Inhibition of cholesterol biosynthesis overcomes enzalutamide resistance in castration-resistant prostate cancer (CRPC)

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Running title: simvastatin overcomes enzalutamide resistance

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Abstract
Enzalutamide, a nonsteroidal second-generation antiandrogen, has been recently approved for the management of castration-resistant prostate cancer (CRPC). Although patients can benefit from enzalutamide at the beginning of this therapy, acquired enzalutamide resistance usually occurs within a short period. This motivated us to investigate the mechanism involved and possible approaches for overcoming enzalutamide resistance in CRPC. In the present study, we found that HMG-CoA reductase (HMGCR), a crucial enzyme in the mevalonate pathway for sterol biosynthesis, is elevated in enzalutamide-resistant prostate cancer cell lines. HMGCR knockdown could re-sensitize these cells to the drug, and HMGCR overexpression conferred resistance to it, suggesting that aberrant HMGCR expression is an important enzalutamide resistance mechanism in prostate cancer cells. Furthermore, enzalutamide-resistant prostate cancer cells were more sensitive to statins, which are HMGCR inhibitors. Of note, a combination of simvastatin and enzalutamide significantly inhibited the growth of enzalutamide-resistant prostate cancer cells in vitro and tumors in vivo. Mechanistically, simvastatin decreased protein levels of the androgen receptor (AR), which was further reduced in combination with enzalutamide. We observed that the decrease in AR may occur through simvastatin-mediated inhibition of the mTOR pathway, whose activation was associated with increased HMGCR and AR expression. These results indicate that simvastatin enhances the efficacy of enzalutamide-based therapy, highlighting the therapeutic potential of statins to overcome enzalutamide resistance in CRPC.

Introduction
Progression of prostate cancer (PCa), the second leading cause of cancer death in American men, is androgen dependent. Consequently, androgen deprivation therapy (ADT) is currently the primary approach for the treatment of PCa. Although patients can benefit from ADT at the beginning of treatment, most of them will relapse with castration-resistant prostate cancer (CRPC), which is
Currently incurable. Enzalutamide is a nonsteroidal second-generation antiandrogen that has recently been approved for the treatment of metastatic CRPC both in the post-docetaxel and chemotherapy-naive settings. It can inhibit androgen binding to the androgen receptor (AR), AR translocation into the nucleus, AR binding to DNA and coactivator recruitment (1). Although enzalutamide can work efficiently at the beginning of treatment, it is only valid for a specified period, after which acquired drug resistance usually occurs. Currently, it is reported that such resistance can be induced by AR splice variants (AR-Vs), AR mutation, autophagy, aberrant glucose metabolism, intracrine androgen biosynthesis, etc. (2-7).

Increasing data suggests that activation of de novo cholesterogenesis induces PCa cell proliferation and promotes cancer development and progression (8-10). Men with a higher level of cholesterol are usually at greater risk of developing high-grade PCa. During cholesterol biosynthesis, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) is the first rate-limiting enzyme, whose inhibition has been traditionally used to lower serum cholesterol as a means of reducing the risk for cardiovascular disease. Drugs that inhibit HMGCR, known collectively as statins, are generic drugs for the treatment of hypercholesterolemia. In addition to statins’ efficacy on treating cardiovascular disease, accumulating evidence suggests that statins also exert an anti-neoplastic effect in many types of cancer, including breast, prostate, ovarian, lymphoma, renal cell carcinoma and colorectal cancer (11). In PCa, both in vitro and in vivo experiments showed that statins could significantly reduce the level of prostate-specific antigen (PSA) (12-14). Further, it was reported that such down-regulated PSA levels might be caused by proteolysis of AR induced by statins (15). All these studies suggest that statins may suppress PCa progression through inhibiting AR. Therefore, we aim to test whether statins can overcome acquisition of enzalutamide resistance in CRPC where AR continues to play an important role.

Herein, RNA-Seq analysis was performed in two PCa cells lines: LNCaP and MR49F, the enzalutamide-resistant derivative of LNCaP. We found that genes involved in cholesterol biosynthesis pathway were upregulated in MR49F in comparison to those in LNCaP cells. Therefore, we hypothesize that cholesterol biosynthesis plays an essential role in the acquisition of enzalutamide resistance and that targeting HMGCR will overcome enzalutamide resistance in CRPC.

