NF-kappaB2/p52 induces resistance to Enzalutamide in Prostate Cancer: Role of androgen receptor and its variants

Nagalakshmi Nadiminty¹¶, Ramakumar Tummala¹, Chengfei Liu¹, Joy Yang¹, Wei Lou¹, Christopher P. Evans¹² and Allen C. Gao¹²¶

¹Department of Urology, ²Comprehensive Cancer Center, University of California at Davis, Sacramento, CA, USA

¶To whom correspondence should be addressed:

Department of Urology

University of California Davis Medical Center

4645 2nd Ave, Research III, Suite 1300

Sacramento, CA 95817

Email: acgao@ucdavis.edu, nnadiminty@ucdavis.edu

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Abstract

Resistance of prostate cancer (CaP) cells to the next generation anti-androgen, Enzalutamide, may be mediated by a multitude of survival signaling pathways. In this study we tested whether increased expression of NF-κB2/p52 induces CaP cell resistance to Enzalutamide and whether this response is mediated by aberrant androgen receptor (AR) activation and AR splice variant production. LNCaP cells stably expressing NF-κB2/p52 exhibited higher survival rates compared to controls when treated with Enzalutamide. C4-2B and CWR22Rv1 cells chronically treated with Enzalutamide were found to express higher levels of NF-κB2/p52. Downregulation of NF-κB2/p52 in CWR22Rv1 cells chronically treated with Enzalutamide rendered them more sensitive to cell growth inhibition by Enzalutamide. Analysis of the expression levels of AR splice variants by qRT-PCR and Western blotting revealed that LNCaP cells expressing p52 exhibit higher expression of AR splice variants. Downregulation of expression of NF-κB2/p52 in VCaP and CWR22Rv1 cells by shRNA abolished expression of splice variants. Downregulation of expression of either full length AR or the splice variant AR-V7 led to an increase in sensitivity of CaP cells to Enzalutamide. These results collectively demonstrate that resistance to Enzalutamide may be mediated by NF-κB2/p52 via activation of AR and its splice variants.
Introduction

Localized prostate cancer (CaP) is dependent on androgens, and the majority of patients respond to androgen ablation. However, virtually every patient will develop castration-resistant prostate cancer (CRPC) and no longer respond to androgen deprivation therapy (ADT). Persistent androgen receptor (AR) activation remains an important player in CRPC progression. CRPC cells often continue to express AR and AR axis genes (1, 2), implying that the AR is active in AR-positive CRPC cells. Such observations form the basis for continued attempts to target the AR axis and for the development of next generation anti-androgens such as Enzalutamide (formerly MDV3100). Enzalutamide binds to the AR with greater affinity than Bicalutamide, inhibits its nuclear translocation and expression of its target genes (3). In spite of initial success, development of resistance is a contraindication for its use in many patients and as demographics change, an increasing number of patients are likely to develop resistance to Enzalutamide. The mechanisms leading to resistance have been poorly understood, even though a recent report showed that AR splice variants play a major role in development of resistance (4). AR splice variants lack the ligand binding domain targeted by Enzalutamide and variants such as AR-V7 are postulated to be constitutively active. The mechanistic aspects of regulation of variant expression leading to resistance against Enzalutamide are unknown. Therefore, an urgent need exists to fully understand the mechanisms of resistance and to devise ways to overcome them.

The classical NF-κB pathway involving the p65/p50 heterodimer has been shown to be constitutively activated in several cancers including CaP (5). The non-canonical NF-κB pathway involves the processing of p100 to NF-κB2/p52 via the recruitment of
NF-κB-inducing kinase (NIK) and subsequent activation of IκB kinase α (IKKα). The processing of p100 to p52 is a tightly controlled event in many cells and tissues (6-9). The functional significance of p100 processing has been confirmed by genetic evidence from humans and mice (10). Overproduction of p52 has been observed in several solid tumors including breast and prostate cancers (11, 12). Our previous studies demonstrated that NF-κB2/p52 induces castration-resistant growth in LNCaP cells (13), that several genes involved in processes such as cell growth, proliferation, cell movement are potential targets of NF-κB2/p52 (14) and that NF-κB2/p52 induces aberrant activation of the AR in a ligand-independent manner and thus promotes castration-resistance (15).

