Title

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Journal
Molecular Cancer Therapeutics, 17(10)

ISSN
1535-7163

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Publication Date
2018-10-01

DOI
10.1158/1535-7163.mct-17-1269

Peer reviewed

Author Manuscript Published OnlineFirst on June 11, 2018; DOI: 10.1158/1535-7163.MCT-17-1269
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Intra vs Inter Cross-Resistance Determines Treatment Sequence between Taxane and AR-Targeting Therapies in Advanced Prostate Cancer

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Key words: prostate cancer resistance, docetaxel, cabazitaxel, enzalutamide, abiraterone

Running title: Cross resistance between taxanes and AR-targeted therapies

Financial information: This work was supported in part by grants NIH/NCI CA140468, CA168601, CA179970 (A.C. Gao), DOD PC150229 (A.C. Gao), and US Department of Veterans Affairs, ORD VA Merits I01BX0002653 (A.C. Gao).

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Conflicts of Interest – C.P. Evans has received speakers bureau honoraria from Astellas, Jansen, and Sanofi. No potential conflicts of interest were disclosed by the other authors.

Author Manuscript Published OnlineFirst on June 11, 2018; DOI: 10.1158/1535-7163.MCT-17-1269
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Abstract

Current treatments for castration resistant prostate cancer (CRPC) largely fall into two classes; AR-targeted therapies such as the next-generation anti-androgen therapies (NGATs), enzalutamide and abiraterone, and taxanes, docetaxel and cabazitaxel. Despite improvements in outcomes, patients still succumb to the disease due to the development of resistance. Further complicating the situation is lack of a well-defined treatment sequence and potential for cross-resistance between therapies. We have developed several models representing CRPC with acquired therapeutic resistance. Here, we utilized these models to assess putative cross-resistance between treatments. We find that resistance to enzalutamide induces resistance to abiraterone and vice versa but resistance to neither alters sensitivity to taxanes. Acquired resistance to docetaxel induces cross-resistance to cabazitaxel but not to enzalutamide or abiraterone. Correlating responses with known mechanisms of resistance indicates that androgen receptor (AR) variants are associated with resistance to NGATs while the membrane efflux protein ABCB1 is associated with taxane resistance. Mechanistic studies show that AR variant-7 (AR-v7) is involved in NGAT resistance but not resistance to taxanes. Our findings suggest the existence of intra cross-resistance within a drug class (i.e., within NGATs or within taxanes), while inter cross-resistance between drug classes does not develop. Furthermore, our data suggests resistance mechanisms differ between drug classes. These results may have clinical implications by showing that treatments of one class can be sequenced with those of another, but caution should be taken when sequencing similar classed drugs. Additionally, the development and use of biomarkers indicating resistance will improve patient stratification for treatment.

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Introduction

Prostate cancer is the third leading cause of cancer-related death in US men. While early stage, localized prostate cancer can be cured with surgical and radiation treatments, advanced and metastatic disease comes with a poor prognosis (1). Advanced prostate cancer is initially treated with androgen deprivation therapy (ADT), but this inevitably fails within ~2-3 years giving rise to castration resistant prostate cancer (CRPC) (2). While there are treatments for CRPC, it remains an incurable disease due to presentation with or the development of treatment resistance (2).

Current treatment options for CRPC fall into two major classes including AR-targeted therapies such as the next-generation anti-androgen therapies (NGATs), enzalutamide and abiraterone, and taxanes such as docetaxel and cabazitaxel. Enzalutamide is a potent anti-androgen which prevents androgen receptor (AR) nuclear translocation and downstream signaling (3). Abiraterone is a CYP17A1 inhibitor which results in attenuated androgen production leading to decreased AR activity (4). Abiraterone is also thought to function in part as a direct inhibitor of the AR and to be metabolized into additional AR inhibitors (5, 6). These NGATs have been shown to provide a survival benefit both before and post-docetaxel treatment (7-10). Taxanes such as docetaxel, and cabazitaxel, a next generation taxane, are also used to treat CRPC and have been shown to provide a survival benefit (11, 12). However, cabazitaxel is only approved to treat docetaxel pre-treated patients.

Although these new therapies have improved treatment and outcomes in the clinic, there are still many unanswered questions. Most notably, the proper sequencing of these agents for maximum patient benefit is poorly understood and a subject of intense study (13). Complicating this issue is the possibility of cross-resistance between these therapies. Potential for cross-resistance takes two forms; intra cross-resistance between drugs of the same class (between NGATs, enzalutamide with abiraterone, or between taxanes, docetaxel with cabazitaxel) and inter cross-resistance between therapies of different classes (NGATs with taxanes). While clinical evidence supports that there is cross-resistance between enzalutamide and abiraterone, cross-resistance

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between docetaxel and cabazitaxel is less understood (14). Additionally, sufficient evidence for inter cross-resistance between taxanes and NGATs is lacking and requires further study.

It has been suggested that inhibition of AR nuclear-trafficking represents a secondary mechanism of action for taxanes in prostate cancer (15, 16). Since NGATs also work through inhibition of AR signaling, it is thought that this common mechanism may allow for the development of a shared means of resistance between these disparate drug classes. One possible common resistance mechanism is the up-regulation and subsequent reliance on signaling from AR-variants (17). Fully understanding whether similar mechanisms of resistance exist between drug classes will lead to improved treatment regimens and avoidance of using therapeutic strategies where they are likely to fail.

We have recently developed and characterized several cell line models which mimic treatment resistance observed in the clinic (18-20). The aim of this study was to utilize these models to assess the potential for both intra and inter cross-resistance between available therapies. We also sought to further explore putative mechanisms of cross-resistance between NGATs and taxanes to better understand how these treatments should be sequenced in the clinic.

Materials and Methods

Cell Culture and Reagents

C4-2 and C4-2B cells were kindly provided and authenticated by Dr. Leland Chung (Cedars-Sinai Medical Center, Los Angeles, CA). CWR22Rv1 cells were obtained from the American Type Culture Collection (ATCC). ATCC uses short tandem repeat (STR) profiling for testing and authentication of cell lines. All cell lines are routinely tested for mycoplasma every 6 months using ABM mycoplasma PCR detection kit (Cat#: G238). All experiments with these cell lines and their derivatives were conducted within 6 months of receipt or resuscitation after cryopreservation. Cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 IU penicillin and 0.1 mg/ml streptomycin. Enzalutamide resistant C4-2B cells

(C4-2B-MDVR), abiraterone resistant C4-2B cells (C4-2B-AbiR), and docetaxel resistant C4-2B cells (C4-2B-TaxR) were characterized and described previously and maintained in complete RPMI 1640 supplemented with either 20µM enzalutamide, 10µM abiraterone, or 5nM docetaxel, respectively (18-20). C4-2B cells were cultured alongside derivative cell lines during their creation as an appropriate control. C4-2-NEO and C4-2-AR-v7 cells were described previously and maintained in complete RPMI 1640 supplemented with 300µg/mL G418 (18). All cells were maintained at 37ºC in a humidified incubator with 5% carbon dioxide. G418 (Cat#: 108321-42-2) was purchased from KSE Scientific. Docetaxel (Ca#: RS019) was purchased from TSZ CHEM. Cabazitaxel (Cat#: S3022) and enzalutamide (Cat#: S1250) were purchased from Selleckchem. Abiraterone Acetate (Cat#: X6144) was purchased from AK Scientific, Inc.