Results
Identification of highly expressed HMGCR in enzalutamide-resistant PCa cell lines
To investigate molecular mechanisms underlying enzalutamide resistance, we performed RNA-Seq analysis with LNCaP and MR49F cells. Based on the RNA-Seq result, higher gene expression of cholesterol biosynthesis pathway was observed in MR49F cells than that in LNCaP cells (Fig. 1A). Herein, HMGCR was selected for further study since it is the first rate-limiting protein that plays an important role in cholesterol biosynthesis. To validate the findings from our RNA-Seq analysis, a series of western blot was performed in LNCaP, MR49F, C4-2 and C4-2R (an enzalutamide resistant cell line derived from C4-2). As shown in Figs. 1B-D, C4-2R and MR49F showed higher expression levels of HMGCR than those of C4-2 and LNCaP. In addition, such a tendency became more significant when all cells were treated with enzalutamide for 4 hours prior to harvest. Meanwhile, the protein level of SREBP2, another critical regulator of cholesterol synthesis, was also examined. Upon enzalutamide treatment, MR49F and C4-2R express higher levels of cleaved SREBP2, the active form of SREBP2, than those of C4-2 and LNCaP. In addition, such a tendency became more significant when all cells were treated with enzalutamide for 4 hours prior to harvest. Meanwhile, the protein level of SREBP2, another critical regulator of cholesterol synthesis, was also examined. Upon enzalutamide treatment, MR49F and C4-2R express higher levels of cleaved SREBP2, the active form of SREBP2, than those of LNCaP and C4-2, respectively (Fig. 1B). Next, we wanted to investigate whether there was more cholesterol synthesized in enzalutamide resistant cell lines by directly measuring total cholesterol levels. As shown in Figs. 1E and 1F, both MR49F and C4-2R show much higher cholesterol levels than those of LNCaP and C4-2, respectively upon enzalutamide treatment. Thus, cholesterol biosynthesis is
indeed elevated by enzalutamide in enzalutamide-resistant PCa cells, but not in enzalutamide-sensitive cells. Collectively, the data described above indicated that a high level of HMGCR activates cholesterol biosynthesis in enzalutamide-resistant PCa cells. To further validate this finding in clinic, we performed bioinformatics analysis with 72 patient specimens (described in the method). Accordingly, we found that cholesterol biosynthesis pathway gene set was enriched in high HMGCR-expressing group, indicating that cholesterol biosynthesis pathway was activated as HMGCR expression was elevated (Figs. 1G and 1H). Furthermore, GSEA of the HMGCR expression profile showed that steroid biosynthesis pathway-related gene set is also positively enriched (Figs. 1I and 1J).

HMGCR expression level affects cellular response to enzalutamide
Having established that HMGCR is highly expressed in enzalutamide-resistant PCa cells, we asked whether alternation of HMGCR protein level could affect cellular response to enzalutamide. To explore this, shRNA was used to knock down (KD) HMGCR in C4-2R cells, and the downregulation of HMGCR was determined by IB (Fig. 2A). Due to the relatively lower KD efficacy of a single shRNA, a mixed pool of both shRNAs was used to construct stable HMGCR-KD cell line. Then, the growth assay was performed to test whether HMGCR-KD cells were more sensitive to enzalutamide. After 3-day treatment of enzalutamide with different concentrations, C4-2R cells were still fairly resistant to enzalutamide, whereas downregulation of HMGCR clearly rendered C4-2R cells more sensitive to enzalutamide (Fig. 2B). Furthermore, after HMGCR was restored in HMGCR-KD cells (Fig. 2C), it apparently rescued cells from enzalutamide-induced growth inhibition (Fig. 2D). In agreement, the similar observation was made in anchorage-independent growth assay (Figs. 2E-F). Compared with control cells which showed no significant difference in colony numbers after enzalutamide treatment (Fig. 2E), significantly fewer colonies were formed when HMGCR-KD cells were treated with enzalutamide (Fig. 2F), and the phenotype was largely reversed upon re-introduction of HMGCR (Fig. 2G). Furthermore, cell apoptosis was examined by IB against cleaved-PARP. C4-2R cells are resistant to enzalutamide and showed almost no apoptosis upon enzalutamide treatment, whereas knockdown of HMGCR restored their sensitivity to enzalutamide (Fig. 2H). Further, we transiently transfected C4-2 cells with HMGCR and asked whether overexpression of HMGCR could confer C4-2 cells resistance to enzalutamide. After HMGCR protein overexpression was confirmed by IB, we performed a 3-day cell growth assay under treatment of enzalutamide with indicated concentrations. As indicated, more C4-2 cells overexpressing HMGCR could survive than C4-2 cells transfected with pcDNA3.0 (Fig. 2I). Collectively, these results demonstrate that downregulation of HMGCR renders enzalutamide resistant PCa cells to be sensitive to enzalutamide, and that upregulation of HMGCR can confer resistance. As overexpression of HMGCR can confer PCa cells resistance to enzalutamide, we finally asked whether enzalutamide-resistant PCa cells are more sensitive to statins, which targets HMGCR. To test this, a 3-day growth assay under treatment of mevastatin with different concentrations was conducted. As expected, MR49F and C4-2R are more sensitive to mevastatin than their parental cell lines, respectively (Figs. 2J and 2K), providing a possible new approach to overcome enzalutamide resistance.

Simvastatin treatment overcomes enzalutamide resistance in vitro
Next, we asked whether simvastatin, one of the marked statins, could enhance the inhibitory effect of enzalutamide on enzalutamide-resistant PCa cells. For that purpose, colony formation assay was performed with MR49F, C4-2R and 22RV1 cells (Figs. 3A, 3B, and 3C). Significantly fewer colonies were observed in the combination groups in all three cell lines, suggesting that simvastatin enhances enzalutamide efficacy of these enzalutamide-resistant cell lines. To further validate this, we also performed proliferation assay in MR49F and C4-2R cells. As shown in Figs. 3D and 3E, simvastatin
alone could slightly slow down cell growth, whereas a combination of enzalutamide and simvastatin significantly inhibited cell proliferation rate. Meanwhile, representative images of four treatment groups were taken on day 5 for C4-2R cells to compare potential morphology change of the cells. As shown in Fig. 3F, there was no apparent difference between control group and enzalutamide monotherapy group, but simvastatin alone was capable of inducing shrinkage of cells. For combination group, the same morphological modification was observed, and cells were clearly sparser. Next, apoptosis level was tested after different treatments with indicated cell lines. 22RV1 or C4-2R cells were treated with simvastatin at indicated concentrations in the presence or absence of enzalutamide and harvested for IB against cleaved PARP, a marker of apoptosis. As shown in Figs. 3G-3I, enzalutamide alone could not induce any apoptosis as cells used are enzalutamide-resistant. While simvastatin treatment alone caused relatively weak cell death, the combination of two drugs clearly led to significantly higher levels of apoptosis. However, such a combinational effect on cell death was not observed in enzalutamide-sensitive LNCaP and C4-2 cells (Fig. 3J). To further confirm the combinatory effect between simvastatin and enzalutamide, CI was measured. As shown in Table 1, IC_{50} values for enzalutamide in C4-2R and MR49F cells are 57.6 μM and 20.0 μM, respectively. However, IC_{50} values for enzalutamide in C4-2R and MR49F cells are reduced to 23.3 μM and 8.2 μM respectively when cells are treated with simvastatin, suggesting that simvastatin renders enzalutamide-resistant cells to be sensitive to enzalutamide. CIs of the two drugs in these two cell lines are 0.9 and 0.91, further indicating that statin and enzalutamide exert a synergistic effect in inhibiting cell growth.

