Therapeutic targeting MDR1 expression by RORγ antagonists resensitizes cross-resistant CRPC to taxane via coordinated induction of cell death programs

Yongqiang Wang¹,⁵, Zenghong Huang¹, Christopher Z. Chen², Chengfei Liu², Christopher P. Evans²,³, Allen C. Gao²,³,⁴, Fangjian Zhou⁵*, and Hong-Wu Chen¹,³,⁴*

¹ Department of Biochemistry and Molecular Medicine, University of California, Davis, School of Medicine, Sacramento, California.
² Department of Urology, University of California, Davis, School of Medicine, Sacramento, California.
³ UC Davis Comprehensive Cancer Center, University of California, Davis, Sacramento, California.
⁴ VA Northern California Health Care System-Mather, Mather, California.
⁵ Department of Urology, Sun Yat-sen University Cancer Center, Guangzhou, Guangdong, China.

Running title: Re-sensitizing taxane-resistant CRPC by RORγ inhibitors

Key words: Chemotherapy resistance, drug efflux, RORC, drug synergy, apoptosis

Conflict of interest: The authors declare no conflict of interest.

Abbreviations

RORγ - retinoic acid receptor-related orphan receptor γ
ABCB1 - ATP-binding cassette subfamily B member 1
MDR1 - multidrug resistance protein 1

* Corresponding author: Hong-Wu Chen, Department of Biochemistry and Molecular Medicine, University of California, Davis, School of Medicine, Sacramento, CA, 95817. Phone: 916-734-7743; Fax:916-734-0190; E-mail: hwzchen@ucdavis.edu). Fangjian Zhou, Department of Urology, Sun Yat-sen University Cancer Center, Guangzhou, Guangdong, 510000, China. Phone: 87343840; Fax: 87343841; E-mail: zhoufj@systucc.org.cn.

Conflict of interest: The authors declare no conflict of interest.

Text excludes reference: 4203 words
Abstract: 176 words
Number of figures: 6
Abstract

Overexpression of ATP-binding cassette subfamily B member 1 (ABCB1)-encoded multidrug resistance protein 1 (MDR1) constitutes a major mechanism of cancer drug resistance including docetaxel (DTX) and cabazitaxel (CTX) resistance in castration-resistant prostate cancer (CRPC). However, no therapeutics that targets MDR1 is available at clinic for taxane sensitization. We report here that RORγ, a nuclear receptor family member, unexpectedly mediates MDR1/ABCB1 overexpression. RORγ plays an important role in controlling the functions of subsets of immune cells and has been an attractive target for autoimmune diseases. We found that its small-molecule antagonists are efficacious in re-sensitizing DTX and CTX cross-resistant CRPC cells and tumors to taxanes in both androgen receptor (AR)-positive and -negative models. Our mechanistic analyses revealed that combined treatment with RORγ antagonists and taxane elicited a robust synergy in killing the resistant cells, which involves a coordinated alteration of p53, Myc and E2F-controlled programs critical for both intrinsic and extrinsic apoptosis, survival and cell growth. Our results suggest that targeting RORγ with small-molecule inhibitors is a novel strategy for chemotherapy resensitization in tumors with MDR1 overexpression.
Introduction

Prostate cancer is still one of the leading causes of cancer-related death among men in the United States (1). Although most patients initially respond to androgen-deprivation therapy (ADT), their tumors eventually become resistant to ADT and the disease progresses to metastatic castration-resistant prostate cancer (mCRPC) (2-4). Docetaxel (DTX) and cabazitaxel (CTX) are microtubule-stabilizing taxane chemotherapy agents. They exert their therapeutic effects mainly through microtubule disassembly blockade-induced G2/M cell cycle arrest and induction of apoptosis. Other effects including suppression the expression or function of the androgen receptor (AR) have also been described for DTX (4-9). DTX and CTX are currently used as the first and second-line treatment for mCRPC. However, only about half of mCRPC patients respond to DTX or CTX and those who initially respond will eventually become resistant to them (4,7).

One of the major mechanisms of taxane resistance in CRPC is the overexpression of drug efflux pumps or transporters of the ATP-binding cassette (ABC) family members, especially ABCB1. Other mechanisms include alterations of beta-tubulin, altered functions of cell survival regulators such as BCL-2, BIRC5, MCL-1, and IAPs (4,7,10,11), the expression of AR variants, tumor cell epithelial-mesenchymal transition (EMT), and aberrant metabolic processes were associated with docetaxel resistance (11-18). ABCB1-encoded MDR1 is involved in multiple drug resistance through pumping drug out of cell and thus diminishing the efficacy of drugs such as DTX (7,10). Cabazitaxel (CTX), a new generation taxane for patients who had progressed after DTX (19,20), is also a substrate of MDR1 (21,22). Importantly, recent studies found that overexpressed MDR1 mediated DTX and CTX cross-resistance in CRPC (7,23). Although several MDR1-specific small-molecule inhibitors were identified, significant complications such as toxicity and poor pharmacological properties contributed to their failure at clinical trials (10). Therefore, further studies are still needed to identify new strategies to overcome MDR1-mediated chemotherapy resistance.