Cell Growth Assay

Cells were plated at a density of 25,000-40,000 cells/well in 24-well plates in complete RPMI 1640 media without any selection agent. After 24 hours, cells were subjected to indicated treatments. Total cells were counted via coulter counter 72 hours post-treatment.

For experiments testing AR-v7 effect on taxane response in C4-2B, cells were first transiently transfected with plasmids expressing AR-v7 or empty control pcDNA3.1 vector using lipofectamine 2000 (Cat#: 11668-019) purchased from Invitrogen. 24 hours later, cells were trypsinized, counted and plated.

For testing 22Rv1 response to NGATs or taxanes with AR-v7 expression inhibition, 22Rv1 cells were plated and first treated 24 hours later with AR-v7 targeting siRNA from Thermo Scientific Dharmacon (Sequence – GUAGUUGUGAGUAUCAUGAUU) or non-targeting control oligonucleotide from Invitrogen (Cat#: 46-5373) using lipofectamine RNAiMAX purchased from Invitrogen (Cat#: 56532).

Data is displayed as percent of control cell growth - treatment group cell number/control group cell number x 100. All conditions were performed either in triplicate or quadruplicate. All experiments were performed at least twice.

Clonogenic Assay

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For all clonogenic assays described, cells were plated at 500 cells/well in 6-well plates in complete RPMI 1640 with no selection agent. Plated cells were subsequently treated 24 hours later as indicated. Colonies formed for 14 days. At the completion of each assay, cell colonies were fixed and stained using the following solution for 20 minutes; 0.05% w/v crystal violet, 1% of 37% formaldehyde, 1% methanol, 1X PBS. After staining, colonies were rinsed, allowed to air dry, and counted. Data is displayed as a percent of control cell colony growth (control is vehicle treatment only). All conditions were performed in duplicate. All experiments were performed at least twice.

Preparation of Whole Cell Lysates

Cells were harvested, washed with PBS, and lysed in high-salt buffer containing 10 mM HEPES [pH 7.9], 250 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM DTT, 1 mM PMSF, 1 mM NaV, 5 mM NaF and supplemented with protease inhibitors (Cat#: 11836153001) purchased from Sigma-Aldrich. Protein concentration was determined with Pierce Coomassie Plus (Bradford) Assay Kit (Cat#: 23236) purchased from Thermo Fisher Scientific.

Western Blot

Protein extracts were resolved by SDS-PAGE and indicated primary antibodies were used. ABCB1 antibody (SC-8313, rabbit-polyclonal, 1:500 dilution) was purchased from Santa Cruz Biotechnology. AR-441 antibody (SC-7305, mouse-monoclonal antibody, 1:1000 dilution) was purchased from Santa Cruz Biotechnology. Tubulin (T5168, mouse monoclonal antibody, 1:6000 dilution) was purchased from Sigma-Aldrich. Tubulin was used to monitor the amounts of samples applied. Proteins were visualized with a chemiluminescence detection system (Cat#: WBLUR0500) purchased from Millipore.

Quantitative PCR (qPCR)

Total RNAs were extracted using TRizol reagent (Cat#: 15596018) purchased from ThermoFisher. RNA was digested with RNase-free DNase 1 (Cat#: EN05216101) purchased from ThermoFisher. cDNAs were prepared using ImProm-II reverse transcriptase (Cat#: M314C) purchased from Promega. The cDNAs were subjected to quantitative-PCR (qPCR) using SsoFast EvaGreen Supermix (Cat#: 172-5205) purchased from Bio-Rad according to the manufacturer's instructions. Triplicates of samples were run on default settings of Bio-Rad CFX-96 real-time cycler. Each reaction was normalized by co-amplification of actin. Data was calculated using the efficiency corrected method. Primers used for qPCR were: AR-V7: 5'- AAC AGA AGT ACC TGT GCG CC, 3'-TCA GGG TCT GGT CAT TTT GA; Actin: 5'- CCC AGC CAT GTA CGT TGC TA, 3'- AGG GCA TAC CCC TCG TAG ATG.

Statistics

All quantitated data is displayed as percent of control mean ± standard deviation. Significance was assessed using a two tailed two sample equal variance students t-test. A p-value of <0.05 was accepted as significant.

Results

Drug resistant cell lines derived from prostate cancer cells exhibit robust resistance to respective drugs

Whether there exists cross-resistance between available therapies for prostate cancer is unclear. We have recently developed several drug resistant cell lines from castration resistant C4-2B cells; C4-2B-MDVR to enzalutamide (MDVR), C4-2B-AbiR to abiraterone (AbiR), and C4-2B-TaxR to docetaxel (TaxR) (18-20). We've characterized these cell lines extensively and determined that each resistant subline is robustly resistant to respective drugs versus parental C4-2B cells. Studies from cell growth assays performed to identify IC_{50} values to respective drugs are summarized in Table 1. Resistance development was higher for docetaxel than either enzalutamide or abiraterone. We sought to use this panel of cell lines with acquired resistance to therapy to explore potential cross-resistance between commonly used therapeutics.

NGAT resistant cells lack inter cross-resistance with taxanes

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Our previous work demonstrated that TaxR cells exhibit robust resistance to docetaxel versus parental C4-2B cells (20). Whether resistance to NGATs confers resistance to taxanes is poorly understood. We compared the response of MDVR and AbiR cells versus that of parental C4-2B cells to increasing doses of docetaxel (Fig. 1A). We found that both enzalutamide resistant MDVR and abiraterone resistant AbiR cells have similar sensitivity to docetaxel compared to parental C4-2B cells, suggesting no cross-resistance to docetaxel in C4-2B-derived cell lines resistant to therapies targeting androgen signaling. Clonogenic assays were performed and further support our findings that cell lines made resistant to enzalutamide and abiraterone exhibit no cross-resistance to docetaxel (Fig. 1B).