In this study, we report that NF-κB2/p52 promotes resistance of CaP cells to Enzalutamide. We demonstrate that increased resistance of CaP cells expressing p52 to Enzalutamidemay be mediated by induction of AR splice variants (such as AR-V7) and by activation of the AR axis by p52.

**Materials and Methods**

**Cell lines and reagents**

LNCaP, CWR22Rv1 and VCaP cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All experiments with cell lines were performed within 6 months of receipt from ATCC or resuscitation after cryopreservation. ATCC uses Short Tandem Repeat (STR) profiling for testing and authentication of cell lines. C4-2B cells were kindly provided and authenticated by Dr. Leland Chung, Cedars-Sinai Medical Center, Los Angeles, CA. Cells were cultured in RPMI containing either 10% complete FBS or 10% charcoal-dextran-stripped FBS (CS-FBS) and penicillin/streptomycin. LNCaP passage numbers <20 were used throughout the study.
VCaP cells were cultured in DMEM supplemented with 10% FBS. NF-κB2/p52 (K-27), AR (441; mouse monoclonal), HA, and Tubulin antibodies were purchased from Santa Cruz biotechnologies (Santa Cruz, CA). Antibodies against AR-V7 splice variant were kindly provided by Dr. Jun Luo (Department of Urology, Johns Hopkins University, Baltimore, MA). All other reagents were of analytical grade and obtained from local suppliers. Sso Fast Eva Green qPCR Supermix was from Bio-Rad.

**Generation of stable cell lines**

Stable cell lines of LNCaP expressing NF-κB2/p52 (LN-p52) were generated by transfection of plasmids containing the cDNA and selection of clones after application of selective pressure with appropriate antibiotics. LNCaP cells expressing p52 under the control of a tetracycline-inducible cassette (LN/TR/p52) were generated using the ViraPower™ lentiviral transduction system (Invitrogen).

**Cell growth assays**

Cells were transfected with plasmids or treated with the indicated reagents and viable cell numbers were determined at various time points using a Coulter cell counter.

**Western Blot Analysis**

Cells were lysed in high salt buffer containing 50 mM Hepes pH 7.9, 250 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 1 mM Na Vanadate, 1 mM NaF and protease inhibitor cocktail (Roche) as described earlier (16). Total protein was estimated using the Coomassie Protein Assay Reagent (Pierce, Rockford, IL). Equal amounts of protein were loaded on 10% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBST (1x PBS+0.1% Tween-20) and probed with
primary antibodies in 1% BSA. The signal was detected by ECL (GE Healthcare) after incubation with the appropriate HRP-conjugated secondary antibodies.

**Real-Time quantitative RT-PCR**

Total RNAs were extracted using TriZOL reagent (Invitrogen). cDNAs were prepared after digestion with RNase-free RQ1 DNase (Promega). The cDNAs were subjected to real-time reverse transcription-PCR (RT-PCR) using Sso Fast Eva Green Supermix (Bio-Rad) according to the manufacturer's instructions and as described previously (15). Each reaction was normalized by coamplification of actin. Triplicates of samples were run on default settings of Bio-Rad CFX-96 real-time cycler.

**Clonogenic Assays**

Anchorage-dependent clonogenic ability assays were performed as described previously (13). Briefly, cells were seeded at low densities (400 cells/dish) in 10 cm culture plates. The plates were incubated at 37°C in media containing either 10% FBS or 10% charcoal-stripped FBS (CS-FBS) and were left undisturbed for 14 days. At the end of the experiment, cells were fixed with methanol, stained with crystal violet and the numbers of colonies were counted.