Control of tumor expression of MDR1/ABCB1 is poorly understood, which may involve
transcription factors such as AP-1, NF-κB, and forkhead transcription factors (24-26). However, so far, none of the major regulators of MDR1/ABCB1 expression are readily tractable for therapeutic targeting. In contrast, members of the nuclear receptor (NR) superfamily of transcription factors are attractive therapeutic targets. Our recent study demonstrated that retinoic acid receptor-related orphan receptor γ (RORγ), an NR member, is overexpressed or amplified in mCRPCs and is a therapeutic target for CRPC (27). RORγ also plays an important role in controlling the functions of subsets of immune cells and has been an attractive target for autoimmune diseases (28,29). In this study, we found that RORγ is an upstream regulator of MDR1. Inhibition of RORγ strongly reduced MDR1 expression, increased drug influx and effectively sensitized DTX and CTX-resistant cells to the taxanes in vitro and in tumor. Our further analysis revealed that the sensitization involves a coordinated alteration of p53, Myc and E2F-controlled cell death and growth programs.

Materials and Methods

Cell culture

C4-2B, DU145, C4-2B-TaxR, and DU145-TaxR prostate cancer cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Hyclone) and penicillin-streptomycin (100 IU/ml), unless indicated otherwise. Cells were grown at 37°C in 5% CO2 incubators. C4-2B was from UroCor Inc. (Oklahoma City, OK). DU145 was from the American Type Culture Collection (ATCC). C4-2B-TaxR and DU145-TaxR were previously described (30,31) and maintained in the medium containing 5 nM DTX. Cell line were regularly tested being negative for mycoplasma and were used for experiments within 3 to 8 passages after initial thawing.

Chemicals

SR2211 was from TOCRIS. GSK805 was from WuXi AppTec. Enzalutamide (MDV3100), docetaxel, cabazitaxel, and elacridar were from Selleckchem.

Cell viability assay

Cells were seeded in 96-well plates at 1,000 cells per well in 100 μl medium. Serially diluted compounds in another 100 μl medium were added to the cells 24 h later. After 72 h of
incubation, Cell-Titer GLO reagents (Promega) were added, and luminescence was measured on GLOMAX microplate luminometer (Promega), according to the manufacturer's instructions. The data was presented as a percentage of cell viability, with vehicle-treated cells set at 100. Synergy distribution maps were constructed by Combenefit Software.(32).

Cell growth assay

Cells were seeded in 6-well plates at about $5 \times 10^4 - 10 \times 10^4$ cells per well in 2 ml medium. Compounds were added to the cells 24 h later at indicated concentrations. Total viable cell numbers were counted with a Coulter cell counter after indicated time points.

Colony formation

C4-2B-TaxR cells were seeded at 500 cells per 10 cm dish in 10 ml medium. DU145-TaxR cells were seeded at 800 cells per well in six-well plate in 2 ml medium. Compounds were added to the cells at indicated concentrations at 24 hrs after the cells were seeded. Medium was changed every 3 days. After about 2 weeks cultured when colonies of cells became visible, the medium was removed. The cells were fixed with 10% formalin for 10 min and were washed with PBS for two times. The cell colonies were then stained with 0.2% crystal violet (in 10% formalin) for 15 min, before being washed with PBS for five times and air-dried. The cell colony numbers were counted by Image J Software.

qRT-PCR and immunoblotting analyses

Total RNA was isolated from cells in six-well plates, and cDNA was prepared, amplified and measured in the presence of SYBR. Briefly, the fluorescence values were collected, and a melting-curve analysis was performed. Fold difference was calculated as described previously (27). Data were presented as mean values ± s.d. Cell lysates were analyzed by immunoblotting with indicated proteins. The PCR primers and all the antibodies used in this study are described in the Supplementary Tables 1 and 2.

Rhodamine-123-based MDR1 efflux assay

Cells were seeded in 12-well plates at $2 \times 10^4 - 15 \times 10^4$ per well in 1 ml medium. The cells
were treated as indicated 24 h later. After the indicated incubation time, the cells were washed with PBS once and changed with fresh medium with 1 μmol/L rhodamine 123 for C4-2B-TaxR or 1 μg/mL rhodamine 123 for DU145-TaxR and incubated for four hours. The cells were subsequently washed three times with PBS. Pictures were taken and analyzed by Image J Software. The data was presented as a percentage of relative rhodamine 123 intensity to cell confluence, with positive control treated cells set at 100.

RORγ overexpression lentivirus production and siRNA transfection

For RORγ overexpression, human RORγ cDNA in pLX304 (DNASU) was amplified, and cloned into a modified pLX304 vector with a V5 tag at the receptor N terminus. Lentiviral particles were produced in 293T cells after co-transfection of the above lentivirus vectors, psPAX2 and pMD2.G in 10-cm dishes, as previously described (27). siRNAs for gene knockdown were purchased from Dharmacon. The siRNA target sequences for RORγ are listed in Supplementary Table 3. Transfections were performed with OptiMEM (Invitrogen) and Dharmafectin 1 (Dharmacon) or jetPRIME® transfection reagent (Polyplus transfection), following the manufacturer’s instructions.

RNA-seq data analysis

C4-2B-TaxR cells were treated with vehicle or SR2211 (2 μM), or DTX (10 nM), or combination (SR2211 2 μM + DTX 10 nM) for 48 h before RNA extraction. RNA-seq libraries from 1 μg total RNA were prepared using Illumina Tru-Seq RNA Sample, according to the manufacturer’s instructions. Libraries were validated with an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Sequencing was performed on an Illumina HiSeq 2000 sequencer at BGI Tech (Hong Kong). The FASTQ-formatted sequence data were analyzed using a standard BWA-Bowtie-Cufflinks workflow. In brief, sequence reads were mapped to the reference human-genome assembly (Feb. 2009, GRCh37/hg19) with BWA and Bowtie software. Subsequently, the Cufflinks package was applied for transcript assembly, quantification of normalized gene and isoform expression in RPKM (reads per kilobase per million mapped reads) or FPKM (fragments per kilobase of exon model per million mapped reads), and testing for differential expression (Cuffdiff). To avoid spurious fold levels resulting
from low expression values, only those genes with expression RPKM or FPKM values of >1 for either the vehicle control cell, or the compound treated cells (but do not have to be both), are included. The RNA-seq data have been deposited at NCBI GEO with accession number GSE138751.

