

SOD Mimetics: A Novel Class of Androgen Receptor Inhibitors That Suppresses Castration-Resistant Growth of Prostate Cancer

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Abstract

Advanced prostate cancer is the second leading cause of cancer-related deaths among American men. The androgen receptor (AR) is vital for prostate cancer progression, even in the face of castrate levels of serum testosterone following androgen ablation therapy, a mainstay therapy for advanced prostate cancer. Down-regulation of superoxide dismutase 2 (SOD2), a major intracellular antioxidant enzyme, occurs progressively during prostate cancer progression to advanced states and is known to promote AR activity in prostate cancer. Therefore, this study investigated the effects of SOD mimetics on AR expression and function in AR-dependent LNCaP, CWR22Rv1, and LAPC-4AD prostate cancer cells. Treatment with Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a SOD mimetic, not only lowered cellular superoxide levels but also concomitantly attenuated AR transcriptional activity and AR target gene expression in a dose- and time-dependent manner, in the presence and absence of dihydrotestosterone, the major endogenous AR agonist. Inhibition of AR by Tempol was mediated, in large part, by its ability to decrease AR protein via increased degradation, in the absence of any inhibitory effects on other nuclear receptors. Inhibitory effects of Tempol on AR were also reproducible with other SOD mimetics, MnTBAP and MnTMPyP. Importantly, effects of Tempol on AR function were accompanied by significant *in vitro* and *in vivo* reduction in castration-resistant prostate cancer (CRPC) survival and growth. Collectively, this study has shown for the first time that SOD mimetics, by virtue of their ability to suppress AR function, may be beneficial in treating the currently incurable CRPC, in which SOD2 expression is highly suppressed. *Mol Cancer Ther*; 11(1); 87–97. ©2011 AACR.

Introduction

The androgen receptor (AR), a transcription factor that mediates the biological effects of androgens, testosterone, and dihydrotestosterone (DHT), is vital for the development and progression of prostate cancer. After an initial response to androgen ablation therapy, which suppresses AR signaling, the majority of advanced tumors eventually transition to the currently incurable androgen-independent or castration-resistant prostate cancer (CRPC; refs. 1, 2). Importantly, CRPC continues to be highly dependent on the persistent expression and function of AR to survive and progress (3, 4). Studies reporting significant inhibition of *in vitro* and *in vivo* growth of CRPC following disruption

of AR expression and/or function (5, 6) have generated much interest in the AR as a key therapeutic target and have intensified efforts to uncover potent AR inhibitors.

Elevated levels of cellular reactive oxygen species (ROS) significantly contribute to the initiation and progression of cancer (7, 8), and the degree of ROS generation correlates with the aggressive phenotype of prostate cancer (8). Cellular ROS levels are normally kept in check by a very efficient cellular detoxifying system, which includes the mitochondrial antioxidant enzyme, superoxide dismutase 2 (SOD2), which catalyzes the conversion of superoxide (O_2^-) to hydrogen peroxide (9). The expression of SOD2 or manganese SOD is commonly downregulated in cancer cells, and restoration of SOD2 activity via SOD2 overexpression significantly inhibits *in vitro* and *in vivo* tumor growth, including prostate cancer growth (10–12). SOD2 levels progressively decline during the transition from prostatic intraepithelial neoplasia to androgen-dependent (AD) prostate cancer to CRPC (13–15). Strikingly, SOD2 levels in CRPC are just 11% of that found in AD prostate cancer (15), supporting the notion that there may be selection for decreased SOD2 expression in advanced prostate cancer. SOD2 downregulation increases AR transcriptional activity, and this effect is reversed with the antioxidant, *N*-acetylcysteine (16).

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These findings raise the possibility that therapies aimed at specifically augmenting SOD2 activity might offer an effective and feasible means of treating CRPC, by directly targeting the key player, the AR.

Use of SOD mimetics to augment the natural antioxidant defenses of the cell has been beneficial in animal models of a number of neoplastic and nonneoplastic diseases, in which oxidative stress is implicated in disease progression (17, 18). As oxidative stress is an integral component of and contributor to cancer progression (7, 8), use of SOD mimetics not only lowers tumor incidence (19–21) but also markedly inhibits *in vitro* and *in vivo* tumor growth (22–26). Although the effects of SOD mimetics on a variety of cancers have been investigated, their effects on AR function and prostate cancer growth was hitherto unknown. Here, we show for the first time that SOD mimetics are effective in suppressing AR activity and *in vitro* and *in vivo* CRPC growth.

Materials and Methods

Reagents, plasmid constructs, luciferase reporter gene assay, cell-cycle analysis, immunoprecipitation, and quantitative reverse transcriptase PCR analysis

See Supplementary Information Materials and Methods.

Tumor cell lines and culture

LNCaP, CWR22Rv1, and PC-3 prostate cancer cells [American Type Culture Collection (ATCC)] were maintained in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells are authenticated by ATCC. LAPC-4AD prostate cancer cells (provided by Dr. Charles Sawyers, Memorial Sloan-Kettering Cancer Center, New York, NY) were maintained in modified IMEM medium (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin.

Western blot analysis

After the indicated treatments, whole-cell lysates were prepared and subjected to Western blot analysis as described previously (27). After incubation with primary antibody, the blots were probed with an IRDye-labeled secondary antibody (LI-COR Biosciences). Scanning of the blots and densitometric analysis of protein bands was done using the LI-COR Odyssey IR Imaging System. The intensity of a target protein band in each sample was normalized to that of β -actin in the same sample and expressed as a fold change, with expression in the control set at 1.

Analysis of cellular superoxide levels

This assay is based on the principle that hydroethidine is oxidized by O_2^- and converted to fluorescent hydroxyethidium (28). After the indicated treatments, cells were washed with PBS and incubated with 15 μ M/L hydroethidine in serum-free medium at 37°C for 2 hours. Fluorescence was read at 510 nm_{Ex}/580 nm_{Em} and represented as relative fluorescence units (RFU).

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Cell viability assay

Cell viability was determined using the CellTiter-Fluor Cell Viability Assay (Promega) as per the manufacturer's protocol. Cell viability was assessed by measuring live cell protease activity, which is determined by measuring fluorescence emitted by the fluorogenic, cell-permeant peptide substrate, GF-AFC. The intensity of fluorescence generated is directly proportional to cell viability and was read at 400 nm_{Ex}/505 nm_{Em}.

