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14. ABSTRACT: Prostate cancer (PrCa) is the second leading cause of cancer death in American men. There is an increasing need to develop effective therapies for advanced stage PrCa due to their limited or no response to androgen ablation therapy. Chemotherapy is an alternative approach for the treatment of advanced stage PrCa. However, the available chemotherapeutic agents used to treat PrCa are non-selective and						
provide only limited response rate Thus, novel treatment modalities are needed to treat advanced stage PrCa. In this proposal, we intend to develop a povel therapeutic modality for advanced stage metastatic prostate cancer.						
There is an urgent need to develop effective therapies for the treatment of advanced stage prostate cancer (PrCa) due to their limited or no response to androgen ablation therapy. In this proposal, we intend to develop a novel therapeutic agent Ormeloxifene (ORM) for the treatment of advanced stage metastatic PrCa. Our results illustrated that ORM treatment effectively inhibited invasion and motility of PrCa cells. Further, we observed that ORM treatment induced the expression of tumor suppressor PKD1 (a modulator of nuclear β-catenin signaling) in PrCa cells. Interestingly. ORM treatment inhibited						
expression of oncogenic isoform of PKD (PKD3) in PrCa cells. We have also observed that ORM mediated overexpression/activation of PKD1 effectively inhibits metastasis associated protein 1 (MTA1) in PrCa cells. MTA1 has been reported to be very tightly associated with cancer metastasis in various cancer types including PrCa. To further investigate association of ORM with MTA1 suppression, we performed molecular docking studies with MTA1 which illustrated potential binding sites of ORM on MTA1 protein. Considering effective therapeutic index of ORM, we are also making more potent analogues of ORM. These findings suggest that ORM could be a potential therapeutic molecule to inhibit growth of advanced stage PrCa and its metastasis.						
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INTRODUCTION

1. BACKGROUND

Prostate cancer (PrCa) is the second leading cause of cancer death in American men. There is an increasing need to develop effective therapies for advanced stage PrCa due to their limited or no response to androgen ablation therapy (1). Chemotherapy is an alternative approach for the treatment of advanced stage PrCa. However, the available chemotherapeutic agents used to treat PrCa are non-selective and provide only limited response rate (2). Thus, novel treatment modalities are needed to treat advanced stage PrCa. In addition, precise understanding of molecular pathogenesis of disease is required to develop novel chemotherapeutic modalities for the treatment of advanced stage PrCa. The androgen receptor (AR) is required for PrCa growth at all stages, including androgen-independent tumors in the presence of very low levels of androgens (3). Recent studies are suggested that in addition to androgens/AR, estrogens/estrogen receptors (ER) may also play crucial role in the development and progression of PrCa. It is shown that the co-administration of both testosterone and E2 is required for the initiation of PrCa. Additionally, the interaction between β -catenin and the AR and ER suggests a possible mechanism of cross talk between Wnt and androgen/estrogen signaling pathways (4). Several lines of evidence have shown that β-catenin can act as an AR/ER co-activator and enhance their transcriptional activity (4). Thus targeting β -catenin-AR/ER signaling pathway by novel chemotherapeutics may have strong clinical implications in developing strategies for PrCa treatment (5). In recent studies, a novel tri-phenyl ethylene molecule, ormeloxifene, has shown potent anti-cancer activity, including in PrCa cells. The central hypothesis of our proposal is that ormeloxifene inhibits growth of advanced stage PrCa cells by modulating β -catenin-AR/ER signaling pathway. Further, it induces cell death via inducing PARP and/or caspase mediated apoptotic pathways. The information gathered from this study will provide insight for developing a novel therapeutic modality for advanced stage PrCa. To test this hypothesis, the following specific aims were proposed:

SPECIFIC AIMS:

AIM 1: To examine the effect of ormeloxifene on β-catenin-AR/ER signaling pathways.

AIM 2: Determine the apoptotic pathways activated by ormeloxifene to induce cell death in PrCa cells.

AIM 3: To evaluate the therapeutic efficacy of ormeloxifene for PrCa treatment in mouse model systems.

Ormeloxifene (ORM): Utilization of clinically approved drugs for other indications as anti-cancer agents (repurposing a drug) appears to be an interesting approach because of their established safety profile in human. The ongoing scenario attracts and welcomes the repositioning and budging of existing established drugs which could complement de novo drug development. Ormeloxifene (**Fig. 1**) is a non-hormonal, non-steroidal synthetic molecule for human use as an oral contraceptive (6,7). Recently, its anti-cancer activity has been reported against advanced breast cancer (8) and head and neck squamous cell carcinoma (HNSCC) (9). Additionally, our recent studies show a potent anti-cancer activity of ormeloxifene (ORM) in various cancer cell lines including



AR sensitive and AR refractory metastatic PrCa cells. *Moreover, ORM is reported to have an excellent therapeutic index and is safe for chronic administration* (10). Therefore, we believe that ORM has a great repurposing potential for PrCa chemoprevention/treatment. Successful examples of drugs repurposing are antidiabetic drug metformin and the birth control hormone medroxyprogesterone acetate. In this study we proposed to investigate effects of ORM on β -catenin-AR/ER signaling pathways (AIM 1), apoptosis (AIM 2) and evaluate its anticancer potential in clinically relevant PrCa cell lines and animal models (AIM 3).

Research work performed during the previous funding cycle (2014-15): we hired the post-doc fellow/Scientist for this project and performed some experiments under specific aims # 1. We performed functional assays to determine the effects of ORM on cell invasion, and cell migration, and colony formation

using androgen-independent C4-2 PrCa cell lines. Additionally, we performed some docking studies of ORM with some potential molecular targets of PrCa. We observed that ORM has some potential binding sites with metastasis associated protein 1 (MTA1). We further investigated the effects of ORM on MTA1 protein levels by Western blot analysis. Our results indicate that ORM treatment of C4-2 cells inhibits the protein levels of MTA1. Our lab investigated that MTA1 protein can be inhibited by *via* ectopic overexpression of protein kinase D1. We observed that ORM induces the expression of PKD1 as determined by Western blot analysis, and qRT-PCR. Key research findings of this year are summarized below.

1. ORM inhibits invasion and motility of C4-2 cells:

2. ORM activates tumor suppressive PKD1 and inhibits oncogenic PKD3 in C4-2 cells:

3. ORM interacts with and inhibits protein levels of Metastasis Associated Protein 1 (MTA1)

4. ORM enhances the sensitivity of chemotherapeutic drug Docetaxel (DTX):

It has been reported that MTA1 is involved in DTX drug resistance (11) and DTX treatment does not inhibit the expression of MTA1 in PrCa cells (Fig. 2A). However, ORM treatment effectively inhibited the expression of MTA1 (Fig. 2A). Thus, we hypothesized that ORM treatment may enhance the DTX sensitivity in PrCa cells. To prove our hypothesis, we performed cell proliferation and colony formation assays in C4-2 cells treated alone or in combination with DTX. Results illustrated that ORM treatment potentiates the effects of DTX as determined by MTS (Fig. 2B). Further our colony formation results also depicted significant (P<0.05) reduction in colony formation compared with alone ORM and DTX treatment. These results indicate that ORM has potential to induce the DTX effects in PrCa. ORM in combination with Overall, these results suggest that ORM which induces PKD1 expression and inhibit MTA1 can sensitize the DTX resistance in PrCa



Research work performed during the previous funding cycle (2015-16): During this funding cycle, we have completed remaining proposed experiments of Task 1 and majority of Task 2. In Task 3, we completed xenograft study in athymic nude mice. We showed functional impact of ORM for inhibiting the growth of hormone refractory prostate cancer cells in vitro and in ectopic xenograft mouse model. We determine the effect of ORM on β-catenin signaling pathways, interaction of β-catenin and AR in PrCa cells. Our results demonstrated that ORM inhibits β -catenin signaling network in PrCa cells. ORM treatment inhibited translocation of β -catenin from cytoplasm into nucleus and inhibited its downstream target gene TCF-4. It has been reported that β -catenin interacts and transactivates AR signaling pathways via non-androgen dependent mechanism (12). Our results have shown that ORM inhibits physical interaction of β -catenin and AR in C4-2 cells and C4-2 cells derived xenograft tumor tissues as determine by immunoprecipitation/Western blot analysis. Next we performed molecular modeling to determine whether ORM directly interacts with AR, ER, GSK3-β, and β-catenin proteins. We observed strong binding pocket of ORM in ER, AR, β-catenin, and GSK3β. ORM treatment showed arrest in G0/G1 phase of cell cycle in PC3 cells and inhibited the expression of various cell cycle regulator protein including MCL1, Cyclin D1, CDK2 and enhanced the expression of p21 and p27. ORM treatment inhibited the expression of various EMT markers (N-Cadherin, Slug and Snail) and enhanced the expression of E-Cadherin. To translate our *in vitro* results into *in vivo*, we performed xenograft studies in athymic nude mice using C4-2 and PC3 cells. ORM administration (100 and 500 µg) i.p thrice a week significantly (P<0.01) inhibited the growth of PC3 cells derived xenograft tumors as determined by decrease in tumor volume and weight in ORM treated mice compared to control groups. We also observed a significant

(P<0.01) inhibition of PC3 cells derived xenograft tumors in ORM treated (500 µg) group. All of these results suggest that ORM is a potent chemotherapeutic drug and could be used for prostate cancer treatment. Our main findings of this vears are summarized below.