We additionally tested response to various doses of cabazitaxel, a next-generation taxane approved to treat docetaxel pre-treated patients. As with docetaxel, cell growth assays demonstrate that MDVR and AbiR cells respond to cabazitaxel similarly to parental C4-2B cells, indicating no existence of cross-resistance (Fig. 1C). Clonogenic assays supported these findings showing no significant cross-resistance to cabazitaxel in either of these cell lines versus control C4-2B cells (Fig. 1D). Our findings suggest that acquired resistance to NGATs does not lead to taxane inter cross-resistance.

Docetaxel resistant cells possess intra cross-resistance to cabazitaxel

Our previous work demonstrated that docetaxel resistant TaxR cells possess robust intra cross-resistance to cabazitaxel (21). Here we confirm using a dose response cell growth assay that TaxR cells are less sensitive to cabazitaxel than parental C4-2B cells (Fig. 1C). Clonogenic assays further support these data (Fig. 1D). Taken together, these results suggest that NGAT resistant cells lack inter cross-resistance to taxanes while intra cross-resistance does exist between the taxanes docetaxel and cabazitaxel.

Taxane resistant cells lack inter cross-resistance to enzalutamide and abiraterone

Our previously published data show that MDVR and AbiR cells exhibit robust resistance to the NGATs enzalutamide and abiraterone respectively (18, 19). Whether resistance to docetaxel leads to resistance to

NGATs is unclear. We used additional cell growth assays to assess the response of TaxR cells to both enzalutamide and abiraterone. TaxR cells were as sensitive to both NGATs as C4-2B cells suggesting that taxane resistance does not confer inter cross-resistance with either enzalutamide or abiraterone treatments (Fig. 2A and 2C). These data were confirmed using clonogenic assays which further demonstrate that TaxR cells continue to respond to enzalutamide and abiraterone treatment (Fig. 2B and 2D).

NGAT resistant cells possess intra cross-resistance to enzalutamide and abiraterone

Next, we determined whether intra cross-resistance exists between enzalutamide and abiraterone. Cell growth and clonogenic assays demonstrate that abiraterone resistant AbiR cells indeed exhibit decreased sensitivity to enzalutamide treatment (Fig. 2A-B). Similar assays confirm that MDVR cells are cross-resistant to abiraterone (Fig. 2C-D). These data strongly suggest the existence of common resistance mechanisms between both enzalutamide and abiraterone. Taken together, our findings suggest the existence of intra cross-resistance (enzalutamide with abiraterone and docetaxel with cabazitaxel), but not inter cross-resistance between the NGAT and taxane drug classes.

AR-variant expression correlates with resistance to NGATs while ABCB1 correlates with resistance to taxanes

Previous study has implicated AR-variants in mediating resistance to NGATs (22). Additionally, we've presented data before that suggests the involvement of AR-v7 in both enzalutamide and abiraterone resistance (18, 19). Western blots were performed to assess correlations between experimentally defined resistance and known markers of resistance (Fig. 3A). As shown via western blot, MDVR and AbiR cells express greatly increased protein levels of AR-variants relative to C4-2B cells. In contrast, TaxR cells do not augment AR-variants expression but do possess increased ABCB1. We've previously shown that ABCB1, a membrane efflux protein, mediates robust resistance to both docetaxel and cabazitaxel in prostate cancer (20, 21). Our data suggest that AR-variants expression is associated with resistance to NGATs but not with taxane resistance, while ABCB1 is associated with robust resistance to taxanes.

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Previous studies suggest that AR-variants, such as AR-v7, are capable of mediating resistance to taxanes thus potentially making them mechanisms of NGAT/taxane inter cross-resistance (23-25). To test this possibility, we used C4-2 cells stably over-expressing AR-v7 and subjected them to cell growth assays using increasing doses of either docetaxel or cabazitaxel (Fig. 3B). We found no resistance to taxane treatment in AR-v7 overexpressing C4-2 cells versus control empty vector containing cells. We next transiently overexpressed AR-v7 into C4-2B cells and again found that increased AR-v7 expression does not induce resistance to taxanes (Fig. 3C). Our data suggest that AR-variants, and specifically, AR-v7, are not involved in taxane resistance.

To further examine the putative role of AR-variants in mediating CRPC therapeutic resistance, we used CWR22Rv1 (22Rv1) cells which express high levels of endogenous AR-variants and exhibit intrinsic resistance to enzalutamide/abiraterone (19, 26). Interestingly, we found that 22Rv1 cells also exhibit intrinsic resistance to both docetaxel and cabazitaxel relative to C4-2B cells (Fig. 4A). Western blot analysis shows that 22Rv1 cells express much higher levels of AR-variants versus C4-2B cells (Fig. 4B). Our previous work suggests that this expression mediates resistance to both enzalutamide and abiraterone (18, 19). Whether this expression is also responsible for resistance to taxanes is unclear. Cell growth assays show that siRNA-mediated inhibition of AR-v7 sensitizes 22Rv1 cells to enzalutamide and abiraterone treatment, but not to docetaxel and cabazitaxel (Fig. 4C). These data taken together suggest that AR-variants are involved in resistance to NGATs but not to taxanes. Thus, while intrinsic resistance may exist in the same cellular context to both NGAT and taxane therapies, our data suggest that separate mechanisms are responsible for mediating sensitivities to these two drug classes.

Discussion

In this study, we show that resistance to NGATs does not induce resistance to taxanes. We further show that taxane resistant prostate cancer cells retain complete sensitivity to enzalutamide and abiraterone. These results argue that acquired resistance to NGATs does not induce inter cross-resistance with taxanes and vice versa. However, our work does show robust intra cross-resistance between therapies of the same category; enzalutamide with abiraterone and vice versa or docetaxel with cabazitaxel and vice versa. Correlating

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responses to known markers/mediators of resistance suggests that AR-variants, specifically AR-v7, may predict response to NGATs while ABCB1 may be a strong predictor of taxane response.

Using experimental resistant cell models, we demonstrate the existence of intra cross-resistance within drug classes, that is NGATs (enzalutamide with abiraterone) and taxanes (docetaxel with cabazitaxel). These data taken together suggest that mechanisms of resistance can often be common for therapies within the same class. Our data is supported by clinical observations which have demonstrated blunted responses when sequencing enzalutamide and abiraterone. Azad et al and Zhang et al demonstrate limited efficacy of enzalutamide post abiraterone and evidence for intra cross-resistance between these two agents (14, 27). The reverse sequence, abiraterone post enzalutamide, was similarly shown to produce blunted responses (28). While clinical studies are largely lacking to assess taxane cross-resistance, the data shown here and before suggest the existence of intra cross-resistance between docetaxel and cabazitaxel (21). The TROPIC clinical trial demonstrated that cabazitaxel does possess meaningful activity in patients previously treated with docetaxel (12). However, responses were modest with a survival benefit of only 2.4 months. Recent results from the FIRSTANA clinical trial testing cabazitaxel against docetaxel in the first line setting showed higher cabazitaxel response rates than those seen in the TROPIC trial testing cabazitaxel post docetaxel (12, 29). While a rigorous statistical retrospective study is needed to make conclusions, this observation suggests that cabazitaxel responses are blunted by prior administration of docetaxel.