GSEA analysis

GSEA was performed utilizing the Java desktop software (http://software.broadinstitute.org/gsea/index.jsp), as described (33). Genes were ranked according to the shrunken limma log2 fold changes, and the GSEA tool was used in ‘pre-ranked’ mode with all default parameters. Previous reported AR activity signature genes (34) were used in the GSEA analysis.

In vivo tumorigenesis assay

Four-week-old male mice (strain: C.B-17/\textit{lcrHsd-Prkdcscid}) were purchased from Envigo. $3 \times 10^6$ cells were suspended in 50% Matrigel and implanted subcutaneously into the dorsal flank on both sides of the mice. Animal group size of six or more was estimated to have a high statistical power, according to the power calculation (http://www.biomath.info/power/) and previous studies involving the same xenograft models. When the tumor volume reached 50 – 100 mm$^3$, the mice were randomized and treatments were initiated as intraperitoneal injection (i.p.). SR2211 and DTX were dissolved in a formulation of 15% Cremophor EL, 82.5% PBS and 2.5% dimethyl sulfoxide (DMSO). Control group: vehicle from day 1 to day 5, five days per week. SR2211 group: SR2211 2.5 mg/kg from day 1 to day 5, five days per week. DTX group: DTX 10 mg/kg at day 4, once per week. Combination group: SR2211 2.5 mg/kg from day 1 to day 5, five days per week; DTX 10 mg/kg at day 4, once per week. Tumor growth was monitored by calipers, and volume was calculated with the equation $V = 0.5 \times \text{length} \times$
Body weight during the course of the study was also monitored. At the end of the study, mice were euthanized and tumors were dissected and weighed. The animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of University of California, Davis.

**Statistical analysis**

Cell culture-based experiments were performed three times or more with assay points triplicated, as indicated. Significance of difference between two groups was determined by the two-tailed Student's t test. The data is presented as mean values ± s.d. p < 0.05 was considered to be significant.

**Results**

**RORγ is required for cell growth and survival of DTX and CTX cross-resistant CRPC**

We recently demonstrated that RORγ plays an essential role in the growth and survival of androgen-sensitive and CRPC cells (27). To examine whether RORγ also plays a similar function in taxane-resistant CRPC, we first performed RORγ knockdown in C4-2B-TaxR cells by different RORC siRNAs. C4-2B-TaxR is a DTX and CTX cross-resistant model of CRPC and was established by a long-term culturing of C4-2B cells in increasing concentrations of DTX. Its taxane sensitivity was dramatically increased with approximately 70 fold higher IC_{50} to DTX and 10 fold higher IC_{50} to CTX than its parental C4-2B (23,31,35). As shown in Fig. 1A, knockdown of RORC markedly inhibited the growth of C4-2B-TaxR cells, which is similar to what we observed with C4-2B cells (27). Several potent RORγ antagonists including SR2211 and GSK805 have been developed for autoimmune diseases (35-37). Next, we treated C4-2B and its derivative TaxR cells with different concentrations of RORγ antagonists SR2211 and found that the cell viability and growth of C4-2B and C4-2B-TaxR were strongly inhibited by the antagonist with essentially the same sensitivity with IC_{50} of 2.5 (Fig. 1B and 1C). We also found that the TaxR cells are also sensitive to GSK805 with IC_{50} of 4.3 (Fig 1C). Consistent with our previous study (27), knockdown of RORγ also inhibited the protein...
expressions of AR, Myc, cyclin D3, and CDC6, and increased activated caspase 7 and cleavage of poly (ADP-ribose) polymerase 1 (PARP1) in C4-2B-TaxR cells (Fig. 1D). The antagonist SR2211 treatment also strongly reduced the expression of AR and the pro-growth and -survival proteins in a dose-dependent manner in C4-2B-TaxR cells (Fig. 1E). Together, these results suggest that RORγ plays an essential role in the growth and survival of taxane-resistant prostate cancer cells.

Inhibition of RORγ re-sensitizes taxane cross-resistant CRPC cells to taxanes

The essential function of RORγ in growth and viability of the taxane-resistant cells prompted us to examine whether targeting RORγ can re-sensitize the resistant cells to DTX or CTX. Thus, we knocked down RORγ partially with a reduced amount (20 nM instead of 50 nM) of siRNAs and treated the resistant cells with a relatively low concentration (20 nM) of DTX. Under this condition, we observed that 20 nM of DTX alone did not cause any significant inhibition of cell growth. In contrast, when combined with a partial RORγ-knockdown, 20 nM of DTX markedly reduced cell growth of C4-2B-TaxR cells (96.1% in siControl vs 72.2% in siRORC-1, or vs 78.9% in siRORC-2). Next, we examined whether combination with RORγ antagonist would sensitize the resistant cells to the taxane. We thus treated the cells with combinations of SR2211 and DTX at a range of concentrations and measured the cell viability. As shown in Fig. 2B, lower left, while 1.25 μM SR2211 alone or 12.5 nM DTX alone only reduced slightly the viability of C4-2B-TaxR (92.3% and 90.1% respectively), their combined treatment reduced the viability to 33.2%, strongly suggesting that the combination is synergistic.

