Acquisition of Chemoresistant Phenotype by Overexpression of the Antiapoptotic Gene Testosterone-repressed Prostate Message-2 in Prostate Cancer Xenograft Models

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ABSTRACT

Testosterone-repressed prostate message-2 (TRPM-2) expression is highly up-regulated in normal and malignant prostate cells after androgen withdrawal. Although recent studies have suggested a protective role of TRPM-2 expression against apoptosis in several experimental models, the functional role of TRPM-2 in chemotherapy-induced apoptosis remains undefined. Here, we demonstrated that overexpression of TRPM-2 in human androgen-dependent LNCaP prostate cancer cells by stable transfection rendered them highly resistant to paclitaxel treatment than control LNCaP cells, with a 20-fold higher IC_{50} through the inhibition of apoptotic cell death. In mice bearing TRPM-2-overexpressing LNCaP tumors, tumor volume and serum prostate-specific antigen increased two to three times faster after castration and paclitaxel treatment compared with mice bearing control tumors. We then tested the efficacy of combined treatment with antisense TRPM-2 oligodeoxynucleotide (ODN) and paclitaxel in the mouse androgen-dependent Shionogi tumor model. Antisense TRPM-2 ODN treatment significantly enhanced paclitaxel chemosensitivity of Shionogi tumor cells in a dose-dependent manner, reducing the IC_{50} by 75%. Combined treatment of Shionogi cells with 500 nM antisense TRPM-2 ODN and 10 nM paclitaxel-induced apoptosis, either agent alone did not. Adjuvant administration of antisense TRPM-2 ODN and polymeric micellar paclitaxel after castration resulted in reduced TRPM-2 levels in vivo and a significant delay of emergence of androgen-independent recurrent Shionogi tumors compared with administration of either agent alone. Furthermore, combined treatment of mice bearing androgen-independent recurrent Shionogi tumors with antisense TRPM-2 ODN and micellar paclitaxel inhibited tumor growth compared with treatment with either agent alone. Collectively, these findings demonstrate that TRPM-2 overexpression helps confer a chemoresistant phenotype through inhibition of apoptosis, and that antisense TRPM-2 ODN may be useful in enhancing the effects of cytotoxic chemotherapy in hormone-refractory prostate cancer.

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer deaths in men in Western industrialized countries. To date, no therapy exists that surpasses androgen withdrawal for men with advanced disease, with symptomatic and/or objective response in ~80% of patients. However, progression to androgen independence ultimately occurs in nearly all of these cases (1). Several hundred clinical studies using traditional cytotoxic chemotherapeutic agents document objective response rates of <10% and no improved survival rates (2). Accordingly, progression to androgen independence remains the main obstacle to improving the survival and quality of life in patients with advanced disease, emphasizing the need for novel therapeutic strategies that target the molecular mechanism of the androgen- and chemoresistant phenotype of prostate cancer.

TRPM-2, also known as clusterin, sulfated glycoprotein-2, or apolipoprotein J, was first isolated from ram rete testes fluid (3) and has been proposed to have various biological functions, including tissue remodeling, reproduction, lipid transport, and apoptotic cell death (4). TRPM-2 was initially regarded as a marker for cell death, because its expression is highly up-regulated in various normal and malignant tissues undergoing apoptosis (5–8). Recent studies, however, report conflicting findings on the association between enhanced TRPM-2 expression and apoptotic activity (9–11). Similarly, TRPM-2 expression is increased in regressing normal prostate after androgen ablation (5, 12), and its up-regulation has been shown to be associated with antiapoptotic activity and disease progression in prostate cancer (13–15). We have recently reported that TRPM-2 expression in prostate cancer cells has a protective role against castration-induced apoptosis (16). However, the functional significance of TRPM-2 expression in apoptosis induced by chemotherapeutic agents has not been investigated.

