Advances in Brief

Antisense TRPM-2 Oligodeoxynucleotides Chemosensitize Human Androgen-independent PC-3 Prostate Cancer Cells Both in Vitro and in Vivo

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Abstract
Although numerous chemotherapeutic regimens have been evaluated for patients with hormone-refractory prostate cancer, none has improved survival. Testosterone-repressed prostate message-2 (TRPM-2), which is highly up-regulated after androgen withdrawal and during androgen-independent progression in prostate cancer, has been shown to inhibit apoptosis induced by various kinds of stimuli. The objectives in this study were to test whether antisense (AS) oligodeoxynucleotides (ODNs) targeted against TRPM-2 enhance chemosensitivity in human androgen-independent prostate cancer PC-3 cells both in vitro and in vivo. Initially, the potency of 10 AS ODNs targeting various regions of the TRPM-2 mRNA were evaluated, and the AS ODN targeted to the TRPM-2 translation initiation site (AS ODN#2) was found to be the most potent sequence for inhibiting TRPM-2 expression in PC-3 cells. Despite significant dose-dependent and sequence-specific suppression of TRPM-2 expression, AS ODN#2 had no effect on growth of PC-3 cells both in vitro and in vivo. However, pretreatment of PC-3 cells with AS ODN#2 significantly enhanced chemosensitivity of Taxol (paclitaxel) and mitoxantrone in vitro. Characteristic apoptotic DNA laddering and cleavage of poly(ADP-ribose) polymerase were observed after combined treatment with AS ODN#2 plus paclitaxel or mitoxantrone but not with either agent alone. In vivo administration of AS ODN#2 plus either paclitaxel or mitoxantrone significantly decreased PC-3 tumor volume by 80 or 60%, respectively, compared with mismatch control ODN plus either paclitaxel or mitoxantrone. In addition, terminal deoxynucleotidyl transferase-mediated nick end labeling staining revealed increased apoptotic cells in tumors treated with AS ODN#2 plus paclitaxel or mitoxantrone. These findings confirm that TRPM-2 overexpression confers resistance to cytotoxic chemotherapy in prostate cancer cells and illustrates the potential utility of combined treatment with AS TRPM-2 ODN plus chemotherapeutic agents for patients with hormone-refractory prostate cancer.

Introduction
Prostate cancer is now the most commonly diagnosed malignancy and the second leading cause of cancer mortality in men in Western industrialized countries. Androgen withdrawal remains the only effective therapy for patients with advanced disease. Approximately 80% of patients achieve symptomatic and/or objective response after androgen ablation; however, progression to androgen independence ultimately occurs in almost all cases (1). Although numerous nonhormonal agents have been evaluated in patients with hormone-refractory prostate cancer, these agents have limited antitumor activity with an objective response rate of <20% and no demonstrated survival benefit (2). Therefore, novel therapeutic strategies targeting molecular mechanisms mediating resistance to conventional agents must be developed to make a significant impact on survival.

Advances in the field of nucleic acid chemistry offers one attractive strategy to design AS ODN-based therapeutic agents that specifically hybridize with complementary mRNA regions of a target gene and thereby inhibit gene expression by forming RNA/DNA duplexes (3). Rapid intracellular degradation of ODNs is a potential disadvantage of AS ODN therapy, but this problem can be overcome by substituting a nonbridging phosphoryl oxygen of DNA with a sulfur to create a phosphorothioate backbone, which stabilizes the ODN to nuclease digestion (4). Recently, several antisense ODNs targeted against specific genes involved in neoplastic progression have been evaluated as potential therapeutic agents (5–8). Collectively, these findings identify AS ODNs as a novel class of antineoplastic agents when designed for appropriate molecular targets. However, because numerous genes are involved in tumor progression, inhibition of a single target gene will likely be insufficient to inhibit tumor progression in a meaningful way. In fact, combined use of AS ODN with other compounds, such as chemotherapeutic...
agents, have been demonstrated to produce more potent antineoplastic effects in some tumor model systems (9–12).