Simvastatin decreases AR protein expression through enhancing protein degradation

Because PCa progression is AR-dependent and enzalutamide resistance can be induced by reactivation of AR (16), we asked whether AR signaling is affected by simvastatin. We found that simvastatin decreased AR protein level in a dose-dependent manner in C4-2R cells (Fig. 5A). To determine whether simvastatin can affect transcription levels of AR and even AR-V7, quantitative real-time PCR was performed. As shown in Fig. 5B, there was no significant difference in mRNA expression of AR or AR-V7 upon simvastatin treatment, suggesting that transcription of AR and AR-V7 is not affected by simvastatin. Next, cycloheximide (CHX), a protein translation inhibitor was utilized to examine the effect of simvastatin on protein turnover of AR. Accordingly, after cycloheximide was added to the cells in the presence of different concentrations of simvastatin, C4-2R cells were harvested at different time points to follow AR degradation. As shown in Fig. 5C, simvastatin treatment clearly accelerated AR degradation in comparison to cells only treated with cycloheximide. Therefore, it is possible...
that simvastatin could induce AR degradation via the ubiquitin-proteasome system instead of downregulating transcription. To confirm this hypothesis, the 26S proteasome inhibitor MG132 was used to ask whether it can reverse the simvastatin-induced AR degradation. As shown in Fig. 5D, MG132 treatment indeed partially rescued AR protein level from degradation induced by simvastatin.

**Enzalutamide and simvastatin decrease AR protein expression synergistically**

Having established that simvastatin can partially enhance AR protein degradation, we then asked whether a combinational treatment of enzalutamide and simvastatin would affect AR protein level synergistically. To investigate this possibility, C4-2R, 22RV1, and MR49F cells were treated with enzalutamide, different concentrations of simvastatin or various combinations of the two drugs, and harvested. As shown in Figs. 6A-6C, AR protein levels were decreased by simvastatin treatment alone in a dose-dependent manner. Further, simvastatin plus enzalutamide led to maximum inhibition of AR (Figs. 6A-6C) and even AR-Vs in 22RV1 cells (Fig. 6B). To further confirm this, immunofluorescence (IF) staining was conducted. As shown in Fig. 6D, enzalutamide alone did not alter the AR protein levels in C4-2R cells. While simvastatin alone partially decreased AR level compared with that of the control group, simvastatin plus enzalutamide almost completely abolished AR signal. We further showed that the reduced AR level in the co-treatment group was not due to a reduced AR mRNA level (Fig. 6E), but due to a shortened protein half-life (Fig. 6F). In addition to these, we aimed to confirm that decreased AR protein level is indeed responsible for apoptosis induced by enzalutamide plus simvastatin. Toward that end, MR49F, C4-2R, and 22RV1 cells were overexpressed with AR, followed by co-treatment with enzalutamide and simvastatin for 48 hours. As shown in Fig. 6G, less apoptosis was observed in cells overexpressing AR in comparison with control cells, indicating that overexpression of AR can protect enzalutamide-resistant cells from apoptosis caused by enzalutamide plus simvastatin. Finally, to test whether the effects seen above are specific to HMGCR, we examined AR protein expression in cells (control or HMGCR-KD) after enzalutamide treatment. As shown in Figs. 6H and 6I, AR level decreases partially in HMGCR-KD cells, but more significantly upon enzalutamide treatment. In summary, our results demonstrate that degradation of AR is induced in PCa cells upon treatment with simvastatin alone or enzalutamide plus simvastatin, and that AR degradation is the mechanism responsible for the synergistic effect of stain plus enzalutamide.