Controlled study of the complex molecular processes associated with progression to androgen independence in prostate cancer has proved difficult, because few animal models exist that reproducibly mimic the clinical course of the disease in men. The AD Shionogi mouse mammary carcinoma model is particularly useful for testing the efficacy of agents targeting castration-induced apoptosis and their effects on time to progression of androgen independence. AD Shionogi tumors in intact male mice undergo complete regression after castration but recur as rapidly growing AI tumors after 1 month in a highly reproducible manner (17). Of the available human prostate cancer cell lines, only the LNCaP tumors are AD when xenografted into male immunodeficient mice, PSA secreting, and immortalized in vitro. As in human prostate cancer, serum PSA levels in the LNCaP tumor model are initially regulated by androgen and directly proportional to tumor volume, with loss of androgen-regulated PSA gene expression after castration as a surrogate end point of progression to androgen independence (18).

In the present study, we evaluated the effects of TRPM-2 overexpression on time to progression of androgen independence after castration and paclitaxel treatment in the LNCaP tumor model. We then evaluated the effects of paclitaxel treatment on TRPM-2 gene expression in Shionogi tumor cells and the effects of antisense TRPM-2 ODN on paclitaxel chemosensitivity using the Shionogi tumor model.

MATERIALS AND METHODS

Paclitaxel. Paclitaxel was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution of paclitaxel (1 mg/ml) was prepared with DMSO and diluted with PBS to the required concentrations before each in vitro experiment.

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3 The abbreviations used are: TRPM-2; testosterone-repressed prostate message-2; AI, androgen-independent; AD, androgen-dependent; PSA, prostate-specific antigen; ODN, oligodeoxynucleotide; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly(ADP-ribose) polymerase; CMV, cytomegalovirus; poly(A)~*~ mRNA, polyadenylated mRNA.

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Polymeric micellar paclitaxel used in the in vivo studies was generously supplied by Dr. Helen M. Burt (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada).

Antisense TRPM-2 ODN. Phosphorothioate ODN used in this study was obtained from Nucleic Acid-Protein Service Unit, University of British Columbia. The sequences of antisense TRPM-2 ODN corresponding to the mouse TRPM-2 translation initiation site were 5’-GCACAGCAGGAGAATCT-3’. A 2-base TRPM-2 mismatch ODN (5’-GCACAGCAGGAGATATTCAT-3’) was used as control.

LNCaP Sublines. LNCaP cells were kindly provided by Dr. Leland Chung (University of Virginia, Charlottesville, VA) and maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% heat-inactivated FCS. Steroid hormone-depleted charcoal-stripped media were prepared as described previously (19). A pRC-CMV expression vector containing the 1.6-kb cDNA fragment encoding human TRPM-2 was kindly provided by Dr. Martin Tenniswood (W. Alton Jones Cell Science Center, Lake Placid, NY). The expression vector was transfected into LNCaP cells by the liposome-mediated gene transfer method as described previously (20). Briefly, 2 x 10^5 LNCaP cells were plated in 6-cm plates. The next day, 5 μg of purified TRPM-2-cloned pRC-CMV or pRC-CMV alone (as a control) were added to LNCaP cells after a preincubation for 30 min with 5 μg of LipofectAMINE reagent and 3 μl of serum-free Opti-MEM (Life Technologies). Drug selection, in 300 μg/ml Geneticin (Sigma), was begun 3 days after the transfection. Colonies were harvested 2 weeks after drug selection using cloning cylinders and expanded to cell lines.

Assessment of in Vivo LNCaP Tumor Growth and Determination of Serum PSA Levels. One million cells of each LNCaP subline were inoculated s.c. with 0.1 ml of Matrigel (Becton Dickinson Labware, Lincoln Park, NJ) in the flank region of 6- to 8-week-old male athymic nude mice (BALB/c strain; Charles River Laboratory, Montreal, Quebec, Canada). Each experimental group consisted of six mice. Mice were castrated via a scrotal approach when tumors reached 200–300 mm^3 in volume, and from 10 to 14 days after...
castration, 0.5 mg of polymeric micellar paclitaxel was administered once daily by i.v. injection. Tumor volume was measured once weekly and calculated by the formula length × width × depth × 0.5236 (19). Blood samples were obtained with tail vein incisions of mice once weekly. Serum PSA levels were determined by an enzymatic immunoassay kit with a lower limit of sensitivity of 0.2 μg/liter (Abbott IMX, Montreal, Quebec, Canada) according to the manufacturer’s protocol. Data points were reported as mean values ± SD.