To further characterize the synergy, we used an open-source algorithm Combenefit (32). As shown in Fig. 2B, lower right, combination of SR2211 and DTX showed a strong synergy in growth inhibition of C4-2B-TaxR cells across the wide range of concentrations of both drugs. In contrast, when we performed similar experiments with the taxane-sensitive C4-2B cells, we did not observe any significant synergy (Fig. 2B, upper panels). Next, we performed clonogenic survival assay to confirm the synergy and also found a highly synergistic inhibitory effects on the survival of the taxane-resistant cells after they were treated by a combination of SR2211 and either DTX or CTX. For example, while 10 nM DTX, 0.25 nM CTX, or 0.25 μM
SR2211 alone did not significantly alter the cell survival, SR2211 combination with either DTX or CTX reduced the cell survival to approximately 15% or 21% respectively (Fig. 2C and 2D). To confirm that the strong synergy is not compound-specific, we used GSK805 and obtained similar synergy with either DTX or CTX in growth inhibition of the taxane-resistant cells (Fig. 2E, and Supplementary Fig. S1). Consistent with the strong inhibition of cell survival, combinations of RORγ antagonist with DTX or CTX markedly induced apoptosis by activating caspase 7 and cleaving PARP1 (Fig 2F). Together, the data strongly suggest that suppression of RORγ expression or function can effectively sensitize taxane-resistant CRPC cells to taxane treatment.

**MDR1/ABCB1 overexpression is controlled by RORγ**

Since MDR1/ABCB1 overexpression is the major mechanism of taxane cross-resistance of C4-2B-TaxR cells (23,31), we next investigated whether RORγ regulated MDR1 expression in those cells. Indeed, RORγ knockdown significantly decreased the expression of MDR1 (Fig. 3A), while ectopic RORγ increased its expression (Fig. 3B). Treatment of the TaxR cells with 1 to 8 μM RORγ antagonist SR2211 also strongly inhibited MDR1 protein and mRNA levels in C4-2B-TaxR cells (Fig. 3C and 3D). A significant reduction of MDR1/ABCB1 mRNA and protein can be observed 24 hours after treatment of the TaxR cells with SR2211 (Fig. 3E and 3F). The strong inhibition of MDR1 expression was also observed with antagonist GSK805 and in cells that were co-treated with taxane DTX (Supplementary Fig. S2). Next, to determine whether inhibition of MDR1 expression by targeting RORγ resulted in changes in the efflux activity of MDR1, we performed MDR1 efflux assay with rhodamine dye as a substrate of the transporter, as in our previous study (30). As shown in Fig. 3G, 36 hours of RORγ antagonist treatment caused a significant increase of cellular rhodamine retention as measured by the relative rhodamine intensity, while longer treatments further increased the rhodamine retention. These temporal effects are very much in agreement with the inhibition on MDR1 expression. Moreover, we also assessed the effects of RORγ overexpression on the transporter activity of MDR1 and found that RORγ overexpression significantly decreased the sensitivity of the cell to the MDR1 inhibitor elacridar, as less rhodamine was retained in the cells when compared to the vector control cells (Fig. 3H).
Together, these results clearly demonstrate that the aberrant expression of MDR1/ABCB1 in the taxane cross-resistant cells is stimulated by RORγ, which is responsible for the elevated pumping activity.

**Taxane re-sensitization by RORγ inhibition is through a robust and coordinated alteration of cell death and growth programs controlled by p53, Myc and E2F**

The strong synergistic inhibition of the TaxR cell viability as shown in Fig. 2 prompted us to examine the underlying mechanisms. We thus performed RNA-seq profiling analysis of C4-2B-TaxR cells treated with 2 μM SR2211, or 10 nM DTX, or their combination to identify gene program changes caused by the combination treatment. As shown in Fig. 4A, the combination altered the expression of over 1,000 genes (with 2.0 fold up or down when compared to vehicle). Similar to our previous findings with taxane-sensitive C4-2B cells (27), gene-set enrichment analysis (GSEA) showed that, in C4-2B-TaxR cells, SR2211 alone strongly suppressed androgen response and AR signature programs (Fig. 4B). Consistent with its inhibitory effect on the TaxR cells and on Myc protein, SR2211 alone also strongly inhibited E2F and Myc-controlled cell proliferation and growth programs (Fig. 4B, and Supplementary Fig. 3A). Interestingly, DTX and SR2211 combination treatment induced program changes that are dominated by E2F-controlled cell proliferation and mitotic progression/checkpoint (Fig. 4C, and Supplementary Fig. S3A). qRT-PCR analysis confirmed the changes of some of the major E2F target genes, including TOP2A, ATAD2 (aka ANCCA) and BIRC5 (Supplementary Fig. S3B).

Importantly, the combination treatment also induced apoptosis signature genes and p53 pathway that are not enriched in the changes by the RORγ inhibitor alone (Fig. 4C and 4D). Indeed, several major pro-apoptotic genes were activated as demonstrated by RNA-seq analysis and qRT-PCR (Fig. 4D and Supplementary Fig. S3C). They include the extrinsic apoptosis pathway genes, such as death receptor FAS and ligand TNFSF10/TRAIL, and the intrinsic pathway genes, such as BBC3/PUMA, PMAIP1/NOXA, BAX and AEN (apoptosis enhancing nuclease). Western blotting also demonstrated a strong induction of p53, FAS and PUMA as well as cleaved caspase 7 at 72 hrs of the combination treatment (Fig. 4E). Interestingly, an induction of p53 and FAS but not cleaved caspase 7 was observed at 24 hrs.
of the treatment (Supplementary Fig. S4A). When we extended our analysis to other cell death-related factors, we found that the expression of BCL-2 and survivin/BIRC5 are significantly inhibited by the combination treatment (Fig. 4E and Supplementary Fig. S4A). Those later proteins are some of the major anti-apoptotic factors which usually function in p53-independent manner. Moreover, treatment of DTX-sensitive C4-2B cells for 24 hrs with either DTX or the RORγ inhibitor revealed that DTX caused an induction of p53 targets BBC/PUMA, FAS and SUSD6 without altering the expression of the E2F targets. In contrast, the RORγ inhibitor decreased the expression E2F targets but did not affect the p53 targets (Supplementary Fig. S4B). Together, the results suggest that taxane re-sensitization by RORγ inhibition involves robust induction of apoptosis program and strong suppression of anti-apoptotic proteins as well as cell cycle arrest effects.