**Gene expression of mTOR pathway is positively correlated with HMGCR and AR expression**

The previous study in our lab showed that a positive feedback loop exists among mTOR pathway, AR signaling, and lipid biosynthesis pathway (17). Thus, we asked whether such an interaction is still active after anti-hormone therapy or even enzalutamide treatment. To explore this possibility, Pearson correlation analysis was performed to detect the gene expression interaction between HMGCR and mTOR, HMGCR and AR, as well as AR and mTOR by using the samples from 72 patients (described in the method). As shown in Fig. 7A-7F, correlations of all three groups’ gene expression were positive, but only the gene co-expression between AR and mTOR was significant with a correlation coefficient value of 0.4288 (Fig. 7C). Moreover, to explore whether mTOR pathway activation is associated with HMGCR and AR expression, GSEA analysis was performed with the same samples. As shown in Figs. 7G and 7H, mTOR pathway gene set was enriched in high HMGCR-expressing group, indicating that mTOR pathway is activated as HMGCR expression increases. Furthermore, GSEA analysis of AR expression profile showed that mTOR pathway-related gene set was positively enriched as well (Figs. 7I and 7J). To validate the observation described above, additional experiments were performed by using of our cell models. As indicated, cleaved SREBP2, HMGCR, AR and critical components of the mTOR pathway were all highly expressed in C4-2R cells in comparison to C4-2 cells (Fig.
In addition, depletion of HMGCR led to downregulation of levels of mTOR, phospho-mTOR, phospho-AKT and AR (Figs. 7L and 6I). Besides HMGCR expression level, we asked whether lower HMGCR activity could affect mTOR pathway as well. As shown in Fig. 7M, both the levels of phospho-AKT and phospho-S6 were decreased upon simvastatin treatment in a dose-dependent manner, suggesting that simvastatin is capable of inhibiting mTOR pathway. Finally, inhibition of AKT with BKM120 induced an apparent degradation of AR in C4-2R cells upon enzalutamide treatment (Fig. 7N), which also explains why a combination of enzalutamide and simvastatin reduces AR synergistically.

Discussion
Enzalutamide has been recently approved as a drug for CRPC by FDA, and it significantly improves the therapy for late-stage CRPC patients (1,18,19). However, drug resistance usually occurs, and patients will relapse with CRPC, which is incurable. In this study, we have identified that HMGCR, a key enzyme for synthesis of cholesterol, is overexpressed in enzalutamide-resistant cell lines including MR49F and C4-2R, suggesting that abnormal cholesterol biosynthesis is likely to be one contributing factor for enzalutamide resistance. We further demonstrate that knockdown of HMGCR re-sensitize C4-2R to enzalutamide and that overexpression of HMGCR confers C4-2 to be more resistant to enzalutamide. Next, simvastatin, an inhibitor of HMGCR, was used to overcome enzalutamide resistance both in vitro and in vivo. Mechanistically, we show that simvastatin downregulates protein level of AR by promoting its degradation and that a combination of simvastatin and enzalutamide exerts a synergistic effect on AR protein turnover, and this process may be through inhibition of mTOR pathway whose activation is found positively associated with HMGCR and AR expression.

Increasing evidence shows that the lethal PCA is associated with deregulation of lipid or cholesterol biosynthesis (20-23), and that lipid biosynthesis induced by AR reactivation can induce resistance to androgen-deprivation therapy and contributes to CRPC progression (24). For enzalutamide resistance, cholesterol is capable of participating in intracrine androgen biosynthesis, conferring PCa cells resistance to enzalutamide (7). Herein, we showed that expression of HMGCR, the first rate-limiting enzyme of cholesterol biosynthesis, was elevated in MR49F and C4-2R cells in comparison to their parental cell lines and that the activity of HMGCR was increased upon enzalutamide treatment, which means more cholesterol was synthesized to support cell survival. Therefore, we propose that aberrant expression of HMGCR is one of enzalutamide resistant mechanisms.

Several studies have been conducted to investigate the effect of statins on PCa cell proliferation. For example, Sekine et al. reported that simvastatin could suppress proliferation and induce apoptosis of PC3 cells (25). Hong et al. showed that LNCaP cell proliferation could be inhibited by lovastatin (26). These findings are consistent with our observation that simvastatin can induce apoptosis of enzalutamide-resistant cells. Meanwhile, we aimed to investigate whether inhibition of HMGCR could overcome enzalutamide resistance. However, we are aware that the effect of combination of enzalutamide and statins on PCa cells was reported by Syvälä et al. during the preparation of our manuscript. The group showed that combination of simvastatin and enzalutamide exerted additive growth inhibition on LNCaP and VCaP cells in vitro (27), indicating that statins had potential to increase the efficacy of enzalutamide-resistant cells. However, whether statin can overcome enzalutamide resistance was not tested in their study, as both LNCaP and VCaP are androgen sensitive cell lines, and no castration-resistant or enzalutamide-resistant cell lines were examined in their study. Herein, we showed that simvastatin could restore enzalutamide’s inhibitory effect on MR49F, C4-2R, and 22RV1 cells, and that the combined treatment could also inhibit proliferation of 22RV1-derived tumor synergistically.

In order to explore the underlying mechanism responsible for the inhibitory effect of statins on
PCa cell proliferation, we investigated the role of AR signaling. Previously, Syvälä et al. revealed that AR level was decreased slightly by simvastatin in LNCaP cells (27), and Yokomizo et al. found mevastatin and simvastatin could downregulate AR protein by proteolysis and decrease proliferation in RWPE-1, 22RV1 and LNCaP cells (15). These results are consistent with our finding that simvastatin led to slight AR protein degradation via the proteasome system without affecting mRNA transcription of AR. Furthermore, we found that combination of enzalutamide and simvastatin could induce more dramatic degradation of AR than simvastatin alone. Such degradation of AR was also observed in HMGCR-KD cells upon treatment with enzalutamide. In addition, we also showed that protein level of AR-Vs was decreased by simvastatin either alone or in combination with enzalutamide in 22RV1 cells. Therefore, all of these data help to explain why statin treatment or HMGCR inhibition overcomes enzalutamide resistance, as it has been documented that formation of AR-Vs, AR mutation, and AR amplification could confer PCa cells resistance to enzalutamide (2,6).