Shionogi Tumor Growth. The Toronto subline of the transplantable SC-115 AD mouse mammary carcinoma was used in all experiments (21). Shionogi tumor cells were maintained in DMEM (Life Technologies) supplemented with 5% heat-inactivated FCS. For in vivo study, ~5 × 10⁶ cells of the Shionogi carcinoma were injected s.c. into adult male DDS/S strain mice. When Shionogi tumors became 1–2 cm in diameter, usually 2–3 weeks after injection, castration was performed through an abdominal incision under methoxyflurane anesthesia. Details of the maintenance of mice, tumor stock, and operative procedures are described in a previous publication (22).

Treatment of Cells with ODN. In vitro–cultured cells were treated with various concentrations of ODN after a preincubation for 20 min with 4 μg/ml Lipofectin (Life Technologies) in serum free Opti-MEM. Media containing ODN and Lipofectin was replaced 4 h later with standard culture medium described above.

Northern Blot Analysis. Total RNA was isolated from in vitro–cultured cells and in vivo tumor tissues by the acid-guanidium thiocyanate-phenol-chloroform method. Poly(A)⁺ mRNAs was then purified from total RNA using oligo(dT)–cellulose (Pharmacia Biotech Inc., Uppsala, Sweden). Five micrograms of poly(A)⁺ mRNA from each sample were subjected to electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nylon membranes (Amersham, Arlington Heights, IL) overnight according to standard procedure (17). The RNA blots were hybridized with a mouse TRPM-2 cDNA probe labeled with [³²P]dCTP by random primer labeling. After stripping, the membranes were rehybridized with a mouse G3PDH cDNA probe. These probes were generated by reverse transcription-PCR from total RNA of mouse brain using primers 5'-AATGAGCTCCAAGAACTG-TCCACT-3' (sense) and 5'-AAAGGACGCTGTCTATGATGCCAGAT-3' (antisense) for TRPM-2 and 5'-ATGGTGAAGGTCGGTGTGAACGGAT-3' (sense) and 5'-AAAGTTGGATCTGAGCATCTT-3' (antisense) for G3PDH. Density of bands for TRPM-2 was normalized against that of G3PDH by densitometric analysis.

Western Blot Analysis. The expression of TRPM-2 and PARP protein in cultured cells and tumor tissues was determined by Western blot analysis as described previously (20). Briefly, samples containing equal amounts of protein (15 μg) were electrophoresed on a SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The filters were blocked in PBS containing 5% nonfat milk powder at 4°C overnight and then incubated for 1 h with an anti-human TRPM-2 goat polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-human PARP mouse monoclonal antibody (PharMingen, Mississauga, Ontario, Canada), or anti-rat β-tubulin mouse monoclonal antibody (Chemicon International Inc., Temecula, CA). The filters were then incubated for 30 min with horseradish peroxidase-conjugated anti-goat or mouse IgG antibody (Amersham), and specific proteins were detected using an enhanced chemiluminescence system (Amersham).

