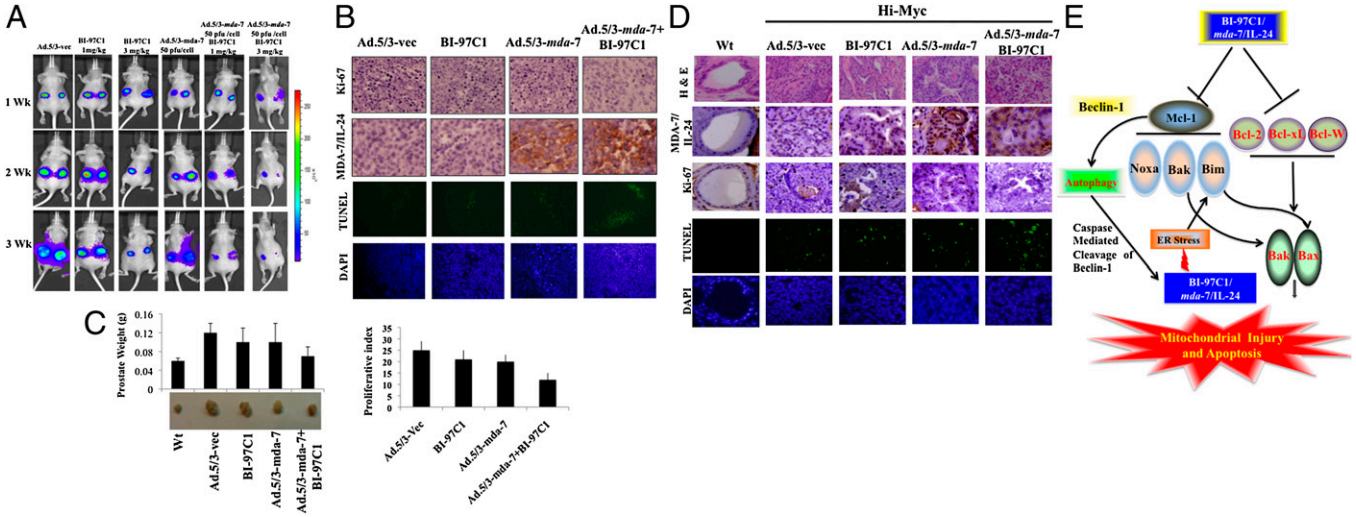

Fig. 6. The combination regimen of *mda*-7/IL-24 and BI-97C1 additively inhibits prostate tumor growth in athymic and immunocompetent mice and a hypothetical model of the combination regimen. (*A*) Athymic nude mice bearing M2812 xenografts stably expressing luciferase in both right and left flanks were treated as indicated in *Materials and Methods*. Tumor growth was visualized by bioluminescence measured by the Xenogen imaging system. (*B*) At the end of the study in *A*, tumors were harvested and stained for Ki-67, MDA-7/IL-24 and TUNEL. (*C*) *Left*: The prostatic region of male Hi-Myc mice were sonoporated for 10 min following tail-vein injection of the indicated complement-treated MB/Ad complexes and treated as described in *Materials and Methods*. At the end of the experiment, the mice were killed and the prostates were harvested, weighed, and photographed. *Right*: 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 *D*. SD (n = 7). (*D*) Hi-Myc mice were treated as described in *C*. Paraffin-embedded sections obtained from the prostate were analyzed by immunohistochemistry using ant-K_i-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 PC cells.

limited by sequestering of the virus in the liver and clearance of the virus by the immune system. We have developed a unique strategy to circumvent both of these problems that employs microbubbles (MB) treated with complement and targeted release of the therapeutic virus at the tumor site using ultrasound, an approach referred to as ultrasound-targeted microbubble-destruction (UTMD) (20, 21). In this context, systemic and targeted delivery of *mda*-7/IL-24 to the prostate of Hi-Myc mice was achieved by i.v. injection of complement-treated MB encapsulated Ad.5/3-*mda*-7 followed by sonoporation in the prostatic area, UTMD (20). A total of nine tail vein injections of MB/Ad.5/3-*mda*-7 were administered over a 4-wk period. BI-97C1 was administered i.p. at 3 mg/kg 3 times/wk 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 was significantly reduced compared with treatment with either agent alone or the vehicle (and Ad.5/3-vec) (Fig. 6*C*). Decreased Ki-67 and increased TUNEL expression demonstrate tumor growth inhibition as observed in sections of the prostates of Hi-myc mice treated with Ad.5/3-*mda*-7 and BI-97C1 compared with each single agent alone (Fig. 6 *C* and *D*).