We also address the critical issue of inter cross-resistance between the NGAT and taxane drug classes. We show that enzalutamide resistant MDVR cells and abiraterone resistant AbiR cells display no cross-resistance to either docetaxel or cabazitaxel. A separate line of study by van Soest et al using PC346C cell based models of enzalutamide and abiraterone resistance suggest there is inter cross-resistance between these NGATs and taxanes (30). However, when this group took their findings in vivo, they found that only docetaxel maintained cross-resistance with enzalutamide-resistant cells (31). Building on our work and theirs, we also demonstrate that docetaxel resistant TaxR cells retain sensitivity to both enzalutamide and abiraterone. Our results suggest that acquired resistance to NGATs does not create resistance to taxanes and that acquired resistance to taxanes similarly does not induce inter cross-resistance to NGATs. A putative treatment scheme based on our findings is presented in figure 5 (Fig. 5). Initial treatment with either enzalutamide, abiraterone, or docetaxel will inevitably result in the development of resistance. Our work suggests that resistance is common between enzalutamide and abiraterone, as detailed in our schematic, but different for taxanes. Due to common resistance mechanisms, we suggest switching to the opposite drug class. However, as research advances, and the mechanisms of resistance and methods to overcome them are discovered and tested, putative combination therapies to re-sensitize to subsequent lines of similar treatments may be possible. Our findings have important clinical implications as it is currently not known what the optimal treatment sequence is for CRPC. The data taken together suggest that patients who receive NGATs can be given taxanes and vice versa but that patients are likely to respond poorly or fail when sequencing drugs with similarly targeted pathways. Additional research and trial data will be needed to optimize treatment schemes.

In support of our findings, many clinical studies suggest a lack of evidence for inter cross-resistance between these therapies. A report from Aggarwal et al suggests a lack of cross-resistance between abiraterone and docetaxel based on their study which demonstrated that patients did not differ in their response to docetaxel regardless of whether they had primary or acquired resistance to abiraterone (32). A study by Azad et al also found that prior response to abiraterone had no bearing on subsequent response to docetaxel (33). In a separate and previously mentioned study, Azad et al found that enzalutamide post abiraterone treatment lead to equally blunted responses irrespective of prior docetaxel exposure (27). These studies suggest a lack of cross-resistance between NGATs and docetaxel. A study by Al Nakouzi et al retrospectively found no statistical difference in cabazitaxel efficacy pre and post abiraterone (34). Pezaro et al found that response to cabazitaxel post docetaxel and either abiraterone or enzalutamide was similar to those in the TROPIC clinical trial which tested cabazitaxel in patients treated with docetaxel but neither NGAT (12, 35). These data in conjunction with our findings suggest that therapies of different classes can be safely and effectively used clinically in sequence. Additionally,

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it has been put forth that a switch between drug classes is not only possible but preferred to prevent the development of resistance (13).

It is thought that inhibition of AR nuclear trafficking by taxanes is a secondary mechanism of action for these drugs in prostate cancer (15, 16). This provides a rationale to hypothesize that cross-resistance could exist between taxanes and AR targeting therapies. In line with this hypothesis, it is thought that low molecular weight isoforms, which no longer require traditional mechanisms to travel to the nucleus, are capable of mediating resistance to taxane therapy, thus providing a mechanism for cross-resistance between drug classes. A study by Zhang et al shows that expression of both AR-v7 and ARv567es can induce resistance to both docetaxel and cabazitaxel (24). Martin et al also find that forced overexpression of ARv567es induces resistance to cabazitaxel (25). In contrast to these findings, Thadani-Mulero et al show data suggesting only AR-v7, not ARv567es, is a meaningful marker of sensitivity to taxanes (23). Our findings suggest that AR-v7 is not a mechanism of taxane resistance. Furthermore, we show no increase in AR-variant expression in docetaxel resistant C4-2B-TaxR cells. Thus, while some evidence has been presented to argue that overexpression of AR-variants may mediate resistance to taxanes, findings conflict suggesting AR-variants may make poor predictors of response to these drugs.

In agreeance with our findings, three independent studies have all found that patients with detectable AR-v7 expression in either circulating tumor cells or exosomes have inferior clinical outcomes on NGATs versus AR-v7 negative patients (36-38). Additionally, two of these studies found that those patients with detectable AR-v7 fared better on taxane treatment than on treatment with either enzalutamide or abiraterone (36, 37). The third of these studies demonstrated that AR-v7 status had no bearing on progression-free survival in taxane treated patients (38). These results suggest that AR-v7 is a meaningful factor in NGAT resistance and response but a poor predictor of response to taxanes. An additional clinical study supporting our work showed that AR-v7 status appears not to predict response to cabazitaxel (39). Thus, our work supports the use of AR-variants to stratify patients between NGATs and taxanes.

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While AR-v7 and more broadly, AR-variants, appear to be able to predict poor outcomes to NGATs and steer clinicians towards taxanes, there are currently no biomarkers to predict response to taxanes. We've previously shown that ABCB1 is a significant mediator of both docetaxel and cabazitaxel resistance (18, 19). Here, we further demonstrate that while TaxR cells express greatly increased levels of ABCB1 versus parental C4-2B cells, MDVR and AbiR cells do not exhibit this expression. Our data suggest that the addition of ABCB1 as a predictor of taxane response could potentially improve treatment decisions. A similar study found that the SLCO1B3 transporter also may be involved in the development of taxane resistance (40). We hypothesize that ABCB1, and other markers such as SLCO1B3, could be developed into biomarkers like AR-v7, capable of informing clinicians on when a patient is unlikely to respond to taxane treatment.

Interestingly, we've found that 22Rv1 cells harbor intrinsic resistance relative to C4-2B cells to both NGATs and taxanes. However, siRNA-mediated inhibition of AR-v7 sensitized Rv1 cells only to enzalutamide and abiraterone, not taxanes. These data suggest that while intrinsic resistance to both drug classes may exist within a tumor, similar mechanisms may not be utilized for all resistance. A previous report demonstrated that patients who were initially refractory to abiraterone therapy were also refractory to docetaxel (41). Thus, these patients appeared to present with resistance to both drugs, providing evidence for our findings that presentation with intrinsic resistance to multiple therapies exists clinically. This result does not however prove that a common mechanism is utilized for resistance to these treatments. It is imperative that we improve our ability to predict response to therapy which will lead to accurate stratification of patients for subsequent treatment.