In Vitro Cell Growth Assays. In the in vitro growth of LNCaP and Shionogi tumor cells was assessed by the in vitro mitogenic assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, respectively, as described previously (19, 20). Briefly, 3 × 10⁴ cells were seeded in each well of 96-well microtiter plates and allowed to adhere overnight. After treatment with various concentrations of paclitaxel and/or ODN, LNCaP cells were fixed with 1% glutaraldehyde (Sigma) and stained with 0.5% crystal violet (Sigma). DNA fragmentation analysis. The nucleosomal DNA degradation was analyzed as described previously with a minor modification (20). Briefly, 1 × 10⁵ cultured cells were seeded in 5-cm culture dishes and allowed to adhere overnight. After the indicated treatment with paclitaxel and/or ODN, 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 3 M sodium acetate 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 3 M 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 Shionogi Tumor Growth. To determine whether combined antisense TRPM-2 ODN and paclitaxel treatment delays time to AI recurrence after castration compared with either agent alone, male DDS/S mice bearing Shionogi tumors were castrated and randomly selected for treatment with antisense TRPM-2 ODN alone (group 1), mismatch control ODN alone (group 2), antisense TRPM-2 ODN plus paclitaxel (group 3), or mismatch control ODN plus paclitaxel (group 4). Each experimental group consisted of seven mice. Beginning the day of castration, 12.5 mg/kg antisense TRPM-2 or
Increased Resistance to Paclitaxel by Overexpression of TRPM-2 in LNCaP Cells in Vitro. Western blot analysis was used to examine TRPM-2 protein expression levels in the LNCaP sublines. As shown in Fig. 1A, abundant levels of both unprocessed (60-kDa) and mature (40-kDa) forms of TRPM-2 protein were detected in TRPM-2-transfected clones (LNCaP/T1 to LNCaP/T4) at almost the same levels, whereas the parental LNCaP (LNCaP/P) and the control vector-transfected cell line (LNCaP/C) did not express detectable TRPM-2 protein levels. The four TRPM-2-transfected clones showed almost the same results in the subsequent experiments; therefore, we hereafter report only the data of LNCaP/P, LNCaP/C, LNCaP/T1, and LNCaP/T2.

To determine whether TRPM-2 overexpression confers a chemoresistant phenotype on LNCaP cells in vitro, the growth rates of LNCaP sublines after paclitaxel treatment were analyzed using normal and charcoal-stripped media. As shown in Fig. 1, B and C, LNCaP/T1 and LNCaP/T2 exhibited significantly higher resistance to paclitaxel compared with LNCaP/P and LNCaP/C both in normal and charcoal-stripped media ($P < 0.01$ for both). Overexpression of TRPM-2 in LNCaP cells increased the IC$_{50}$ of paclitaxel 5-fold (from 1.5 to 8 nM) in normal media and 20-fold (from 0.01 to 0.4 nM) in charcoal-stripped media (Fig. 1B).

The induction of apoptosis in LNCaP sublines in normal media treated with 1 nM paclitaxel for 72 h was assessed by DNA degradation assay and Western blot analysis of PARP protein, a substrate of the caspases activated during the process of apoptotic execution (23). The characteristic apoptotic DNA ladders were detected in LNCaP/P and LNCaP/C but not in LNCaP/T1 and LNCaP/T2 (Fig. 1D). Similarly, the $M_p$ 116,000 intact form of PARP was observed in all of LNCaP sublines, whereas the $M_p$ 85,000 PARP cleavage fragment was detected after paclitaxel treatment only in LNCaP/P and LNCaP/C (Fig. 1E).

Acquisition of Resistant Phenotype to Paclitaxel by Overexpression of TRPM-2 in the LNCaP Tumor Model in Vivo. To determine whether TRPM-2 overexpression confers resistance to paclitaxel treatment in vivo, 1 x 10$^6$ cells of each cell line (LNCaP/P, LNCaP/C, LNCaP/T1, or LNCaP/T2) were inoculated s.c. in male nude mice. When tumors reached 200–300 mm$^3$, mice were castrated. Beginning 10 days after castration, 0.5 mg of polymeric micellar paclitaxel was administered i.v. once daily for 5 days. LNCaP/P and LNCaP/C tumor growth decreased by 61 and 57%, respectively, by 4 weeks after castration and remained below precastrate volumes by 10 weeks after castration. In contrast, LNCaP/T1 and LNCaP/T2 tumor volume decreased by 11 and 28%, respectively, by 4 weeks after castration and thereafter increased 2.4- and 1.9-fold, respectively, by 10 weeks after castration (Fig. 2A). Serum PSA in mice bearing LNCaP/P and LNCaP/C tumors decreased by 77 and 75%, respectively, by 1 week after castration and remained below precastrate levels by 10 weeks after castration. In comparison, serum PSA in mice bearing LNCaP/T1 and LNCaP/T2 tumors decreased by 51 and 55%, respectively, before increasing 1.6- and 1.4-fold above precastrate levels, respectively, by 10 weeks after castration (Fig. 2B).