TRPM-2, also known as clusterin or sulfated glycoprotein-2, was first isolated from ram rete testes fluid (13) and plays important roles in various pathophysiological processes, including tissue remodeling, reproduction, lipid transport, complement regulation, and apoptosis (14). Because TRPM-2 expression is increased in various benign and malignant tissues undergoing apoptosis, it has been regarded as a marker for cell death (15–18). Recent studies, however, provide conflicting findings regarding the relationship between TRPM-2 up-regulation and increased apoptotic activity (19–21). Similarly, TRPM-2 expression increases in regressing normal prostatic epithelial cells (22, 23) and prostate cancer xenografts (24, 25) after treatment with various apoptotic stimuli and is associated with cell survival and disease progression in prostate cancer (26–30). We have reported recently that an increase in TRPM-2 expression after androgen ablation accelerates tumor progression by inhibiting castration (31)- and chemotherapy4-induced apoptosis. However, the functional significance of TRPM-2 expression in established AI human prostate cancer has not been examined.

In the present study, we designed and screened 10 phosphorothioate AS ODNs targeted against the human TRPM-2 gene to identify potent ODN sequences that specifically inhibit TRPM-2 expression in human AI prostate cancer PC-3 cells. We then tested whether AS TRPM-2 ODN can enhance the response of PC-3 cells to either Taxol (paclitaxel) or mitoxantrone both in vitro and in vivo.

Materials and Methods

Tumor Cell Line. PC-3, derived from human prostate cancer, was purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% heat-inactivated FCS.

Chemotherapeutic Agents. Paclitaxel and mitoxantrone were purchased from Sigma Chemical Co. (St. Louis, MO) and Wyeth-Ayerst, Inc. (Montreal, Canada), respectively. Stock solutions of paclitaxel and mitoxantrone (1 mg/ml) were prepared with DMSO and diluted with PBS to the required concentrations before each in vitro experiment. Polymeric micellar paclitaxel used in these in vivo studies was generously supplied by Dr. Helen M. Bart (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada).

AS TRPM-2 ODN. Phosphorothioate ODNs used in this study were obtained from the Nucleic Acid-Protein Service Unit, University of British Columbia (Vancouver, Canada). The sequences of 10 AS TRPM-2 ODNs (AS ODN#1 to AS ODN#10) and a two-base TRPM-2 MM ODN used as a control (MM Co. ODN) were as follows: AS ODN#1, 5′-ATCAAGCTGC-GAGTCTCCCGGCACTT-3′; AS ODN#2, 5′-TGCAGAACA-GATCTCCCGGACATT-3′ (AS) for TRPM-2; and 5′-TGC-TTTAAC-TCTTGTTAAAT-3′ (sense) and 5′-ATATTGGGCAGGTITTTCTAGA-3′ (AS) for GAPDH. The density of bands for TRPM-2 was normalized against that of GAPDH by densitometric analysis.

MTT Assay. The in vitro growth-inhibitory effects of AS TRPM-2 ODN plus paclitaxel or mitoxantrone on PC-3 cells were assessed using the MTT assay as described previously (32). Briefly, 1 × 10^4 cells were seeded in each well of 96-well microtiter plates and allowed to attach overnight. Cells were then treated once daily with 500 nm ODN for 2 days. After ODN treatment, cells were treated with various concentrations of paclitaxel or mitoxantrone. After 48 h of incubation, 20 μl of 5 mg/ml MTT (Sigma Chemical Co.) in PBS were added to each well, followed by incubation for 4 h at 37°C. The formazan crystals were then dissolved in DMSO. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware, Lincoln Park, NJ) at 540 nm. Absorbance values were normalized to the values obtained for the vehicle-treated cells to

Fig. 1 Effects of various AS human TRPM-2 ODNs on TRPM-2 mRNA expression in PC-3 cells. A, schematic representation of the relative position of 10 phosphorothioate AS ODNs designed to hybridize with various regions of the TRPM-2 gene. UTR, untranslated region. B, PC-3 cells were treated daily with AS TRPM-2 ODN or a two-base TRPM-2 MM ODN for 2 days. Total RNA was extracted from cultured cells and analyzed for TRPM-2 and GAPDH levels by Northern blotting. No Tx, untreated cells. C, quantitative analysis of TRPM-2 mRNA levels after normalization to GAPDH mRNA levels in PC-3 cells with AS TRPM-2 or MM Co. ODN was performed using a laser densitometer. Columns, means; bars, SD. *, differs from control (P < 0.01) by Student’s t test.
Determine the percentage of survival. Each assay was performed in triplicate.