The proposed model described in our previous study (17) summarized how mTOR pathway, cholesterol biosynthesis, and AR signaling cooperated to drive PCa progression. In agreement, we found that positive correlations among HMGCR, AR and mTOR pathway exist in hormone therapy-resistant patient specimens, as well as in enzalutamide-resistant PCa cells. Therefore, mTOR pathway is likely to play a critical role in the enzalutamide resistance, and it could be affected by HMGCR expression. In addition, we showed that simvastatin could inhibit mTOR pathway as well, consistent with previous studies (28,29). Considering that inhibition of AKT induced apparent degradation of AR in C4-2R cells upon enzalutamide treatment, it is likely that simvastatin induces AR degradation via inhibiting mTOR pathway.

In summary, our study shows the aberrant expression of HMGCR is one of enzalutamide resistant mechanisms. In addition, proliferation of enzalutamide-resistant cells is inhibited by simvastatin both alone as well as combined with enzalutamide in vitro and in vivo. Mechanistically, the combination of the two drugs induces maximum degradation of AR and AR-Vs, potentially through inhibition of mTOR pathway. Recently, it has been reported that statins could be used to delay progression of PCa in patients under ADT (30,31). Therefore, the new combination strategy can be considered for clinical trials to overcome enzalutamide resistance in PCa.

**EXPERIMENTAL PROCEDURES**

**Cell culture and drug**
LNCaP, C4-2, 22RV1, MR49F and C4-2R cells were used in the study. While LNCaP cells are androgen dependent, C4-2 cells were derived from LNCaP but androgen independent. MR49F cells were derived from LNCaP but enzalutamide resistant. C4-2R cells were derived from C4-2 but enzalutamide resistant. We ordered LNCaP and 22RV1 from ATCC. While C4-2 cells were obtained from MD Anderson Cancer Center, MR49F and C4-2R cells were kindly provided by Dr. Amina Zoubeidi at The Vancouver Prostate Cancer Center and Dr. Allen Gao at University of California at Davis, respectively. LNCaP, C4-2 and 22RV1 cells were cultured in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 U/mL penicillin, 100 U/mL streptomycin at 37°C in a humidified incubator with 5% carbon dioxide. C4-2R and MR49F cells were maintained in previously described medium containing 20 μM and 10 μM enzalutamide, respectively. Enzalutamide and Simvastatin were purchased from Medchem Express.

**Antibodies**
Antibodies against androgen receptor (5153S), cleaved-PARP (9541L), p-AKT (S473) (4051S), AKT (4691S), p-S6 (S235/236) (2211S), p-S6 (S240/244) (5364S), S6 (2317S), p-4EBP1 (T37/46) (2855S), GAPDH (2118L) and cleaved-caspase 3 (9661S) were purchased from Cell Signaling Technology. Antibody against HMGCR (ABS229) was ordered from...
Millipore. Antibody against SREBP-2 (sc-5603) was obtained from Santa Cruz Biotechnology.

**Immunoblotting (IB)**

Upon harvest, cells were suspended with TBSN buffer (20 mmol/L Tris-HCl, pH 8.0, 0.5% NP-40, 5 mmol/L EGTA, 1.5 mmol/L EDTA, 0.5 mmol/L sodium vanadate and 150 mmol/L NaCl) with protease inhibitors and phosphatase inhibitors, sonicated and then collected. Protein concentrations were determined by Protein Assay Dye Reagent from Bio-Rad. Equal amounts of protein from each sample were mixed with SDS loading buffer and separated by SDS-PAGE. After transferring to PVDF membranes, proteins were probed with appropriate first and second antibodies. All western blotting were repeated at least three times and protein bands were quantified by Image J and normalized to their respective GAPDH or β-Actin.

**Clonogenic assay**

Cells were seeded with equal density in 6-well plate and treated with DMSO or different drugs with designated concentrations for 14 days. The medium was changed every 7 days. After the colonies were fixed by 10% formalin and stained with 5% crystal violet, colony numbers were determined by Image J.

**Combination index (CI)**

The equation CI = (Am)_{50}/(As)_{50} + (Bm)_{50}/(Bs)_{50} was used to calculate CI of enzalutamide and simvastatin (32). While (As)_{50} is the concentration of enzalutamide that will exert 50% inhibitory effect by itself, (Bs)_{50} is the concentration of simvastatin that will exert 50% inhibitory effect by itself. (Am)_{50} is the concentration of enzalutamide to achieve a 50% inhibitory effect in combination with simvastatin. (Bm)_{50} is the concentration of simvastatin that will produce a 50% inhibitory effect in combination with enzalutamide. Antagonism is indicated when CI > 1, CI = 1 indicates an additive effect and CI < 1 means synergy (33).

**Quantification of total cholesterol in cells**

Total cholesterol was measured by a commercial kit (ab65359) following the manufacturer's instruction. Cholesterol was extracted from 1×10^6 cells and dried at 50°C before dissolved in supplied assay buffer. OD was measured in a 96-well plate reader in duplicate at 570 nm, and total cholesterol amount was calculated by standard curve.

**HMGCR shRNA transduction**

The kit (TL312393V) with HMGCR - Human shRNA lentiviral particles was purchased from Origene. A mixed pool of particles was applied to knock down HMGCR. The selection was performed by using puromycin following manufacturer's instruction.