**Luciferase Assays**

Cells were transfected with reporters along with plasmids and AR and AR-V7 siRNAs as indicated in the figures. Cell lysates were subjected to luciferase assays with the Luciferase Assay System (Promega).

**Statistical Analyses**
Data are shown as means ± SD. Multiple group comparison was performed by one-way ANOVA followed by the Scheffe procedure for comparison of means. $P \leq 0.05$ was considered significant.

**Results**

*CaP cells expressing NF-κB2/p52 are resistant to Enzalutamide and Bicalutamide*

LN-neo (LNcaP cells expressing the empty vector) and LN-p52 cells (LNCaP cells stably expressing p52) were treated with 0, and 20 μM Enzalutamide or Bicalutamide in media containing either complete FBS or charcoal-stripped FBS and cell growth was examined after 48 h. DMSO was used as the vehicle control. As shown in Fig. 1A, cells stably expressing p52 exhibited better cell survival ability when exposed to Enzalutamide or Bicalutamide compared to control LN-neo cells. To confirm these experiments, we treated LN-neo or LN-p52 cells with 0, 20 and 40 μM Enzalutamide or Bicalutamide and performed clonogenic assays. As shown in Fig. 1B, LN-neo cells were highly sensitive to both Enzalutamide and Bicalutamide and formed fewer colonies, whereas the number of colonies formed by cells expressing p52 was significantly higher, indicating that NF-κB2/p52 may induce resistance to Enzalutamide and Bicalutamide in CaP cells. To further confirm these results, we used the Tetracycline-inducible system to induce p52 expression in LNCaP cells and tested Enzalutamide and Bicalutamide sensitivity. We treated LN/TR/Con and LN/TR/p52 cells with 0, 20 and 40 μM Enzalutamide or Bicalutamide and performed growth assays. As shown in Fig. 1C, induction of expression of p52 by DOX significantly enhanced the ability of LN/TR/p52 cells to survive in the presence of Enzalutamide or Bicalutamide compared to control LN/TR/Con cells. These results collectively demonstrate that CaP cells expressing higher levels of NF-κB2/p52
are more resistant to Enzalutamide and Bicalutamide compared to cells which do not express p52.

**CaP cells chronically treated with Enzalutamide exhibit higher levels of NF-κB2/p52**

Our previous studies showed that most androgen-dependent CaP cell lines do not express detectable levels of endogenous NF-κB2/p52 (13). Hence, to test whether CaP cells resistant to Enzalutamide exhibit higher levels of p52, we treated CWR22Rv1 cells with 5-10 μM Enzalutamide chronically for >10 months. The resultant cells showed higher cell survival rates when treated with Enzalutamide. We examined the expression levels of NF-κB2/p52 in these cells by qRT-PCR and by Western blotting. As shown in Fig. 2A, CWR22Rv1 cells treated chronically with Enzalutamide exhibited higher levels of both precursor p100 as well as p52, indicating that CaP cells resistant to Enzalutamide may upregulate the endogenous levels of NF-κB2/p52. To test whether downregulation of p52 resensitizes these cells to Enzalutamide, we transfected shRNAs specific to p52 into CWR22Rv1 cells treated chronically with Enzalutamide (expressing higher levels of p52) and examined cell growth after 24 and 48 h. Downregulation of p52 after transfection was confirmed by qRT-PCR. As shown in Fig. 2B, cells transfected with p52 shRNA were increasingly sensitive to Enzalutamide compared to control CWR22Rv1-Enza-R cells, indicating that expression of p52 may be necessary for the survival of cells treated chronically with Enzalutamide. These results collectively demonstrate that NF-κB2/p52 may regulate the induction of resistance to Enzalutamide in CaP cells.