While our findings are intriguing and shed light on cross-resistance in CRPC, we are aware of the limitations of our *in vitro* models and studies. These limitations include absence of key heterotypic signaling interactions and lack of systemic function of these drugs on the body (ie. abiraterone's effect on testosterone production beyond intracrine androgen synthesis). Additional clinical trials and study of resistance markers in clinically derived specimens are needed to expand and validate our findings.

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In conclusion, our findings suggest the existence of intra cross-resistance within a drug class (i.e., within NGATs or within taxanes), while inter cross-resistance between drug classes does not develop. We also provide evidence that AR-variants are capable of signaling the presence of and mediating resistance to NGATs but not to taxanes, while ABCB1 may be useful in predicting taxane response. Our data supports many clinical studies and suggests that taxanes and NGATs can be sequentially administered without risk of diminished efficacy while further work to elucidate the mechanisms of resistance will increase our understanding and lead to both 1) novel biomarkers to aid patient stratification, such as AR-variants and ABCB1, and 2) methods to overcome cross-resistance which can restore efficacy to subsequent lines of treatment.

Acknowledgments

This work was supported in part by grants NIH/NCI CA168601, CA179970, DOD PC150229, and the U.S. Department of Veterans Affairs, Office of Research & Development BL&D grant number I01BX0002653 (A.C. G), a Research Career Scientist Award (A.C. G). A.C.G is also a Research Career Scientist at VA Northern California Health Care System, Mather, California.

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Tables

Table 1 – IC_{50} values to enzalutamide, abiraterone, and docetaxel in C4-2B and C4-2B-derived resistant cell lines to enzalutamide (C4-2B-MDVR), abiraterone (C4-2B-AbiR), and docetaxel (C4-2B-TaxR).

Table 1: IC_{50} values to enzalutamide, abiraterone, and docetaxel in models of CRPC therapeutic resistance

	Enzalutamide	**Abiraterone**	**Docetaxel**
C4-2B	31 μM	8 μM	0.8 nM
C4-2B-MDVR	56 μM		
C4-2B-AbiR		16 μM	
C4-2B-TaxR			>100 nM

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Figure Legends

Figure 1 – Assessment of response to docetaxel and cabazitaxel in resistant cell lines. A. Cell growth assays were performed to determine response to docetaxel in MDVR and AbiR cells versus parental C4-2B cells. B. Clonogenic assays were performed to determine response to docetaxel in MDVR and AbiR cells versus parental C4-2B cells. Colonies were counted and normalized to control colony formation. C. Cell growth assays were performed to determine response to cabazitaxel in MDVR, AbiR, and TaxR cells versus parental C4-2B cells. D. Clonogenic assays were performed to determine response to cabazitaxel in MDVR, AbiR, and TaxR cells versus parental C4-2B cells. Colonies were counted and normalized to control colony formation. All growth assays were done in triplicate and performed at least twice. All colony formation assays were done in duplicate and performed at least twice. DTX = docetaxel. CTX = cabazitaxel. * = p-value < 0.05

Figure 2 – Assessment of response to enzalutamide and abiraterone in resistant cell lines. A. Cell growth assays were performed to determine response to enzalutamide in TaxR and AbiR cells versus parental C4-2B cells. B. Clonogenic assays were performed to determine response to enzalutamide in TaxR and AbiR cells versus parental C4-2B cells. Colonies were counted and normalized to control colony formation. C. Cell growth assays were performed to determine response to abiraterone in TaxR and MDVR cells versus parental C4-2B cells. D. Clonogenic assays were performed to determine response to abiraterone in MDVR and TaxR cells versus parental C4-2B cells. Colonies were counted and normalized to control colony formation. All growth assays were done in triplicate and performed at least twice. All colony formation assays were done in duplicate and performed at least twice. Enz = enzalutamide. Abi = abiraterone. * = p-value < 0.05

Figure 3 – AR-variants correlate with NGAT resistance while ABCB1 correlates with taxane resistance. A. Western blots for expression of AR and ABCB1 were performed in C4-2B, MDVR, AbiR, and TaxR whole cell lysates. Tubulin served as a loading control. B. C4-2-NEO and C4-2-ARv7 cells were subjected to cell growth assays testing dose response to either docetaxel or cabazitaxel. Western blot was used to show that C4-2-ARv7

cells overexpress AR-v7 versus control C4-2-NEO cells. Tubulin served as a loading control. C. C4-2B cells were transiently transfected with an AR-v7 expressing construct and subjected to cell growth assays testing response to either docetaxel or cabazitaxel versus control construct (pcDNA3.1) expressing cells. qPCR was used to determine successful transfection and overexpression of AR-v7. All growth assays were done in triplicate and performed at least twice. FL-AR = full-length androgen receptor. AR-V = androgen receptor variants. Ab = antibody used. C = control (vehicle). DTX = docetaxel. CTX = cabazitaxel. * = p-value < 0.05

Figure 4 – AR-v7 does not mediate NGAT/Taxane inter cross-resistance in 22Rv1 cells. A. Cell growth assays were used to determine response to docetaxel and cabazitaxel in 22Rv1 cells versus C4-2B cells. B. Western blots were done to assess AR and AR-variant levels in 22Rv1 cells versus C4-2B cells. Tubulin served as a loading control. C. 22Rv1 cells were treated with AR-v7 targeting siRNA (si-v7) or a control non-targeting oligonucleotide (siControl) and subjected to cell growth assays testing response to either enzalutamide and abiraterone or docetaxel and cabazitaxel. Western blot was used to show successful inhibition of AR-v7 via siRNA. Tubulin served as a loading control. All growth assays were done in triplicate and performed at least twice. FL-AR = full-length androgen receptor. AR-V = androgen receptor variant. Ab = antibody used. DTX = docetaxel. CTX = cabazitaxel. Enz = enzalutamide. Abi = abiraterone. * = p-value < 0.05

Figure 5 - Development of resistance and potential sequencing and treatment strategies to overcome intra-cross resistance. NGAT drugs and taxanes are approved for CRPC. The development of resistance is inevitable. If an NGAT is given initially, our data suggests switching to a taxane or using another NGAT in combination with a re-sensitizing agent. If docetaxel is given initially, our data suggests 1) switching to an NGAT, 2) using cabazitaxel, or 3) using a taxane with a re-sensitizing agent.