**Real-time quantitative reverse transcription-PCR**

Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. The same amount of RNA was used for reverse transcription that was performed by iScript cDNA Synthesis Kit (Bio-Rad), followed by gene amplification using FastStart Universal SYBR Green (Roche Applied Science) and a Roche LightCycler 96 thermocycler (Roche Diagnostics Corp.). All individual reactions were performed in triplicate, and all genes were normalized to GAPDH or 18S RNA. Primers used for Real-time PCR are: AR full length, 5’-AAGCCAGAGCTGTGCAGATGA-3’ (forward) and 5’-TGTCCTGCAGCCACTGTGTC-3’ (reverse); AR-V7, 5’-AACAGAAGTGACTGTGCAGATGA-3’ (forward) and 5’-TCAGGGTCTGGTCATTTTGA-3’ (reverse); 18S RNA, 5’-GAGAAGGCTGGGGCTCAT-3’ (forward) and 5’-TGTCCTGCAGCCACTGTGTC-3’ (reverse); GAPDH, 5’-GTAACCCGTTGAACCACATT-3’ (forward) and 5’-GAGAAGGCTGGGGCTCAT-3’ (reverse).

**In vivo tumorigenesis assay**

22RV1 cells (2 x 10^5 / mouse) were mixed with Matrigel (1:1) and injected subcutaneously into the right flank of nude mice. Tumor-bearing mice were randomized into 4 groups for indicated treatment as follows: for the first week, enzalutamide (25 mg/kg body weight) was gavaged and simvastatin was intraperitoneally injected every two days; from the second week, both drugs were...
administered every day. Tumor size was measured by calipers every two days and volumes were calculated using length × width²/2. The mouse experiments have been approved by the Purdue Animal Care and Use Committee.

**Histology and IFC**

Xenograft tumors were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned to 5 mm, and stained using conventional hematoxylin and eosin (H&E) staining. Immunofluorescent chemistry (IFC) staining was accomplished with the M.O.M.™ kit from VECTOR LABORATORIES.

**Gene Set Enrichment Analysis**

For TCGA prostate adenocarcinoma patient data, the RNA-seq information was collected from Level 3 (for Segmented or Interpreted Data, IlluminaHiSeq_RNASeqV2 of TCGA. For hormone-sensitive and -resistant high Gleason score signature in TCGA PCa, cancer samples were divided into low-grade (Gleason scores <8) group and high-grade (Gleason scores ≥ 8) group. Samples with a high-grade Gleason score plus anti-hormone treatment were considered to be potentially resistant to anti-hormone therapy. To integrate patient demography and drug treatment annotation together, 72 out of 497 cases with clinical anti-hormone treatment were selected as observed objectives. After 72 patients were separated into two groups based on the gene expression level of our target proteins, GSEA (gene set enrichment analysis) software was applied to detect the gene pathway enrichment variation between the groups we studied.

**Statistical analysis**

Standard 2-tailed Student t tests were performed to analyze statistical significance of the results. A P value of less than 0.05 indicates statistical significance.

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**CONFLICT OF INTEREST**

The authors declare no potential conflicts of interest.

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Figure Legends

Figure 1. Cholesterol biosynthesis is elevated in enzalutamide-resistant PCa cells

(A) Heat map of gene expression patterns in LNCaP vs MR49F cells upon RNA-seq. (B) LNCaP, MR49F, C4-2 and C4-2R cells were treated with DMSO or enzalutamide (Enza) for 4 hours and harvested for immunoblotting (IB) with antibodies indicated. (C and D) Quantification of the relative HMGCR band intensities from three experiments. The intensities of HMGCR bands were normalized to GAPDH first, then normalized to those of LNCaP and C4-2 cells with DMSO treatment, respectively. Results are represented as mean ± SD, n = 3. (E and F) After cells were treated with enzalutamide for 4 hours, total cholesterol was extracted and measured by a kit following the manufacturer's instruction. Results are presented as means ± SD of two experiments in duplicate. (G) GSEA confirms that cholesterol biosynthesis pathway gene set is enriched in the high HMGCR-expressing group. The enrichment plot shows the distribution of genes in the set that are correlated with HMGCR expression. (H) The heat map shows where gene expression is high (red) or low (blue) for each gene within the cholesterol biosynthesis pathway. (I) GSEA confirms that steroid biosynthesis pathway gene set is enriched in the high HMGCR-expressing group. The enrichment plot shows the distribution of genes in the set that are correlated with HMGCR expression. (J) The heat map shows where gene expression is high (red) or low (blue) for each gene within the steroid biosynthesis pathway.

Figure 2. HMGCR confers resistance to enzalutamide in PCa cells

(A) C4-2R cells were stably transfected with sh-Control, sh-HMGCR #1, sh-HMGCR #2 or a mixed pool of #1 and #2, and then harvested for IB, followed by quantification. (B) HMGCR-KD (knock down) C4-2R cells and control cells were treated with enzalutamide at indicated concentrations for three days, followed by cell number determination. Results are presented as means ± SD of three experiments. (C and D) HMGCR-KD C4-2R cells were transfected with control or shRNA-resistant HMGCR plasmid, treated with different concentrations of enzalutamide for three days, and harvested for cell number determination. (E and F) HMGCR-KD C4-2R cells and control cells were cultured in the medium with enzalutamide (20 µM) for 14 days with the medium being changed on the seventh day. After cells were fixed in 10% formalin, colony formation was subjected to crystal violet staining and quantified with results being presented as means ± SD of three experiments. (G) HMGCR-KD C4-2R cells were transfected with control or shRNA-resistant HMGCR plasmid, and cultured in the medium containing enzalutamide (20 µM), followed by a 14-day anchorage-independent growth assay. (H) C4-2R cells (control or HMGCR-KD) were treated with 20 µM enzalutamide for 48 hours and harvested for IB against cleaved PARP. IB results are quantified and presented as means ± SD of one experiment in triplicate. Meanwhile, cell lysates were collected after three days for IB to determine the expression of HMGCR. (J and K) The indicated PCa cells were treated with different concentrations of mevastatin (Meva) for 72 hours, followed by MTT assay to measure cell viability. Results are presented as means ± SD of two experiments in triplicate. *, p < 0.05.