**NF-κB2/p52 enhances expression of AR splice variants**

It has been shown that higher levels of AR splice variants may be responsible for the resistance to Enzalutamide in CaP (4), hence we tested whether NF-κB2/p52 regulates
the expression of AR splice variants. Total RNAs from LNCaP and C4-2B cells transfected with either empty vector or p52 in media containing either complete or charcoal-stripped FBS (CS-FBS) were analyzed by qRT-PCR for the expression levels of full length AR as well as the major splice variant AR-V7. As shown in Fig. 3A, expression of p52 enhanced the expression levels of the splice variant AR-V7 in both FBS and CS-FBS, while expression of full length AR remained unchanged in LNCaP cells (left panel). These results were confirmed by Western blotting using antibodies specific for full length AR and AR-V7 (right panel). Similar results were observed in C4-2B cells, in which expression of p52 enhanced the expression levels of AR-V7 while expression levels of full length AR were unaffected (Fig. 3B). To substantiate these results, we examined expression levels of full length AR and AR-V7 in LN-neo and LN-p52 cells by qRT-PCR and Western blotting and found that expression levels of AR-V7 were elevated in LN-p52 cells compared to LN-neo cells (Fig. 3C). We also analyzed expression levels of AR-V7 in xenografts of LNCaP cells expressing p52 and found that xenografts expressing p52 exhibited significantly higher levels of AR-V7 mRNA compared to control LNCaP cell xenografts (Fig. 3D). These findings demonstrate that NF-κB2/p52 may induce upregulation of the expression of AR-V7.

**Downregulation of NF-κB2/p52 abrogates expression of AR splice variants**

Next, we tested whether NF-κB2/p52 was necessary for the enhanced expression of AR splice variants. VCaP and CWR22Rv1 CaP cells express endogenous levels of AR splice variants, AR-V1, AR-V5, AR-V7, AR-1/2/2b and AR-1/2/3/2b. We transfected shRNA specific to p52 into VCaP and CWR22Rv1 cells and examined the expression levels of these splice variants by qRT-PCR using specific primers. As shown in Fig. 4A and B (left
panels), downregulation of p52 reduced the expression levels of most of the splice variants significantly, while levels of full length AR remained unaffected. These results were confirmed for AR-V7 expression by Western blotting using antibodies specific against AR-V7 and FL AR in VCaP and CWR22Rv1 cells (Fig. 4A and B, right panels), indicating that expression of p52 may be necessary for the synthesis of AR splice variants.