- **1.** ORM treatment inhibits the growth of hormone-refractory prostate cancer cells.
- **2.** ORM treatment arrests cell cycle in G0/G1 phase.
- **3.** ORM treatments modulates cell cycle regulatory proteins (p21, p27 cyclin D1 and MCL1) in PrCa cells.
- **4.** ORM treatment inhibits EMT, MMPs, invasion, and migration of PrCa cells.
- 5. ORM inhibits and rogen receptor (AR) signaling and its interaction with β -catenin.
- 6. ORM docks with β -catenin, GSK3 β , and AR/ER.
- 7. ORM treatment inhibits the growth of PrCa cells derived xenograft tumors in athymic nude mice (Fig. 3).
- ORM treatment inhibited the expression of PCNA, AR and β-catenin in xenograft tumors



Figure 3: ORM inhibits tumor growth of PrCa cells in athymic nude mice. A. Effect of ORM on C4-2 xenograft tumors in athymic nude mice. Ai Line graph is showing mean tumor volume of 4 mice in each group at 1-5 weeks. Aii. Representative C4-2 xenograft tumor bear mouse in each group. Aiii. Bar graph representing tumor weight of each group mice. Value in graph represents Mean±SE of 4 mice in each group. **B.** Effect of ORM on PC3 xenograft tumors in athymic nude mice. A total of 10 mice were used in this experiment and were divided into two groups (n=5). 2X10⁶ PC3 cells were injected subcutaneously and ORM treatment (250 µg) was administered 3 times a week till 6 weeks. Mice of both the group were sacrificed when control mice showed a targeted tumor volume 1000 mm³. **Bi.** Average tumor volume of each group mice at different weeks. **Bii.** Representative mouse picture of control and ORM treated tumor bearing mouse. **Biii.** Bar graph representing tumor weight of each group mice. Value in graph represents Mean±SE of 5 mice in each group.

Research Progress during the 2016-17 Fiscal Period:

We made significant progress during the 2016-17 academic year. We have completed the remaining proposed experiments of **Tasks 1.2**, and **3**. Since then, we have observed cell cycle arrest in G0-G1 phase, and we investigated the effect of ORM on apoptotic induction in PrCa cells. We noted that ORM treatment induces apoptosis in both PC3 and DU145 cells as determined by enhanced Annexin V staining in cells and PARP cleavage. We next examined the effect of ORM on mitochondrial membrane potential ($\Delta \psi m$) using TMRE staining, which is a marker of apoptosis. ORM decreased TMRE staining in both DU145 and PC3 cells as determined by fluorescence microscopy and flow cytometry respectively. These results suggest the apoptosis inducing potential of ORM in PrCa cells. We also determined the molecular mechanism of ORM induced inhibition of β -catenin targeting pathways in cancer cells. ORM treatment was found to inhibit nuclear β catenin in DU145. GSK3β-dependent phosphorylation of β-catenin enhances its proteasomal degradation and inhibits its translocation into the nucleus, thus regulating its various downstream target oncogenes. Our results indicate that ORM activates GSK3 β , thereby degrading β -catenin in the cytoplasm and also inhibiting nuclear β-catenin translocation and repressing TCF-4 promoter activity. ORM also inhibited the expression of various motility markers (integrin §5, vinculin, vimentin and phosphorylation of cofflin) in PrCa cells. ORM administration also showed significant (P<0.01) inhibition of prostate tumor growth in TRAMP mice. Histopathological analysis further confirmed significant decrease of poorly differentiated adenocarcinoma in

TRAMP mice. ORM treatment inhibited expression of PCNA, vimentin and slug in excised prostate tumor tissues as compared to control group mice. Androgen receptor signaling plays a key role in the development as well as the progression of PrCa. Accumulating evidence suggests that anti-androgen therapy is effective to reduce the growth of primary as well as castration resistant prostate tumors in humans. However, this therapy still has limitations since after castration, AR signaling is still active in target cells *via* non-androgen activation of AR or the presence of AR splicing variants. ORM has the ability to suppress the function of AR in both LNCaP and 22Rv1 cells. This might be because ORM competes with the LBD of AR. ORM inhibited the expression of the AR splicing variant ARv7 in 22Rv1 cells. More importantly, a known anti-androgen drug (Enzalutamide) for castration resistant prostate cancer does not target AR splicing variants and have combinatory effect with Enzalutamide. This novel observation suggests that ORM can be used to improve the therapeutic efficacy of Enzalutamide in castration resistant PrCa. These study are ongoing in our lab and will be performed from no-cost extension funds. We are working to determine the molecular mechanisms of ORM-induced sensitization of docetaxel therapy of PrCa *in vitro* and *in vivo*.

Task 1: To examine the effect of ormeloxifene on β -catenin/AR/ER signaling

1. ORM inhibits nuclear **β-catenin** in DU145 cells. We observed that ORM inhibits βthrough catenin its sequestration in the cytoplasm. ORM treatment (10 µM) inhibited nuclear β -catenin in DU145 cells (Fig. 4Ai) through its sequestration in the cytoplasm as determined by Western blot analysis. This result was further confocal confirmed by ORM microscopy as showed inhibition of β catenin translocation into the nucleus of PrCa cells as compared to control (Fig. 4Aii). We next evaluated the effect of ORM on lithium chloride (LiCl)induced β-catenin/TCF promoting activity bv transiently co-transfecting the DU145 cells with TCFfirefly luciferase reporter constructs (pTOP-FLASH) and *Renilla* luciferase or (pFOP-FLASH) and Renilla luciferase. ORM treatment (10 μ M) for 6 hrs, significantly (P<0.01) inhibited lithium chloride



Figure 4: Effect of ORM on β -catenin and GSK3 β . A. Effect of ORM on β -catenin distribution in cytoplasm and nucleus of DU145 cells. Briefly, cells were treated with indicated concentrations of ORM for 24 hrs, nuclear extracts were prepared and subjected for Western blot analysis to detect the protein levels of β -catenin. Results demonstrated a decreased expression of nuclear β -catenin and increased expression of β -catenin in the cytoplasm (Ai) of DU145 cells. (Aii)Effect of ORM on β -catenin localization in PC3 cells as determined by confocal microscopy. Yellow arrows indicate localization of β -catenin in control and ORM treated cells after 24 hrs treatment (Original magnification 40X). (Aiii) Effect of ORM on TCF-4 promoter activity. Cells were transiently co-transfected with TCF-firefly luciferase reporter constructs (pTOP-FLASH) (1 µg) and Renilla luciferase (200 ng) or (pFOP-FLASH) (1 µg) and Renilla luciferase (200 ng). After 24 hrs, cells were treated with LiCl (50 µM) alone or in combination with ORM (10 µM). Cell lysates were prepared 6 hrs post-treatment and firefly and *Renilla* luciferase activity were analyzed via Dual luciferase kit (Promega). The βcatenin/TCF transcription activity was determined by normalizing the firefly luciferase activity to that of Renilla luciferase activity and calculating the ratio of TOP-FLASH signal to FOP-FLASH signal. Values in bar graph indicates mean±SE of three wells reading in each group. Asterisk (*) denotes the significant value p<0.01. B. (Bi) Effects of ORM on protein levels of phospho GSK3 β in DU145 as determined by Western blot analysis. (**Bii**)Effect of ORM on β catenin degradation as analyzed by pulse chase experiment. Briefly, DU145 cells were treated with CHX (50 µg) alone or in combination with ORM (15 µM) at indicated time points. Protein lysates were prepared and subjected for Western blot analysis to analyze the protein levels of β -catenin. Results indicates protein levels of β -catenin in alone CHX-treated (upper blot) and in CHX and ORM-treated (Lower blot). (Biii)Line graph showing quantification of Western blots of Figure Bii. $T_{1/2}$ denotes time point for 50% β -catenin degradation.

(LiCl)-induced TCF-4 promoter activity in DU145 cells (**Fig. 4iii**). We also examined the effect of ORM on activation of GSK3 β by Western blot analysis which illustrated a marked increase in phosphorylated GSK3 β protein levels in DU145 cells (**Fig. 4Bi**). We have since observed activation of GSK3 β protein by ORM treatment; thus, we next examined the effect of ORM on β -catenin degradation after using translational inhibitor (cyclohexamide). Results revealed a time-dependent decrease in the protein levels of β -catenin in DU145 cells compared to cyclohexamide treatment alone (**Fig. 4Bii-iii**).

Task 2: To examine the mode of cell death triggered by ORM in PrCa cells

2. ORM treatment induces apoptosis in PrCa cells. Since we observed cell cycle arrest in the G0-G1

phase, we investigated the effect of ORM on apoptotic induction in PrCa cells by flow cytometry analysis. ORM (10-20 µM) doseincreased dependently apoptotic cell populations in both PC3 (Fig. 5Ai) and DU145 (Fig. 5Aii) cells as determined by enhanced Annexin V positive cells. ORM at 20 µM showed 55.6% and 50% apoptotic PC3 and DU145 cells respectively compared to control group (Fig. 5Ai-ii). We next examined the effect of ORM on mitochondrial membrane $(\Delta \psi m)$ potential using TMRE staining (Fig. 5Biii), which is a marker of apoptosis induction through intrinsic pathway. ORM (10-20)μM) dosedependently decreased TMRE staining in both DU145 and PC3 cells as determined by fluorescence microscopy (Fig. 5Bi) and flow cytometry (Fig. 5Biii), respectively. We also examined the effect of ORM on PARP cleavage which is a marker for apoptosis. ORM (20 µM) induced PARP cleavage as determined by western blot analysis (Fig. 5C). These results suggest the apoptosis-inducing

potential of ORM in PrCa cells.