In vivo xenograft tumor growth

Animal procedures were in accordance with UT Southwestern Medical Center Institutional Animal Care and Use Committee-approved animal protocol. Ten to 12 week old male, nonobese diabetic severe-combined immunodeficient mice were injected subcutaneously in the flank region with 10⁷ LAPC-4AD cells suspended in Matrigel. Tumor volume was calculated by the formula: length \times width²/2 (29). Once tumor volume reached 275 \pm 75 mm³, the mice were castrated. Following castration, once tumors exhibited the first measurable increase in growth, mice were randomized into 2 groups: (i) Control diet (n = 6) and (ii) Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) diet (n = 4). Tempol treatment was in the form of bacon-flavored mouse chow mixed with powdered Tempol at a concentration of 58 mmol/L (10 mg/g; Bioserv). Control animals received bacon-flavored mouse chow minus Tempol. The animals were individually caged and food intake monitored at regular intervals. Individual relative tumor volume (RTV) at any given time (x) was calculated as follows: V_x/V_1 , in which V_x is the tumor volume at time x and V_1 is the tumor volume at the start of treatment. Mean RTV and SD for control and Tempol groups were calculated at the end of each week of treatment and statistical significance assessed by unpaired t test. Animals were euthanized 9 weeks after commencement of control or Tempol diet and tumors excised and processed for Western blotting.

Statistical analyses

Data were tested for statistical significance by 1-way ANOVA followed by Tukey post hoc test or by unpaired t test where appropriate (as indicated in the figure legends) using the Graph-Pad InStat software. Data were considered statistically significant only if P < 0.05.

Results

Tempol-mediated decline in cellular O_2^- levels is accompanied by a significant reduction in AR transcriptional activity

Tempol (Supplementary Diagram S1) is a cell membrane permeable, water soluble compound belonging to the nitroxide class of SOD mimetics (30). Tempol dose dependently attenuated O_2^- levels in AR-dependent

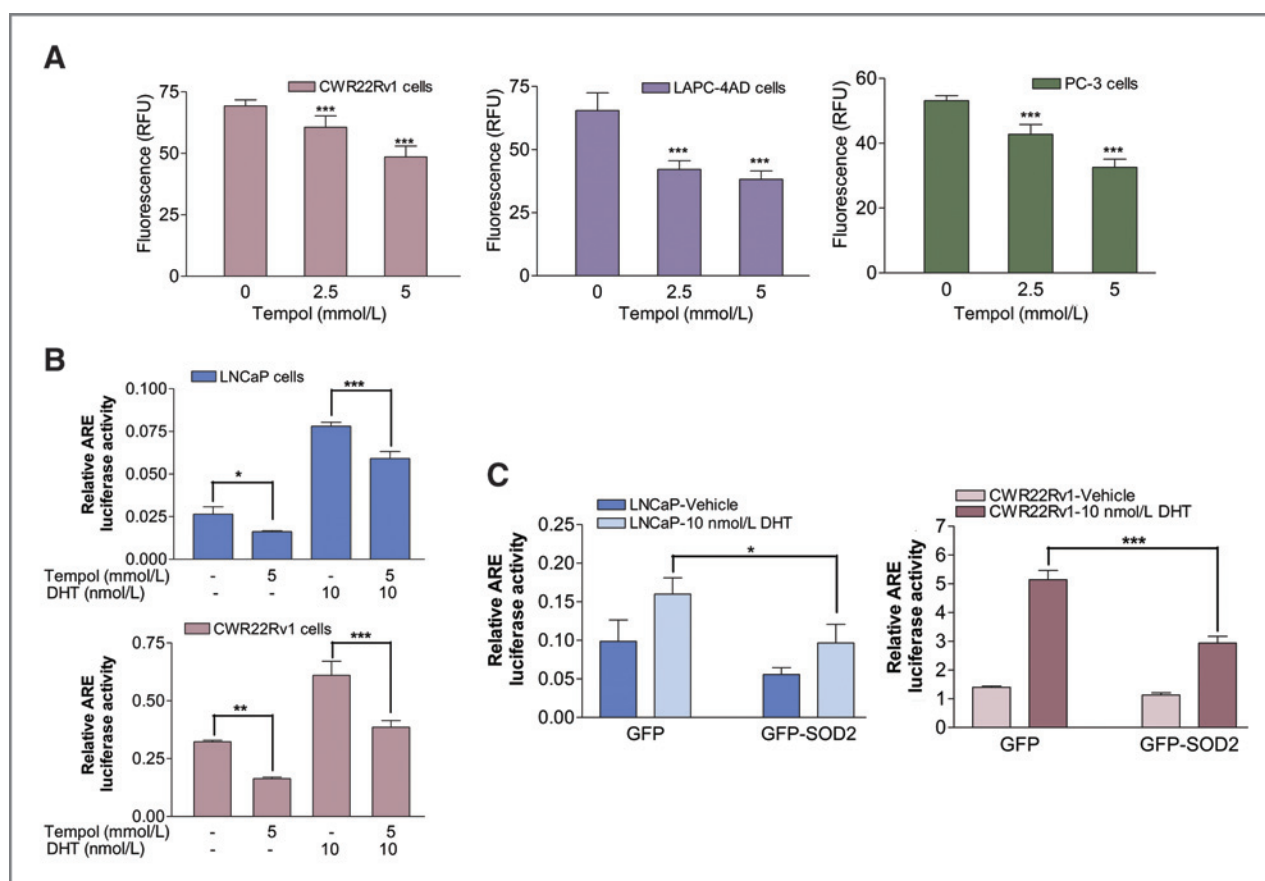


Figure 1. Tempol-mediated decline in cellular O_2^- levels is accompanied by a significant reduction in AR transcriptional activity. **A**, Tempol decreases cellular O_2^- levels. Cells were treated with 0 to 5 mmol/L Tempol in phenol red-free (PR free), serum-free medium for 24 hours, followed by O_2^- analysis with hydroethidine. Data are mean \pm SD. Statistical significance assessed by 1-way ANOVA is versus the untreated cells. **B**, Tempol inhibits ARE-driven luciferase reporter activity, both in the presence and absence of DHT. Cells were transiently cotransfected with plasmids pGL3-TK-3xARE-FLuc and pGL4.75-RLuc, followed by treatment with 5 mmol/L Tempol \pm 10 nmol/L DHT for 24 hours. Relative luciferase activity was determined and represented as mean \pm SD. Statistical significance was assessed by unpaired *t* test. **C**, effect of Tempol on ARE-driven luciferase reporter activity is closely mimicked by SOD2 overexpression. Cells were transiently cotransfected with GFP-SOD2 or GFP plasmids, along with pGL3-TK-3xARE-FLuc and pGL4.75-RLuc constructs, and subsequently treated for 24 hours with vehicle or 10 nmol/L DHT. Relative luciferase activity was determined and represented as mean \pm SD. Statistical significance was assessed by unpaired *t* test.

CWR22Rv1 and LAPC-4AD cells, as well as in AR-negative PC-3 prostate cancer cells (Fig. 1A).