**Downregulation of full length AR and AR-V7 increase sensitivity of p52-expressing CaP cells to Enzalutamide**

LNCaP cells stably expressing p52 (LN-p52) exhibit higher levels of AR-V7. We also assessed expression levels of other members of the NF-κB family by Western blotting and found that their levels were not altered (Suppl. Fig. 1), indicating that the effect on AR-V7 expression was mainly due to the expression of NF-κB2/p52. Next, we analyzed expression levels of anti-apoptotic proteins such as Bcl-xL, Survivin and Cyclin D1 in LN-p52 cells compared to LN-neo cells and found that LN-p52 cells express higher levels of Bcl-xL, Survivin and Cyclin D1 (Suppl. Fig. 2), indicating that activation of anti-apoptotic genes may play an important role in resistance against Enzalutamide. As reported previously, the cells also exhibit aberrant activation of AR in the absence of androgen and exhibit castration-resistant growth (15). In the current study, we also demonstrated that LN-p52 cells are resistant to enzalutamide-induced growth inhibition compared to control LN-neo cells. Hence, to test whether full length AR or AR-V7 play a role in the p52-induced resistance to Enzalutamide, we transfected siRNAs specific against either full length AR or AR-V7 into LN-neo and LN-p52 cells and monitored cell growth in response to Enzalutamide. As shown in Fig. 5A, downregulation of either FL
AR or AR-V7 reduced growth of control LN-neo cells by ~20%, and enzalutamide itself reduced growth of LN-neo cells by ~50%. No additional reduction of growth was observed in LN-neo cells when FL AR or AR-V7 was downregulated in the presence of enzalutamide, showing that inhibition of either FL AR or AR-V7 had no effect on the sensitivity of LN-neo cells to Enzalutamide. In LN-p52 cells which express higher levels of AR-V7, downregulation of either FL AR or AR-V7 reduced growth by ~50% in the presence of Enzalutamide, thus resensitizing LN-p52 cells to Enzalutamide. In other words LN-p52 cells are more sensitive to Enzalutamide when expression of either FL AR or AR-V7 was inhibited. These results suggest that resistance of LN-p52 cells to Enzalutamide is mediated by alterations in the AR signaling pathway and demonstrate that activation of the AR axis by p52 plays an important role in the p52-induced resistance to Enzalutamide. To confirm these results and test whether downregulation of full length AR or AR-V7 modulates p52-induced AR activation, we co-transfected a luciferase reporter containing the enhancer and promoter regions of PSA (PSA-E/P-Luc) along with p52 and siRNAs against full length AR or AR-V7 into VCaP and CWR22Rv1 cells. The cells were treated with either vehicle or 20 μM Enzalutamide and luciferase assays performed. VCaP and CWR22Rv1 cells express higher endogenous levels of p52 and our previous studies demonstrate that p52 induces ligand-independent activation of AR (13, 15). As shown in Fig. 5B and as shown in our previous studies (15), p52-induces activation of AR-mediated target gene transcription, which was abolished by downregulation of either FL AR or AR-V7. p52-induced activation of AR was unaffected by Enzalutamide treatment. Treatment with Enzalutamide further enhanced the suppressive effect of siRNAs against FL AR or AR-V7 on p52-induced AR-mediated
target gene transcription. These results demonstrate that activation of AR signaling is necessary for the p52-induced resistance against Enzalutamide. Similar results were obtained in CWR22Rv1 cells (Fig. 5C), demonstrating that the interplay between full length AR, AR-V7 and NF-κB2/p52 may be critical in the development of resistance to Enzalutamide in CaP cells. These results implicate the activation of the AR signaling axis by p52 via full length AR and its splice variants as being responsible for the induction of resistance against enzalutamide.

**Discussion**

Next generation anti-androgens such as Enzalutamide and inhibitors of androgen synthesis such as Abiraterone have revolutionized the standard of care for both early stage and late-stage prostate cancer patients. Despite their successes and continuing widespread use, threat of development of resistance looms large (17, 18). The understanding of mechanisms by which resistance against these agents may develop in CaP cells may be critical for early intervention strategies in the event of development of resistance. Enzalutamide binds to the ligand-binding domain of AR and inhibits its nuclear translocation, DNA-binding and transactivation of target genes (3). In the current study, we demonstrate that NF-κB2/p52 may play a crucial role in the development of resistance to Enzalutamide and that the interplay between p52 and the AR signaling axis may be one of the underlying mechanisms. Even though Enzalutamide and Bicalutamide have similar mechanisms of action, clinically patients that progress on Bicalutamide may respond to Enzalutamide, indicating the existence of different mechanisms of resistance and that NF-κB2/p52 may be one of the many mediators of resistance. Our studies also show that CaP cells treated chronically with Enzalutamide may develop resistance
against the agent via upregulation of expression of p52. These findings have important implications for therapeutic regimen in which patients are treated for long periods of time with Enzalutamide. Further studies are warranted to test whether blocking of cellular signaling pathways in combination with anti-androgens may prove beneficial.

Our current data demonstrate that signaling networks such as p52 and AR interactions can mediate resistance to therapies targeting full-length AR, including the next-generation anti-androgen, Enzalutamide. The significance of these studies lies in the fact that resistance, either de novo or acquired, is one of the major clinical limitations for new AR inhibitors. The majority of patients that display disease progression on Enzalutamide also display rising PSA, indicating that Enzalutamide-resistant tumors remain driven by persistent AR activity. AR-Variants are overexpressed in a subset of CRPC metastases and correlate with poor survival (19, 20). As AR splice variants lack the ligand-binding domain, they may be insensitive to inhibition by both Bicalutamide and Enzalutamide. One of the pioneering studies about Enzalutamide showed that it may be effective against cells expressing higher levels of AR splice variants, though this fact remains to be substantiated (21). Even though AR variants have been hypothesized to be independent mediators of castration resistance (4), full length AR may still be necessary and may augment the castration-resistant response (21). Conflicting results have been obtained about the distinct transcriptional programs activated by AR variants and full length AR in CaP cells (4, 22). It is probable that such perceived differences are due to experimental platforms utilized and do not reflect physiological deviations. It is also possible that AR variants execute a “tumor-specific” program, which is a part of the broader transcriptional program of the full length AR, and hence are enriched in tumors.
This may not necessarily mean that the full length AR is no longer a player in the progression of CaP. It would be more likely that co-operation between full length AR and its splice variants is the driver behind CRPC progression, rather than a distinct and dominant transcriptional program driven by the splice variants alone.

