Molecular Cancer Therapeutics

Recombinant MDA-7/IL24 Suppresses Prostate Cancer Bone Metastasis through Downregulation of the Akt/Mcl-1 Pathway

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Abstract

Prostate cancer is a principal cause of cancer-associated morbidity in men. Although 5-year survival of patients with localized prostate cancer approaches 100%, survival decreases precipitously after metastasis. Bone is the preferred site for disseminated prostate cancer cell colonization, altering the equilibrium of bone homeostasis resulting in weak and fragile bones. Currently, no curative options are available for prostate cancer bone metastasis. Melanoma differentiation associated gene-7 (MDA-7)/IL24 is a well-studied cytokine established as a therapeutic in a wide array of cancers upon delivery as a gene therapy. In this study, we explored the potential anticancer properties of MDA-7/IL24 delivered as a recombinant protein. Using bone metastasis experimental models, animals treated with recombinant MDA-7/IL24 had significantly less metastatic lesions in their femurs as compared with controls. The inhibitory effects of MDA-7/IL24 on bone metastasis resulted from prostate cancer–selective killing and inhibition of osteoclast differentiation, which is necessary for bone resorption. Gain- and loss-of-function genetic approaches document that prosurvival Akt and Mcl-1 pathways are critically important in the antibone metastatic activity of MDA-7/IL24. Our previous findings showed that MDA-7/IL24 gene therapy plus Mcl-1 inhibitors cooperate synergistically. Similarly, an Mcl-1 small-molecule inhibitor synergized with MDA-7/IL24 and induced robust antibone metastatic activity. These results expand the potential applications of MDA-7/IL24 as an anticancer molecule and demonstrate that purified recombinant protein is nontoxic in preclinical animal models and has profound inhibitory effects on bone metastasis, which can be enhanced further when combined with an Mcl-1 inhibitory small molecule. *Mol Cancer Ther; 17(9); 1951–60. ©2018 AACR.*

Introduction

Prostate cancer is one of the most common cancers affecting men worldwide with a strong propensity for bone metastases, which are refractory to conventional therapeutic approaches (1). Currently, advanced prostate cancer is incurable and results in significant disease morbidity and mortality (2). Bone metastasis begins with the dissemination of tumor cells toward bone, adherence to bone marrow cells, penetration/invasion into bone marrow to the mineralized matrix, and growth of micrometastatic lesions (3). Colonization of cancer cells in bone is regulated by a variety of factors that determine the extent to which cancer cells can engage and communicate with the bone marrow, particularly with osteoblast and osteoclast cells, which are the two major components of tumor bone modeling (4). Understanding the molecular factors influencing this multistep process and the associated signaling pathways remain critical to designing effective therapeutics to inhibit and treat bone metastasis.

Mcl-1 is a member of the Bcl-2 family of proteins that is abundantly expressed in cancer lineages including prostate cancer (5). With well-established antiapoptotic functions, increased expression of Mcl-1 is also associated with the acquisition of chemoresistance and tumor relapse (5, 6). Treatment of rabbit osteoclasts with RANKL increased expression of Mcl-1 (but not the other members of the Bcl-2 family, e.g., Bcl-2) and enhanced osteoclastic activity (bone resorption; ref. 7). Recently, more definitive evidence indicates that without affecting differentiation, Mcl-1 expression prolongs osteoclast survival and suppresses bone-resorbing activity (8). Thus, targeting Mcl-1 is beneficial from both perspectives, including direct killing of tumor cells as well as maintaining cellular/organ homeostasis, which is deregulated during bone metastasis. Among various strategies for targeting Mcl-1, either genetic or chemical, BH3 mimetics have been one of the most promising translational strategies. In this approach, small molecules that fit into the hydrophobic pocket of the antiapoptotic proteins are being developed. However, many of the compounds synthesized to date only effectively inhibit Bcl-2 and Bcl-xL, but not Mcl-1, e.g., ABT-737 and its clinical counterpart ABT-263. Using NMR binding assays and computational parameters, an Apogossypol derivative, Sabutoclax, was identified and shown to exhibit highly potent antitumor efficacy with little cytotoxicity, therefore representing a promising drug for novel apoptosis-based cancer therapies (9–14).

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Melanoma differentiation associated gene-7/Interleukin 24 (MDA-7/IL24), a member of the IL10 cytokine gene family, is now established as a broad-spectrum anticancer gene capable of inducing apoptosis or toxic autophagy selectively in transformed cells of diverse origin, including prostate cancer (15–17), without harming normal or nontransformed cells. A phase I/II clinical trial in advanced cancers has established safety and therapeutic efficacy when *mda-7/IL24* was administered intratumorally multiple times by means of a replication incompetent adenovirus, Ad.-*mda-7* (INGN 241). We also evaluated Ad.5/3-*CTV*, a tropism-modified, conditionally replication competent oncolytic adenovirus carrying *mda-7/IL24*, in comparison with Ad.5-*CTV* in low Coxsackievirus and Adenovirus Receptor (CAR) human prostate cancer cells (17, 18), demonstrating higher efficacy in suppressing *in vivo* tumor growth in a nude mouse xenograft model and in spontaneously developed prostate cancer in Hi-*myc* transgenic mice. Ad.5/3-*CTV* also exerted a marked "*bystander*" antitumor effect *in vivo* (19). In addition, Mcl-1 inhibitors [Sabutoclax (BI-97C1 and BI-97D6)] sensitized prostate cancer cells to MDA-7/IL24-induced toxicity by enhancing the stability of MDA-7/IL24, thus rationalizing MDA-7/IL24 in combination with Mcl-1 inhibitors as a potential therapeutic option for the treatment of metastatic prostate cancer.

We now document that purified MDA-7/IL24 recombinant protein inhibits the metastasis of prostate cancer cells to bone. Treatment with recombinant MDA-7/IL24 significantly reduced the occurrence of bone metastasis in an experimental *in vivo* model, and the effect was more vigorous when combined with an Mcl-1 inhibitor. *In vitro* studies suggest that MDA-7/IL24 reduced osteoclast differentiation induced by RANKL, partly through inhibition of phosphorylated-Akt and Mcl-1. Accordingly, MDA-7/IL24 protein and an Mcl-1–targeted small-molecule inhibitor hold potential as efficacious therapeutics against prostate cancer bone metastasis.