The AR-dependent prostate cancer cell lines used in this study express AR variants that represent the spectrum found in CRPC: LNCaP cells harbor AR with a mutation in the ligand-binding domain (LBD), CWR22Rv1 cells express constitutively active, LBD-lacking, truncated AR and full-length AR with a mutated LBD, and LAPC-4AD cells predominantly express wild-type AR (31, 32). Tempol significantly decreased androgen response element (ARE)-driven luciferase reporter activity, in the presence and absence of DHT, the major AR agonist (Fig. 1B), suggesting that AR transcriptional activity is attenuated by Tempol. To determine how closely inhibition of AR transcriptional activity by Tempol mimics the effect of SOD2, we assessed ARE-driven luciferase reporter activity after SOD2 overexpression. Inhibition of ARE-driven luciferase reporter activity after SOD2 overexpression

(Fig. 1C) closely paralleled that following Tempol treatment, both in the presence and absence of DHT (Fig. 1B).

The Tempol-mediated decrease in AR transcriptional activity was reflected in a significant decline in mRNA levels of AR target genes, *PSA* and *FKBP5*. Inhibitory effects of Tempol on AR target gene expression were evident in the presence and absence of DHT (Fig. 1D, Supplementary Fig. S1), as well as in a dose-dependent (Fig. 1E, Supplementary Fig. S2) and time-dependent manner (Supplementary Fig. S3). Dose-response studies revealed that Tempol decreased *PSA* mRNA levels by 83%, 80%, and 60% in LNCaP, CWR22Rv1, and LAPC-4AD cells, respectively, relative to that after vehicle treatment (Fig. 1E). A similar trend was seen with *FKBP5* mRNA levels (Supplementary Fig. S2). A 2.5 mmol/L dose of Tempol elicited a significant, time-dependent decline in *PSA* and *FKBP5* mRNA levels in LNCaP, CWR22Rv1, and LAPC-4AD cells at each time point under

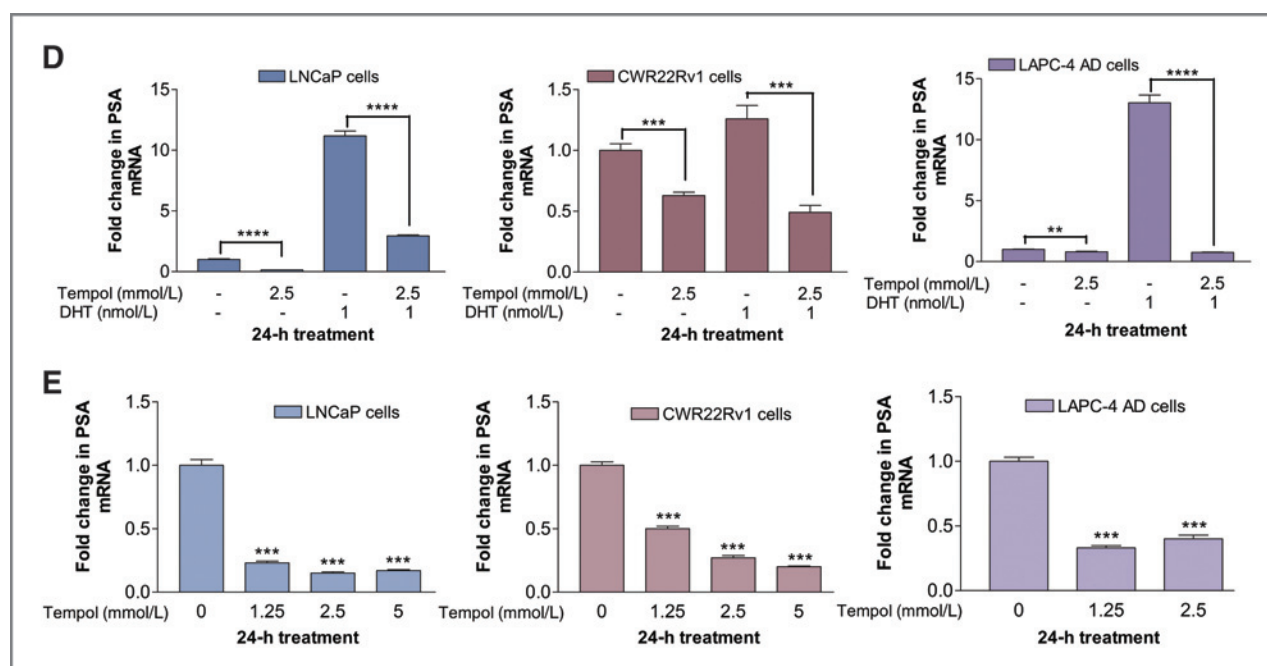


Figure 1. (Continued) D, Tempol decreases PSA mRNA, both in the presence and absence of DHT. Cells were treated for 24 hours with 1 nmol/L DHT \pm 2.5 mmol/L Tempol in PR-free medium containing 5% charcoal-stripped (C/S) FBS. PSA mRNA was assessed by qPCR and expressed as fold change \pm SD ($\Delta\Delta C_t$ method), with mRNA levels in the vehicle-treated control set at 1. Statistical significance was assessed by unpaired *t* test. E, Tempol elicits a dose-dependent decline in PSA mRNA. Cells were treated for 24 hours with increasing doses of Tempol in serum-free medium, followed by qPCR analyses of PSA mRNA, which was expressed as fold change \pm SD, with mRNA levels in the untreated control set at 1. Statistical significance assessed by 1-way ANOVA is versus the control. For Fig. 1A–E, *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

study, relative to that in the vehicle-treated cells at the same time points (Supplementary Fig. S3).

These data suggest that Tempol is effective in suppressing AR transcriptional activity in prostate cancer cells, and this suppression is closely tied to its ability to lower cellular O_2^- levels.

Tempol decreases AR protein without significantly altering the expression of other nuclear receptors

To determine whether the Tempol-mediated inhibition of AR transcription was due to a decrease in AR protein, we next assessed changes in AR protein following treatment with Tempol. Although DHT is known to increase AR protein (33), Tempol markedly decreased AR protein, both in the absence and presence of DHT (Fig. 2A). Remarkably, Tempol not only significantly reduced protein levels of full-length AR (high molecular weight AR band in LNCaP, CWR22Rv1, and LAPC-4AD cells) but also decreased levels of truncated AR (lower molecular weight AR band in CWR22Rv1 cells; Fig. 2A). For all AR Western blot analyses, we used an antibody targeting the N-terminal domain of AR; hence, it can detect full-length AR and truncated ARs that lack the C-terminal LBD (31, 34).