Our earlier studies showed that NF-κB2/p52 promotes castration-resistant progression of CaP by activating the AR in conditions of androgen deprivation (13, 15). In this study we showed that p52 also induces expression of AR splice variants. Since the mechanism of action of Enzalutamide is the inhibition of AR activation, we hypothesized that CaP cells expressing higher levels of p52 may be resistant to Enzalutamide. Our current results confirm the hypothesis and point to the role of the interaction between AR and p52 as being one of the critical turns during the progression to castration resistance.

In summary, our study demonstrates a link between persistent activation of the AR by NF-κB2/p52 and development of resistance to enzalutamide in CaP. Future points of interest would be whether overcoming these networks and improving the efficacy of currently available clinical agents represents a viable area of research.

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References


**Figure Legends**

**Figure 1.** NF-κB2/p52-expressing CaP cells are resistant to Enzalutamide. A) LNCaP cells stably expressing p52 (LN-p52) and control LNCaP cells (LN-neo) were treated with 0 and 20 μM Enzalutamide or Bicalutamide in media containing either FBS or CS-FBS and cell numbers were counted after 48 h. Results are presented as means±SD of 3 experiments performed in triplicate. LN-p52 cells exhibited higher survival rates when treated with Enzalutamide or Bicalutamide compared to LN-neo cells. B) LN-neo and LN-p52 cells were treated with 0, 20 or 40 μM Enzalutamide or Bicalutamide and clonogenic assays were performed. Results are presented as means±SD of 2 experiments performed in triplicate. LN-p52 cells formed higher numbers of colonies compared to LN-neo cells when treated with Enzalutamide or Bicalutamide. C) LN/TR/p52 cells (expressing p52 under the control of a tet-inducible promoter) and control LN/TR/Con cells were treated with 0, 20 or 40 μM Enzalutamide or 20 μM Bicalutamide in the presence or absence of 0.5 μM doxycycline (DOX) and cell numbers were counted after
48 h. Results are presented as means±SD of 3 experiments performed in triplicate. * denotes $P \leq 0.05$. LN/TR/p52 cells displayed higher survival rates when p52 expression was induced with DOX, compared to uninduced LN/TR/p52 cells as well as LN/TR/Con cells.

**Figure 2.** CaP cells treated chronically with Enzalutamide upregulate the expression of NF-κB2/p52. A) CWR22Rv1 cells treated chronically with Enzalutamide exhibit higher endogenous levels of both p100 and p52. B) CWR22Rv1 cells treated chronically with Enzalutamide were transfected with either control shRNA or shRNA against NF-κB2/p52 and were treated with 0, 20 or 40 μM Enzalutamide. Cell numbers were counted after 24 and 48 h. Results are presented as means±SD of 2 experiments performed in triplicate. * denotes $P \leq 0.05$. Cells transfected with shRNA against p52 exhibited lower cell survival when treated with Enzalutamide.