Materials and Methods

Cell lines, plasmids, and Mcl-1 inhibitor

PC3-ML, a metastatic variant of the prostate cancer cell line PC3, was grown as described previously (20). This cell line was used to produce prostate cancer–induced bone metastasis in athymic male nude mice. DU-145, PC3, RWPE-1 (immortalized normal human prostate epithelial cells), and RAW 264.7 (murine macrophage) cells were obtained from the ATCC and maintained as suggested by the vendor. ARCaP-E and ARCaP-M cell lines and specific media were purchased from Novicure Biotechnology. For osteoclast differentiation assays, the RAW 264.7 cell line was used. Immortal primary human fetal astrocytes (IM-PHFA) were developed and maintained as described earlier (21). All the cell lines from the ATCC and other vendors were purchased during 2012–2016 and authenticated by using short tandem repeat analysis. Experiments were done with early passage cells. Cells were monitored routinely for contamination including mycoplasma using a mycoplasma detection kit (Sigma-Aldrich, Inc.). Myr-Akt, DN-Akt, and Mcl-1 plasmids were from Addgene. LY294002, an inhibitor of PI3 kinase, was purchased from Sigma-Aldrich, Inc. Mcl-1 inhibitor, BI-97D6 compound, was synthesized and evaluated as described earlier (22, 23) and provided by Dr. Maurizio Pellecchia (University of California Riverside, CA). BI-97D6 inhibits the binding of BH3 peptides to Bcl-2, Bcl-xL, and Mcl-1 (22).

Purification of recombinant His-MDA-7 protein

IM-PHFA cells were infected with Ad.5-His-*mda*-7 using a standard protocol (24). Cell supernatant was mixed with Ni-NTA (a nickel-nitrilotriacetic) acid slurry to allow binding of MDA-7/IL24 to the Ni-NTA beads. Twenty-four hours after incubation, the Ni-NTA beads were collected and washed, and the purified MDA-7/IL24 protein was eluted in imidazole buffer. The protein was validated by Western blotting using anti–MDA-7 antibody (Genhunter Corporation). Biological activity was checked in appropriate cells (PC3-ML) by MTT assay (25).

MTT cell proliferation and clonal assays

Cell proliferation assays were done as described previously (25). Briefly, 2,000 cells were seeded in each well of a 96-well plate and allowed to attach overnight. Cells were treated with complete media in the presence or absence of different concentrations of MDA-7/IL24. At appropriate time points, cells were further incubated with MTT (3-(4, 5-di methyl thiazol-2-yl)-2, 5 diphenyl tetrazolium bromide) reagents. Finally, DMSO was applied to dissolve the blue salt, and the optical density was measured at 560 nm (25). For determining long-term effects, colony formation (cloning) assays were performed as described earlier (26). Briefly, 200 cells were plated and allowed to grow in the presence or absence of MDA-7/IL24 for an additional 15 days. Culture media were replaced with fresh media containing MDA-7/IL24 once a week, 2 times in total.

In vivo metastasis studies

All animal studies were approved by the Institutional Animal Care and Use Committee (Virginia Commonwealth University). For experimental bone metastasis assays, 6- to 8-week-old male athymic nude mice (purchased from Harlan) were injected with 1×10^5 PC3-ML cells stably expressing firefly luciferase gene through an intracardiac route. For determining the therapeutic activity of MDA-7/IL24, mice received an intravenous injection of recombinant protein (5 mg/kg), 1 day after implantation of cells. Animals were treated with therapeutics for a total of 6 times (2× a week for first 3 weeks). In combinatorial treatment studies, MDA-7/IL24 (5 mg/kg) and BI-97D6 (1.5 mg/kg) were delivered through tail vein and intraperitoneal route, respectively. Image of the bone region was monitored by a bioluminescence imaging (BLI) method using an IVIS imaging system (15, 19, 20).

Real-time q-PCR

Total RNA was isolated from cells with the RNA isolation Kit from Qiagen. RQ-PCR was performed using taqman probes and master mix from Applied Biosystems. Data were analyzed using the graph pad prism software.

Live–dead cell assay

Live and dead cells were observed by confocal laser microscopy (Zeiss) after staining with live/dead staining reagent (Invitrogen) as per the manufacturer's instructions. The images were analyzed by Zeiss software.

Western blotting

Standard protocols were followed for Western blotting assays (24, 27). The primary antibodies used were pAkt, Akt, pGSK3β, GSK3β, NFATc1, cyclin D1 (Cell Signaling Technology), and EF1α (Abcam). Appropriate secondary antibodies were purchased from Sigma-Aldrich, Inc.

Osteoclast formation assays

Bone marrow cells were induced for osteoclast differentiation using previously described protocols (28). Briefly, bone marrow cells were cultured in minimal essential medium (α-MEM) with 10% FBS with macrophage colony-stimulating factor (MCSF) (10 ng/mL) for 24 hours. They were subsequently treated with RANKL (100 ng/mL) for 5 days. Cultured cells were fixed and stained for tartarate-resistant acid phosphatase (TRAP). TRAP staining was performed following the specific protocol provided with the kit (Sigma-Aldrich, Inc.). Multinucleated cells, considered as differentiated osteoclasts, were counted manually under bright field microscope. TRACP enzymatic assays were done as per the manufacturer's instructions (R&D).

Statistical analyses

Statistical analyses were performed using Graph pad prism software. The Student *t* test was used to compare the mean differences between groups.

Results

Recombinant MDA-7/IL24 selectively inhibits prostate cancer cell growth

MDA-7/IL24 is established as a tumor suppressor regardless of tumor anatomic site and has been shown to promote cancer-selective antitumor activity *in vitro, in vivo* in preclinical animal models, and in clinical studies (29, 30). Adenoviral-mediated delivery of *mda-7/IL24* induces cancer-selective cytotoxic cell death without affecting survival of normal cells. However, the therapeutic properties of purified MDA-7/IL24 recombinant protein have not been evaluated in prostate cancer or in metastasis. Recombinant MDA-7/IL24 was purified using a His-based protein purification system as described in Materials and Methods (Fig. 1A). After purification, the quality of the purified protein was confirmed using anti–MDA-7 antibody by Western blotting (Fig. 1B). To confirm biological activity, we treated PC3-ML cells with MDA-7/IL24 and checked the antiproliferative and potential molecular changes by MTT assays and Western blotting analyses, respectively (Fig. 1C and D). Cell proliferation was significantly impaired following MDA-7/IL24 treatment (Fig. 1C). Significant increases were observed in the levels of p27, GRP78, and Beclin-1 (Fig. 1D), which is consistent with our previous studies where *mda-7/IL24* was delivered using an Adenovirus (25). Next, to check the long-term effect of MDA-7/IL24 on cell proliferation, colony formation (clonal) assays were performed in an assortment of prostate cancer cell lines. Results shown in Fig. 1E confirm that MDA-7/IL24 significantly reduced the proliferation of prostate cancer cells without affecting the proliferation capacity of immortalized normal primary human prostate epithelial cells (RWPE-1).