Task 1: To examine the effect of ormeloxifene on β -catenin/AR/ER signaling

ORM inhibits motility markers in 3. PrCa cells. Since we observed that ORM inhibits the migratory potential of PrCa cells, we investigated the effect of ORM on various cytoskeletal proteins which help in lamellipodia formation during the migratory process of cancer cells. Results demonstrated that ORM (10 µM) inhibited the expression of integrin $\beta 5$, vinculin, vimentin and phosphorylation of cofilin in PrCa cells (Fig. 6A). ORM treatment also inhibited the expression of Integrin $\beta 5$? (receptor for fibronectin) as determined by confocal microscopy (Fig. 6B). These results suggest that ORM has the ability to suppress cancer cell migratory potential via inhibiting these cell motility markers.



Task 3: To evaluate the therapeutic efficacy of ORM for PrCa treatment in TRAMP mouse model

4. ORM inhibits tumor growth in TRAMP mice. Transgenic adenocarcinoma of mouse prostate (TRAMP) mice closely mimics human prostate tumors in many ways. For example, focal adenocarcinoma in TRAMP mice develops rapidly within 10 to 20 weeks. Adenocarcinoma arises in the dorsal lateral lobe, which is considered analogous to the peripheral zone, where the human PrCa originates (13). These mice have been used to identify new molecular targets and to test new therapeutic modalities against PrCa. Moreover, these mice overexpress key oncogenic signaling pathways involved during the development, progression and metastasis of PrCa (14). The main objective of this experiment was to investigate whether ORM treatment

inhibits prostate tumor growth and metastasis in an intact model. this mouse In experiment, a total of 22 TRAMP mice (6-7 week old) were purchased from Jackson Laboratory and divided into two groups. ORM treatment (200 µg IP three days a week) was started when mice were 9 weeks old and continued until end points. Vehicle group mice received 0.2 ml vehicle only. None of the mice showed apparent toxicity with ORM administration during entire period of the experiment. Four mice from each group were examined at 18 week of age for prostate tumor development. At this point, none of the mice showed prostate tumor growth in either group (Data not shown). Remaining mice were sacrificed



Figure 7: Effect of ORM on prostate carcinogenesis in TRAMP mice. A. Representative pictures of control and ORM treated TRAMP mice and their excised prostate tumors at 35 week. B. Bar graph showing mean \pm SE of Genito-urinary tracts (i) and prostate tumor (ii) weight of control and ORM treatment group(4 mice each). C. Histopathological analysis of prostate tumor of control and ORM treated group. Mouse #1 and 2 in control group are showing poorly differentiated adenocarcinoma while mouse #3 moderately to widely differentiated adenocarcinoma. Representative picture of ORM treatment mice #1, 2, and 3 shows low differentiation. One mouse show moderately differentiated carcinoma. None of the mice in ORM group show poorly differentiated carcinoma.

at 35 weeks. As shown in Figure 7, ORM administration inhibited the growth of prostate tumor in TRAMP

mice as determined by a significant (P<0.01) decrease in weight of prostate tumors and genito-urinary tracts (CI) compared to vehicle-treated mice. Data shown here are mean±SE of prostate tumor of 4 mice in each group. We excluded the remaining three mice of both groups as they did not develop prostate tumors. However, these mice showed presence of SV40-T antigen in tail genotyping by Jackson Laboratory. Histopathological examination of control mice showed mainly poorly differentiated adenocarcinoma (PDAC) at 35 weeks (**Fig. 7 C**). The PDC exhibited marked polymorphism and high levels of mitosis and apoptosis with neuroendocrine phenotype of large nuclei with fine chromatin and inconspicuous nucleoli and scant cytoplasm within apparent cell membranes (**Fig. 7C**). ORM treatment inhibited progression of PDAP in TRAMP mice. At 35 week, ORM treatment resulted in focal PIN in 3 mice (**Fig. 7C**), and diffuse PIN in one mice (**Fig. 7C**). At the same time point (35 weeks), one of the four animals showed a small PDAC in the ORM treated group. These data suggest the potential chemotherapeutic effect of ORM against progression of PCa.

5. ORM inhibits the expression of PCNA, vimentin, CD63, and MMP3 in excised tumor tissues of TRAMP mice. We next evaluated the key oncogenic signaling components involved in aggressive prostate tumor microenvironments. Accumulating evidence suggests that Tumor associated macrophages is one of the

components of tumor microenvironments (TMEs) involved in development and metastasis of PrCa (15). CD63 is one of the markers for TAMs which was shown to be overexpressed in aggressive prostate tumor types. Thus, we examined the expression of CD63 in excised prostate tumor tissues of TRAMP mice. ORM treatment inhibited the expression of CD63



compared to control as determined by immunohistochemistry (**Fig. 8**). Since we observed that ORM treatment inhibits the metastatic phenotypes of PrCa cells *via* targeting EMT markers and MMPs, thus, we examined the effect of ORM on the expression of vimentin and MMP3 in excised prostate tumor tissues. Our results demonstrated effective inhibition of both vimentin and MMP3 expression with ORM treatment as compared to control. These findings indicate that ORM targets componets of prostate TME.

6. ORM inhibits androgen-induced growth of PrCa cells. Since we observed that ORM binds LBD domain of AR and inhibits AR transactivation in C4-2 cells, we examined whether ORM has the ability to inhibit the androgen-induced growth of PrCa cells. In this experiment, LNCaP cells were grown in charcoal-treated hormone free medium. After 70% confluency, cells were treated with synthetic androgen (R1881,1 μ M), ORM (10 μ M) or a combination of these agents. Twenty four hrs post-treatment, cell proliferation was examined by MTS assay. Results demonstrated that ORM treatment signmificantly (P<0.01) inhibited androgen- induced growth of LNCaP cells (Fig.9A). These results suggest that ORM competes with androgens to repress the growth of AR positive PrCa cells. We also tested the effect of ORM on the growth of androgen-independent 22Rv1 cells. These cells express multiple AR splicing variants that remain constitutively active, leading to ligand-independent induction of target gene expression and cell growth (16,17). Emerging evidence supports the concept that development of metastatic castration resistant PrCa (mCRPC) is largely due to expression of AR splicing variants (ARVs) which are missing variable portions of the C-terminal domain and ligand binding domain (18-20). Among all splicing variants ARV7 and AR^{v567es} have shown more clinical

relevance indeveloping mCRPC and ENZ therapy resistance (16,17). A recent study indicates that higher expression of ARV7 and AR^{v567es} in CRPCs are associated with CRPC developing more rapidly and reduced patient survival (20). ARV7 protein was found to be overexpressed in bone metastatic samples (21), circulating tumor cells (20) of CRPC patients treated with ENZ. These results suggest that AR splicing variants are potential molecular target for the management of mCRPC to overcome enzalutamide resistance. We observed that ORM (2.5-20 μ M) treatment also significantly inhibited the growth of 22Rv1 cells (**Fig. 9D**). These results prompted us to explore the effect of ORM on the expression of ARV7 and its combinatory effects with Enzalutamide. Western blot results demonstrated that ORM inhibits the protein levels of both full length AR and ARV7 in 22Rv1 cells (**Fig. 9B**). However, no effect was observed at mRNA expression of AR, ARV7, and PSA (**Fig. 9Ci-iii**). ORM treatment showed additive effect with Enzalutamide in 22Rv1 cells (**Fig. 9D**). These results indicate that ORM has the potential to inhibit mCRPC and overcome enzalutamide resistance. We are exploring in detail the molecular mechanism of ORM targeting ARV7 and its combinatory effect with Enzalutamide in xenograft mice using 22Rv1 cells.



Figure 9: ORM treatment inhibits the androgen-induced growth of PrCa cells and inhibits the expression of AR and ARV7 in PrCa cells. A. Effect of ORM on R1881-induced growth of androgen-dependent LNCaP cells. Briefly, cells were treated with R1881 (10 nM) alone or in combination with ORM (10 μ M). MTS assay was performed to analyze the cell growth. Data in bar graph represents mean ±SE of 5 wells in each group. Asterics represent significant value P<0.01. **B.** Effect of ORM (12 hrs treatment) on protein levels of full length AR and ARV7 in 22Rv1 cells as determined by Western blot analysis. **C.** Effect of ORM on mRNA expression of AR (i), ARV7 (ii), and PSA (iii) after 12 hrs cells treatment as determined by qPCR analysis. **D.** Effect of ORM on the expression of AR in xenograft tumors. **E.** Effect of ORM alone or in combination with Enzalutamide on growth of 22Rv1 cells (24 hrs treatment) as determined by MTT assay.

Ongoing Experiments for AIM 3: Exploring the effects of ORM on various components of TME and CSCs in excised prostate tumor tissues of TRAMP mice.

Ongoing Experiments From No Cost Extention Funds For 2017-18:

> To identify the detailed molecular mechanisms for ORM-specific targeting of ARV7

- > To investigate the therapeutic effects of ORM alone or in combination with Enzalutamide *in vivo*.
- To determine the chemosensitizing effect of ORM in docetaxel resistant PrCa (PC3 and DU145) cells in vivo.