**Figure 3.** NF-κB2/p52 induces higher expression of AR splice variants. Total RNAs from LNCaP (A) and C4-2B (B) cells transfected with empty vector or p52 were analyzed by qRT-PCR for the expression of FL AR and AR-V7 in media containing either FBS or CS-FBS. Expression of p52 enhanced the levels of AR-V7 while levels of FL AR remained unchanged. Right panels, immunoblotting of above lysates with antibodies specific against either FL AR or AR-V7. C) Total RNAs from LN-p52 and LN-neo cells were analyzed by qRT-PCR for the expression levels of FL AR or AR-V7. LN-p52 cells showed higher levels of expression of AR-V7 compared to LN-neo cells, while FL AR levels were unaffected. Right panel, immunoblotting of above lysates with antibodies against FL AR or AR-V7. D) Expression levels of AR-V7 were enhanced in xenografts from LNCaP cells expressing p52 compared to xenografts from parental
LNCaP cells. Results are presented as means±SD of 2 experiments performed in triplicate. * denotes $P \leq 0.05$.

**Figure 4.** Downregulation of NF-κB2/p52 in CaP cells reduces expression of AR splice variants. VCaP (A) and CWR22Rv1 (B) cells were transfected with either control shRNA or shRNA against p52 and expression levels of the indicated AR splice variants were analyzed by qRT-PCR. Downregulation of p52 led to a decrease in synthesis of AR splice variants while expression levels of FL AR remained unchanged. Right panels show immunoblots of above lysates with antibodies against FL AR or AR-V7. Results are presented as means±SD of 3 experiments performed in triplicate. * denotes $P \leq 0.05$.

**Figure 5.** Downregulation of FL AR and AR-V7 increase sensitivity of p52-expressing CaP cells to Enzalutamide. A) LN-p52 and LN-neo cells were transfected with siRNAs specific to either FL AR or AR-V7 and were treated with 0 or 20 μM Enzalutamide. Cell numbers were counted after 48 h. Results are presented as means±SD of 3 experiments performed in triplicate. Downregulation of either FL AR or AR-V7 increased sensitivity of LN-p52 cells to Enzalutamide. VCaP (B) and CWR22Rv1 (C) cells were transfected with PSA-E/P-Luc reporter, empty vector or p52 together with siRNAs against FL AR or AR-V7. Cells were treated with 0 or 40 μM Enzalutamide and luciferase assays were performed after 48 h. Results are presented as means±SD of 2 experiments performed in triplicate. * denotes $P \leq 0.05$. Downregulation of either FL AR or AR-V7 suppressed p52-induced activation of AR in both VCaP and CWR22Rv1 cells.
Figure 1

A

![Graph A showing cell number for LN-neo and LN-p52 with and without DOX and different compounds]

B

![Graph B showing number of colonies for LN-neo and LN-p52 with and without DOX and different compounds]

C

![Graph C showing cell number for LN/Tr/Con and LN/Tr/p52 with and without DOX and different compounds]
Figure 2

A

22Rv1  22Rv1-Enza-R

p100

p52

Tubulin

B

CWR22Rv1

DMSO

Enza 20

Enza 40

Cell Number

24 h  48 h

shCon  shp52

24 h  48 h

*  *
Figure 3

A

Relative mRNA levels

FBS, AR-V7 CS-FBS, AR-V7 FBS, FL AR CS-FBS, FL AR

B

Relative mRNA levels

FBS, AR-V7 CS-FBS, AR-V7 FBS, FL AR CS-FBS, FL AR

C

Relative mRNA levels

FL AR AR-V7

D

Relative AR-V7 mRNA levels

LNCaP
**Figure 4**

(A) VCaP

(B) CWR22Rv1

Bar graphs showing relative mRNA levels for FL AR, AR-V7, AR-V1, AR-V5, AR-1/2/2b, and AR-1/2/3/2b in response to shCon and shp52 treatments. Western blots for IB: AR-V7, IB: FL AR, IB: Tubulin, and IB: p52 are also shown.
Figure 5

A

Cell Number

DMSO Enza 20

LN-neo

siCon

siAR

siAR-V7

LN-p52

B

VCaP

Con

p52

p52+siAR

p52+siV7

DMSO

Enza

C

CWR22Rv1

Con

p52

p52+siAR

p52+siV7

DMSO

Enza

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