Recombinant MDA-7/IL24 decreases *in vivo* prostate cancer bone metastasis

Previous studies using viral-based delivery of MDA-7/IL24 demonstrated a strong antitumor role in prostate cancer (24). In order to evaluate the possible therapeutic role of recombinant MDA-7/IL24 to suppress prostate cancer bone metastasis, we used an experimental metastasis model. In this model, stable luciferase-expressing PC3-ML cells were injected in male athymic nude mice through an intracardiac route to produce bone metastases. The expansion, invasion, and migration of PC3-ML cells were monitored by IVIS imaging (15, 20, 23). Based on preliminary studies, which defined the duration of treatment, optimum dose of MDA-7/IL24, and the number of injections to achieve a maximum effect (Supplementary Fig. S1), animals were treated with MDA-7/IL24 for 3 weeks with a total of 6 doses at 5 mg/kg through tail vein injection. BLI followed development of metastatic lesions in bone. We observed robust bone metastasis in the

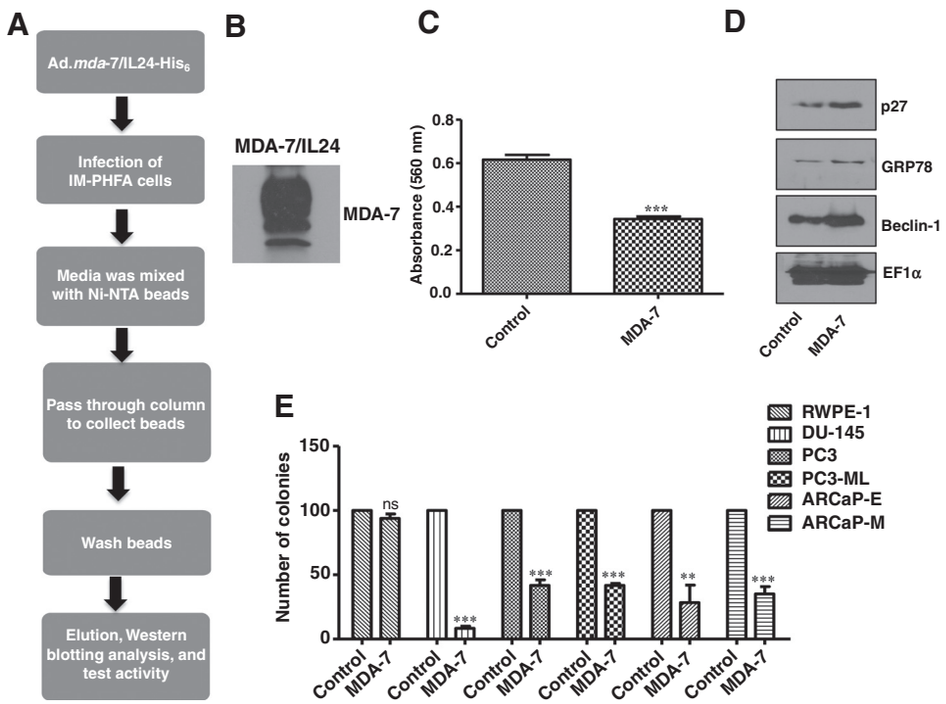

Figure 1.
Production and characterization of His-Tagged recombinant MDA-7/IL24 protein. **A,** Schematic diagram of production of His-tagged recombinant MDA-7/IL24 protein. **B,** Confirmation of MDA-7/IL24 protein by Western blotting. **C,** MTT assays examined the activity of MDA-7/IL24 on PC3-ML cells. Data showed a significant inhibition in cell proliferation. **D,** Expressions of downstream MDA-7/IL24 signaling cascade molecules, including p27, Beclin-1, and BiP/GRP78, were confirmed using Western blotting, which are upregulated in MDA-7/IL24-treated cells. EF1α was used as a loading control. **E,** Colony formation (clonal) assays were performed with different prostate cancer cells and RWPE-1 cells in triplicates. Approximately 200 cells were plated and treated with His-MDA-7/IL24, and 2 weeks after treatment, they were stained with crystal violet. Numbers of colonies were counted, and the data were plotted. Data represent mean ± SD of two independent experiments; **, $P < 0.01$; ***, $P < 0.001$ versus control.

control group, whereas there was significantly less evidence of metastasis in the MDA-7/IL24-treated animals as indicated by decreased BLI signals using IVIS imaging (Fig. 2A). The luciferase intensities in the different groups of animals are shown in Fig. 2B. These results, together with survival, which increased in MDA-7/IL24-treated animals (Fig. 2C), support a therapeutic role of recombinant MDA-7/IL24 in suppressing prostate cancer–induced bone metastasis. To determine the effect of MDA-7/IL24 on primary bone marrow in animals, cells from the bone cavity were isolated and treated with His-MDA-7 at different doses *in vitro*. No apparent toxicity was observed in primary bone marrow cells treated with His-MDA-7 (Supplementary Fig. S2).

MDA-7/IL24 inhibits RANKL-induced osteoclast differentiation

Bone homeostasis is maintained by an equilibrium between osteoblasts (bone formation) and osteoclasts (bone resorption; ref. 4). Osteoblasts play a central role in bone formation, and the microenvironment secretes factors for osteoclast maturation, which help in bone resorption. Disturbances in the equilibrium between osteoblasts and osteoclasts causes several bone disorders and promotes the growth of cancer cells (osteoblastic or osteolytic), which is an outcome of tumor cells in bone. Increased osteoclastic activity makes bone fragile; osteoclasts also play a role in the early dissemination of cancer cells in the bone marrow niche (31).

To determine the osteoclastic activity in tumor-bearing animals, either treated or untreated with therapeutic, bone marrow cells were isolated from the femur, and osteoclast differentiation was experimentally induced. MDA-7/IL24-treated animals had significantly less osteoclasts as compared with the control group. This was quantified by counting the number of osteoclasts (Fig. 2D) and also measuring TRACP osteoclastic enzymatic activity (Fig. 2E). These initial results indicated a potential function of MDA-7/IL24 in regulating osteoclast differentiation.