**DNA Fragmentation Analysis.** Nucleosomal DNA degradation was analyzed as described previously with a minor modification (32). Briefly, $1 \times 10^5$ PC-3 cells were seeded in 5-cm culture dishes and allowed to adhere overnight. After treatment with ODN plus paclitaxel or mitoxantrone using the same schedule described above, cells were harvested and then lysed in a solution containing 100 mM NaCl, 10 mM Tris (pH 7.4), 25 mM EDTA, and 0.5% SDS. After the centrifugation, the supernatants were incubated with 300 µg/ml proteinase K for 5 h at 65°C and extracted with phenol-chloroform. The aqueous layer was treated with 0.1 volume of 3M sodium acetate, and the DNA was precipitated with 2.5 volumes of 95% ethanol. After treatment with 100 µg/ml RNase A for 1 h at 37°C, the sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

**Assessment of in Vivo Tumor Growth.** Approximately $1 \times 10^6$ PC-3 cells were inoculated s.c. with 0.1 ml of Matrigel (Becton Dickinson Labware, Bedford, MA) in the flank region of male athymic nude mice (BALB/c strain; Charles River Laboratory, Montreal, Quebec, Canada), 6–8 weeks of age, under methoxyfluorane anesthesia. When PC-3 tumors grew to 1 cm in diameter, usually 2–3 weeks after injection, mice were randomly selected for treatment with AS TRPM-2 ODN alone, MM Co. ODN alone, AS TRPM-2 ODN plus paclitaxel, MM Co. ODN plus paclitaxel, AS TRPM-2 ODN plus mitoxantrone, or MM Co. ODN plus mitoxantrone. Each experimental group consisted of eight mice. After randomization, 10 mg/kg AS TRPM-2 or MM Co. ODN was injected i.p. once daily into each mouse for 28 days. From days 10 to 14 and from days 24 to 28, 0.5 mg polymeric micellar paclitaxel or 0.03 mg mitoxantrone was administered once daily by i.v. injection. Tumor volume was measured once weekly and calculated by the formula: length $\times$ width $\times$ depth $\times$ 0.5236. Points, mean tumor volume in each experimental group containing eight mice; bars, SD.

**Fig. 2** Sequence-specific and dose-dependent inhibition of TRPM-2 expression by AS TRPM-2 ODN in PC-3 cells. A, PC-3 cells were treated daily with various concentrations of AS TRPM-2 ODN (AS ODN#2: CAGCAGCAGA-GTCTTCATCAT) or a two-base TRPM-2 MM ODN (MM Co. ODN: CAGCA-GCAGAGTATTTATCAT) as a control for 2 days; total RNA was extracted from culture cells, and TRPM-2 and GAPDH levels were analyzed by Northern blotting. No Tx, untreated cells. B, quantitative analysis of TRPM-2 mRNA levels after normalization to GAPDH mRNA levels in PC-3 cells after treatment with various concentrations of AS ODN#2 or MM Co. ODN was performed by using laser densitometry. Points, means of triplicate analyses; bars, SD. ** and *, differ from control ($P < 0.01$ and $P < 0.05$, respectively) by Student’s $t$ test. C, PC-3 cells were treated daily with 1 µM AS ODN#2 or MM Co. ODN for 4 days, protein was extracted from culture cells, and TRPM-2 and β-tubulin protein levels were analyzed by Western blotting. No Tx, untreated cells.
As shown in Fig. 1, treatment with these AS ODNs on TRPM-2 mRNA expression. Northern blot analyses were used to evaluate the effects of 10 phosphorothioate ODNs designed to hybridize with various regions of TRPM-2 mRNA were synthesized (Fig. 1A). Seven ODNs exhibited varied degrees of activity. Seven AS ODNs had little or no effect on TRPM-2 mRNA expression levels, whereas three ODNs had moderate effects. The most potent AS sODN identified from this series was AS ODN#2 (5'-CAGCAGCAGATCTCATAT-3'), which targets the human TRPM-2 translation initiation site, reducing TRPM-2 expression levels by 80% compared with MM Co. ODN treatment. AS ODN#2 was used in all subsequent experiments.