**RORγ antagonist re-sensitizes taxane-resistant CRPC tumor to DTX**

We next examined whether inhibition of RORγ by its antagonist can re-sensitize taxane-resistant CRPC tumors to taxane. In mice bearing C4-2B-TaxR tumors, we observed that intraperitoneal administration of 2.5 mg/kg of SR2211 suppressed the tumor growth significantly, consistent with the *in vitro* effect shown in Fig. 2. In contrast, dosing the mice with 10 mg/kg of DTX did not show significant effect. However, a combination of 10 mg/kg DTX and 2.5 mg/kg SR2211 caused a robust tumor growth inhibition (in both tumor volume and weight) that was significantly stronger than the effect by either alone (Fig. 5A and 5B). Western blotting analysis demonstrated that tumor MDR1 protein expression was strongly reduced in the combination treatment group (Supplementary Fig. S5). As previously reported (38), DTX treatment can affect mouse body weight. However, compared to those of the DTX alone group, the mouse body weight in the combination treatment did not change significantly (Fig. 5C). These results indicate that small molecule targeting of RORγ can effectively re-sensitize taxane-resistant CRPC tumors to DTX.

**Targeting RORγ to re-sensitize taxane-resistant CRPC can be independent of AR**

Our above study was performed with an AR-positive CRPC model. To determine whether targeting RORγ as a strategy to re-sensitize taxane-resistant CRPC can be effective in
AR-negative context, we performed similar experiments using an AR-negative, taxane-resistant model, namely DU145 derived DU145-TaxR (30). Data in Fig. 6A and B clearly show that, like what observed with C4-2B-TaxR cells, RORγ antagonist SR2211 displayed strong synergistic growth inhibition with DTX or CTX in DU145-TaxR but not in regular DU145 cells. Strong synergistic inhibition of survival was also observed in colony formation assay with DU145-TaxR cells treated by a combination of SR2211 and DTX or CTX (Fig. 6C and D). Moreover, similar synergistic growth inhibition was seen with the other RORγ antagonist GSK805 (Fig. 6E and F). Moreover, similar to its inhibition of MDR1 in C4-2B-TaxR cells, the RORγ antagonist also strongly reduced MDR1 mRNA and protein expression in the presence or absence of DTX (Supplementary Fig. S6A-S6D). Finally, we used DU145-TaxR cells to establish xenograft tumors and treated the tumor-carrying mice as above in the C4-2B-TaxR model. Results in Supplementary Fig. S6E and 6F demonstrate that the combinatorial treatment significantly inhibited DU145-TaxR tumor growth and tumor MDR1 protein expression while DTX alone had no significant effect. Therefore, these results suggest that inhibition of MDR1 by RORγ to re-sensitize taxane-resistant CRPC can be independent of AR status in the taxane-resistant CRPC.

Discussion

The pivotal role of overexpressed MDR1 in tumor taxane resistance in many types of cancer has prompted many studies on inhibition of MDR1 enzymatic activities for re-sensitization of resistant tumors to taxane (10,39). Unfortunately, the development of highly potent and specific MDR1 inhibitors, such as dofequidar and elacridar, did not lead to successful clinical trials, likely due to their toxicity elicited when normal tissue MDR1 is also targeted. Other re-sensitization strategies aimed at individual anti-apoptotic factors such as BCI-2 or MCL-1, AR, STAT3, or GR and targeting cancer stem cells (7,13,14,30,40). Here, we demonstrated that targeting nuclear receptor RORγ by either genetic means or pharmacological agents is highly effective in strong inhibition of MDR1 expression and its drug pumping activity. Such inhibitory effects led to a strong synergy with taxane in inhibition of growth and survival of the resistant cells and the tumors in AR-positive and AR-negative preclinical models. Furthermore, we demonstrated that the synergy with taxane was obtained.
with different RORγ antagonists and in combination with DTX and CTX. Therefore, we think that suppression of MDR1 expression by targeting its key regulators such as RORγ represents a novel and effective strategy in re-sensitizing tumors to taxane.

In exploring the mechanisms underlying the strong synergy by RORγ antagonists and taxane in killing the TaxR cells, we found that their combination induces extensive changes in cell growth and survival gene programs. The changes include a strong up-regulation of a number of pro-apoptotic genes such as FAS, TNFSF10/TRAIL, PUMA, NOXA, BAX and AEN, and down-regulation of anti-apoptotic proteins such as survivin/BIRC5 and BCL-2. FAS and TRAIL are components of the extrinsic pathway of apoptosis and themselves are attractive therapeutic targets (41,42), while the others are key regulators of the intrinsic pathway. PUMA, NOXA, BAX, AEN, FAS and TRAIL are established direct targets of p53 (43), thus indicating a strong activation of p53 pro-apoptosis function by the combination treatment. However, the inhibition of survivin/BIRC5 and BCL-2 likely involves other transcriptional factors such as E2Fs and epigenetic regulators such as the bromodomain protein ANCCA/ATAD2 (44-46). Indeed, a number of E2F targets including TOP2A, BIRC5/survivin and ANCCA/ATAD2 are strongly down-regulated by the combination treatment. Interestingly, ANCCA/ATAD2 is a critical coactivator of E2Fs and AR, and is also a direct target of AR and E2Fs (44,47,48). We previously demonstrated that ANCCA/ATAD2 activates the expression of TOP2A and BIRC5 (44,49). Therefore, the combined RORγ antagonists and taxane treatment caused a coordinated inhibition of a network of gene programs that are controlled by p53 and E2Fs, which is likely responsible for the robust synergistic killing of TaxR cells.