To investigate the effect of MDA-7/IL24 on osteoclast differentiation, bone marrow cells from athymic nude mice were isolated and induced to differentiate with RANKL in the presence or absence of MDA-7/IL24. Five days after induction with RANKL, osteoclast differentiation was measured by counting multinucleated cells. Cells positively stained for TRAP and multinucleated cells were quantified under a light microscope (Supplementary Fig. S3A). The number of osteoclasts and its activity was significantly reduced in the MDA-7/IL24-treated group in comparison with controls (Supplementary Fig. S3B and S3C). To provide molecular insights into this inhibitory effect, we used a mouse macrophage cell line RAW 264.7, which can be induced to differentiate into osteoclasts when incubated with RANKL (32). Using RAW 264.7 cells, different genetic markers associated with osteoclastic differentiation were monitored (33). RAW 264.7 cells were treated with RANKL or MDA-7/IL24 alone and in

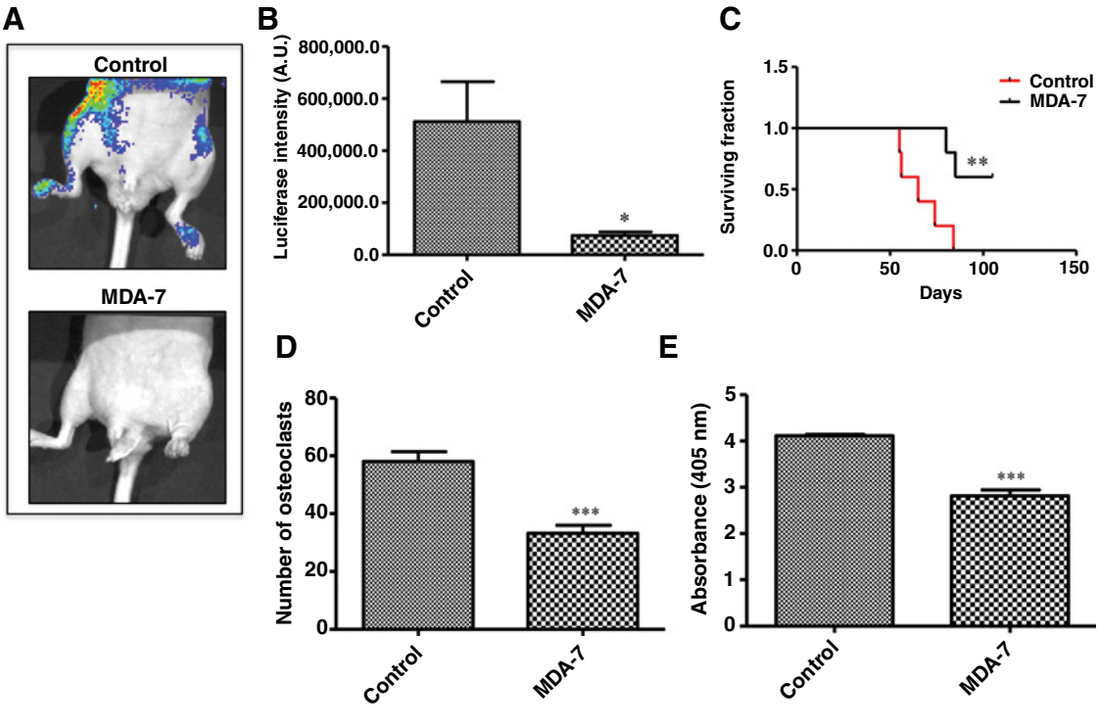

Figure 2.

Antimetastatic activity and inhibition of osteoclast differentiation by MDA-7/IL24 protein. **A,** *In vivo* bone metastasis assay evaluating the effect of His-MDA-7 on bone metastasis development (5 mg/kg, 2× a week for 3 weeks); $n = 5$ in each group. **B,** Luciferase intensity was quantified, and bar graph showing the significant inhibition in luciferase intensity in MDA-7/IL24-treated animals. **C,** Survival plot showing the role of MDA-7/IL24 in the enhancement of survival of animals. Data represent mean ± SD of two independent experiments: **, $P < 0.01$ versus control. Bone marrow cells were collected at the end of the study, and osteoclast differentiation was induced. Mature osteoclasts were stained using a TRAP staining kit, and osteoclast activity was measured by TRACP enzymatic assay kit as described in Materials and methods. Number of osteoclasts (**D**) and osteoclast activities (**E**) in control and MDA-7/IL24-treated samples are as shown in the graphs. Five replicates were done for each group. Data represent mean ± SD of two independent experiments; *, $P < 0.05$; ***, $P < 0.001$ versus control.

combination. RNA was isolated after 5 days of treatment, and real-time PCR quantified expression of TRAP, Cathepsin K (CTSK), and Calcitonin Receptor (CTR) genes. As described earlier, RANKL induced the expression of TRAP, CTSK, and CTR gene expression. Addition of MDA-7/IL24 attenuated RANKL-induced regulation of these genes (Supplementary Fig. S4A). These data validate the hypothesis that MDA-7/IL24 can mediate inhibition in RANKL-induced osteoclastic differentiation. MTT assays were performed to determine if MDA-7/IL24 caused any growth suppression in this cell line. Proliferation of RAW 264.7 was not affected by MDA-7/IL24, further confirming the tumor specificity of this cytokine (Supplementary Fig. S4B). DU-145 (prostate cancer) cells were used as a positive control (Supplementary Fig. S4C).

MDA-7/IL24 regulates AKT signaling in mouse macrophage cells

Multiple signaling cascades regulate osteoclast differentiation. Previous studies suggest that increased expression of the pAkt-activated NF-κB pathway in PC3 cells up regulates the level of RANKL, consequently inducing osteoclastogenesis (34). Also, RANKL treatment induces p-Akt (35). Inoue and colleagues previously demonstrated that *mda-7/IL24* negatively regulates p-Akt, which affects tumor progression (36). To determine whether MDA-7/IL24 exerts any effect on Akt activation in our model system, we treated RAW 264.7 cells with MDA-7/IL24 in the presence or absence of RANKL. As observed in previous reports (34, 35), we found that RANKL induces Akt activation in RAW 264.7 cells, and this activated Akt was suppressed by MDA-7/IL24 (Fig. 3A; Supplementary Fig. S5A). These data suggest a likely role of Akt inhibition in MDA-7/IL24-mediated downregulation of osteoclast differentiation. The effect of Akt inhibition by a PI3K inhibitor (LY294002) was also studied in the signaling cascade involving NFAT, Mcl-1, and Akt (Fig. 3B; Supplementary Fig. S5B). The data show the cellular signaling of Akt pathway–related genes mediated by RANKL and MDA-7/IL24.

To further confirm the above data, a constitutively active form of Akt (MYR-Akt) was used. Cells were transfected with MYR-Akt and treated with MDA-7/IL24. As shown in Fig. 3C and Supplementary Fig. S5C, treatment with MDA-7/IL24 profoundly inhibited Mcl-1 expression, which was rescued by overexpression of a constitutively active Akt (MYR-Akt). These results confirm the role of Akt in MDA-7/IL24-mediated suppression in osteoclastic differentiation.