Sequence-specific and Dose-dependent Inhibition of TRPM-2 Expression by AS TRPM-2 ODN. To further define the specificity and potency of AS ODN#2-mediated inhibition of TRPM-2 gene expression, the effects of AS ODN#2 and MM Co. ODN on TRPM-2 mRNA and protein levels were determined by Northern and Western blot analyses, respectively. As shown in Fig. 2, A and B, daily treatment of PC-3 cells with AS ODN#2 (100, 500, or 1000 nM) for 2 days reduced TRPM-2 mRNA levels by 17, 39, or 78%, respectively, whereas TRPM-2 mRNA expression was not affected by the MM Co. ODN at any of the used concentrations. Inhibition of TRPM-2 protein levels in PC-3 cells was also observed after daily treatment with AS ODN#2 for 4 consecutive days (Fig. 2C).

Effects of AS TRPM-2 ODN Treatment on the Growth of PC-3 Cells Both in Vitro and in Vivo. To determine whether the reduction of TRPM-2 expression affects the growth of PC-3 cells in vitro, the growth rates of PC-3 cells after treatment with various concentrations of AS ODN#2 or MM Co. ODN once daily for 2 days were examined using the MTT assay. No significant difference in PC-3 cell growth was observed between AS ODN#2 and MM Co. ODN treatment (data not shown).

We then evaluated the effects of AS ODN#2 treatment on the growth of PC-3 tumors in vivo. Male nude mice bearing PC-3 tumors ~1 cm in diameter were randomly selected for treatment with AS ODN#2 versus MM Co. ODN, and 10 mg/kg ODN was administered once daily by i.p. injection for 28 days. As shown in Fig. 3, there was no significant difference in PC-3 tumor growth between these two groups.

To determine whether AS ODN#2 treatment inhibits TRPM-2 expression in PC-3 tumors in vivo, each of three tumor-bearing nude mice were given 10 mg/kg AS ODN#2 or MM Co. ODN i.p. once daily for 5 days, and TRPM-2 mRNA expression levels in harvested tumor tissues were then analyzed by Northern blotting. Treatment with AS ODN#2 resulted in a 68% reduction in TRPM-2 mRNA levels in PC-3 tumor tissues compared with MM Co. ODN-treated tumors (Fig. 4, A and B).

Enhanced Chemosensitivity of PC-3 Cells in Vitro with AS TRPM-2 ODN Treatment. To determine whether treatment with AS ODN#2 enhances the cytotoxic effects of paclitaxel and mitoxantrone, PC-3 cells were treated with 500 nM AS ODN#2 or MM Co. ODN once daily for 2 days and then incubated with medium containing various concentrations of either paclitaxel or mitoxantrone for 2 days. The MTT assay was then performed to determine cell viability. As shown in Fig. 5, A and B, AS ODN#2 treatment significantly enhanced chemosensitivity of paclitaxel and mitoxantrone in a dose-dependent manner, reducing the IC50 of paclitaxel and mitoxantrone by more than 60 and 50%, respectively.

The DNA fragmentation assay was performed to compare induction of apoptosis after treatment with 500 nM AS ODN#2 either alone or in combination with 5 nM paclitaxel or 1 nM mitoxantrone. Using the same treatment schedule described above, the characteristic apoptotic DNA ladder was observed only with combined treatment of AS ODN#2 plus paclitaxel or mitoxantrone (Fig. 5C). We further evaluated the effects of combined AS ODN#2 plus chemotherapy by using Western blot
analysis to identify cleavage of PARP protein, a substrate of the caspases activated during the process of apoptotic execution (35). The Mr 116,000 intact form of PARP was observed in all samples examined, whereas the Mr 85,000 PARP cleavage fragment was detected only after combined treatment with AS ODN#2 plus paclitaxel or mitoxantrone (Fig. 5D).