Publications Pertinent to this Grant:

- Hafeez BB, Ganju A, Sikander M, Kashyap VK, Hafeez ZB, Chauhan N, Malik S, Massey AE, Tripathi MK, Halaweish FT, Zafar N, Singh MM, Yallapu MM, Chauhan SC, Jaggi M. Chauhan SC. Ormeloxifene Suppresses Prostate Tumor Growth and Metastatic Phenotypes via Inhibition of Oncogenic β-catenin Signaling and EMT Progression. *Mol Cancer Ther.* 2017 Oct;16(10):2267-2280.
- 2. Sikander, M, Hafeez BB, Malik S, Ganju A, Jaggi M, **Chauhan SC.** Cucurbitacin inhibits the growth of hormone refractory prostate cancer cells *via* modulating glucose metabolism. **Prostate 2016** (Under Communication).
- 3. Hafeez BB, Sikander, Ganju A, Jaggi M, **Chauhan SC.** Ormaloxifene enhances chemosensitization potential of docetaxel in prostate cancer cells *via* suppressing p-glycoprotein. (**Oncotarget 2017** Under preparation)
- 4. Hafeez BB, Sikander, Ganju A, Jaggi M, **Chauhan SC.** ORM suppress the growth of prostate tumor and metastasis in TRAMP mice. **Carcinogenesis 2017** (Under preparation)

Grants Submitted for Extramural Funding on Prostate Cancer:

- DOD Idea development award 04/01/2018 to 03/31/2021
 PI: Chauhan SC
 Title: Anti-cancer activity of a novel cucurbitacin D analogue
- 2. DOD Idea development award 04/01/2018 to 03/31/2021
 PI: Hafeez BB, Co-Investigator: Chauhan SC
 Title: Role of WAVE2 in Tumor Microenvironment and Metastasis of Prostate Cancer
- **DOD Health disparity award** 06/01/2018 to 05/31/2021
 PI: Jaggi M, **Co-Investigator:** Chauhan SC
 Title: Molecular and Cellular Aspects of Prostate Cancer Health Disparity
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Ormeloxifene Suppresses Prostate Tumor Growth and Metastatic Phenotypes via Inhibition of Oncogenic β -catenin Signaling and EMT Progression

Molecular Cancer Therapeutics



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Abstract

Ormeloxifene is a clinically approved selective estrogen receptor modulator, which has also shown excellent anticancer activity, thus it can be an ideal repurposing pharmacophore. Herein, we report therapeutic effects of ormeloxifene on prostate cancer and elucidate a novel molecular mechanism of its anticancer activity. Ormeloxifene treatment inhibited epithelial-to-mesenchymal transition (EMT) process as evident by repression of N-cadherin, Slug, Snail, vimentin, MMPs (MMP2 and MMP3), β -catenin/TCF-4 transcriptional activity, and induced the expression of pGSK3 β . In molecular docking analysis, ormeloxifene showed proficient docking with β -catenin and GSK3 β . In addition, ormeloxifene induced apoptosis, inhibited growth and metastatic potential of prostate cancer cells and arrested cell cycle in G₀-G₁ phase via modulation of

Introduction

Prostate cancer is the second leading cause of cancer-related death among American men. The American Cancer Society projected that a total of 161,360 new cases of prostate cancer would be diagnosed and approximately 26,730 men will die in the United States alone in the year of 2017 (1). Despite the initial success of androgen-ablation therapy, resistance to anti-androgen therapy manifests by progression to androgen-independent pros-

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cell-cycle regulatory proteins (inhibition of Mcl-1, cyclin D1, and CDK4 and induction of p21 and p27). In functional assays, ormeloxifene remarkably reduced tumorigenic, migratory, and invasive potential of prostate cancer cells. In addition, ormeloxifene treatment significantly (P < 0.01) regressed the prostate tumor growth in the xenograft mouse model while administered through intraperitoneal route (250 µg/mouse, three times a week). These molecular effects of ormeloxifene were also observed in excised tumor tissues as shown by immunohistochemistry analysis. Our results, for the first time, demonstrate repurposing potential of ormeloxifene as an anticancer drug for the treatment of advanced stage metastatic prostate cancer through a novel molecular mechanism involving β -catenin and EMT pathway. *Mol Cancer Ther*; 16(10); 2267–80. ©2017 AACR.

tate cancer, which is the end stage that accounts for the majority of cancer deaths (2). Current chemotherapeutic drugs such as docetaxel, cabazitaxel, and mitoxantrone provide moderate treatment benefits for the management of advanced prostate cancer, but all of them suffer from severely toxic side effects. Moreover, these drugs do not target major oncogenic signaling pathways such as β -catenin/epithelial-to-mesenchymal transition (EMT), which lead to tumor growth and metastasis of prostate cancer (3).

Studies have shown that loss of E-cadherin and overexpression of N-cadherin is involved in EMT, leading to aggressive and metastatic prostate cancer phenotypes (4, 5). Altered β -catenin expression/subcellular localization also plays a major role in EMT process in various malignancies including prostate cancer (6). Studies have suggested involvement of β-catenin in the development, progression, and therapy resistance of advanced prostate cancer (3). β -catenin is present in the cytoplasm as a heterodimeric protein complex that includes glycogen synthase kinase 3β $(GSK3\beta)$, axin, and adenomatosis polyposis coli (APC; ref. 7). GSK3β-dependent phosphorylation of β-catenin enhances its proteasomal degradation and inhibits its translocation into the nucleus, where it binds to T-cell Factor (Tcf) family of transcription factors, leading to transcriptional activation of various downstream target oncogenes. Various studies including ours have reported increased nuclear β-catenin expression correlates with higher prostate tumor grades as compared with normal adjacent tissues (5, 8). The stabilization of the transcriptional co-activator



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 β -catenin regulates expression of many genes which are involved in cell proliferation, differentiation, and the EMT (9). These studies suggest that β -catenin appears to be a very important molecular target in cancer therapy, and its targeting may lead to successful therapeutic approach for the management of metastatic prostate cancer. Thus, there is an urgent need to develop nontoxic agents/pharmacologic inhibitors that target aforementioned signaling pathways. These agent(s) could be used alone or in combination with conventional chemotherapy for the treatment of advanced prostate cancer.

Ormeloxifene has demonstrated excellent anticancer activity in many different tumor types such as breast cancer (10), head and neck squamous cell carcinoma (HNSCC; ref. 11), and ovarian cancer (12). We have recently demonstrated potent therapeutic efficacy of ormeloxifene in pancreatic cancer via inhibiting sonic hedgehog (SHH) signaling pathway, and modulation of tumor microenvironment (13). However, its effects on EMT processes and Wnt/ β -catenin signaling are not investigated thus far. Herein, we have shown that ormeloxifene effectively inhibits molecular signatures of EMT, β-catenin/TCF-4 transcriptional activity, and induces phosphorylation of GSK3β, and degrades β-catenin leading to the suppression of prostate tumor growth in xenograft mouse model. Because ormeloxifene is reported to have an excellent therapeutic index and is safe for human use for antifertility (contraception) purpose (14), ormeloxifene appears to be an ideal pharmacologic agent for its repurposing as an anticancer agent against metastatic prostate cancer.

Materials and Methods

Cell lines

The human prostate cancer cells (PC3 and DU145) were the kind gift from Dr. Rajesh Singh, Assistant Professor, Morehouse School of Medicine, Atlanta, GA. They purchased these cells from ATCC in January, 2016. Upon receipt cells were expanded and frozen aliquots (passage < 6) were stored in liquid nitrogen. When needed, cells were thawed and grown for less than 6 months. These cell lines were propagated in RPMI1647 media supplemented with 10% FBS and 1× antibiotic and antimycotic solution. The media components were purchased from Lonza (Basel Switzerland).

Chemicals and antibodies

Specific monoclonal and polyclonal antibodies of β -actin (cat. no. 3700), cyclin D1 (cat. no. 2922), CDK4 (cat. no. 12790), p21 (cat. no. 2947), p27 (cat. no. 3686), Mcl-1 (cat. no. 5453), pGSK3β (cat. no. 5558), Histone H3 (cat. no. 4499), GAPDH (cat. no. 5174), N-cadherin (cat. no. 4061), Slug (cat. 9585), Snail (cat. no. 3879), Vimentin (cat. no. 5741), PARP (cat. no. 9532S), and MMP2 (cat. no. 4022) were obtained from Cell Signaling Technology Inc. β-catenin (cat. no. SC-7199), E-cadherin (cat. no. SC-7870), and MTA1 (cat. no. SC-17773) antibody was obtained from Santa Cruz Biotechnology. MMP3 (cat. no. IM36) antibody was procured from Calbiochem, Merck Biosciences, Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were acquired from Promega. Anti-mouse Cy3 secondary antibody was purchased from Thermo Fisher Scientific. Ormeloxifene was synthesized and characterized in Dr. Fathi Halaweish laboratory at South Dakota State University, Brookings, SD. The detail procedure for synthesis and characterization is described in our previous published manuscript (12).

MTT assay

Cell proliferation was determined by using MTT assay. Briefly, 5×10^3 cells of PC3 or DU145 were plated in 96-well plates and incubated for 24 hours in incubator at 37°C containing 5% CO₂. Cells were treated with ormeloxifene (5–40 µmol/L) for 24 and 48 hours. Twenty microliter of 5 mg/mL MTT was added in each well containing 100 µL of cell media. The cells were then further incubated for 6 hours in incubator and media was replaced with 150 µL of DMSO. Plates was vigorously shaked for 15 minutes and absorbance was taken at 570 nm on microplate reader (Cytation 3; BioTek).