Mcl-1 inhibitor, BI-97D6, synergizes with MDA-7/IL24 in suppressing prostate cancer bone metastasis

Since our prior studies indicated that inhibition of Mcl-1 enhances the antitumor activity of MDA-7/IL24 when

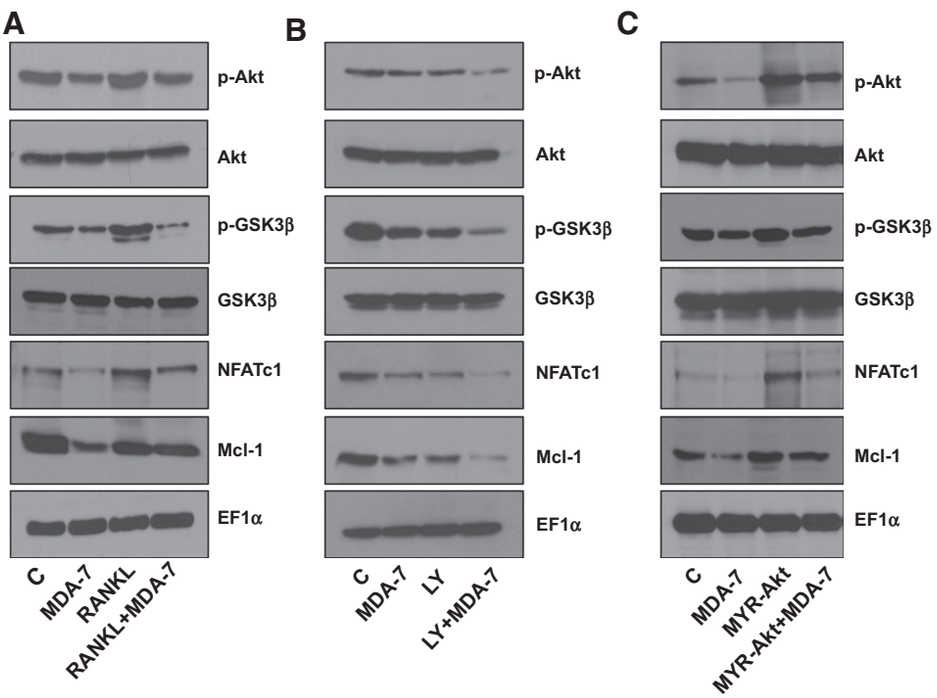

Figure 3.

Effect of MDA-7/IL24 on signaling cascades in RAW 264.7 cells. Cells were treated with the indicated reagents/constructs, and Western blotting was performed to investigate the signaling cascades. **A,** Cells were treated with RANKL (100 ng/mL) and His-MDA-7 (10 μg/mL). After 5 days, cells were lysed, and Western blotting was done. Akt phosphorylation decreased with MDA-7/IL24 treatment, which was reversed upon RANKL treatment. Mcl-1, NFATc1, and phosphor-GSK3β, which are downstream signaling molecules, followed a similar pattern. **B,** Cells were treated with a PI3 kinase inhibitor LY294002 (10 μmol/L) and His-MDA-7 (10 μg/mL). Treatment with a PI3K inhibitor or MDA-7/IL24 attenuated the level of Phosphor-Akt. Combined treatment with a PI3K inhibitor and MDA-7/IL24 further downregulated Phosphor-Akt and downstream molecules. **C,** Cells were transfected with MYR-Akt (constitutively active Akt) and treated with His-MDA-7 (10 μg/mL). Western blotting was performed to check the expression of the indicated proteins. Phosphor-GSK3β, NFATc1, and Mcl-1 expression increased with the overexpression of a constitutively active Akt, which were inhibited by treatment with His-MDA-7. EF1α was used as a loading control in all the experiments. The densitometric quantification of p-Akt/Akt, p-GSK3β/GSK3β, NFATc1/EF1α, and Mcl-1/EF1α under different experimental conditions in RAW 264.7 cells is shown in Supplementary Fig. S5A–S5C.

administered by an adenovirus (17), we tested the effect of MDA-7/IL24 protein in combination with BI-97D6, a small-molecule Mcl-1 inhibitor, in male athymic nude mice injected with PC3-ML cells by the intracardiac route, resulting in bone metastases. Animals were treated through tail vein injection with MDA-7/IL24 for 3 weeks with a total of 6 doses at 5 mg/kg. BI-97D6 was administered through intraperitoneal route at 1.5 mg/kg body weight with a total of 6 doses (Fig. 4A). A significant level of bone metastasis was evident in the control group, whereas MDA-7/IL24 treatment resulted in significantly less metastatic lesions (Fig. 4A). Treatment with BI-97D6 alone also showed some inhibitory effects on bone metastasis development; however, when combined with MDA-7/IL24, a dramatic inhibition in bone metastasis development was seen suggesting a combinatorial therapeutic role of MDA-7/IL24 with an Mcl-1 inhibitor in prostate cancer–induced bone metastasis (Fig. 4A). The luciferase intensities are as shown (Fig. 4B). This combination also reduced osteoclast differentiation. Dose–response assays showed a downregulation in the number of osteoclasts when primary bone marrow cells were induced with MCSF and RANKL (Supplementary Fig. S6A) and treated with Mcl-1 inhibitors. Synergy in the inhibition of osteoclast differentiation was also evident following treatment with the combination of MDA-7/IL24 and BI-97D6 (Supplementary Fig. S6B).

Suppression of osteoclast differentiation was also confirmed using an *in vivo* metastatic model (Fig. 4C). Bone marrow cells were isolated after sacrifice of mice, and osteoclast differentiation was induced. The MDA-7/IL24- or BI-97D6–treated groups of animals had significantly less osteoclasts as compared with control groups. The bone marrow cells isolated from the MDA-7/IL24 and BI-97D6 animals formed statistically fewer osteoclasts following induction with RANKL. This was evident by counting osteoclasts (Fig. 4C) and also by measuring TRACP osteoclastic enzymatic activity (Fig. 4D).

Akt regulates bone metastasis of prostate cancer cells *in vivo*

Earlier data suggest a critical role of the Akt pathway in bone metastasis of prostate cancer cells, and *mda-7/IL24* inhibits Akt activation (37). To provide further confirmation of a role of Akt in prostate cancer bone metastasis, we inhibited the activity of Akt using the PI3 kinase inhibitor LY294002 (38) and determined effects on osteoclast differentiation. The PI3 kinase inhibitor LY294002 synergized with MDA-7/IL24 to reduce osteoclast differentiation (Supplementary Fig. S7A). In addition, treatment with LY294002 in combination with MDA-7/IL24 resulted in decreased expression of downstream molecules including NFAT and Mcl-1 (Fig. 3B; Supplementary Fig. S5B). As further confirmation, RAW 264.7 cells were stably transfected with CA-Akt, DN-Akt, and Mcl-1, and osteoclast differentiation was determined. Akt- and Mcl-1–overexpressing clones formed more osteoclasts as compared with controls, whereas DN-Akt–overexpressed RAW 264.7 cells formed fewer osteoclasts (Supplementary Fig. S7B).