Enhanced Cytotoxic Effects of Chemotherapy in PC-3 Tumors in Vivo by Systemic Administration of AS TRPM-2 ODN. Athymic male mice bearing PC-3 tumors ~1 cm in diameter were randomly selected for treatment with AS ODN#2 plus paclitaxel, MM Co. ODN plus paclitaxel, AS ODN#2 plus mitoxantrone, or MM Co. ODN plus mitoxantrone. Mean tumor volume was similar at the beginning of treatment in each of these groups. After randomization, 10 mg/kg AS ODN#2 or MM Co. ODN were injected i.p. once daily for 28 days. From days 10 to 14 and from days 24 to 28, 0.5 mg polymeric micellar paclitaxel or 0.3 mg mitoxantrone was administered once daily by i.v. injection. As shown in Fig. 6, A and B, AS ODN#2 significantly enhanced the apoptotic effects of micellar paclitaxel and mitoxantrone in PC-3 tumors, reducing mean tumor volume by more than 80 and 60%, respectively, by 8 weeks after initiation of treatment. In addition, TUNEL staining detected a 5- or 3-fold increase in the numbers of apoptotic cells in the PC-3 tumors treated with AS ODN#2 plus micellar paclitaxel or mitoxantrone, respectively, compared with those treated with MM Co. ODN plus micellar paclitaxel or mitoxantrone. Under the experimental conditions used in the above in vivo experiments, no side effects associated with ODN treatment and/or chemotherapy were observed.

Discussion

In the prostate gland, despite the original hypothesis that TRPM-2 is a marker of programmed cell death (12, 23–25), several experimental and clinical studies have provided conflicting findings showing the dissociation of TRPM-2 expression from apoptosis (26–30). For example, increased TRPM-2 expression in dysplastic lesions of rat prostate was not associated with enhanced apoptotic activity (29). Introduction of the TRPM-2 gene into LNCaP prostate cancer cells renders them...
Fig. 6  Effects of combined treatment with AS TRPM-2 ODN plus chemotherapy on PC-3 tumor growth. A and B, mice bearing PC-3 tumors were randomly selected for treatment with AS ODN#2 plus micellar Taxol (paclitaxel) or MM Co. ODN plus micellar paclitaxel (A), or AS ODN#2 plus mitoxantrone or MM Co. ODN plus mitoxantrone (B). When PC-3 tumors became ~1 cm in diameter, 10 mg/kg AS ODN#2 or MM Co. ODN were daily injected i.p. for 28 days. From days 10 to 14 and from days 24 to 28, 0.5 mg micellar paclitaxel or 0.3 mg mitoxantrone was daily administered by i.v. injection. Tumor volume was measured once weekly and calculated by the formula: length $^3$ width $^3$ depth $^3$ 0.5236. Points, mean tumor volume in each experimental group containing eight mice; bars, SD. ** and *, differ from control ($P < 0.01$ and $P < 0.05$, respectively) by Student’s $t$ test. C–F, after completion of the same treatment schedule described in A and B, PC-3 tumors were harvested from each treatment group for detection of apoptosis using TUNEL staining. Sections of paraffin-embedded PC-3 tumors were stained with digoxigenin-dUTP antibody to identify apoptotic cells. C, PC-3 tumor after treatment with AS ODN#2 and micellar paclitaxel. D, PC-3 tumor after treatment with MM Co. ODN and micellar paclitaxel. E, PC-3 tumor after treatment with AS ODN#2 and mitoxantrone. F, PC-3 tumor after treatment with MM Co. ODN and mitoxantrone.
highly resistant to tumor necrosis factor-α-induced apoptosis (27). Furthermore, a close correlation between intracellular levels of TRPM-2 and tumor grade in human prostate cancer specimens has been reported (28). We also demonstrated previously that overexpression of TRPM-2 helps mediate AI progression against castration (31)- and chemotherapy-mediated apoptosis in androgen-dependent prostate cancer models. Collectively, these findings suggest that TRPM-2 up-regulation plays a protective role in normal and malignant prostate tissues against apoptosis induced by various kinds of stimuli and thereby may confer an aggressive phenotype during prostate cancer progression.