Colony forming assay

To investigate the effects of ormeloxifene on clonogenic potential of PC3 and DU145 cells, colony formation assay was performed. In brief, 500 cells were seeded per well in six-well plate and allowed to stand for next 3 days. The cells were treated with ormeloxifene (2.5–7.5 μ mol/L) for 7 days. Control cells were treated with DMSO (0.1%) as a vehicle control. The cells were maintained under standard cell culture conditions at 37°C and 5% CO₂ in a humid environment. Colonies were fixed in methanol, stained with hematoxylin, and counted using UVP 810 software.

Western blot analysis

Western blot analysis was performed to investigate the effect of ormeloxifene on protein levels of various oncogenes linked to prostate carcinogenesis. Briefly, prostate cancer cells (70–80% confluent) were treated with ormeloxifene (10–20 μ mol/L) concentrations for 24 hours. Control cells were treated with vehicle (0.1% DMSO). Total cell lysates were prepared as described (15). Cytoplasmic and nuclear lysates were prepared using Nuclear Extract Kit (Active Motif). Forty microgram of protein lysates were subjected for Western blot analysis using 4% to 20% SDS-PAGE gels, blotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad), and blocked with 10% BSA 1 hour at room temperature. The membranes were then incubated with the indicated primary antibodies followed by a HRP secondary antibody and developed with enhanced chemiluminescence reagent (Roche) using a UVP gel documentation system.

Immunofluorescence analysis

To determine the effect of ormeloxifene on β -catenin localization, 30,000 cells were seeded in four wells chamber slide. Next day, ormeloxifene (10 µmol/L) treatment was given for 24 hours. Localization of β -catenin was monitored by performing immunofluorescence analysis using confocal microscopy as described (16).

TCF luciferase assay

To investigate the effect of ormeloxifene on TCF promoter activity, we performed TCF luciferase assay. The reporter constructs were a generous gift from Dr. R. Moon (University of Washington, Seattle, WA). In this experiment, DU145 cells (1.5×10^5 cells/well) were plated in triplicate in 12-well plates. Cells were transiently co-transfected with TCF-firefly luciferase reporter constructs (pTOP-FLASH; 1 µg) and *Renilla* luciferase (200 ng) or (pFOP-FLASH; 1 µg) and *Renilla* luciferase (200 ng). After 24 hours, cells were treated with ormeloxifene (10 and 20 µmol/L) and lysates prepared at 6 hours posttreatment. Firefly and *Renilla* luciferase activity was analyzed by using Dual Luciferase Kit (Promega). The β -catenin/TCF transcription activity was determined by normalizing the *Firefly* luciferase activity to that of *Renilla* luciferase activity and calculating the ratio of TOP-FLASH signal to FOP-FLASH signal.

Pulse chase assay

Pulse chase experiment was performed to determine the effect of ormeloxifene on β -catenin degradation using translational inhibitor cyclohexamide. Briefly, 70% confluent DU145 cells were treated with cyclohexamide (50 µg/mL) alone or in combination with ormeloxifene (15 µmol/L) for 1 to 24 hours. Cell lysates was prepared and protein levels of β -catenin was analyzed by Western blot analysis.

Molecular docking

Molecular docking experiments were conducted to know whether and where ormeloxifene binds in β -catenin (PDB ID: 4DJS; ref. 17) and GSK3β (PDB ID: 4ACH; ref. 18) proteins. The 2D and 3D structure of ormeloxifene were taken from (https:// pubchem.ncbi.nlm.nih.gov/compound/154413#section=Top) Pub Chem. These experiments were performed using autodock 4.2 suit by employing Lamarckian genetic algorithm (19). The grid map illustrating the active site pocket for ligands were calculated by autogrid and the dimension of the grid for 4DJS and 4ACH were 56 \times 50 \times 90 and 60 \times 62 \times 70 grid points respectively with a spacing of 0.375 A° between the grid points and centered on the ligand. Docking was accomplished by each cycle with an initial population of 150 individuals and the remaining parameter set as default. Ten conformational docking poses were created and the best docked confirmation was selected based on the autodock binding energy (20). The confirmations with the most favorable free binding energy were selected for analyzing the interactions between the target receptor and ligands by visualization with Discovery Studio Software (version 3.5).

Chemoinvasion assay

Cell invasion assay was performed using a Cell Invasion Kit (BD Biocoat Matrigel Invasion Chambers; BD Biosciences). All procedures were followed as per the manufacturer's instructions. In brief, PC3 cells (50,000 cells/well) were seeded in an upper chamber containing serum free medium and further treated with ormeloxifene (5–15 μ mol/L) or 0.1% DMSO as vehicle for 24 hours. The lower chamber was filled with 500 μ L of media containing 20% FBS. Forty-eight hours posttreatment, cells were completely removed from inside the upper chamber by cotton swab. Cells were fixed with methanol and stained with Crystal Violet. Invaded cells were observed by using a light microscope at 100× magnification. Cells that had invaded the matrix membrane were counted in three random fields of view and the experiments were performed in triplicate.

Cell migration assay

Cell migration assay was performed using Boyden's Chambers (BD Biosciences), as per manufacturer's protocol. After 48 hours incubation, the migrating cells were fixed with methanol and stained with crystal violet and photographed under light microscope. Migratory cells in ormeloxifene-treated group were compared with control.

Cell proliferation, invasion, and migration by real-time xCELLigence system

To further confirm the functional impact of ormeloxifene on migration, invasion, and proliferation of prostate cancer cells, real-time proliferation, invasion, and migration assays were performed using the xCELLigence system as described (13). Briefly, 5×10^3 prostate cancer cells were seeded per chamber of cell proliferation or invasion and 7×10^4 cells/well for migration. After 24 hours, ormeloxifene (5–15 µmol/L) or the vehicle control (0.1% DMSO) was added and the experiment was allowed to run for 48 hours. Average baseline cell index of ormeloxifene-treated cells was calculated and compared with vehicle-treated control cells.

Agarose bead assay

Effect of ormeloxifene on cellular motility was determined by an agarose bead-based cell motility assay as described (21).

Cell-cycle analysis

In this experiment, approximately 70% confluent PC3 cells were synchronized by overnight starvation of cells in serum-free media. Cells were treated with ormeloxifene $(10-20 \mu mol/L)$ for 24 hours and cell-cycle analysis was performed by flow cytometry as described (12).

Apoptosis analysis

Effect of ormeloxifene treatment on apoptosis induction in prostate cancer cells was analyzed using Annexin V-7AAD Apoptosis Kit (BD Biosciences) as described (15). In brief, PC3 and DU145 cells (200,000 cells per well) were plated in six-well plates and allowed to attach overnight. Next day, cells were treated with ormeloxifene (10–20 μ mol/L) concentrations for 24 hours. Both floating and adherent cells were collected, washed twice with cold PBS, and stained with Annexin V-7AAD (5 μ L) each/100 μ L of cell suspension for 20 minutes in dark at room temperature. Number of apoptotic cells were analyzed by setting FL2 (Annexin V) and FL3 (7AAD) channels in BD Accuri flow cytometer (BD Biosciences). To analyze the effect of ormeloxifene on mitochondrial membrane potential (Δ ψ m), we utilized tetramethyl rhodamine ethyl ester (TMRE) as described (12).

Xenograft study

A total of 12 athymic nude male mice were used to investigate the effect of ormeloxifene on PC3 cells derived xenograft tumors. The mice were maintained in a pathogen-free environment and all were carried out as per our approved protocol by the UTHSC Institutional Animal Care and Use Committee (IACUC). Briefly, PC3 cells (2 \times 10⁶) were dispersed in 100 µL 1:1 ratio of 1 \times PBS and 100 µL Matrigel (BD Biosciences) and injected subcutaneously into the dorsal flank of each mouse. The mice were periodically monitored for tumor development and the tumor volume was measured using a digital Vernier caliper. The tumor volume was calculated using the ellipsoid volume formula: tumor volume (mm³) = $\pi/6 \times L \times W \times H$, wherein L is length, W is width, and H is height. The mice were given intraperitoneal injection of ormeloxifene (250 µg/mice) and 0.2% ethanol in PBS three times a week for 6 consecutive weeks starting from week 1st. The tumor volume was regularly monitored and allowed to grow until the tumor burden reached a maximum volume of 1,100 mm³. At the time of sacrifice, the mice tumors were

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removed, fixed in formalin, embedded in paraffin, and sliced into 5 μ m sections for further biochemical analysis.

Immunofluorescence and immunohistochemistry analyses

Immunofluorescence analysis was performed to detect changes occur in the expression of β -catenin in excised xenograft tumor tissues of control and ormeloxifene-treated mice as described (21).

Statistical analysis

Statistical analysis was performed using an unpaired two-tailed Student t test and employed to assess the statistical significance between the control and ormeloxifene-treated groups. P value < 0.05 was considered as significant.

Results

Ormeloxifene treatment inhibits the growth of prostate cancer cells

Metastatic prostate cancer is the significant cause of mortality and morbidity in prostate cancer patients (22). Thus, we examined the anticancer effects of ormeloxifene on human prostate cancer cell lines (LNCaP, C4-2, PC3, and DU145) by cell counting assay (Supplementary Fig. S1). We performed additional cell proliferation assays (MTS assay and real-time xCELLigence assay) in two highly metastatic prostate cancer cell lines (PC3 and DU145). Ormeloxifene treatment inhibited viability of both PC3 and DU145 cells in a (10–40 μ m) dose-dependent manner. The IC₅₀ of ormeloxifene in PC3 and DU145 cells was 22 and 17 μ mol/L, respectively (Fig. 1Ai–ii), after 24 hours treatment,



Figure 1.