Changes in TRPM-2 Expression in Shionogi Tumor Cells after Antisense TRPM-2 ODN and Paclitaxel Treatment. Northern blot analysis was used to determine the effects of paclitaxel treatment on TRPM-2 mRNA expression in Shionogi tumor cells. As shown in Fig. 3A, TRPM-2 mRNA induction increased in a dose-dependent manner by paclitaxel treatment at concentrations up to 10 nM. Time course experiments demonstrated that paclitaxel-induced TRPM-2 mRNA up-regulation peaked by 48 h after treatment and began decreasing by 72 h after treatment (Fig. 3B).

We then examined the effects of combined treatment with antisense TRPM-2 ODN and paclitaxel on TRPM-2 mRNA expression in
Shionogi cells. As shown in Fig. 3C, 500 nM antisense TRPM-2 ODN combined with 10 or 50 nM paclitaxel decreased TRPM-2 mRNA levels by 85 or 70%, respectively, compared with 500 nM mismatch control ODN treatment.

Synergistic Effects of Antisense TRPM-2 ODN and Paclitaxel Treatment on Induction of Apoptosis in Shionogi Tumor Cells. To examine whether treatment with antisense TRPM-2 ODN enhances the paclitaxel-induced cytotoxicity, Shionogi tumor cells were treated with various concentrations of antisense TRPM-2 ODN once daily for 2 days and then incubated with various concentrations of paclitaxel for 2 days. As shown in Fig. 4A, antisense TRPM-2 ODN treatment significantly enhanced paclitaxel chemosensitivity in a dose-dependent manner \((P < 0.01)\), reducing the IC\(_{50}\) of paclitaxel from 100 to 25 nM, whereas mismatch control ODN had no effect. Dose-dependent synergy between antisense TRPM-2 ODN and paclitaxel was also observed by increasing the antisense ODN concentration when paclitaxel concentration was fixed at 10 nM \((P < 0.01)\). Treatment of Shionogi tumor cells with antisense TRPM-2 ODN significantly enhanced the sensitivity to paclitaxel \((P < 0.01)\).

DNA fragmentation assay and Western analysis of PARP protein were used to evaluate effects of combined antisense TRPM-2 ODN (500 nM) and paclitaxel (10 nM) treatment on apoptosis induction. After the same treatment schedule described above, characteristic apoptotic DNA laddering was observed only after combined treatment with antisense TRPM-2 ODN and paclitaxel (Fig. 4C). Similarly, cleavage of PARP protein was detected only after combined antisense TRPM-2 ODN and paclitaxel treatment (Fig. 4D).