A consistent dose-dependent decline in full-length AR (LNCaP, CWR22Rv1, and LAPC-4AD cells) and truncated AR (CWR22Rv1 cells) was observed following treatment with Tempol (Fig. 2B). A 2.5 mmol/L Tempol elicited a

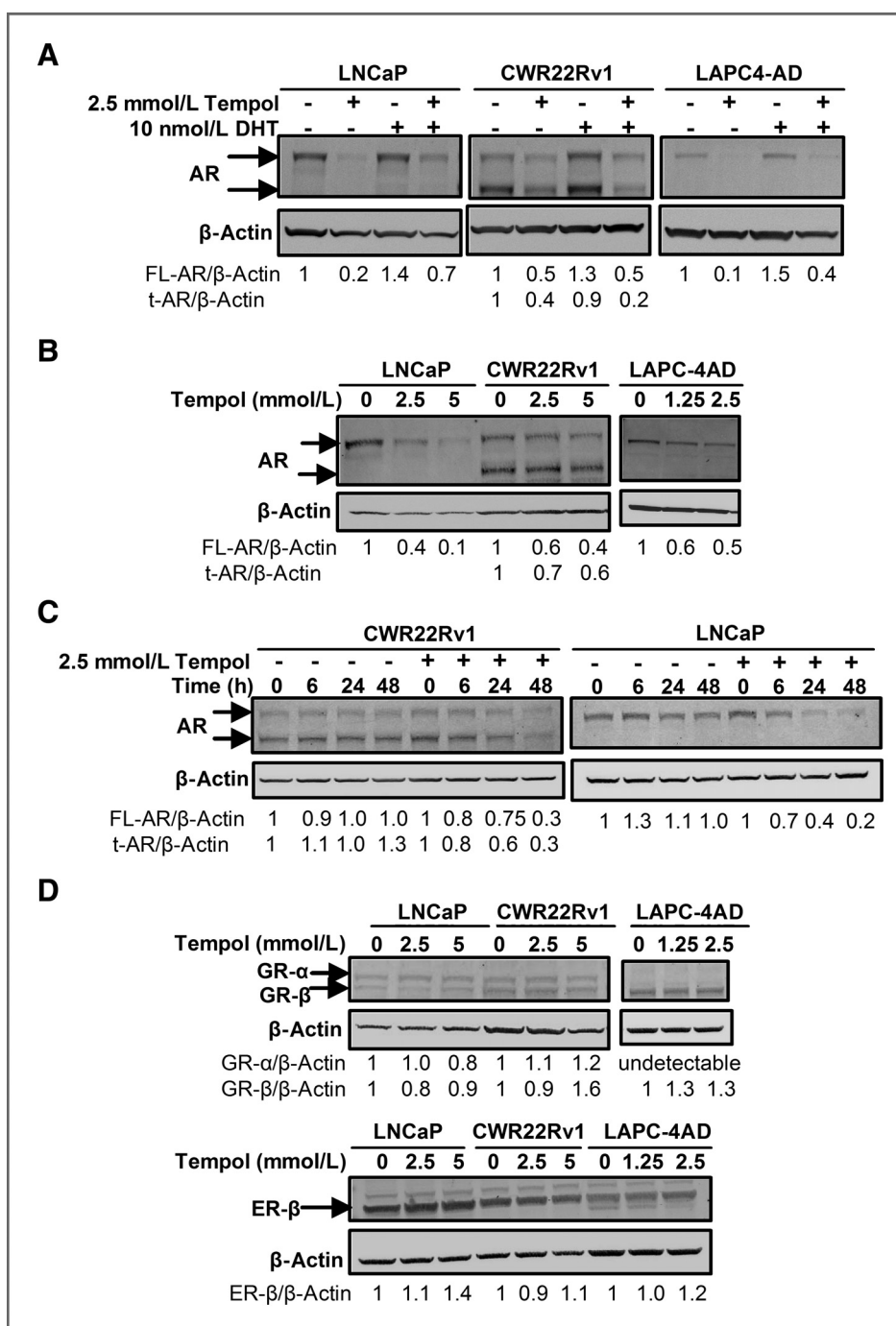
significant time-dependent decline in both full-length and truncated AR over the course of 48 hours, relative to that following vehicle treatment (Fig. 2C, Supplementary Fig. S4). In contrast to its ability to dose dependently decrease AR protein across all prostate cancer cell lines under study, Tempol did not have similar suppressive effects on other nuclear receptors, such as glucocorticoid receptors- α and - β (GR- α and GR- β) or estrogen receptor- β (ER- β ; Fig. 2D), indicating that Tempol selectively decreases AR protein in prostate cancer cells.

Tempol accelerates degradation of AR protein and decreases AR mRNA levels

To dissect the mechanism by which Tempol decreases AR protein, we assessed whether Tempol promotes AR protein degradation. To this end, prostate cancer cells were treated with the protein synthesis inhibitor, cycloheximide (CHX), in the presence or absence of Tempol (Fig. 3A). We reasoned that once new protein synthesis is inhibited with CHX, levels of residual AR protein would reflect the rate of AR degradation; therefore, more rapid degradation of AR would result in lower residual AR protein levels. Concomitant treatment with CHX and Tempol decreased AR protein levels more rapidly than did treatment with either CHX or Tempol alone (Fig. 3A), suggesting that Tempol enhances AR protein degradation.

Next, to explore whether the enhanced degradation of AR by Tempol is due to increased ubiquitination, prostate

Figure 2. Tempol decreases AR protein without significantly altering the expression of other nuclear receptors. **A**, Tempol decreases AR protein, both in the presence and absence of DHT. Cells were grown in PR-free medium containing 5% C/S FBS for 2 days before 24-hour treatment with 10 nmol/L DHT \pm 2.5 mmol/L Tempol. Cell lysates were prepared and AR protein analyzed by Western blotting. **B**, Tempol elicits a dose-dependent decline in AR protein. Cells were treated for 24 hours with increasing doses of Tempol in serum-free medium, followed by cell lysate preparation and AR protein analysis by Western blotting. **C**, Tempol elicits a time-dependent decline in AR protein. Cells were treated with vehicle or 2.5 mmol/L Tempol for 0 to 48 hours in complete medium, followed by cell lysate preparation and AR protein analysis by Western blotting. AR expression was normalized to that of β -actin and expressed as fold change, relative to that at the 0-hour time point for vehicle or 2.5 mmol/L Tempol, respectively. **D**, Tempol does not decrease protein levels of other nuclear receptors. Cells were treated for 24 hours with increasing doses of Tempol in serum-free medium. Cell lysates were prepared and protein levels of nuclear receptors GR- α , GR- β , and ER- β were assessed by Western blot analyses. For all Western blot analyses, nuclear receptor expression in each sample was normalized to that of the loading control, β -actin.



cancer cells were treated with the proteasomal inhibitor, MG-132, in the presence or absence of Tempol, and AR was immunoprecipitated and probed for ubiquitin (Fig. 3B, Supplementary Fig. S5). Tempol increased AR ubiquitination, relative to that in the control. Also, the level of ubiquitinated AR after cotreatment with MG-132 and Tempol was significantly higher than that with either MG-132 or Tempol alone (Fig. 3B, Supplementary Fig. S5). These findings suggest that Tempol promotes AR degradation via increased ubiquitination of AR.