Ormeloxifene (ORM) inhibits the growth of hormone refractory prostate cancer cells. **A**, Effect of ORM on cell viability of PC3 (i) and DU145 (ii) cells. Briefly, cells (2,500) were seeded in each well of 96well plate and after overnight incubation, cells were treated with the

indicated concentrations of ormeloxifene for 24 and 48 hours. Cell viability was assessed by MTT assay. The line graph represents the percent viable cells compared with the vehicle-treated group cells. Each concentration value is the mean $\pm\,\text{SE}$ of triplicate wells of each group. B, Effect of ormeloxifene on prostate cancer cells proliferation Briefly, prostate cancer cells (5,000 cells/well) were seeded in E-plate (xCELLigence) following the xCELLigence Real-Time Cell Analyzer (RTCA) DP instrument manual as provided by the manufacturer. After 38 hours, ormeloxifene or the vehicle control was added and the experiment was allowed to run for 80 hours. Average baseline cell index for ormeloxifene treated PC3 (Bi) and DU145 (Bii) cells was compared with vehicle-treated group. C and D, Effect of ormeloxifene on clonogenic potential of prostate cancer cells. Representative colony images of control and ormeloxifenetreated PC3 (Ci) and DU145 (Di) cells. Bar graphs indicating quantification of colony formation in PC3 (Cii) and DU145 (Dii) cells. Asterisk (*) denotes the significant value P < 0.05

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whereas IC₅₀ of ormeloxifene was 20 µmol/L in PC3 cells and 15 µmol/L in DU145 cells after 48 hours treatment, respectively (Fig. 1Ai-ii). We next evaluated the effect of ormeloxifene treatment on prostate cancer cell proliferation using xCELLigence assay for the duration of 72 hours (Fig. 1Bi-ii). This assay monitors cell growth in real time by measuring changes in electric impedance between two golden electrodes embedded in the bottom of the cell culture wells. The impedance, which is converted to a cell index value, is directly proportional to the number of cells and also reflects the cells viability, morphology, and adhesion strength (23). The growth curve, which is presented as a baseline cell index, showed that ormeloxifene (10-20 µmol/L) reduced the baseline cell index in PC3 (Fig. 1Bi) and DU145 (Fig. 1Bii) cells in a dose-dependent manner compared with vehicle-treated cells. Ormeloxifene treatment inhibited clonogenic potential of PC3 (Fig. 1Ci-ii), DU145 (Fig. 1Di-ii), and C4-2 (Supplementary Fig. S2Ai-ii) cells as determined by independent colony formation assay. Moreover, ormeloxifene also inhibited anchorage-dependent growth of C4-2 cells (Supplementary Fig. S2Bi-ii). These results indicate that ormeloxifene effectively inhibits growth of prostate cancer cells including highly aggressive metastatic prostate cancer cells.

Ormeloxifene represses β -catenin signaling in prostate cancer cells

As ormeloxifene inhibited proliferation of prostate cancer cells, thus, we examined the effect of ormeloxifene on β -catenin signaling which is a major oncogenic pathway involved in tumorigenesis and metastasis (3, 24-27). Ormeloxifene treatment (10 μ mol/L) inhibited nuclear β -catenin in DU145 cells (Fig. 2Ai) through its sequestration in the cytoplasm (Fig. 2Ai) as determined by Western blot analysis. This result was further confirmed by confocal microscopy as ormeloxifene showed inhibition of β -catenin translocation into the nucleus of prostate cancer cells as compared with control (Fig. 2Aii and Supplementary Fig. S3Bi-ii). We next evaluated the effect of ormeloxifene on lithium chloride (LiCl)-induced β-catenin/TCF promoting activity by transiently cotransfecting the DU145 cells with TCF-firefly luciferase reporter constructs (pTOP-FLASH) and Renilla luciferase or (pFOP-FLASH) and Renilla luciferase. Ormeloxifene treatment (10 μ mol/L) for 6 hours, significantly (P < 0.01) inhibited lithium chloride (LiCl)-induced TCF-4 promoter activity in DU145 cells (Fig. 2iii). We also examined the effect of ormeloxifene on activation of GSK3ß by Western blot analysis which illustrated a marked increase in phosphorylated GSK3ß protein levels in DU145 cells (Fig. 2Bi). Because we observed activation of GSK3β protein by ormeloxifene treatment, thus, we next examined the effect of ormeloxifene on β-catenin degradation after using translational inhibitor (cycloheximide). Results revealed a time-dependent decrease in the protein levels of β -catenin in DU145 cells compared with cyclohexamide treatment alone group (Fig. 2Bii-iii). We next performed molecular docking studies to determine the orientation of ormeloxifene bound in the active sites of $\beta\text{-}catenin$ and GSK3 β using Discovery Studio software (version 3.5) as described (17). This study revealed that ormeloxifene binds with both β-catenin and GSK3β with a considerably high binding energy. Ormeloxifene binds into the active site of β -catenin (4DJS) and GSK3 β (4ACH) with minimum binding energy (ΔG), -6.2 and -8.5 kcal/mol, respectively (Table inserted as Fig. 2C). The docking results also confirmed that ormeloxifene strongly binds with amino acid residue of β-catenin at ARG: 469, ARG: 612 (Fig. 2Di-ii), and GSK3β at LYS: 85, ARG 96 (Fig. 2Diii–iv). These amino acid residues actively participate in hydrophobic, hydrophilic, and π – π interactions. Overall, these results suggest that ormeloxifene is a potent inhibitor of WNT/ β -catenin signaling pathway.

Ormeloxifene treatment effectively attenuates metastatic potential of prostate cancer cells

EMT is the basic characteristic of cancer cell in which epithelial cells undergo morphologic and molecular changes that transform these cells to mesenchymal, highly metastatic (invasive and motile) and drug-resistant phenotype (28). Thus, targeting EMT will reduce the invasive phenotypes of a cancer cell and have significant advantage to overcome drug resistance. It has been reported that β-catenin is involved in invasion and metastasis via inducing EMT in various tumor cells including prostate cancer (29, 30). Because ormeloxifene effectively inhibits β -catenin signaling, therefore, we evaluated the effect of ormeloxifene treatment on various EMT markers in prostate cancer cells. Ormeloxifene treatment revealed marked inhibition of N-cadherin, and Snail expressions in PC3 and DU145 cells (Fig. 3Ai-ii), whereas it induced the expression of E-cadherin in PC3 prostate cancer cells (Fig. 3Ai). Activation of MMPs are involved in matrix degradation that facilitate invasion of cancer cells (31), thus we sought to investigate the effect of ormeloxifene treatment on MMPs and found that ormeloxifene inhibited the expression of MMP2 and MMP3 (Fig. 3Ai-ii). We next performed functional assays (invasion and migration) by Boyden's chamber to determine whether ormeloxifene inhibits the invasive and migratory potential of prostate cancer cells. Our results revealed that ormeloxifene treatment (5-15 µmol/L) effectively inhibited both invasion (Fig. 3Bi) and migration (Fig. 3Ci) of PC3 cells and C4-2 cells (Supplementary Fig. S2Ci-ii). We further investigated the impact of ormeloxifene on real-time invasion and migration of PC3 cells using xCELLigence system. Ormeloxifene treatment also effectively decreased invasion (Fig. 3Bii) and migration (Fig. 3Cii) of PC3 cells. We further confirmed effect of ormeloxifene on PC3 cells migration (Fig. 3D) by beads assay. Our results revealed a marked decrease in number of migratory PC3 cells after 48 hours ormeloxifene treatment compared with control group (Fig. 3D).

Ormeloxifene treatment arrests cell cycle via modulation of cell-cycle regulatory proteins

Various studies have shown that agents which arrest cell cycle in G_0 - G_1 phase have potential chemotherapeutic effects (32, 33). Because, we observed that ormeloxifene inhibits the growth of prostate cancer cells, we sought to determine the effect of ormeloxifene on prostate cancer cell-cycle distribution. For this, we synchronized PC3 cells and treated with ormeloxifene (10-20 µmol/L) for 24 hours and cell-cycle analysis was performed by flow cytometry. Ormeloxifene treatment arrested PC3 cells cycle in G₀-G₁ phase in a dose-dependent manner (Fig. 4Ai-ii). Ormeloxifene treatment resulted in 5%, 20%, and 20% increase in cell-cycle arrest in G_0 – G_1 phase at 10, 15, and 20 μ mol/L dose, respectively, compared with vehicle-control treated cells (insert Table in Fig. 4Aii). Similar result was also observed in DU145 cells (Supplementary Fig. S3). We then evaluated the effect of ormeloxifene on cell-cycle regulatory proteins in prostate cancer cells. Ormeloxifene (10-20 µmol/L) inhibited expression of Mcl-1 in both PC3 and DU145 cells (Fig. 4Bi-ii). However, ormeloxifene (10-20 µmol/L) treatment showed more effect on cyclin D1 inhibition in DU145-treated cells as compared with PC3-treated

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cells. Ormeloxifene treatment induced expression of cell-cycle inhibitory proteins (p21 and p27) in both PC3 and DU145 cells (Fig. 4Bi-ii). These results suggest that ormeloxifene arrests cell cycle via modulating key cell-cycle regulatory proteins.