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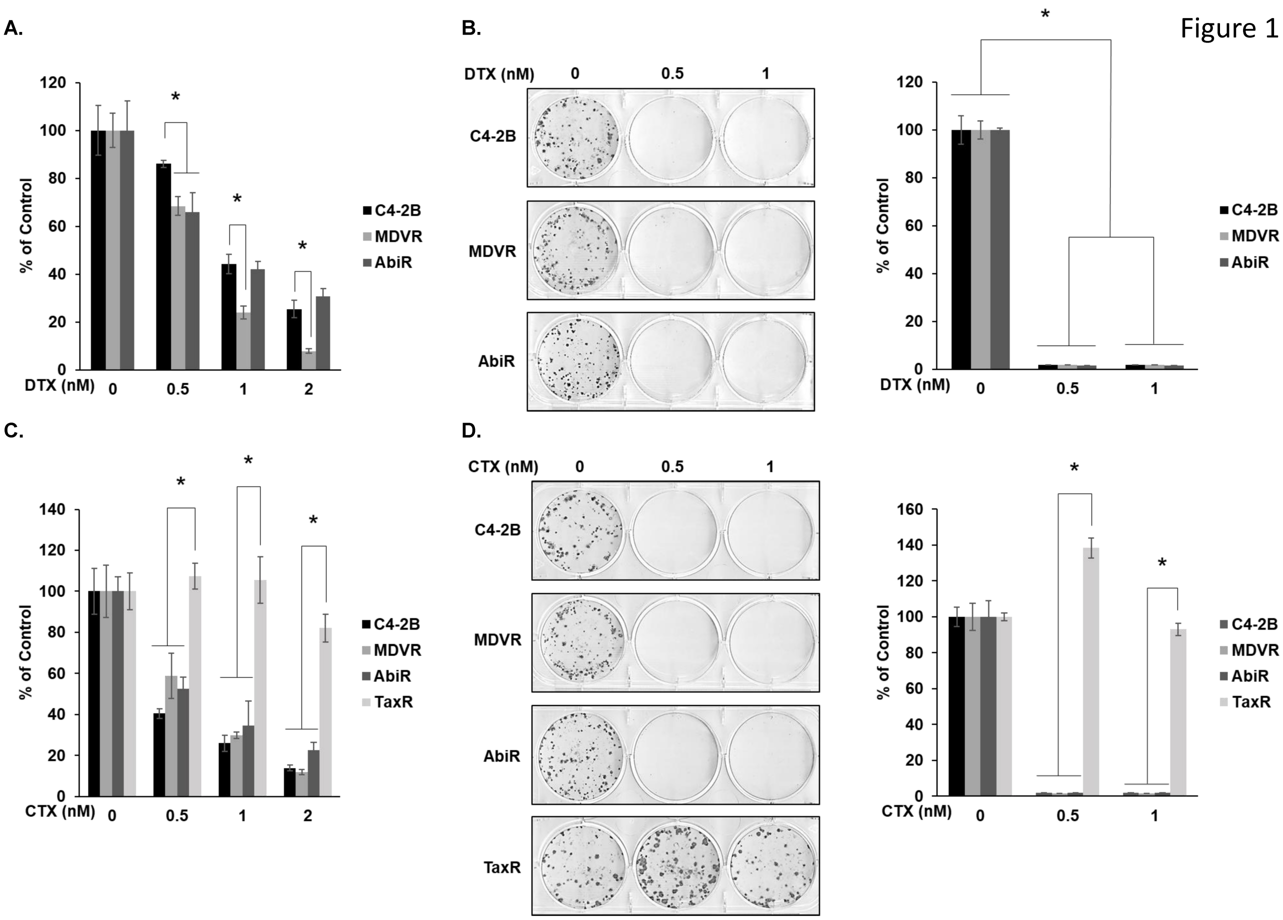