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-x_L, 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-x_L, 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-x_L, which is resistant to Ad.*mda*-7–mediated apoptosis (15). Inhibiting Mcl-1 expression was able to overcome resistance of Du-Bcl-x_L cells to Ad.*mda*-7–induced apoptosis. In contrast, siRNA-mediated reduction of Bcl-x_L in *mda*-7/IL-24–resistant Du-Mcl-1–8 cells (4) did not sensitize them to Ad.*mda*-7–induced apoptosis (Fig. S2 *A* and *B*). These findings indicate that Mcl-1, rather than Bcl-x_L, plays a more definitive role in generating resistance to *mda*-7/IL-24–induced apoptosis. Treatment with unique BI-97C1 in nanomolar range is sufficient to sensitize *mda*-7/IL-24–resistant cells, which overexpress Bcl-2, Bcl-x_L, or Mcl-1, to *mda*-7/IL-24–dependent apoptosis. Because the Bcl-2/Bcl-x_L 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 (Fig. S3 *A* and *B*).

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 (Fig. 6*E*). Although the mechanism by which Bim promotes apoptosis remains unclear, it has been shown that up-regulation of Bim results in the release of proapoptotic 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*, apoptosis inducing factor, and Smac/DIABLO (16). Evidence for direct activation of Bax by Bim also exists (22). 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 (23). Alternatively, activation of Bak/Bax by BI-97C1 and *mda*-7/IL-24 may inhibit Bcl-2 and Bcl-x_L. It is important to note that in the present combination studies, the concentration of BI-97C1 used was 520 nM, which also inhibits Bcl-2 and Bcl-x_L. 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 Beclin-1 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 (Fig. 6*E*).

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 unique 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, and $Bax^{-/-}/Bak^{-/-}$ MEF were cultured as described (15). DU-145 clones stably overexpressing Bcl-2, Bcl-x_L, Mcl-1, and M2182-Luc were generated as described (4, 15, 11).

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) (15), 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-x_L siRNAs, or control siRNA (siControl) (Dharmacon), siATG5, siBeclin-1, or siATG-7 (Santa Cruz Biotechnology) (24, 25).

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), PARP, Bcl-x_L, total and p-ERK1/2 (Thr-202/Tyr-204), total and p-Akt (Thr-183/Tyr-185), Bid, p-Bad (Cell Signaling Technology), MDA-7/IL-24 (GenHunter), anti-EF1α (Upstate Biotechnology), 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), ABT-737 (SYNthesis Med Chem), and 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 PC Xenografts. M2182-Luc (1×10^6) 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 0.5×10^8 pfu in 100 μL. The injections were given 3 times the first week and then 2 times/wk 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 subtoxic level (11) either at 1 mg/kg or 3 mg/kg 3 times per week throughout the study (total nine injections).

Preparation of MBs, Ultrasound Platform, and UTMD. Hi-Myc (19) mice were obtained from the Mouse Repository at NCI 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. Ultrasound (sonoporation) was performed (20) in the ventral side of mice in the prostatic area. BI-97C1 was administered i.p. at a dose level of 3 mg/kg 3 times per week for the duration of the study (total nine injections).

Immunohistochemical Staining. For immunohistochemical analysis, formalin-fixed and paraffin-embedded specimens of 3- to 4-μm thickness were sectioned and stained with ant-MDA-7/IL-24 and K_i-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 ^1H-^{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 ± SD and analyzed for statistical significance using one-way ANOVA followed by Newman-Keuls test as a post hoc test. $P < 0.05$ was considered significant.

ACKNOWLEDGMENTS. The present study was supported in part by National Institutes of Health Grants R01 CA097318, R01 CA127641, and P01 CA104177; the National Foundation for Cancer Research (P.B.F.); National Institute of Health Grant R01 CA149668 (to M.P.); and Coronado Biosciences, Inc., San Diego Grant CSRA-08 (to M.P.). R.D. is a recipient of a Postdoctoral Prostate Cancer Training Award from the Department of Defense.