To evaluate the role of Akt *in vivo*, we injected metastatic PC3-ML cells and PC3-ML cells overexpressing constitutively active Akt (PC3-ML^{Akt}; Fig. 5A) carrying firefly luciferase into

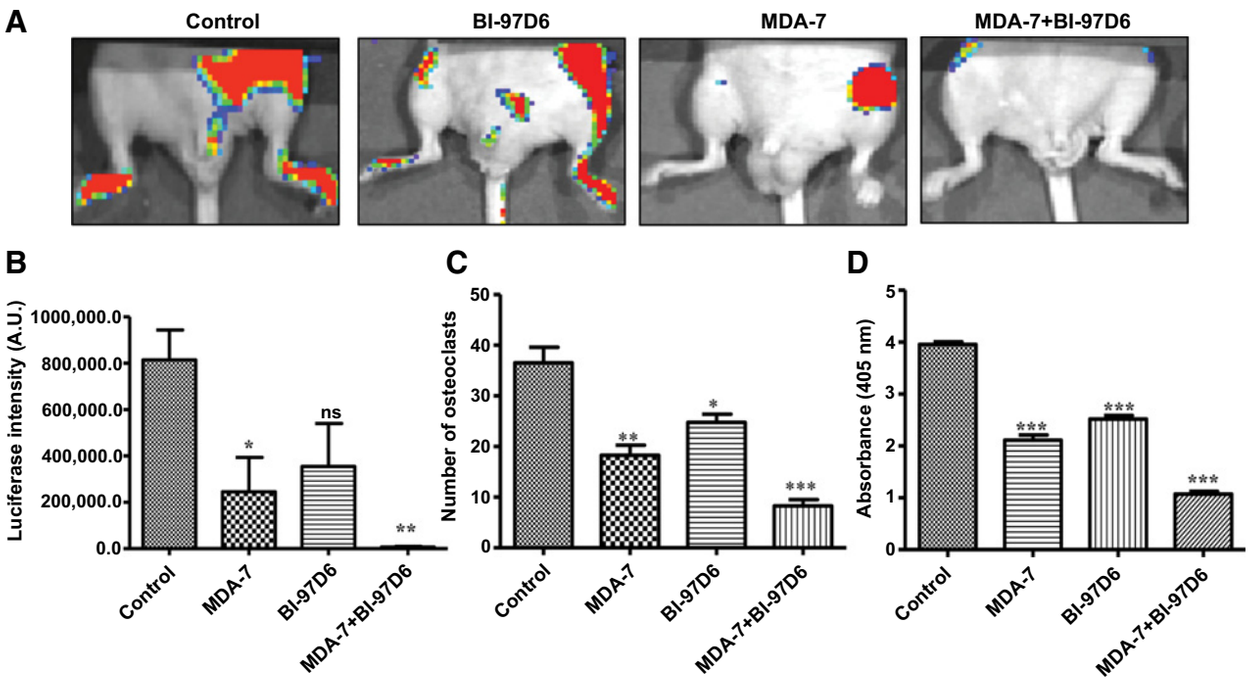

Figure 4.

Combinatorial effect of His-MDA-7 and BI-97D6 on metastasis of prostate cancer in bone and osteoclast differentiation. **A,** *In vivo* bone metastasis assays evaluated the effects of His-MDA-7 and BI-97D6. Experimental treatment protocol is described in Materials and Methods. **B,** Luciferase intensity was quantified in triplicates, and the bar graph shows the significant inhibition in luciferase intensity in MDA-7/IL24-treated animals. Addition of BI-97D6 further enhanced the inhibitory effects of MDA-7/IL24 on bone metastasis development. Osteoclasts were stained with a TRAP staining kit, and osteoclastic activity was measured using a TRACP enzymatic assay kit. Number of osteoclasts (**C**) and osteoclastic activity was measured (**D**), which is represented graphically. Four replicates were taken for each group. Data represent mean ± SD of two independent experiments; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus control.

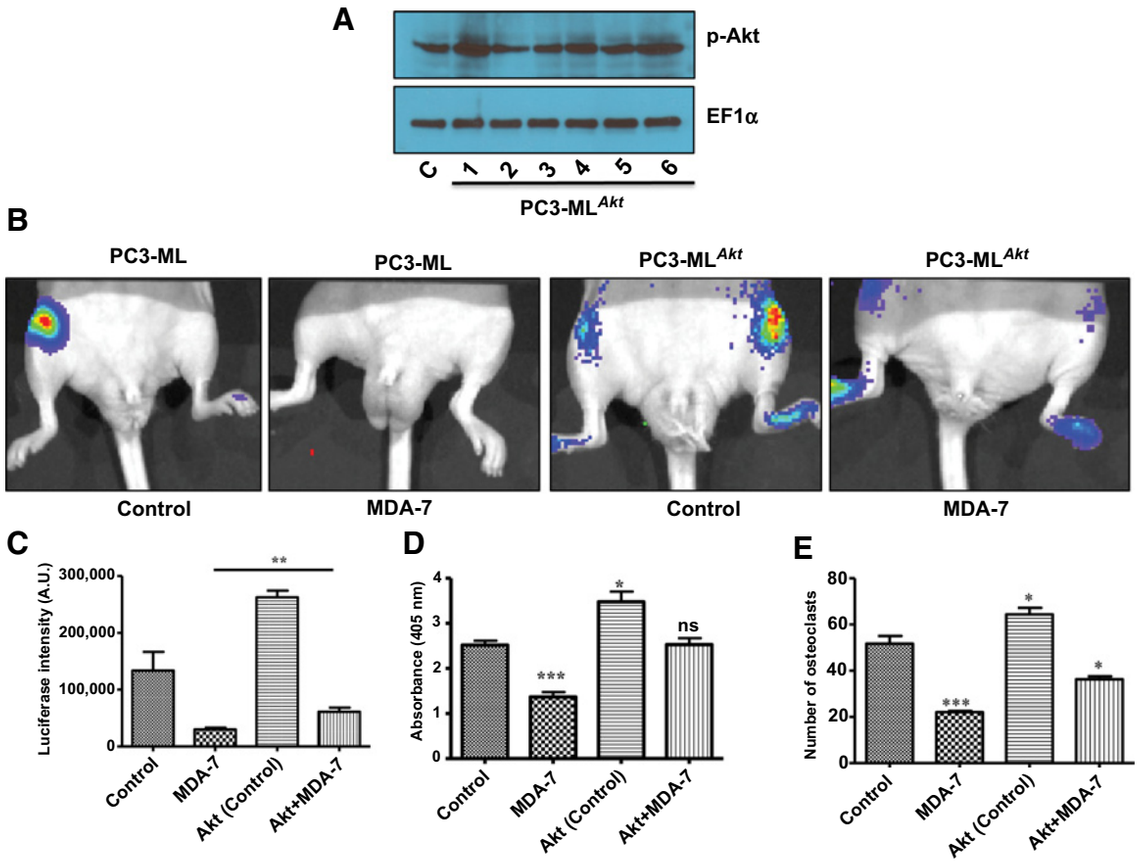

Figure 5.