Delayed Hormone-refractory Recurrence of Shionogi Tumors in Vivo by Combined Antisense TRPM-2 ODN and Paclitaxel Treatment. Male mice bearing Shionogi tumors between 1 and 2 cm in diameter were randomly selected for treatment with either antisense TRPM-2 ODN alone, mismatch control ODN alone, antisense TRPM-2 ODN plus micellar paclitaxel, or mismatch control ODN plus micellar paclitaxel. Mean tumor volume was similar at the beginning of treatment in all four treatment groups. Beginning the day of castration, 12.5 mg/kg antisense TRPM-2 ODN was administered i.p. once daily for 15 days. Beginning 10 days after castration, 0.5 mg of polymeric micellar paclitaxel was administered i.v. once daily for 5 days. During an observation period of 60 days after castration, AI tumors recurred in four of seven mice after a median of 53 days in antisense TRPM-2 ODN plus micellar paclitaxel treatment group, whereas AI tumors recurred in all mice after a median of 28, 39, and 42 days in the mismatch control ODN treatment group, antisense TRPM-2 ODN treatment group, and mismatch control ODN plus micellar paclitaxel treatment group, respectively \((P < 0.01)\).
Mean tumor volume at day 60 after castration was 2789, 3481, 712, and 1276 mm$^3$ in the antisense TRPM-2 ODN, mismatch control ODN, antisense TRPM-2 ODN plus micellar paclitaxel, and mismatch control ODN plus micellar paclitaxel. Beginning the day of castration, 12.5 mg/kg antisense TRPM-2 ODN or mismatch control ODN was injected i.p. once daily for 15 days. Beginning 10 days after castration, 0.5 mg of micellar paclitaxel was injected i.v. once daily for 5 days. Tumor volume was measured twice weekly and calculated by the formula length $\times$ width $\times$ depth $\times$ 0.5236. Each point represents the mean tumor volume in each experimental group containing seven mice $\pm$ SD. Mean tumor volume in mice treated with antisense TRPM-2 ODN alone was significantly smaller than that in mice treated with antisense TRPM-2 ODN alone or mismatch control ODN plus micellar paclitaxel ($P < 0.001$). Begin the time to progression of androgen independence in mice treated with antisense TRPM-2 ODN alone or mismatch control ODN plus micellar paclitaxel was significantly delayed compared with mice treated with antisense TRPM-2 ODN alone or mismatch control ODN plus micellar paclitaxel ($P < 0.01$).

Time to sacrifice was delayed in the other two treatment groups. Combined antisense TRPM-2 ODN plus paclitaxel treatment resulted in the most significant delay in tumor progression, producing a mean tumor volume $\sim$30–40% lower than in the mismatch control ODN plus paclitaxel treatment group ($P < 0.05$).

Northern and Western blot analyses were used to examine the effects of combined in vivo treatment with antisense TRPM-2 ODN and paclitaxel on TRPM-2 mRNA expression and cleavage of PARP protein in AI Shionogi tumors, which were harvested after completion of the same treatment schedule described above. Consistent with the in vitro results, antisense TRPM-2 ODN treatment resulted in a substantial reduction in TRPM-2 mRNA in AI Shionogi tumors (Fig. 6B). Furthermore, the $M_r$ 85,000 PARP cleavage fragment was de-
tectable in AI Shionogi tumors only after combined treatment with antisense TRPM-2 ODN and micellar paclitaxel (Fig. 6C).

**DISCUSSION**

TRPM-2 expression is highly up-regulated in several tissues undergoing apoptosis, including normal prostate, and prostate and breast cancer xenograft models after hormone withdrawal (5–8, 12). Although TRPM-2 expression was initially regarded as a marker for cell death, its biological function in this process is poorly defined (9–11, 13–15, 25). Accumulating evidence suggests that TRPM-2 is a cell survival gene that protects cells from apoptotic death. For example, overexpression of TRPM-2 in LNCaP prostate cancer cells enhances resistance to apoptosis induced by tumor necrosis factor-α (13). Furthermore, Steinberg et al. (14) reported a close correlation between staining intensity of TRPM-2 by immunohistochemical analysis and Gleason pattern in human prostate cancer specimens. We also demonstrated that TRPM-2 expression renders prostate cancer cells more resistant to androgen ablation and helps mediate progression of androgen independence after castration (16). Collectively, these findings suggest a protective role of TRPM-2 against apoptosis induced by various types of stimuli; however, the significance of TRPM-2 expression in chemotherapy-induced apoptosis has not been evaluated.