1. Damber JEAG, Aus G (2008) Prostate cancer. *Lancet* 371:1710–1721.
2. Dash R, et al. (2010) mda-7/IL-24: a unique member of the IL-10 gene family promoting cancer-targeted toxicity. *Cytokine Growth Factor Rev* 21:381–391.
3. Jiang H, Lin JJ, Su ZZ, Goldstein NI, Fisher PB (1995) Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression. *Oncogene* 11: 2477–2486.
4. Dash R, et al. (2010) Mechanism by which Mcl-1 regulates cancer-specific apoptosis triggered by mda-7/IL-24, an IL-10-related cytokine. *Cancer Res* 70:5034–5045.
5. Cunningham CC, et al. (2005) Clinical and local biological effects of an intratumoral injection of mda-7 (IL24; INGN 241) in patients with advanced carcinoma: a phase I study. *Mol Ther* 11:149–159.
6. Gupta P, et al. (2006) BiP/GRP78 is an intracellular target for MDA-7/IL-24 induction of cancer-specific apoptosis. *Cancer Res* 66:8182–8191.
7. Yacoub A, et al. (2010) Cisplatin enhances protein kinase R-like endoplasmic reticulum kinase- and CD95-dependent melanoma differentiation-associated gene-7/interleukin-24-induced killing in ovarian carcinoma cells. *Mol Pharmacol* 77:298–310.
8. Hamed HA, et al. (2010) Inhibition of multiple protective signaling pathways and Ad.5/3 delivery enhances mda-7/IL-24 therapy of malignant glioma. *Mol Ther* 18: 1130–1142.
9. Day CL, et al. (2005) Solution structure of prosurvival Mcl-1 and characterization of its binding by proapoptotic BH3-only ligands. *J Biol Chem* 280:4738–4744.
10. Oltersdorf T, et al. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 435:677–681.
11. Wei J, et al. (2010) BI-97C1, an optically pure Apogossypol derivative as pan-active inhibitor of antiapoptotic B-cell lymphoma/leukemia-2 (Bcl-2) family proteins. *J Med Chem* 53:4166–4176.
12. Wei J, et al. (2009) Apogossypol derivatives as pan-active inhibitors of antiapoptotic B-cell lymphoma/leukemia-2 (Bcl-2) family proteins. *J Med Chem* 52:4511–4523.
13. Wei J, et al. (2009) Apogossypol derivatives as antagonists of antiapoptotic Bcl-2 family proteins. *Mol Cancer Ther* 8:904–913, and erratum (2009) 8:2076.
14. Dash R, et al. (2010) Enhanced delivery of mda-7/IL-24 using a serotype chimeric adenovirus (Ad.5/3) improves therapeutic efficacy in low CAR prostate cancer cells. *Cancer Gene Ther* 17:447–456.
15. Lebedeva IV, et al. (2003) Bcl-2 and Bcl-x(L) differentially protect human prostate cancer cells from induction of apoptosis by melanoma differentiation associated gene-7, mda-7/IL-24. *Oncogene* 22:8758–8773.
16. Willis SN, et al. (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* 19:1294–1305.
17. She QB, et al. (2005) The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. *Cancer Cell* 8:287–297.
18. Djavaheri-Mergny M, Maiuri MC, Kroemer G (2010) Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin 1. *Oncogene* 29:1717–1719.
19. Ellwood-Yen K, et al. (2003) Myc-driven murine prostate cancer shares molecular features with human prostate tumors. *Cancer Cell* 4:223–238.
20. Greco A, et al. (2010) Eradication of therapy-resistant human prostate tumors using an ultrasound-guided site-specific cancer terminator virus delivery approach. *Mol Ther* 18:295–306.
21. Dash R, et al. (2011) Developing an effective gene therapy for prostate cancer: New technologies with potential to translate from the laboratory into the clinic. *Discov Med* 11:46–56.
22. Kuwana T, et al. (2005) BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 17:525–535.
23. Han J, Goldstein LA, Hou W, Rabinowich H (2007) Functional linkage between NOXA and Bim in mitochondrial apoptotic events. *J Biol Chem* 282:16223–16231.
24. Yacoub A, et al. (2008) Caspase-, cathepsin-, and PERK-dependent regulation of MDA-7/IL-24-induced cell killing in primary human glioma cells. *Mol Cancer Ther* 7:297–313.
25. Rahmani M, et al. (2009) The BH3-only protein Bim plays a critical role in leukemia cell death triggered by concomitant inhibition of the PI3K/Akt and MEK/ERK1/2 pathways. *Blood* 114:4507–4516.