Ormeloxifene treatment induces apoptosis in prostate cancer cells

Because we observed arrest of cell cycle in G_0 - G_1 phase, thus we investigated the effect of ormeloxifene on apoptotic induction in prostate cancer cells by flow cytometry analysis. Ormeloxifene (10-20 µmol/L) dose-dependently increased apoptotic cell populations in both PC3 (Fig. 5Ai) and DU145 (Fig. 5Aii) cells as determined by enhanced Annexin V positive cells. Ormeloxifene at 20 µmol/L showed 55.6% and 50% apoptotic PC3 and DU145 cells respectively compared with control group (Fig. 5Ai-ii). We next examined the effect of ormeloxifene on mitochondrial membrane potential ($\Delta \psi m$) using TMRE staining (Fig. 5Bi-ii), which is a marker of apoptosis induction through intrinsic pathway. Ormeloxifene (10-20 µmol/L) dose-dependently decreased TMRE staining in both DU145 and PC3 cells as determined by fluorescence microscopy (Fig. 5Bi) and flow cytometry (Fig. 5Biii), respectively. We also examined the effect of ormeloxifene on PARP cleavage which is a marker for apoptosis. Ormeloxifene (20 µmol/L) induced PARP cleavage as determined by Western blot analysis (Fig. 5C). These results suggest apoptosis inducing potential of ormeloxifene in prostate cancer cells.

Ormeloxifene suppresses tumor growth in xenograft mouse model

To evaluate clinical relevance of our *in vitro* findings, we subcutaneously implanted PC3 cells in a preclinical xenograft mouse model. Intraperitoneal administration of ormeloxifene (250 µg/mice/thrice weekly) significantly (P < 0.01) reduced prostate cancer tumor growth (Fig. 6Ai–iii). To confirm if this tumor growth inhibition is mediated through the suppression of β -catenin, we performed immunofluorescence analysis of excised xenograft tumors for β -catenin. Both cytoplasmic and nuclear β -catenin expression was decreased in ormeloxifene-treated xenograft tumor cells compared with control tumor cells (Fig. 6B). This result was validated by immunohistochemistry analysis (Fig. 6C). We further determined the expression of EMT markers in excised tumor tissues of control and ormeloxifene-treated mice. Orme

loxifene treatment also inhibited the expression of N-cadherin, Slug, Snail, and Vimentin and induced the expression of E-Cadherin (Fig. 6C). Metastasis-associated protein 1 (MTA1) is known to be upregulated in many cancer types and is an important metastatic marker for tumor aggressiveness and metastasis (34, 35). Interestingly, we observed a decreased expression of MTA1 in ormeloxifene-treated xenograft tissues as compared with control. Next, we examined the expression of cell proliferative markers, proliferative cell nuclear antigen (PCNA), in control and ormeloxifene-treated tumor tissues by immunohistochemistry analysis. We found a marked decrease in nuclear PCNA staining in ormeloxifene-treated tumors compared with the tumors in vehicle-treated mice (Fig. 6C). These results reaffirm that ormeloxifene has potent therapeutic efficacy against prostate cancer and could be used for the treatment of metastatic prostate cancer. The possible molecular mechanisms of ormeloxifene to inhibit prostate tumor growth and metastasis have been summarized in a schematic diagram (Supplementary Fig. S4), which shows ormeloxifene inhibits Wnt/ β -catenin and EMT signaling and it induces activation of GSK3B, E-cadherin, p21, and p27 signaling pathways, thus decreasing prostate tumor growth and metastasis.

Discussion

Prostate cancer continues to remain the most common cancer and the second leading cause of cancer-related deaths, in American men. Metastatic prostate cancer is the end-stage and accounts for the majority of cancer deaths (22). Moreover, men with metastatic prostate cancer are at a higher risk of developing bone metastasis, which results in clinical skeletal morbidity (36). Although prostate cancer is frequently curable in its early stage by surgical and/or radiation therapy, many patients present locally advanced or metastatic disease for which there are currently no curative treatment options (37). Docetaxel and Cabazitaxel are FDA approved chemotherapeutic drugs for the treatment of metastatic prostate cancer, but these drugs have severe toxic side effects (38, 39). Accumulating evidence suggest that β-catenin signaling pathway and its related oncogenic events play a major role during the development, progression, and metastasis of cancer including prostate cancer (30, 40). Thus, there is an urgent need to identify more effective and nontoxic agents or drugs, which can target Wnt/ β-catenin and related oncogenic pathways.

Figure 2.

Effect of ormeloxifene on β-catenin signaling pathway and molecular docking of ormeloxifene (ORM) with β-catenin and GSK3β. A, Effect of ormeloxifene on β-catenin distribution in cytoplasm and nucleus of DU145 cells. Briefly, cells were treated with indicated concentrations of ormeloxifene for 24 hours, nuclear extracts were prepared and subjected for Western blot analysis to detect the protein levels of β -catenin. Results demonstrating decreased expression of nuclear β-catenin and increased expression of β-catenin in the cytoplasm (Ai) of DU145 cells. Blots were reprobed with Histone H3 and GAPDH antibodies as an internal control. Effect of ormeloxifene on β-catenin localization in PC3 cells as determined by confocal microscopy (Aii). Yellow arrows indicate localization of β-catenin in control and ormeloxifene-treated cells after 24 hours treatment (original magnification 40×) (Aii). Effect of ormeloxifene on TCF-4 promoter activity (Aiii). Cells were transiently cotransfected with TCF-firefly luciferase reporter constructs (pTOP-FLASH; 1 µg) and Renilla luciferase (200 ng) or (pFOP-FLASH; 1 µg) and Renilla luciferase (200 ng). After 24 hours, cells were treated with LiCl (50 µmol/L) alone or in combination with ormeloxifene (10 µmol/L). Cell lysates were prepared 6 hours posttreatment and firefly and Renilla luciferase activity was analyzed by using Dual Luciferase Kit (Promega). The β-catenin/TCF transcription activity was determined by normalizing the firefly luciferase activity to that of Renilla luciferase activity and calculating the ratio of TOP-FLASH signal to FOP-FLASH signal. Values in bar graph indicates mean ± SE of three wells reading in each group. Asterisk (*) denotes the significant value P < 0.01. B, Effects of ormeloxifene on protein levels of phospho GSK3β in DU145 as determined by Western blot analysis (Bi). Effect of ormeloxifene on β-catenin degradation as analyzed by pulse chase experiment. Briefly, DU145 cells were treated with cycloheximide (CHX; 50 µg) alone or in combination with ormeloxifene (15 µmol/L) at indicated time points. Protein lysates were prepared and subjected for Western blot analysis to analyze the protein levels of β -catenin. Results indicates protein levels of β-catenin in alone cyclohexamide-treated (top blot) and in cycloheximide and ormeloxifene-treated (bottom blot) (Bii). Line graph showing quantification of Western blots of Bii. 7_{1/2} denotes time point for 50% β-catenin degradation. C and D, Molecular docking studies of ormeloxifene with β-catenin and GSK3β. C, Table showing docking score of ormeloxifene with β-catenin and GSK38, **D.** Stereo view of ormeloxifene binding with β-catenin (**Di**) and GSK38 (**Diii**) showing hydrogen bond donor and acceptor residues around component. Schematic diagram of ormeloxifene docking with β-catenin (Dii) and GSK3β (Div) showing residues involved in hydrogen-bonding, Pi interactions, charge or polar interactions, Van der Waals interactions, which are represented by respective colors.

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Figure 3.

Effect of ormeloxifene (ORM) on cell invasion, migration, and EMT markers. Briefly, 70% confluent prostate cancer cells were treated with ormeloxifene (10-20 μ mol/L) for 24 hours. Cell lysates were prepared and subjected for Western blot analysis for EMT markers and MMPs analysis. **A,** Effect of ormeloxifene on EMT markers (E-cadherin, N-cadherin, and Snail) and MMPs (MMP2 and MMP3) in PC3 (**i**) and DU145 (**ii**) cells. Values shown above the blots are the densitometry analysis of each protein band normalized with respective β -actin value. **B,** Effect of ormeloxifene on invasion of PC3 cells as determined by Boyden chamber and xCELLigence assays. Representative photographs (20× original magnification) of invaded cells of control and ormeloxifene-treated PC3 cells as determined by Boyden Chamber Kit (**i**). Effect of ormeloxifene on real-time cell invasion (**ii**). Briefly, PC3 cells (7 × 10⁴) were seeded in invasion plate and invasion potential of these cells was determined by xCELLigence instrument as described in material and methods. Results indicate dose-dependent decrease in Cell Index, which correlates inhibition of real-time cell invasion by ormeloxifene treatment (**ii**). C, Effect of ormeloxifene on real-time cell invasion (**i**). C, Effect of ormeloxifene on cell migration by Boyden chamber and xCELLigence assays. Representative images (20× original magnification) showing inhibition of PC3 cells migration by Boyden chamber and xCELLigence assays. Representative images (20× original magnification) showing inhibition of PC3 cells as determined by Boyden chamber and xCELLigence assays. Representative images (20× original magnification) showing inhibition of PC3 cells migration by Boyden chamber assay (**i**). Effect of ormeloxifene on real-time cell migration as determined by xCELLigence assay (**i**). Briefly, PC3 cells (7 × 10⁴) were seeded in migration plate and ormeloxifene treatment (**5**-15 μ mO/L) was given after 15 hours and allowed the plate at 37 °C and 5% CO₂ for real-time migration assay up

Ormeloxifene has an excellent the rapeutic index and is safe for chronic administration in humans (14). The maximum serum concentration ($C_{\rm max}$) of ormeloxifene in humans is dose dependent ($C_{\rm max}$ of 55.53 \pm 15.43 ng/mL for 30 mg dose and $C_{\rm max}$ of 122.57 \pm 6.25 ng/mL for 60 mg dose) and is reached within 4 to 6 hours (41). Similar $C_{\rm max}$ values for ormeloxifene has also been detected in breast cancer patients treated with either 30 mg, twice a week for 12 weeks ($C_{\rm max}$ 54.98 \pm 14.19 ng/mL) or 60 mg of ormeloxifene on alternate days for 1 month (C_{max} 135 \pm 15.5 ng/mL). These studies indicate that ormeloxifene is a nontoxic, highly bioavailable, and shown potent anticancer effects against breast cancer (10), HNSCC (11), ovarian (12), and pancreatic cancer (13). However, molecular mechanisms of its anticancer properties are not well understood. In this study, we identified a novel molecular mechanism of ormeloxifene's anticancer action as it effectively targets Wnt/ β -catenin and EMT-related oncogenic signaling pathways in prostate cancer cells.