Finally, although this study focused on CRPC tumor cell–intrinsic function of RORγ, therapeutic targeting of RORγ will likely elicit anti-tumor effect also through other mechanisms. One recent study showed that the tumorigenic signaling by tumor-infiltrating myeloid-derived suppressor cells (MDSC) and their cytokines involves the function of RORγ (50). On the other hand, several orally bioavailable RORγ antagonists including ones being tested at clinical trials have recently been developed for human autoimmune diseases (28). Therefore, future studies can be directed to examine the resensitization efficacy of newly developed RORγ antagonists in immune-competent models of CRPC and other cancer types and their potential for clinic trials.
Acknowledgements

We thank Dr. Xinbin Chen for providing antibodies and members of Genomics Shared Resources of UC Davis Cancer Center for their technical help. This work was supported in part by grants from the NIH (R01 CA206222), the Prostate Cancer Foundation (16CHAL02), the US Department of Defense (W81XWH-16-1-0583), and the US Department of Veterans Affairs, Office of Research & Development BL&D (I01 BX004271) to H-W. C. The UCDCCC Genomics Shared Resource is funded by the UC Davis Comprehensive Cancer Center Support Grant (CCSG) awarded by the National Cancer Institute (NCI P30 CA093373).

References


Figure legends

Fig. 1. Inhibition of RORγ suppresses taxane-resistant CRPC cell growth and survival. A, C4-2B-TaxR cells were transfected with 50 nM siRNAs against RORC (siRORC-1 or siRORC-2) or control siRNA (siCont). Total cell numbers were determined at 0, 2, 4, and 6 days. B and C, C4-2B and C4-2B-TaxR cells were treated with different concentrations of RORγ antagonists (SR2211 or GSK805) at indicated concentrations in RPMI 1640 supplemented with 9% cds-FBS, plus 1% regular FBS. After 96 h, cell viabilities and cell numbers were determined. D, C4-2B-TaxR cells were transfected with RORC siRNAs. Ninety-six hours later, total cell lysates were prepared and subjected to immunoblotting. E, C4-2B-TaxR cells were treated with different concentrations of SR2211 for 72 h. Total cell lysates were prepared and subjected to immunoblotting. ns, not significant statistically when
Fig. 2. Inhibition of RORγ resensitizes C4-2B-TaxR cells to taxanes. A, C4-2B-TaxR cells were transfected with 20 nM RORC siRNAs and treated with DMSO or DTX for another 96 h at 48 h after transfection. Total cell numbers were determined. B, C4-2B and C4-2B-TaxR cells were treated with different concentrations of SR2211, also with or without different concentrations of DTX, the cell viabilities were measured at 72 h (left). The synergy distribution maps were generated using Combenefit (right). C, C4-2B-TaxR cells were treated with SR2211, also with or without DTX or CTX treatment, and subjected to clonogenic assay. D, The colonies were quantified. E, C4-2B-TaxR cells were treated with SR2211 or GSK805, also with or without DTX or CTX for 72 h and cell numbers were determined. F, C4-2B-TaxR cells were treated with SR2211, also with or without DTX or CTX for 72 h, the whole cell lysates were prepared and subjected to immunoblotting. ns, not significant statistically when compared to control; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Fig. 3. RORγ regulates ABCB1/MDR1 overexpression in C4-2B-TaxR cells. A, C4-2B-TaxR cells were transfected with RORC siRNA for 96 h, whole cell lysates were prepared and subjected to immunoblotting. B, Immunoblotting analysis of C4-2B-TaxR cells ectopically expressing RORγ and control cells. C, C4-2B-TaxR cells were treated with different concentrations of SR2211 for 72 h, whole cell lysates were prepared and subjected to immunoblotting. D, C4-2B-TaxR cells were treated with different concentrations of SR2211 for 48 h, total RNA was extracted and ABCB1 mRNA was determined by qRT-PCR. E and F, C4-2B-TaxR cells were treated with 5 μM SR2211, at different time point, whole cell lysates were prepared and subjected to immunoblotting, total RNA was extracted and ABCB1 mRNA was determined by qRT-PCR. G, C4-2B-TaxR cells treated with 5 μM SR2211 for indicated hours, the rhodamine efflux assay was performed. H, C4-2B-TaxR cells ectopically expressing RORγ and the control cells were treated with 20 nM elacridar for two hours and rhodamine intensities were measured. ns, not significant statistically when compared to control; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Fig. 4. Combination of SR2211 and DTX effectively change cell death and growth programs controlled by p53, Myc and E2F. A, Venn diagram display of the number of genes with expression changed (> 2 fold and FDR < 0.001, compared to vehicle) detected by RNA-seq analysis of C4-2B-TaxR cells treated with 2 μM SR2211, or 10 nM DTX, or their combination. B and C, Significantly enriched up-regulated and down-regulated gene/data sets (FDR q val < 0.001) obtained by GSEA in C4-2B-TaxR cells between different treatments. D, Heat map of the expression of p53 pathway genes in C4-2B-TaxR cells treated as indicated. E, C4-2B-TaxR cells were treated with DMSO, DTX, SR2211 or their combination for 72 h. Whole cell lysates were prepared and subjected to immunoblotting.