Stable prostate cancer cells overexpressing Akt and effect of Akt expression on prostate cancer bone metastasis, response to MDA-7/IL24, and osteoclast differentiation. **A,** Phosphor-Akt expression in control PC3-ML and stable Akt overexpressing PC3-ML^{Akt} clones. **B,** *In vivo* bone metastasis study using PC3-ML cells and PC3-ML cells overexpressing CA-Akt (PC3-ML^{Akt}). Constitutive activation of Akt diminished the inhibitory effects of MDA-7/IL24 on prostate cancer–induced bone metastasis. **C,** Luciferase intensities are as represented. Effect of constitutive Akt expression on osteoclast activity (**D**) and number of osteoclasts (**E**) were measured in triplicates and are represented graphically. Bone marrow cells from mice (described in **B**) were collected, and 5×10^5 cells were induced for osteoclast differentiation. Data represent mean ± SD of two independent experiments; *, $P < 0.05$; ***, $P < 0.001$ versus control.

male athymic nude mice through the intracardiac route. Clone 1 was used for the *in vivo* studies, which was validated for expression of Akt downstream pathway gene expression by Western blotting (Supplementary Fig. S8). Proliferation, invasion, and migration of the tumor cells were monitored by IVIS imaging. Treatment of MDA-7/IL24 was continued for 3 weeks with a total of 6 doses at 5 mg/kg through the tail vein. Significant bone metastasis was apparent in the control group, whereas there was a significant decrease in metastasis in MDA-7/IL24-treated animals. The Akt-overexpressing PC3-ML cells were more metastatic than the control PC3-ML cells. Treatment with MDA-7/IL24 inhibited metastasis by the PC3-ML^{Akt} group; however, this effect was reduced in comparison with His-MDA-7–treated parental PC3-ML cells. These observations confirm the significance of Akt in prostate cancer–mediated bone metastasis development, which can be partially abrogated by MDA-7/IL24 treatment (Fig. 5B). These data were further substantiated by quantification of luciferase intensities in the different experimental animal groups (Fig. 5C). To investigate osteoclastic differentiation in these animals, bone marrow cells were isolated after completion of the study. Osteoclast differentiation was induced as described in the Materials and Methods section. Bone marrow isolated from MDA-7/IL24-treated animals showed less osteoclastic activity as compared with the control group. Bone marrow from animals injected with elevated Akt-stable expression cells showed more osteoclastic activity, which decreased following MDA-7/IL24 treatment. Osteoclast differentiation assays further supported the importance of Akt in prostate cancer–mediated bone metastasis (Fig. 5D and E). A schematic representation of the proposed role of MDA-7/IL24 in prostate cancer–mediated bone metastasis through modulation of the bone microenvironment is presented in Fig. 6. Additional effects of MDA-7/IL24 that may contribute to inhibition of prostate cancer–induced bone metastasis include direct killing of prostate cancer cells (through apoptosis or toxic autophagy), inhibition of angiogenesis, and immune-mediated antiprostate cancer activity (29, 30).

Discussion

Bone metastasis in patients with prostate cancer is the leading cause of morbidity and death (39). Prostate cancer is a global health problem affecting men, and no current cure is available for

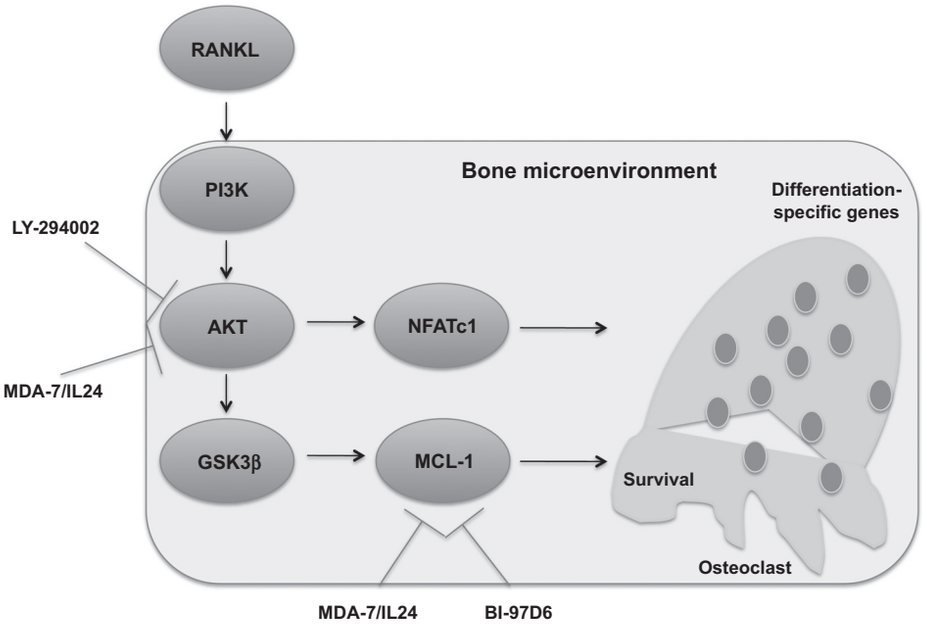

Figure 6.
Schematic representation of MDA-7/IL24-mediated inhibition in progression of prostate cancer–induced bone metastasis through modulation of the bone microenvironment.

this eventual stage of prostate cancer progression (39). In this context, there is an imperative to develop improved therapeutics to target advanced prostate cancer, which has metastasized predominantly to bone and other sites in the body. The precise genetic mechanisms and signaling pathways mediating the metastatic process, particularly secondary colonization and growth in the microenvironment, represent works in progress. Advanced cases of prostate cancer are difficult to treat, and existing modes of therapy induce side effects that can exacerbate the clinical condition. Our present study highlights a novel, specific role of recombinant MDA-7/IL24 protein in dramatically inhibiting prostate cancer–induced bone metastasis, through effects on the bone microenvironment, which is enhanced further when combined with an Mcl-1 inhibitor (BI-97D6).