A close assessment of AR protein levels in the MG-132 \pm Tempol input lysates revealed that despite the presence of MG-132, Tempol appreciably decreased AR protein relative to that in the vehicle control (Fig. 3B, Supplementary Fig. S5). This observation pointed to the possibility that Tempol may also be decreasing AR synthesis. Indeed, Tempol dose dependently decreased AR mRNA in LNCaP, CWR22Rv1, and LAPC-4AD cells (Fig. 3C). Collectively, we show that Tempol decreases AR via accelerated degradation of AR protein and a reduction in AR mRNA.

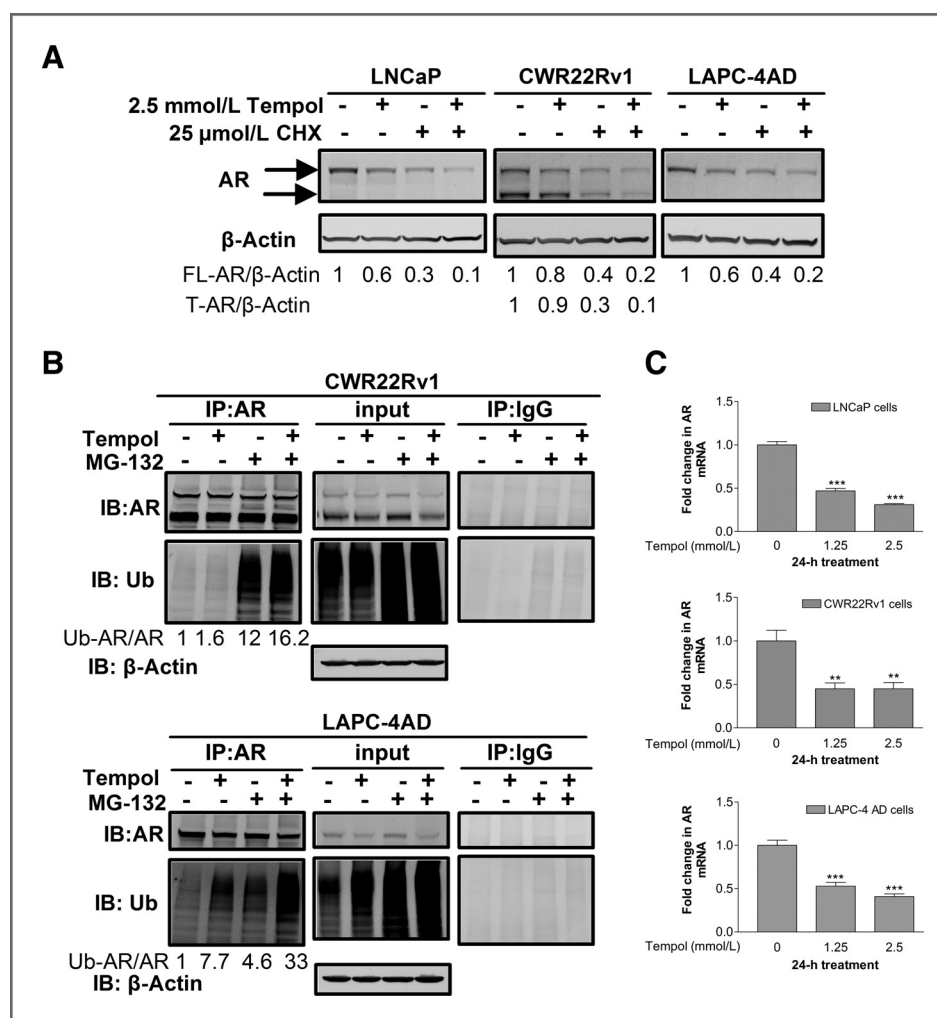


Figure 3. Tempol accelerates degradation of AR protein and reduces AR mRNA levels. **A**, Tempol promotes AR protein degradation. Cells were treated with 25 μmol/L CHX ± 2.5 mmol/L Tempol for 18 hours in complete medium. CHX-treated samples were pretreated with CHX for 6 hours. AR expression was analyzed by Western blotting and expressed as fold change, relative to vehicle control. **B**, Tempol promotes ubiquitination of AR. Cells were treated with 5 mmol/L Tempol ± 2.5 μmol/L MG-132 for 8 hours, and AR was immunoprecipitated from the cell lysates. Samples immunoprecipitated with an isotype-matched control antibody were run in parallel. The immunoprecipitates and input lysates were subjected to Western blot analyses with antibodies specific for AR and ubiquitin (Ub). β-Actin served as a loading control for the input. The intensity of ubiquitin in each immunoprecipitation sample was normalized to that of AR in the same sample and expressed as a fold change, with the ubiquitinated AR/AR ratio in the untreated control set at 1. **C**, Tempol dose dependently decreases AR mRNA. Cells were treated for 24 hours with increasing doses of Tempol in serum-free medium, followed by qPCR analyses of AR mRNA. AR mRNA levels were expressed as fold change ± SD ($\Delta\Delta C_t$ method), with mRNA levels in the untreated control set at 1. **, $P < 0.01$; ***, $P < 0.001$ versus the control (1-way ANOVA). IB, immunoblotting; IP, immunoprecipitation.