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Figure 4.

Effect of ormeloxifene (ORM) on cellcycle progression of prostate cancer cells. Ormeloxifene arrests PC3 cell cycle in G_0 - G_1 phase as determined by flow cytometry. A, Histogram (i) and table (ii) represent the cell-cycle distribution in PC3 cells. B, Effect of ormeloxifene on protein levels of cell-cycle regulatory proteins (cyclin D1, Mcl-1, p21, and p27) in both PC3 (Bi) and DU145 (Bii) cells. Briefly, cells were treated with indicated concentrations of ormeloxifene for 24 hours, total cell lysates were prepared. and subjected for Western blot analysis. Equal loading of protein in each lane was determined by probing the blots with β -Actin antibody. Values shown above the blots are the densitometry analysis of each protein band normalized with respective β -Actin value.

It is well documented that constitutive activation of β -catenin signaling pathway plays a major role in cancer progression and metastasis (3, 24) and drug resistance (42). Accumulating evi-

dences also suggest that β -catenin cross-talks with other oncogenic signaling components leading to more aggressive phenotype of prostate cancer cells (43). GSK3 β -dependent phosphorylation of

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Figure 5.

Ormeloxifene (ORM) treatment induces apoptosis in prostate cancer cells. PC3 and DU145 cells were treated with indicated concentration of ormeloxifene for 24 hours and processed for apoptosis analysis using Annexin V-7AAD Apoptosis Kit. **A**, Representative FL3-A and FL2-A plots showing dose-dependent increase of apoptosis in DU145 (**i**) and PC3 cells (**ii**). **B**, Effect of ormeloxifene on mitochondrial membrane potential ($\Delta \psi m$) as determined by TMRE staining. Representative fluorescence images showing dose-dependent effect of ormeloxifene on TMRE staining in PC3 and DU145 cells (**i**). Bar graph indicating dose-dependent inhibition of mitochondrial membrane potential ($\Delta \psi m$) in ormeloxifene-treated PC3 and DU145 cells as determined by flow cytometry. Asterisk (*) denotes the significant value *P* < 0.01. **C**, Effect of ormeloxifene on PARP cleavage. Briefly, 70% confluent prostate cancer cells were treated with ormeloxifene (10-20 µmol/L) for 24 hours. Whole cell lysates was prepared and subjected for Western blot analysis for full and cleaved PARP.

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Figure 6.

Ormeloxifene (ORM) inhibits prostate tumor growth in xenograft mouse model. A, Effect of ormeloxifene on PC3 cells derived xenograft tumors in athymic nude mice. In brief, a total of 12 mice were used in this experiment and were divided into two groups. A total of 2 \times 10⁶ PC3 cells were injected subcutaneously on dorsal flank of each mouse. Ormeloxifene (250 µg) was administered (intraperitoneal: 250 µg/ mouse) thrice a week till 6 weeks and control group mice received 0.2% ethanol in PBS as vehicle control. Mice of both the groups were sacrificed when control mice reached a targeted tumor volume of 1.000 mm³ Representative mouse picture of control and ormeloxifene-treated tumor bearing mouse (i). Average tumor volume of each group mice at different weeks (ii). Bar graph representing tumor weight of each group mice (iii). Value in graph represents mean \pm SE of six mice in each group. Asterisk (*) denotes the significant value P < 0.01. **B.** Effect of ormeloxifene on β -catenin expression in xenograft tumors of control and ormeloxifene-treated mice as determined by immunofluorescence (IF) analysis. White arrows indicate β-catenin accumulation in nucleus of the xenograft tissues. C, Effect of ormeloxifene on the expressions of β-catenin, N-cadherin, MTA1, Slug, Snail, Vimentin, and E-cadherin and PCNA in excised tumors of control (i) and ormeloxifene (ii) treated mice as determined by IHC analysis. All images of IF and IHC analyses were captured at $20 \times$ magnification.



β-catenin enhances its proteasomal degradation and inhibits its translocation into the nucleus, thus regulates its various downstream target oncogenes. Our results indicate that ormeloxifene activates GSK3β, thereby, degrades β-catenin in the cytoplasm and also inhibits nuclear β-catenin translocation and represses TCF-4 promoter activity. It has been reported that ARG 469 is one of the important amino acids involved in β-catenin interaction with TCF4 (44). Our molecular docking results indicate that ormelox-ifene potentially binds with ARG 469 amino acids of β-catenin (Fig. 2Di). It is possible that ormeloxifene inhibits β-catenin induced TCF4 promoter activity *via* binding to this cavity and inhibiting β-catenin/TCF4 interaction. Both LYS 85 and ARG 96 are important amino acids for GSK3β, which plays an important role in ATP binding and phosphoryl transfer (45, 46). It has been documented that mutation of LYS85 with ARG inhibits binding of Axin to GSK3 β , which resulted in inactivation of GSK3 β (47). Mutation in ARG 96 leads to complete loss of GSK3 β catalytic activity (46). Consistent with this observation, our molecular docking study reveals that ormeloxifene effectively docks with LYS 85 and ARG 96 (Fig. 2Dii). It may be possible that ormeloxifene induces activation of GSK3 β through increased ATP binding at LYS 85 and blocking mutation in these amino acids. More biological studies are warranted to further confirm the importance of these amino acid residues in ormeloxifene-induced activation of GSK3 β . β -catenin also regulates EMT-related oncogenic signaling though which cancer cells gain mesenchymal and metastatic

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characteristics (48). EMT is typically accompanied by loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as N-cadherin, Snail, and Vimentin in prostate cancer (49, 50). Our results indicate that ormeloxifene induces the expression of E-cadherin and inhibits Snail and N-cadherin in prostate cancer cells. These results suggest that ormeloxifene effectively blocks EMT progression. MMPs are secreted proteins which help cancer cell to invade through basal lamina by degrading extracellular matrix (51). MMP2/3 plays an important role in progression and metastasis of prostate cancer (31). Our results indicate that ormeloxifene has the ability to inhibit metastatic potential of prostate cancer cells, through repression of MMP2 and MMP3. In this study, we have also shown that ormeloxifene can effectively inhibit the cell proliferation and clonogenic potential of highly aggressive prostate cancer cells. Abnormal regulation of cell-cycle progression is one of trademark of cancer cells (52). G_0 - G_1 abrogation of the cell cycle prevents cancer cells from repairing DNA and inhibits them from entering the S phase. Thus, the G₀-G₁ checkpoint has emerged as an attractive therapeutic target for cancer therapy (32). Interestingly, ormeloxifene treatment arrested prostate cancer cells in in G₀-G₁ phase of cell cycle and induced apoptosis. These results are consistent with similar previous findings in other cancer cells (12, 13). It has been shown that cyclins and cyclin-dependent kinases (CDK) play a critical role in cell-cycle progression; their deregulation leads to cell-cycle arrest (53). The observed inhibitory effects of ormeloxifene on cyclin D1 in prostate cancer cells clearly demonstrates interference in cell-cycle regulatory proteins. It is known that p21/WAF1 and p27/KIP1 regulates CDK activity and our results illustrate increased expression of both p21 and p27 proteins in prostate cancer cells. These results suggest that ormeloxifene has the ability to arrest the prostate cancer cells in G_0 - G_1 phase via modulating cell-cycle regulatory proteins. Thus, ormeloxifene is expected to have high therapeutic efficacy against prostate cancer. Our in vivo therapeutic study showed that ormeloxifene significantly (P <0.01) reduces the prostate tumors burden in the preclinical athymic nude mouse model with no apparent toxicity. We also observed effective inhibition of β-catenin and EMT related markers (vimentin, N-cadherin, snail, and slug) in excised xenograft tumors of ormeloxifene-treated mice. It has been reported that MTA1 and β-catenin reciprocally activate each other during cancer/metastasis progression (54, 55). Inhibition of MTA1 expression correlates with improved clinical outcome in various cancer types. Ormeloxifene-mediated repression of MTA1 in xenograft tumors further indicates its potential to inhibit prostate cancer metastasis. Taken together, these results confirm that ormeloxifene is a potent inhibitor of β-catenin and EMT-related signaling pathways and has a potential to inhibit the metastatic phenotype of prostate cancer cells. These results also suggest that ormelox-

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ifene can be repurposed for the treatment of metastatic prostate cancer. For that, human trials, however, are warranted in near future.

Conclusion

In summary, we have shown potential anticancer effects of ormeloxifene against prostate cancer using cell lines and preclinical mouse models. ormeloxifene efficiently targets β -catenin and EMT-related signaling pathways to repress prostate tumor growth and metastatic phenotypes. We conclude that ormeloxifene could be used alone or in combination with current therapeutic regimen for the treatment of human prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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