Adenovirus-mediated *mda-7/IL24* expression has profound anticancer activity in a wide variety of cancer cells *in vitro* and *in vivo* in preclinical animal models (30). Moreover, intratumoral injection with a replication incompetent adenovirus-expressing *mda-7/IL24* (INGN 241) had no significant toxicity with definitive clinical (apoptosis induction) responses in patients with advanced cancers, including melanomas and carcinomas (40). MDA-7/IL24 is a multifunctional cytokine, which selectively induces cell death in cancer cells without harming their normal counterparts. *mda-7/IL24* has been efficacious in killing a broad array of cancers by regulating multiple downstream target molecules. It regulates many biological processes in cancer cells including apoptosis (25), angiogenesis (41, 42), autophagy (25, 43), invasion (44), and metastasis (45), which are very relevant processes in cancer progression. In addition, MDA-7/IL24 displays synergistic therapeutic activity when combined with several therapeutic modalities (radiotherapy, chemotherapy, and monoclonal antibody therapy) used in the clinic (30).

To comprehend the biological properties of MDA-7/IL24 in normal and cancer cells, we generated recombinant protein (both GST- and His-tagged MDA-7/IL24; refs. 46, 47). In leukemia, renal carcinoma and non–small cell lung carcinoma cells, GST-tagged MDA-7/IL24 stimulated apoptosis, suggesting potential application of recombinant MDA-7/IL24 as a therapeutic (29). Previously, using His-tagged MDA-7/IL24 and different receptor mutant cells, we demonstrated that His-MDA-7/IL24 interacts with specific surface receptors and activates an autocrine/paracrine loop causing production and secretion of endogenous MDA-7/IL24 (46). In the present study, we evaluated the preclinical activity of His-tagged MDA-7/IL24 against prostate cancer bone metastasis. The results of this study indicate that recombinant MDA-7/IL24 can be delivered repeatedly systemically in mice without promoting toxicity and can inhibit the development of prostate cancer bone metastasis. The effect of His-tagged MDA-7/IL24 on metastasis is more global, because treatment of animals receiving intracardiac delivery of PC3-ML cells also suppressed development of lung metastases (Supplementary Fig. S9). These more general effects of MDA-7/IL24 reflect the multifunctional antitumor properties of this therapeutic cytokine, which can directly induce cancer cell death through apoptosis or toxic autophagy, promote cancer cell toxicity through "bystander antitumor activity," inhibit tumor angiogenesis, and enhance immune therapy of cancer (29, 30).

Advanced and metastatic cancers are complex genetic and epigenetic disorders where a number of genetic loci are amplified or activated leading to abnormal expression of oncogenic proteins, i.e., Bcl-2, Bcl-xL, or Mcl-1. Elevated expression of Mcl-1 is associated with advanced prostate cancer in humans (17). In addition, other carcinomas and leukemias over express Mcl-1 (48). We have now studied the role of inhibition of Mcl-1 in RANKL-induced osteoclast differentiation and progression of bone metastasis. Over expression of Mcl-1 inhibits the intrinsic mode of apoptosis that is mediated by the mitochondrial pathway (49). A pan-Bcl2 antagonist (BI-97C1 or Sabutoclax) can block prostate cancer tumor development in transgenic and human xenograft mice (17). A significant role of Mcl-1 in osteoclast differentiation has been documented (8). RANKL induces enhanced levels of Mcl-1, which increase the survival of

preosteoclasts leading to an increased number of osteoclasts (7). Inhibition of osteoclasts prevents spontaneous bone tumors in transgenic mouse models. Because Mcl-1 inhibitors stabilize MDA-7/IL24 protein (23), we used this inhibitor in combination with MDA-7/IL24 to determine if its prostate cancer antibone metastasis activity could be enhanced further. A combination of BI-97D6 with MDA-7/IL24 significantly ablated the progression of bone metastasis induced by prostate cancer cells. *In vitro* studies indicate that genetic inhibition of Mcl-1 or treatment with BI-97D6, a pharmacologic inhibitor of Mcl-1, obstructs osteoclast differentiation.

Akt regulates osteoclast differentiation through the GSK3β/NFATc1 signaling pathway (34). *mda-7/IL24* down regulates Akt phosphorylation, thereby regulating cellular proliferation and cell death (37). We now demonstrate experimentally that treatment of RAW 264.7 cells with MDA-7/IL24 down regulates pAkt levels, which in turn reduces the levels of NFATc1 and phospho-GSK3β. Conversely, high levels of Akt promote more aggressive prostate cancer bone metastasis and resistance to MDA-7/IL24 therapy. These observations indicate that Akt plays a seminal role in bone metastasis development, which can be partially abrogated by MDA-7/IL24. Targeted therapy by MDA-7/IL24 also reduces Mcl-1 expression. Interesting links between *mda-7*/IL24 and several downstream signaling pathways have emerged recently. *mda-7*/IL24 has been shown to mediate AIF-mediated cell death specifically in neuroblastoma (50) and miR-221/beclin-1–mediated toxic autophagy (25). In the present study, we establish that MDA-7/IL24 inhibits prostate cancer–mediated bone metastasis by eliminating cancer cells and inhibiting the osteoclast differentiation pathway via downregulation of Akt and Mcl-1.

In summary, we demonstrate that MDA-7/IL24 may provide an important therapeutic reagent for inhibiting prostate cancer–induced bone metastasis. In addition, antimetastatic properties of MDA-7/IL24 toward prostate cancer bone metastasis can be enhanced further by using a combinatorial approach with an Mcl-1 or Akt inhibitor. Mechanistically, MDA-7/IL24 can directly target prostate cancer cells for apoptosis and can block osteoclast differentiation and inhibit the Akt/Mcl-1 pathway mediating prostate cancer bone metastasis. Considering the lack of toxicity and profound multifunctional and near ubiquitous anticancer properties, MDA-7/IL24 protein, particularly when used in combination with other therapeutic modalities, may offer significant benefit in the treatment of metastasis in prostate cancer and other neoplastic diseases.

Disclosure of Potential Conflicts of Interest

P.B. Fisher is President and Interim CEO of, has ownership interest (including stock, patents, etc.) in, and is a consultant/advisory board member for InterLeukin Combinatorial Therapies. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: A.K. Pradhan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.K. Pradhan, P. Bhoopathi, S. Talukdar, X.-N. Shen, P.B. Fisher
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.K. Pradhan, S. Talukdar, L. Emdad, S.K. Das, P.B. Fisher
Writing, review, and/or revision of the manuscript: A.K. Pradhan, L. Emdad, S.K. Das, D. Sarkar, P.B. Fisher
Study supervision: P.B. Fisher

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