AR inhibition is also a property common to other SOD mimetics

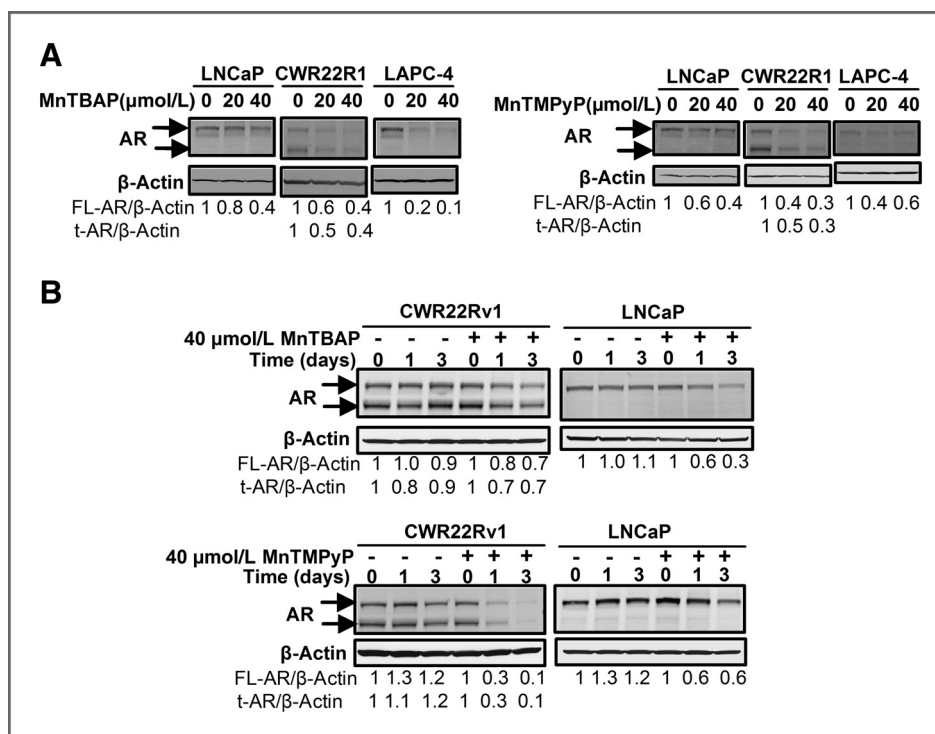
To determine whether the suppressive effects of Tempol on AR is a property also shared by other SOD mimetics, we tested the effects of MnTBAP and MnTMPyP, both of which belong to the class of SOD mimetics termed manganese (III) metalloporphyrins (Supplementary Diagram S1). MnTBAP and MnTMPyP have significant anticancer activities against cancers of the colon, liver, esophagus, and prostate (24–26). Both MnTBAP and MnTMPyP dose dependently decreased full-length and truncated AR protein levels (Fig. 4A). Forty μmol/L MnTBAP and MnTMPyP elicited a signi-

ficant time-dependent decline in both full-length and truncated AR protein in LNCaP and CWR22Rv1 cells (Fig. 4B). The above results closely paralleled those seen with Tempol (Fig. 2B and C), thereby indicating that the inhibitory effects of Tempol on AR are due to characteristics also shared by other SOD mimetics and are not unique to Tempol alone.

Tempol decreases the viability of prostate cancer cells

Studies have shown marked *in vitro* and *in vivo* declines in CRPC growth following inhibition of AR expression and/or function (5, 6). Because our data showed that

Figure 4. AR inhibition is also a property common to other SOD mimetics. A, SOD mimetics, MnTBAP and MnTMPyP, elicit a dose-dependent decrease in AR protein. Cells were treated for 24 hours with 0 to 40 $\mu\text{mol/L}$ MnTBAP or MnTMPyP in serum-free medium. AR protein was assessed by Western blotting. B, MnTBAP and MnTMPyP decrease AR protein in a time-dependent manner. Cells were treated with vehicle or 40 $\mu\text{mol/L}$ MnTBAP or MnTMPyP, respectively, for 0 to 3 days in complete medium and AR protein analyzed by Western blotting. The intensity of the AR protein band in each sample was normalized to that of β -actin in the same sample and expressed as a fold change, relative to that at the 0-day time point for vehicle and 40 $\mu\text{mol/L}$ MnTBAP or MnTMPyP treatments, respectively.



Tempol significantly decreases AR protein levels and AR transcriptional activity in prostate cancer cells (Figs. 1–3), we next investigated the effects of Tempol on prostate cancer cell survival and proliferation. First, we determined the cell-cycle phases that were altered by Tempol (Fig. 5A). The most consistent trend that emerged across prostate cancer cell lines after treatment with 2.5 mmol/L Tempol was a significant increase in the Sub G₀ (dead cell) fraction, relative to that after vehicle treatment. In addition, Tempol significantly decreased the G₂-M fraction of CWR22Rv1 cells (Fig. 5A).

To test whether the Tempol-mediated suppression of AR leads to a selective decline in viability of AR-dependent over AR-negative cells, we assessed prostate cancer cell viability after treatment with 0 to 2.5 mmol/L Tempol (Fig. 5B). Tempol significantly decreased the viability of AR-expressing prostate cancer cells at all doses. Dosages of 0.25, 0.5, and 2.5 mmol/L Tempol decreased CWR22Rv1 cell viability by 18%, 23%, and 57%, and LAPC-4AD cell viability by 21%, 33%, and 57%, respectively, relative to that after vehicle treatment. Although 0.25 and 0.5 mmol/L Tempol had significant suppressive effects on viability of AR-positive prostate cancer cells, it only elicited a 5% and 16% decrease in viability of AR-negative PC-3 cells, relative to vehicle treatment. However, 2.5 mmol/L Tempol decreased PC-3 cell viability by 46% (Fig. 5B). Our data (Fig. 5A and B) indicate that Tempol significantly decreases prostate cancer cell viability, with a more pronounced effect on viability of AR-dependent cells.

In addition, AR overexpression significantly blunted the ability of Tempol to decrease prostate cancer cell viability, suggesting that the Tempol-mediated decline in viability of AR-dependent cells is predominantly via suppression of AR function (Fig. 5C).

Tempol decreases AR protein *in vivo* and suppresses growth of CRPC xenografts

Because our *in vitro* studies revealed a significant Tempol-mediated decrease in AR protein levels and prostate cancer cell viability, we next evaluated the effect of Tempol on CRPC growth *in vivo*. To this end, subcutaneous LAPC-4AD tumor-bearing mice were surgically castrated and placed on control diet or Tempol-containing diet for 9 weeks. This form of Tempol treatment has been shown to produce an average serum Tempol concentration of 90 to 100 $\mu\text{mol/L}$ and to selectively decrease ROS and oxidative damage *in vivo* without selectively inducing cytotoxicity (21). The average RTV and cellularity in the Tempol group was consistently and significantly lower than that in the control group (Fig. 6A; Supplementary Fig. S7). After 9 weeks of treatment, 5 of the 6 mice in the control group had relatively large tumors, with individual RTVs of 3.34, 3.18, 1.93, 1.86, and 1.55; 1 control mouse had a regressing tumor with an individual RTV of 0.75. In contrast, all 4 mice in the Tempol group had comparably smaller tumors, with 3 being regressing tumors (individual RTVs of 0.39, 0.51, and 0.66), and 1 slow-growing tumor (individual RTV of 1.35; Supplementary Fig. S6). After 9 weeks of treatment, the average RTV of tumors in the Tempol