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Figure 2

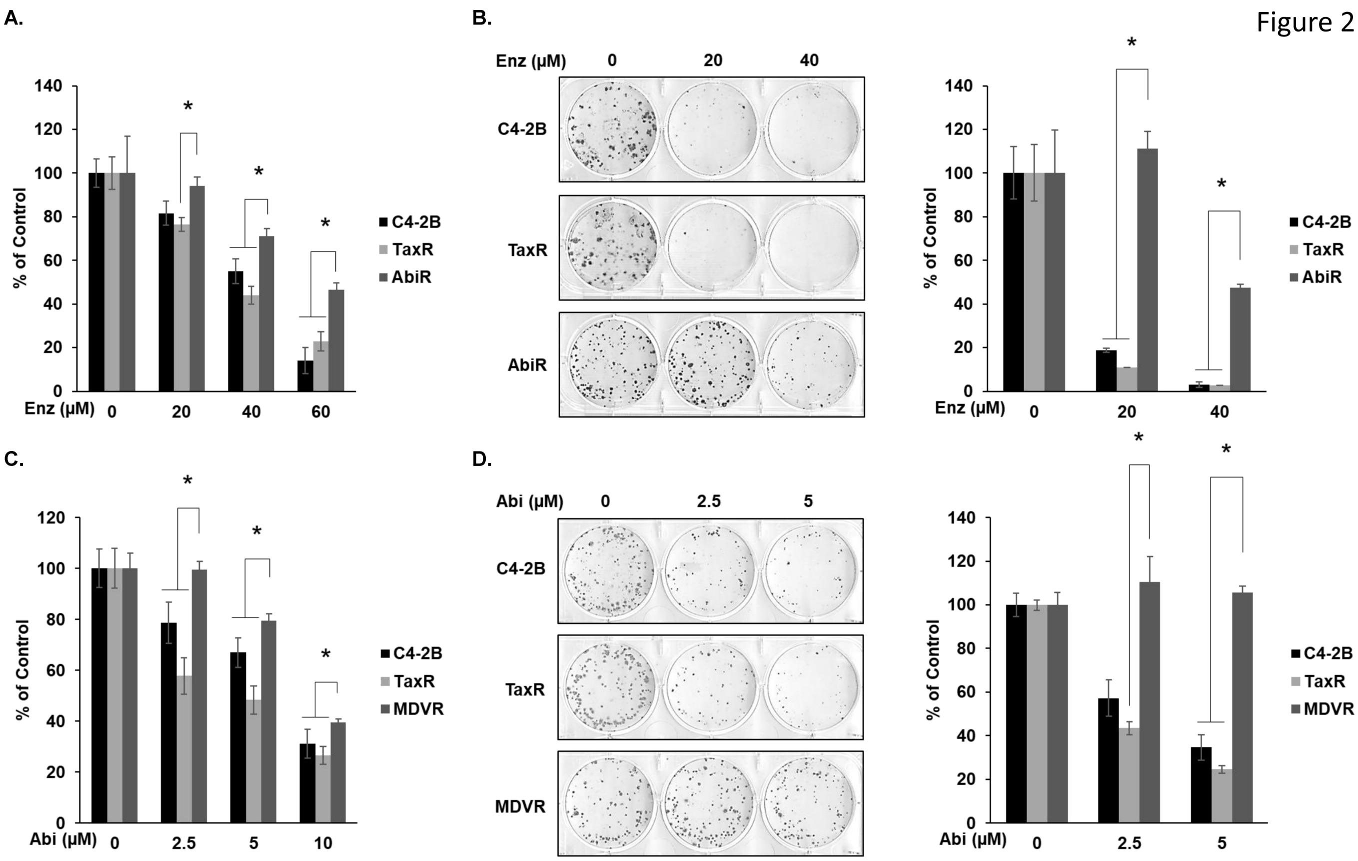

Figure 3

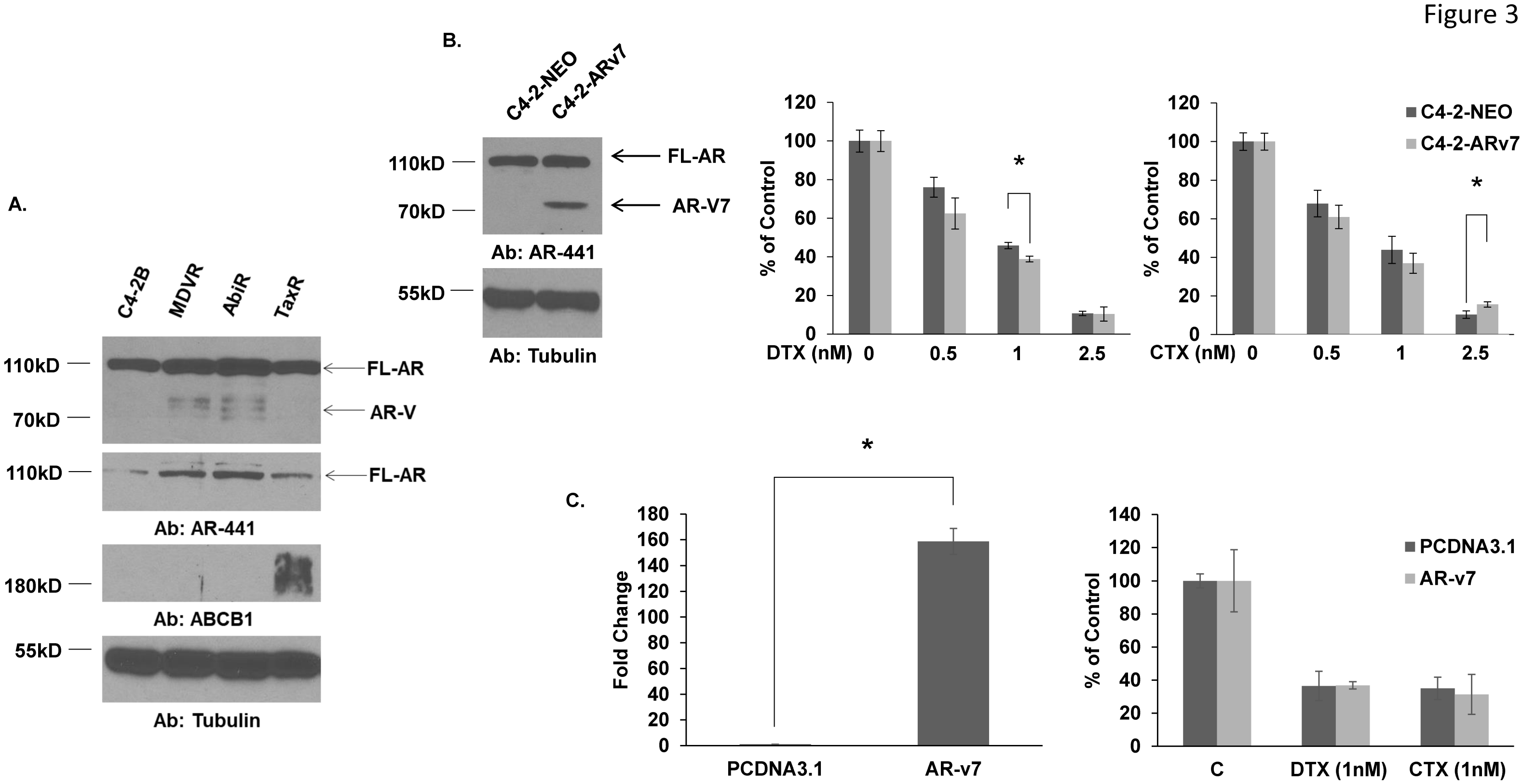
A.
C4-2B
MDVR
AbiR
TaxR
110kD
70kD
FL-AR
AR-V
110kD
FL-AR
Ab: AR-441
180kD
Ab: ABCB1
55kD
Ab: Tubulin
B.
C4-2-NEO
C4-2-ARv7
110kD
70kD
FL-AR
AR-V7
Ab: AR-441
55kD
Ab: Tubulin
% of Control
DTX (nM) 0 0.5 1 2.5
CTX (nM) 0 0.5 1 2.5
C4-2-NEO
C4-2-ARv7
C.
Fold Change
PCDNA3.1
AR-v7
% of Control
PCDNA3.1
AR-v7
C
DTX (1nM)
CTX (1nM)