The limited efficacy of cytotoxic chemotherapy remains a major problem for the treatment of patients with advanced hormone refractory prostate cancer.

The lack of survival benefits with traditional cytotoxic chemotherapy in patients with hormone-refractory prostate cancer results from intrinsic chemoresistance and the limitation of toxicity on an elderly population. The chemoresistant phenotype in hormone-refractory prostate cancer is attributable, in part, to high levels of antiapoptotic genes, including Bcl-2, Bcl-xL, and TRPM-2, all of which are increased after androgen ablation and remain constitutively overexpressed in AI tumors (18, 36, 37).

Although no chemotherapeutic agent has demonstrated improved survival in patients with advanced prostate cancer, recent Phase II reports are documenting improved response rates in hormone-refractory disease (2, 36–38). For example, >50% of patients responded in one Phase II study of combined paclitaxel plus estramustine treatment (38). In two randomized trials, mitoxantrone in combination with prednisone was shown to produce significant palliative benefit compared with steroids alone (39, 40). Furthermore, recent preclinical studies have provided proof of principle evidence that targeting antiapoptotic genes using AS ODN enhances apoptosis induced by conventional cytotoxic chemotherapy (9–12). Therefore, in this study, we set out to screen for potent and sequence-specific ODNs targeted against human TRPM-2. The AS TRPM-2 ODN corresponding to the human TRPM-2 translation initiation site was the most potent sequence of the 10 AS ODN targeting various regions of TRPM-2 gene. To clarify the functional role of TRPM-2 expression in AI prostate cancer, we then tested the effects of AS TRPM-2 ODN on human prostate cancer PC-3 cell growth and whether AS TRPM-2 ODN could enhance the cytotoxic effects of paclitaxel and mitoxantrone in this model system.

Phosphorothioate AS TRPM-2 ODN used in this study significantly inhibited expression of TRPM-2 mRNA and protein in PC-3 cells both in vitro and in vivo. Sequence specificity was confirmed using a two-base TRPM-2 MM ODN, which had no effects on TRPM-2 expression in PC-3 cells. Despite a significant decrease in TRPM-2 expression after AS ODN treatment, no differences in PC-3 cell growth in vitro or PC-3 tumor growth in vivo was observed after AS TRPM-2 ODN treatment. These findings suggest that targeting and inhibiting TRPM-2 expression have no significant effects on cell proliferation in the absence of other apoptotic stimuli or cell death signals.

The administration of AS TRPM-2 ODN with chemotherapeutic agents, however, inhibited PC-3 cell growth both in vitro and in vivo through enhanced apoptosis. Pretreatment of PC-3 cells with AS enhanced apoptosis induced by these agents. Consistent with these in vitro studies, synergistic effects of combined use of AS TRPM-2 ODN plus chemotherapeutic agents were also observed in in vivo studies. Systemic administration of AS TRPM-2 ODN plus polymeric micellar paclitaxel or mitoxantrone suppressed the PC-3 tumor growth by 90 and 50%, respectively, compared with treatment with MM Co. ODN plus either agent. Detection of increased apoptotic cells after combined AS ODN and chemotherapy by TUNEL staining in PC-3 tumors suggests that decreased tumor progression rates after combined AS TRPM-2 ODN plus paclitaxel or mitoxantrone resulted from enhanced chemotherapy-induced apoptosis rather than decreased cell proliferation.

The results in the present study suggest that increased TRPM-2 helps mediate prostate cancer progression by inhibiting apoptotic cell death induced by several kinds of therapy, including cytotoxic chemotherapy. Decreasing TRPM-2-mediated chemoresistance by AS TRPM-2 ODN may provide a feasible and safe strategy to enhance chemosensitivity in hormone-refractory prostate cancer. The preclinical data presented here provide proof of principle support for designing clinical studies with combined AS TRPM-2 plus paclitaxel and/or mitoxantrone therapy for patients with hormone-refractory disease.

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References


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