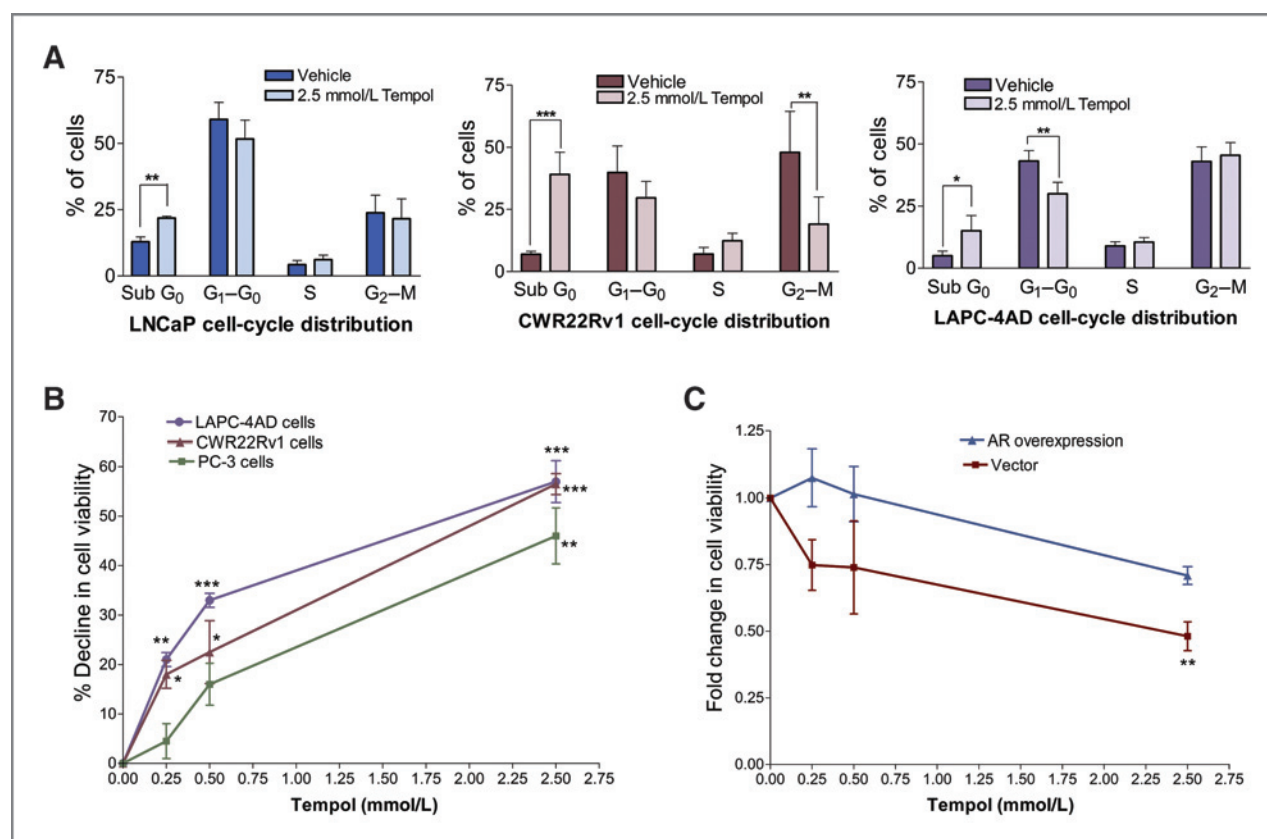


Figure 5. Tempol decreases prostate cancer cell viability. **A**, cell-cycle analysis of Tempol-treated cells reveals an increase in the dead cell fraction. Cells were treated with vehicle or 2.5 mmol/L Tempol in PR-free medium containing 5% C/S FBS for 3 days and cell-cycle distribution assessed by flow cytometry. Data are mean \pm SD, and statistical significance was assessed by unpaired *t* test. **B**, Tempol elicits a dose-dependent decline in cell viability. Cells were treated with 0 to 2.5 mmol/L Tempol in PR-free medium containing 5% C/S FBS for 2 days. Thereafter, cell viability was assessed and represented as % decline, relative to vehicle control. Data are mean \pm SD, and statistical significance was assessed by 1-way ANOVA. **C**, AR overexpression blunts Tempol-mediated decrease in prostate cancer cell viability. LAPC-4AD cells were transfected with AR cDNA or empty vector and treated for 1 day with 0 to 2.5 mmol/L Tempol in PR-free medium containing 5% C/S FBS. Cell viability was assessed and expressed as fold change relative to vehicle treatment. Data are mean \pm SD, and statistical significance was assessed by 1-way ANOVA. For Fig. 5A–C, *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 vs. control.

group was 0.72, versus 1.89 in the control group (Fig. 6A). We did not observe a statistically significant difference in food intake or body weight between mice in the control and Tempol groups (Fig. 6B, left and right panels), suggesting that differential food intake was probably not a factor influencing tumor growth in the Tempol group.

We next assessed the extent to which 9 weeks of Tempol treatment modulated AR protein in the prostate cancer xenografts (Fig. 6C). Tempol treatment elicited a 20% decrease in full-length AR. Interestingly, the LAPC-4AD xenografts also expressed a truncated AR variant that had the same mobility as the truncated AR in the CWR22Rv1 cell lysate, which was run in parallel. Notably, expression of the truncated AR variant was suppressed by more than 50% in the Tempol group, relative to that in the control group (Fig. 6C).

Discussion

Current androgen deprivation strategies to treat advanced, metastatic prostate cancer have been unable

to adequately suppress AR function, resulting in a vast majority of patients failing treatment and progressing to the lethal CRPC (1). Clearly, there is an urgent need for the discovery of AR inhibitors that will remain highly effective, notwithstanding the numerous current-therapy-evading AR molecular alterations that crop up both during and following the development of castration resistance. This study is the first to identify SOD mimetics as effective AR inhibitors in prostate cancer cells and also the first to document significant *in vivo* tumor growth suppressive effects with the use of a SOD mimetic in castrated, tumor-bearing mice.

Several reports (31, 34, 35) on the intriguing discovery of novel AR splice variants that lack the LBD in CRPC have necessitated a reevaluation of strategies aimed at targeting the AR in advanced prostate cancer. Targeting truncated AR variants is critical because (i) they are constitutively active (31, 34, 35); (ii) their expression is significantly increased during prostate cancer progression, and they confer androgen-independent prostate cancer growth (31, 34, 35), and (iii) their expression correlates with the