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Figure 4

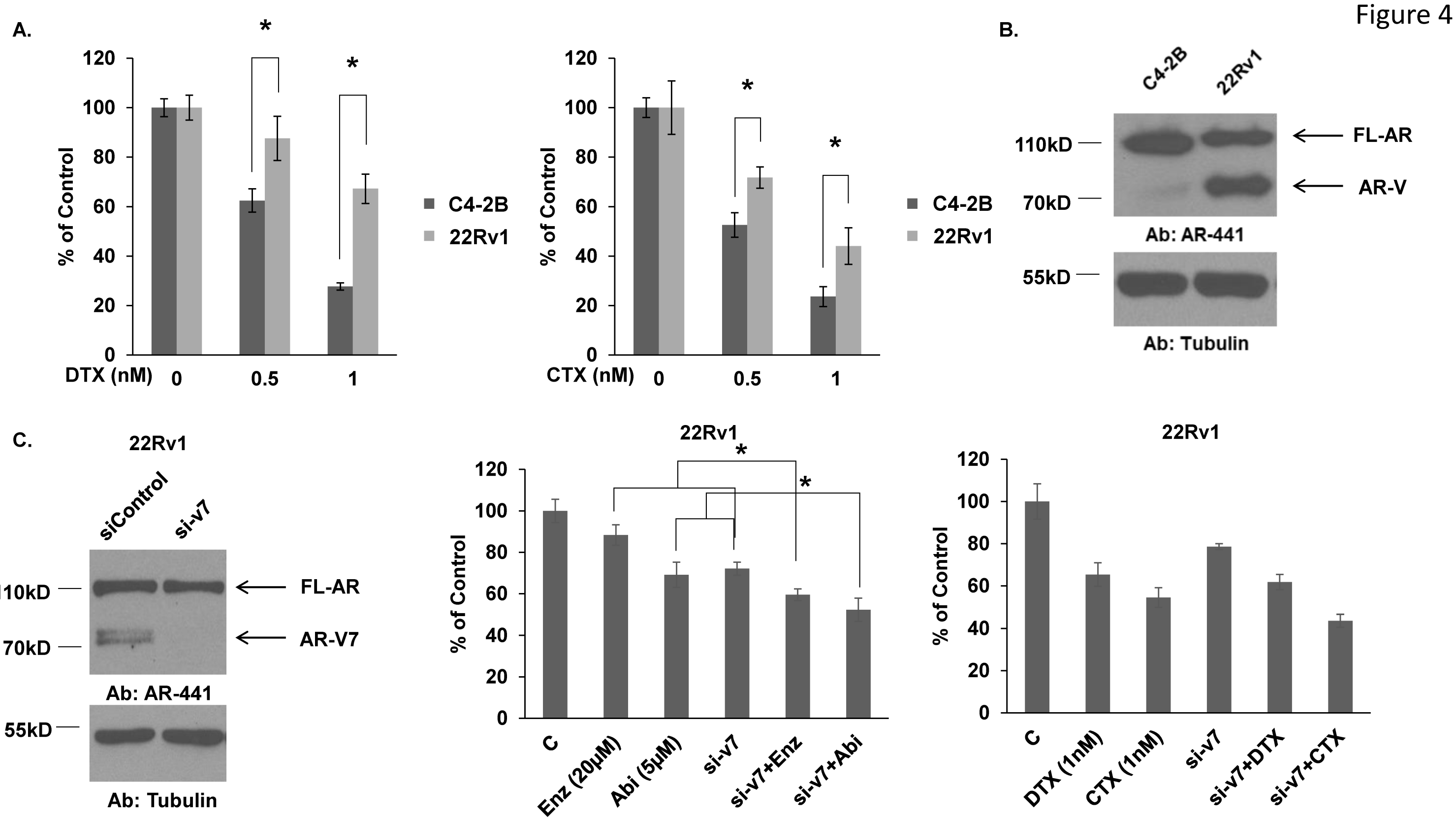

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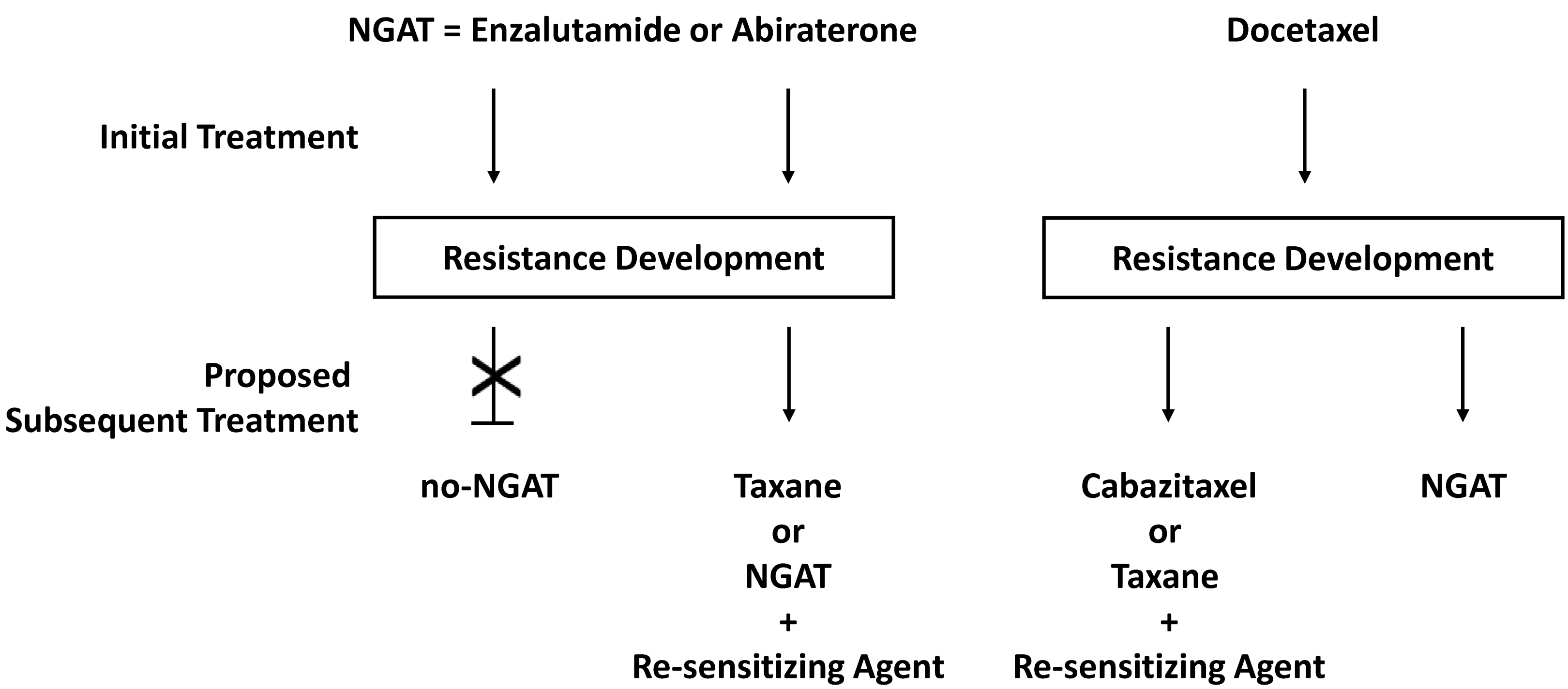
Figure 5



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Molecular Cancer Therapeutics

Intra vs Inter Cross-Resistance Determines Treatment Sequence between Taxane and AR-Targeting Therapies in Advanced Prostate Cancer

Alan P. Lombard, Liangren Liu, Vito Cucchiara, et al.

Mol Cancer Ther Published OnlineFirst June 11, 2018.