Fig. 5. SR2211 resensitizes taxane-resistant CRPC tumors to DTX. A, Mice bearing C4-2B-TaxR xenograft tumors were treated via i.p. injection with vehicle, 2.5 mg/kg SR2211, 10 mg/kg DTX or their combination for 17 days. Tumor volumes were measured twice weekly. B and C, Tumor weight and mouse body weight were determined. ns, not significant
statistically when compared to control; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Fig. 6. RORγ inhibitors resensitize AR-negative DU145-TaxR cells to taxanes. A and B, DU145 and DU145-TaxR cells were treated with different concentrations of SR2211, also with or without different concentrations of DTX or CTX, the cell viabilities were measured at 72 h (left). The synergy distribution maps of the combinations were generated using Combenefit (right). C and D, DU145-TaxR cells were treated with SR2211, also with or without DTX or CTX treatment, and subjected to clonogenic assay. E, DU145-TaxR cells were treated with SR2211 or GSK805, also with or without DTX or CTX for 72 h and total cell numbers were determined. F, DU145-TaxR cells were treated with SR2211, also with or without DTX or CTX for 72 h, the whole cell lysates were prepared and subjected to immunoblotting. ns, not significant statistically when compared to control; **, p < 0.01; ***, p < 0.001.
Fig. 2

A. 

B. 

C. 

D. 

E. 

F.
**Fig. 3**

**A**

<table>
<thead>
<tr>
<th>SR2211 (μM)</th>
<th>RORγ</th>
<th>MDR1</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>siControl</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>siRORC-1</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>siRORC-2</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>SR2211 (μM)</th>
<th>RORγ</th>
<th>MDR1</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>RORC #1</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>RORC #2</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
</tr>
<tr>
<td>RORC #3</td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
<td><img src="image21" alt="Image" /></td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th>SR2211 (μM)</th>
<th>MDR1</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="image22" alt="Image" /></td>
<td><img src="image23" alt="Image" /></td>
</tr>
<tr>
<td>1</td>
<td><img src="image24" alt="Image" /></td>
<td><img src="image25" alt="Image" /></td>
</tr>
<tr>
<td>2</td>
<td><img src="image26" alt="Image" /></td>
<td><img src="image27" alt="Image" /></td>
</tr>
<tr>
<td>4</td>
<td><img src="image28" alt="Image" /></td>
<td><img src="image29" alt="Image" /></td>
</tr>
<tr>
<td>8</td>
<td><img src="image30" alt="Image" /></td>
<td><img src="image31" alt="Image" /></td>
</tr>
</tbody>
</table>

**D**

**E**

<table>
<thead>
<tr>
<th>Relative mRNA Level</th>
<th>MDR1</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="image32" alt="Image" /></td>
<td><img src="image33" alt="Image" /></td>
</tr>
<tr>
<td>1</td>
<td><img src="image34" alt="Image" /></td>
<td><img src="image35" alt="Image" /></td>
</tr>
<tr>
<td>Relative mRNA Level</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**F**

**G**

<table>
<thead>
<tr>
<th>Relative rhodamine intensity/cell confluence (%)</th>
<th>Vector</th>
<th>RORC #1</th>
<th>RORC #2</th>
<th>RORC #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="image36" alt="Image" /></td>
<td><img src="image37" alt="Image" /></td>
<td><img src="image38" alt="Image" /></td>
<td><img src="image39" alt="Image" /></td>
</tr>
<tr>
<td>12</td>
<td><img src="image40" alt="Image" /></td>
<td><img src="image41" alt="Image" /></td>
<td><img src="image42" alt="Image" /></td>
<td><img src="image43" alt="Image" /></td>
</tr>
<tr>
<td>24</td>
<td><img src="image44" alt="Image" /></td>
<td><img src="image45" alt="Image" /></td>
<td><img src="image46" alt="Image" /></td>
<td><img src="image47" alt="Image" /></td>
</tr>
<tr>
<td>36</td>
<td><img src="image48" alt="Image" /></td>
<td><img src="image49" alt="Image" /></td>
<td><img src="image50" alt="Image" /></td>
<td><img src="image51" alt="Image" /></td>
</tr>
<tr>
<td>48</td>
<td><img src="image52" alt="Image" /></td>
<td><img src="image53" alt="Image" /></td>
<td><img src="image54" alt="Image" /></td>
<td><img src="image55" alt="Image" /></td>
</tr>
<tr>
<td>72</td>
<td><img src="image56" alt="Image" /></td>
<td><img src="image57" alt="Image" /></td>
<td><img src="image58" alt="Image" /></td>
<td><img src="image59" alt="Image" /></td>
</tr>
</tbody>
</table>

**H**

<table>
<thead>
<tr>
<th>Relative rhodamine intensity/cell confluence (%)</th>
<th>Vector</th>
<th>RORC #1</th>
<th>RORC #2</th>
<th>RORC #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="image60" alt="Image" /></td>
<td><img src="image61" alt="Image" /></td>
<td><img src="image62" alt="Image" /></td>
<td><img src="image63" alt="Image" /></td>
</tr>
<tr>
<td>50</td>
<td><img src="image64" alt="Image" /></td>
<td><img src="image65" alt="Image" /></td>
<td><img src="image66" alt="Image" /></td>
<td><img src="image67" alt="Image" /></td>
</tr>
<tr>
<td>100</td>
<td><img src="image68" alt="Image" /></td>
<td><img src="image69" alt="Image" /></td>
<td><img src="image70" alt="Image" /></td>
<td><img src="image71" alt="Image" /></td>
</tr>
</tbody>
</table>