Figure 3. Simvastatin treatment overcomes enzalutamide resistance in vitro

(A-C) MR49F, C4-2R and 22RV1 cells (500 – 1,000 cells/well) were cultured in the media with enzalutamide, simvastatin, or both drugs at the indicated concentrations. Media containing drug(s) every 3 days for 10 days, cells were fixed, followed by colony formation.
staining with crystal violet. Colonies were counted and results are presented as means ± SD of three experiments. (D) MR49F cells (5,000 cells/well) were cultured in the media with enzalutamide (10 μmol/L), simvastatin (1 μmol/L), or both drugs. Growth assay was carried out for 5 days, with cell numbers being counted every day. (E and F) C4-2R cells (5,000 cells/well) were cultured in the media with enzalutamide (20 μmol/L), simvastatin (1 μmol/L), or both drugs. Growth assay was carried out for 5 days, with cell numbers being counted every day. Representative images were taken on day 5 to show the morphology of the cells in 4 groups. (G) C4-2R cells were treated with simvastatin, enzalutamide or combination of the two drugs at the indicated concentrations for 48 hours, followed by IB against cleaved PARP. (H) Quantification of relative band intensities of cleaved PARP from three independent experiments. The intensities of cleaved PARP bands were normalized to those of β-actin and then normalized to the first lane. Data are represented as means ± SD, n=3. (I) 22RV1 cells were treated with simvastatin (SIM), enzalutamide or combination of the two drugs at the indicated concentrations for 48 hours, followed by anti-cleaved PARP IB to measure apoptosis. (J) LNCaP and C4-2 cells were treated with simvastatin (5 μmol/L), enzalutamide (20 μmol/L) or a combination of two drugs for 48 hours, and harvested for anti-cleaved PARP IB.

**Figure 4. Simvastatin treatment overcomes enzalutamide resistance in vivo**

(A-D) Mice bearing 22RV1 tumors were treated with enzalutamide, simvastatin or a combination of two drugs for 4 weeks as described in Materials and Methods. Tumor volumes were measured every 2 days (mean ± SD; n = 4 mice for each group). Mice body weights were measured before sacrifice, and fresh tumors were weighed immediately after sacrifice. **, P < 0.01 compared with enzalutamide or simvastatin monotherapy. (E) Representative images of H&E staining on formaldehyde-fixed, paraffin-embedded 22RV1 xenograft tumor sections from groups with different treatments. (F and H) Representative images of anti-Ki67 and anti-cleaved caspase 3 IFC staining of tumor sections. (G and I) Quantification of Ki67 and cleaved caspase 3 staining. *, P<0.05. **, P<0.01.

**Figure 5. Simvastatin treatment suppresses the level of AR protein expression**

(A) C4-2R cells were treated with simvastatin at indicated concentrations for 48 hours, followed by anti-AR IB. Quantification of relative AR band intensities from three independent experiments. AR bands were normalized to GAPDH and then normalized to the first lane. Data are represented as means ± SD, n=3. (B) 22RV1 cells were treated with simvastatin at indicated concentrations for 48 hours and harvested for quantitative RT-PCR. (C) C4-2R cells were treated with 50 μg/ml cycloheximide (CHX) and different concentrations of simvastatin and harvested at indicated time points, followed by anti-AR IB. The data were plotted relative to AR level at time 0. (D) C4-2R cells were treated with different concentrations of simvastatin for 48 hours, further incubated with 5 μM MG132 for 8 hours, and harvested for IB.

**Figure 6. Combination of enzalutamide and simvastatin further decreases AR protein level**

(A) C4-2R cells were treated with 20 μM enzalutamide, indicated concentrations of simvastatin or various combinations of the two drugs for 48 hours, and harvested for IB to measure AR and PSA expression. (B) 22RV1 cells were treated with 20 μM enzalutamide, indicated concentrations of simvastatin or different combinations of the two drugs for 72 hours, and harvested for IB. (C) MR49F cells were treated with 10 μM enzalutamide, indicated concentrations of simvastatin or different combinations of the two drugs for 48 hours, and harvested for IB. (D) Representative
images of IF staining for AR in C4-2R cells treated with 20 µM enzalutamide, 5 µM simvastatin or a combination of the two drugs for 48 hours. (E) C4-2R cells were treated with enzalutamide (20 µmol/L), simvastatin (5 µmol/L) or a combination of two drugs for 48 hours and harvested for quantitative RT-PCR. (F) C4-2R cells were treated with 50 µg/ml cycloheximide (CHX) in the presence of simvastatin (5 µmol/L) or enzalutamide (20 µmol/L) plus simvastatin (5 µmol/L), and harvested at indicated time points, followed by anti-AR IB. (G) MR49F, C4-2R, and 22RV1 cells were transiently transfected with AR or pcDNA3.0, cultured in medium with the combination of 20 µM enzalutamide and 5 µM simvastatin for 48 hours, and harvested for IB against AR and cleaved PARP. (H) C4-2R cells (control or HMGCR-KD) were treated with 20 µM enzalutamide or DMSO for 48 hours and harvested. (I) Quantification of relative AR band intensities from three independent experiments. AR bands were normalized to β-Actin and then normalized to the first lane. Data are represented as means ± SD, n=3.