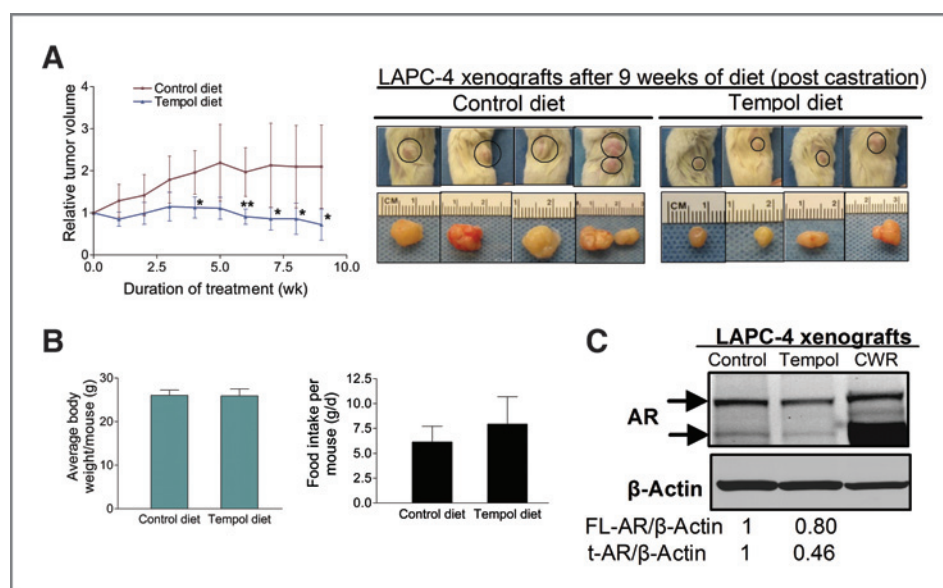


Figure 6. Tempol decreases AR protein *in vivo* and suppresses growth of CRPC xenografts. **A**, left, Tempol significantly suppresses growth of castration-resistant LAPC-4 prostate cancer xenografts. Subcutaneous LAPC-4AD tumor-bearing mice were surgically castrated and placed on control diet ($n = 6$) or Tempol diet ($n = 4$) for 9 weeks. Mean relative tumor volume \pm SD for each group was calculated at the end of each week of treatment. *, $P < 0.05$; **, $P < 0.01$ versus the control for each week (unpaired t test). Right, representative photographs of subcutaneous LAPC-4AD tumor-bearing mice that were on control or Tempol diet for 9 weeks following surgical castration, and the corresponding harvested tumors are shown. **B**, left, no significant difference in average body weight per mouse after 9 weeks of treatment with control or Tempol diet. Data are mean \pm SD of body weight of 6 mice in the control group and 4 in the Tempol group. Right, no statistically significant difference in daily food intake between mice on control diet ($n = 6$) or Tempol diet ($n = 4$). Data are mean \pm SD. **C**, Tempol decreases both full-length and truncated AR protein in castration-resistant LAPC-4 xenografts. Pooled lysates of LAPC-4 xenografts from castrated mice on control diet ($n = 4$) or Tempol diet ($n = 4$) were analyzed for AR protein by Western blotting. CWR22Rv1 (CWR) cell lysate was run in parallel.

risk of tumor recurrence after radical prostatectomy (31, 35). We show that SOD mimetics downregulate full-length and truncated AR protein levels, both *in vitro* and *in vivo*, without significantly altering levels of other nuclear receptors. In CWR22Rv1 cells, which express high levels of truncated AR, all 3 SOD mimetics under study, that is, Tempol, MnTBAP, and MnTMPyP, significantly decreased both full-length and truncated AR forms *in vitro*. Truncated AR protein was not readily detectable in LAPC-4AD cells *in vitro*; however, the LAPC-4AD xenografts accumulated appreciable levels of truncated AR. This finding is in conformity with findings by Dehm and colleagues, which showed that xenograft-based models of CRPC were enriched with truncated AR splice variants (34). Notably, the accumulation of truncated AR in LAPC-4AD xenografts was significantly blunted in mice that were on Tempol-containing diet. Interestingly, Tempol had a more pronounced suppressive effect on truncated AR rather than full-length AR *in vivo* (Fig. 6C). This finding raises an interesting question about the relative contributions of full-length and truncated AR forms to tumor growth *in vivo*. Although our study has not specifically addressed this question, Guo and colleagues have previously shown that specific short hairpin RNA-mediated knockdown of truncated AR expression, without altering full-length AR, was sufficient to attenuate prostate cancer growth in xenograft models (31). Furthermore, truncated AR regulates a unique set of genes that are not

regulated by full-length AR (31). Together, these observations, as well as ours, imply that expression of the truncated AR is critical for regulating prostate cancer growth.

Our data reveal a Tempol-mediated decline in viability of AR-positive, as well as AR-negative, prostate cancer cells. However, it is noteworthy that AR-positive prostate cancer cells are more susceptible to the growth inhibitory effects of Tempol and exhibit a greater decline in viability when treated with lower doses of Tempol than the AR-negative PC-3 cells at the corresponding dose (Fig. 5B). Furthermore, AR overexpression blunts suppressive effects of Tempol on viability of AR-dependent prostate cancer cells (Fig. 5C). Together, these observations suggest that (i) effect of Tempol on AR-dependent prostate cancer cell viability is predominantly mediated via AR, and (ii) the AR-expressing prostate cancer cells depend on the growth-promoting effects of AR signaling (4–6, 36), and hence, even a minor suppression of AR function causes them to become acutely sensitive. On the other hand, AR-negative prostate cancer cells may have developed alternative AR-independent, but ROS-dependent means of survival and proliferation, and hence they require a higher dose of SOD mimetic to lower ROS levels to an extent that will sufficiently suppress those ROS-dependent cell survival pathways. This notion is in partial agreement with a finding by Venkataraman and colleagues (10) that shows that SOD2 overexpression inhibits growth of AR-negative PC-3 cells, suggesting that

reduced cellular ROS levels inhibit tumor growth, independent of the AR. In line with this view, we have shown a significant Tempol-mediated reduction in cellular O_2^- levels in AR-positive and AR-negative prostate cancer cells (Fig. 1A). Collectively, our data suggest that Tempol may be exerting its antiproliferative effects via AR-dependent, as well as AR-independent, but ROS-dependent mechanisms (37, 38). Safety and toxicity studies conducted by many research groups have confirmed that concentrations of Tempol needed to suppress tumor growth *in vivo* do not elicit signs of general or organ toxicity and that Tempol is significantly more effective in inhibiting the growth of a range of neoplastic than nonneoplastic cell lines (20–23). The above findings, together with our demonstration of significant AR suppressive effects by Tempol, provide a mechanistic rationale for the use of Tempol and other SOD mimetics in prostate cancer treatment.

In summary, we have discovered SOD mimetics to be a promising, novel class of AR inhibitors with the ability to suppress CRPC growth. Given that SOD2 expression progressively declines in the spectrum from benign prostatic epithelium to CRPC (13–15), and that ROS and AR play a critical role in every stage of prostate cancer development (4, 8, 10, 16, 39, 40), this new class of AR inhibitors could be beneficial in prostate cancer treatment, with maximal beneficial effects seen in CRPC, in which SOD2 expression is decreased the most. Our demonstration of

significant suppression of both full-length and truncated ARs with SOD mimetics could also open up the prospect for the use of SOD mimetics in prostate cancers that are not amenable to treatment with therapeutic modalities that inhibit the synthesis of androgens and those that competitively block access to the AR LBD.

Disclosure of Potential Conflicts of Interest

N. Sharifi has been compensated as a consultant for Ortho Biotech.

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