Downloaded from mct.aacrjournals.org on May 16, 2021. © 2019 American Association for Cancer Research.
Fig. 4

A

Up-regulated genes

SR2211

94

DTX

4 9

6

189

Combination

9

4

5

6

606

Down-regulated genes

SR2211

74

DTX

4

7

11

4

290

Combination

B

SR2211 vs. Control

<table>
<thead>
<tr>
<th>Dataset</th>
<th>NES</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANDROGEN_RESPONSE</td>
<td>-2.89</td>
</tr>
<tr>
<td>E2F_TARGETS</td>
<td>-2.43</td>
</tr>
<tr>
<td>MYC_TARGETS_V2</td>
<td>-2.36</td>
</tr>
<tr>
<td>MYC_TARGETS_V1</td>
<td>-2.10</td>
</tr>
<tr>
<td>AR SIGNATURE.GRP</td>
<td>-2.07</td>
</tr>
<tr>
<td>G2M_CHECKPOINT</td>
<td>-1.94</td>
</tr>
</tbody>
</table>

C

Combination vs. Control or DTX or SR2211

<table>
<thead>
<tr>
<th>Dataset</th>
<th>NES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>E2F_TARGETS</td>
<td>-3.47</td>
</tr>
<tr>
<td>G2M_CHECKPOINT</td>
<td>-3.26</td>
</tr>
<tr>
<td>ANDROGEN_RESPONSE</td>
<td>-2.74</td>
</tr>
<tr>
<td>MYC_TARGETS_V1</td>
<td>-2.61</td>
</tr>
<tr>
<td>MYC_TARGETS_V2</td>
<td>-2.36</td>
</tr>
<tr>
<td>MITOTIC_SPINDLE</td>
<td>-2.22</td>
</tr>
<tr>
<td>P53 PATHWAY</td>
<td>2.11</td>
</tr>
<tr>
<td>INTERFERON_GAMMA_RESPONSE</td>
<td>2.00</td>
</tr>
<tr>
<td>AR SIGNATURE.GRP</td>
<td>-1.99</td>
</tr>
<tr>
<td>INTERFERON_ALPHA_RESPONSE</td>
<td>1.91</td>
</tr>
<tr>
<td>TNFA_SIGNALING_VIA_NFKB</td>
<td>1.85</td>
</tr>
<tr>
<td>APOPTOSIS M5902</td>
<td>1.81</td>
</tr>
</tbody>
</table>

D

p53 pathway genes

E

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Control</th>
<th>DTX 10nM</th>
<th>SR2211 2μM</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUSD6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFSF10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMAIP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fold Change (log2)

BBC3 (PUMA)

FAS

SUSD6

TNFSF10

PMAIP1

BAX

AEN

≥2

Fas

c-Caspase-7

Bcl-2

Survivin

GAPDH

Downloaded from mct.aacrjournals.org on May 16, 2021. © 2019 American Association for Cancer Research.
**Fig. 5**

**A**

Graph showing tumor volume (mm$^3$) over days for Control, DTX 10 mg/kg, SR2211 2.5 mg/kg, and Combination treatments.

**B**

Bar graph showing tumor weight (mg) for Control, DTX 10 mg/kg, SR2211 2.5 mg/kg, and Combination treatments.

**C**

Graph showing body weight (g) over days for Control, DTX 10 mg/kg, SR2211 2.5 mg/kg, and Combination treatments.
Fig. 6

A

DU145

<Diagram showing cell viability for Docetaxel (nM) vs. DMSO, SR2211 0.625 μM, SR2211 1.25 μM, SR2211 2.5 μM, and SR2211 5 μM.>

DU145-TaxR

<Diagram showing cell viability for Docetaxel (nM) vs. DMSO, SR2211 0.625 μM, SR2211 1.25 μM, SR2211 2.5 μM, and SR2211 5 μM.>

B

DU145

<Diagram showing cell viability for Cabazitaxel (nM) vs. DMSO, SR2211 0.625 μM, SR2211 1.25 μM, SR2211 2.5 μM, and SR2211 5 μM.>

DU145-TaxR

<Diagram showing cell viability for Cabazitaxel (nM) vs. DMSO, SR2211 0.625 μM, SR2211 1.25 μM, SR2211 2.5 μM, and SR2211 5 μM.>

C

<Graph showing colony number for DMSO, SR2211 0.5 μM, SR2211 1 μM, DTX 5 nM, DTX 10 nM, CTX 0.25 nM, and CTX 0.5 nM.>

D

<Bar graph showing relative cell number (%): DMSO, SR2211 1 μM, GSK805 2 μM.>

E

<Bar graph showing relative cell number (%): DMSO, DTX 25 nM, CTX 2 nM.>

F

<Western blot showing PARP, c-PARP, c-Caspase-7, and β-Actin expression for DMSO, DTX 25 nM, CTX 2 nM, SR2211 1 μM.>

Downloaded from mct.aacrjournals.org on May 16, 2021. © 2019 American Association for Cancer Research.
Molecular Cancer Therapeutics

Therapeutic targeting MDR1 expression by RORγ antagonists resensitizes cross-resistant CRPC to taxane via coordinated induction of cell death programs

Yongqiang Wang, Zenghong Huang, Christopher Z. Chen, et al.

Mol Cancer Ther Published OnlineFirst November 11, 2019.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-19-0327

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2019/11/09/1535-7163.MCT-19-0327.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/early/2019/11/09/1535-7163.MCT-19-0327.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.

Downloaded from mct.aacrjournals.org on May 16, 2021. © 2019 American Association for Cancer Research.