Figure 7. Gene expression of mTOR pathway is positively correlated with HMGCR and AR expression

(A and B) Correlation between expression of HMGCR and mTOR in 72 patient samples. (C and D) Correlation between expression of AR and mTOR in 72 patient samples. (E and F) Correlation between expression of AR and HMGCR in 72 patient samples. (G) GSEA confirms that mTOR pathway gene set is enriched in high HMGCR-expressing group. The enrichment plot shows the distribution of genes in the set that are correlated with HMGCR expression. (H) The heat map shows where gene expression is high (red) or low (blue) for each gene of the mTOR pathway in the samples with different levels of HMGCR. (I) GSEA confirms that mTOR pathway gene set is enriched in high AR-expressing group. The enrichment plot shows the distribution of genes in the set that are correlated with AR expression. (J) The heat map shows where gene expression is high (red) or low (blue) for each gene of the mTOR pathway in the samples with different levels of AR. (K) LNCaP, MR49F, C4-2 and C4-2R cells were cultured in RPMI-1640 media containing 10% FBS for 2 days and harvested for IB with antibodies against indicated proteins. (L) The cell lines described in Fig. 2C were cultured in RPMI-1640 media containing 10% FBS for 2 days and harvested for IB with antibodies indicated. (M) C4-2R cells were treated with enzalutamide and simvastatin at indicated concentrations for 48 hours, followed by IB. (N) C4-2R cells were treated with enzalutamide and BKM120 at indicated concentrations for 48 hours, followed by IB.
Figure 1
HMGCR/GAPDH relative to C4-2

Cholesterol/cell levels relative to LNCaP

Enrichment plot:
REACTOME_CHOLESTEROL_BIOSYNTHESIS

Enrichment plot:
KEGG_STEROID_BIOSYNTHESIS

Low HMGCR gene expression
High HMGCR gene expression

Low HMGCR gene expression
High HMGCR gene expression

**NS**

-Enza +Enza

-Enza +Enza

SREBP-2 (60 KDa)
SREBP-2 (120 KDa)
HMGCR
GAPDH

55 kDa
37 kDa
100 kDa
100 kDa

1.00 0.67 0.85 0.60 3.07 1.61 1.96 0.73
1.00 1.37 1.28 1.63 0.76 1.32 1.40 3.25
Figure 2

A

B

C

D

E

F

G

H

I

J

K

Relative C-PARP/β-Actin

Cell survival rate (%)
**Figure 4**

A. Tumor volume (mm$^3$) over time with Ctrl, Enza, SIM, and Combo treatments.

B. Photographs of tumor samples after treatment.

C. Tumor weight (g) comparison among Ctrl, Enza, SIM, and Combo treatments.

D. Body weight (g) comparison among Ctrl, Enza, SIM, and Combo treatments.

E. Histological images showing tumor sections for Ctrl, Enza, SIM, and Combo treatments.

F. Immunofluorescence images showing DAPI, Ki67, and Merge for Ctrl, Enza, SIM, and Combo treatments.

G. Ki67-positive tumor cells (%) comparison among Ctrl, Enza, SIM, and Combo treatments.

H. Immunofluorescence images showing DAPI and Cleaved caspase-3 for Ctrl, Enza, SIM, and Combo treatments.

I. Cleaved caspase-3 positive cells (%) comparison among Ctrl, Enza, SIM, and Combo treatments.
Figure 5

A

B

C

D

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Figure 6

C
Ctrl
DAPI AR Merge
EnzaSIMCombo
50 µm
50 µm
50 µm
50 µm

B
AR
AR-Vs
GAPDH
22RV1
Enza (20 µM)
SIM (µM)
-- - ++ +
12 . 5
12 . 5
12 . 5
12 . 5

GAPDH
AR/GAPDH
AR-V/GAPDH
AR/β-Actin
β-Actin
PSA/β-Actin

MR49F
Enza (10 µM)
SIM (µM)
-- - ++ +
1.00 1.01 0.88 0.71 0.66 0.48 0.61 0.29
1.00 0.56 3.31 3.31 2.88 0.52 0.53 0.26

AR/GAPDH
AR-V/GAPDH

D
DAPI
AR
Merge
Ctrl
Enza
SIM
Combo

H
Enza
HMGCR-KD

Relative AR/β-Actin level

E
Relative AR mRNA level

Ctrl
ENZA
SIM
Combo

F
0h 12h 24h 36h
AR
GAPDH
AR/GAPDH

AR
GAPDH
AR/GAPDH

Ctrl
SIM
Combo

Enza
Sim
Combo

AR
C-PARP
GAPDH

AR/GAPDH
C-PARP/GAPDH

HMGCR
AR
PSA
β-Actin

AR
PSA
β-Actin

Relative AR/β-Actin

Ctrl
HMGCR-KD

NS
*
Table 1. Combination index analysis of enzalutamide combined with simvastatin. CI = Combination Index

|                  | C4-2BR | MR49F |
|------------------|--------|-------|
| Enza (As)_50     | 57.6 µM| 20.0 µM|
| SIM (Bs)_50      | 6.4 µM | 2.4 µM |
| Enza (Am)_50 (combined with half (Bs)_50 SIM) | 23.3 µM | 8.2 µM |
| CI               | 0.9    | 0.91  |
Inhibition of cholesterol biosynthesis overcomes enzalutamide resistance in castration-resistant prostate cancer (CRPC)

Yifan Kong, Lijun Cheng, Fengyi Mao, Zhuangzhuang Zhang, Yanquan Zhang, Elia Farah, Jacob Bosler, Yunfeng Bai, Nihal Ahmad, Shihuan Kuang, Lang Li and Xiaoqi Liu

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