The efficacy of chemotherapy for patients with prostate cancer remains limited for various reasons, including inherent chemoresistance, pharmaceutical mechanism of chemotherapeutic action, and inability of elderly patients to tolerate its toxicity (2, 24). To date, no chemotherapeutic agent has demonstrated improved survival in patients with advanced prostate cancer, emphasizing the need for novel therapeutic strategies that target the molecular basis of androgen resistance and chemoresistance of prostate cancer. We have recently shown that antisense Bcl-2 ODN delayed progression to androgen independence (17) and enhanced paclitaxel chemosensitivity in the Shionogi tumor model (24). These findings illustrate that targeting an antiapoptotic gene with sequence-specific antisense ODN can result in enhanced apoptosis after androgen withdrawal and conventional cytotoxic chemotherapy. The objectives of this study were to examine whether TRPM-2 overexpression confers resistance to paclitaxel and to determine whether antisense TRPM-2 ODN could enhance paclitaxel chemosensitivity and delay emergence of AI tumors beyond that achieved with either agent alone.

We initially evaluated the effects of TRPM-2 overexpression on paclitaxel chemosensitivity using TRPM-2-transfected LNCaP cells and observed that TRPM-2 transfectants were more highly resistant to paclitaxel both *in vitro* and *in vivo* through the inhibition of apoptotic cell death. These findings provide the first evidence that TRPM-2 overexpression protects prostate cancer cells from paclitaxel-induced apoptosis, and its up-regulation may contribute to the chemoresistant phenotype in prostate cancer. Increased expression of TRPM-2 after paclitaxel treatment and androgen withdrawal is likely an adaptive response, which helps the cell survival against a cell death signal. It follows that inhibition of TRPM-2 up-regulation precipitated by castration and paclitaxel treatment may delay progression of androgen independence through enhanced castration- and paclitaxel-induced apoptosis. Antisense ODNs are chemically modified single-stranded DNA fragments complementary to mRNA regions of a target gene, which form RNA-DNA duplexes and thereby reduce gene expression (26). The potential problems of rapid intracellular degradation can be overcome by phosphorothioate modification of ODNs, which are more resistant to nuclease digestion. After parenteral administration, phosphorothioate ODN becomes associated with high-capacity, low-affinity serum-binding proteins (27). Antisense ODNs therefore offer one strategy to specifically target TRPM-2 gene expression.

Phosphorothioate antisense TRPM-2 ODN corresponding to the mouse TRPM-2 translation initiation site used in this study inhibited TRPM-2 mRNA expression in a dose- and sequence-dependent manner, even after paclitaxel treatment, which increases TRPM-2 expression. Furthermore, treatment of Shionogi cells with antisense TRPM-2 ODN reduced the IC₅₀ of paclitaxel by 75% and enhanced paclitaxel-induced apoptosis. Systemic administration of antisense TRPM-2 ODN and micellar paclitaxel *in vivo* significantly delayed time to emergence of AI tumors compared with either agent alone and also enhanced regression of established AI tumors. Although early adjuvant antisense TRPM-2 ODN treatment after castration delayed progression of androgen independence, treatment with antisense TRPM-2 ODN alone had no effect on growth rates of established AI tumors. However, combined treatment with antisense TRPM-2 ODN plus paclitaxel decreased TRPM-2 mRNA expression and accelerated apoptosis induction in AI Shionogi tumors *in vivo*. These findings illustrate the efficacy of combined antisense TRPM-2 ODN and paclitaxel treatment for cooperatively delaying progression to androgen independence.

Integration and appropriate timing of combination therapies, based on changes in expression of functionally relevant genes after androgen ablation, may help delay progression to androgen independence. The results in the present study provide proof of principle for two potential strategies to delay emergence of the AI phenotype. The first strategy would initiate treatment earlier to enhance castration-induced apoptosis by targeting the antiapoptotic TRPM-2 gene up-regulation by androgen ablation with antisense TRPM-2 ODN. The second strategy would attempt to enhance sensitivity to conventional chemotherapy by reduction of TRPM-2-mediated chemoresistance with antisense TRPM-2 ODN. The preclinical data presented here provide support for clinical studies with combined antisense TRPM-2 ODN and paclitaxel therapy for advanced prostate cancer.

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