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14. ABSTRACT. Our goal was to identify via a high-throughput screen compounds that inhibit androgen receptor (AR) target genes driving cancer but not normal cell growth. We hypothesized that genes with consensus response elements (cAREs) would drive proliferation but genes with selective response elements (sAREs) would drive differentiation. Doxorubicin showed this activity by acting on DNA rather than directly with AR, as shown by protein-DNA interaction assays. The differential effect of dox was obtained at low dose, in multiple prostate cancer cell lines, and shown in vivo by chromatin immunoprecipitation (ChIP) studies. ChIP-seq provided a global view of genes responsive to low dox treatment in LNCaP cells. Binding to cAREs decreased with increasing dox concentration. For a subset of genes at low dox, AR binding increased at pre-existing sites overlapping sites for prostate-specific factors, such as FOXA1. The transcriptome viewed by RNA-seq revealed cell cycle genes to be downregulated by low dox and DNA damage response increased by high dox. The differential effect occurred in xenograft tumors notably at the level of gene expression. Thus doxorubicin provides proof of our hypothesis and unique opportunities for prostate cancer treatment.

15. SUBJECT TERMS
androgen receptor, prostate cancer, antiandrogens, high-throughput screen, differential promoter activation, selective response elements, doxorubicin

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Selective AR Modulators that Distinguish Proliferative from Differentiative Gene Promoters

1. INTRODUCTION:

Prostate cancer (PCa) initially depends on androgens acting via the androgen receptor (AR). Potent new drugs block androgen synthesis and AR function, but resistance inevitably arises and AR regains control to drive disease [1]. To reduce resistance, we have sought selective AR modulators (SARMs) that prevent expression of genes involved in cancer cell proliferation but allow expression of genes for normal cell growth and differentiation. The consensus androgen response element (cARE) that drives most AR-responsive genes is an inverted repeat also recognized by other steroid receptors, but some genes involved in differentiation rely on a selective ARE (sARE) comprised of a half site or direct repeat element [2, 3]. We developed a high-throughput screen for compounds that elicit differential AR regulation based on these distinct promoter elements. The strongest hit was doxorubicin (dox), one of the earliest chemotherapeutics, which inhibited cARE-driven reporters in preference to sARE-driven reporters. In prostate tumor cell lines, dox treatment inhibited cARE-driven endogenous AR target genes but, at low dose, selectively increased the expression of sARE-driven AR targets. We showed by *in vitro* protein-DNA interaction studies that this selectivity relies on the greater disruption of AR binding to cAREs than to sAREs upon intercalation of doxorubicin into DNA. Furthermore, differential recruitment of AR to chromatin for a small set of proliferative versus differentiative AR target genes was demonstrable by ChIP-PCR. To obtain a genome-wide view of distinct AR target gene sets and the extent to which they drive differentiative versus proliferative cell growth, we performed RNA-seq and ChIP-seq experiments. The data provide a foundation demonstrating the ability of AR *in vivo* to distinguish selective from consensus response elements in chromatin and the importance of these elements in driving differential patterns of gene expression. Finally, we tested the effect of dox on xenograft tumor growth in mice - remarkably tumor gene expression recapitulated the differential effect of low dose dox that is seen in tissue culture. This report describes in greater detail these results, which are being prepared for publication. Ultimately, results of this project provide a basis for development of new prostate cancer therapies that modulate rather than block AR activity, thus delaying resistance and producing fewer side effects.

2. KEYWORDS:

androgen receptor, prostate cancer, antiandrogens, high-throughput screen, selective androgen receptor modulator, doxorubicin, selective response elements, proliferation versus differentiation

3. ACCOMPLISHMENTS:

The major goals of this project were to perform a high-throughput compound screen and characterize resulting hits for selective anti-AR effects. Doxorubicin was validated early in these studies, while other hits were not strongly reproducible. Therefore, in order to highlight positive outcomes, we revised our Statement of Work on 12/30/2016 to focus on doxorubicin as follows:

Task 1. Perform a larger screen for novel compounds using an improved protocol.
Task 2. Probe modulatory effect of doxorubicin on AR control and cell phenotype.
Task 3. Examine mechanism of the selective effect of doxorubicin on AR action.
Task 4. Determine differential antitumor effects of low vs. high doses of doxorubicin in mice.

Below I will briefly summarize results presented in the previous annual reports and then present new results completed in the no-cost extension. Overall results remarkably confirm our general two-fold hypothesis that: 1) sets of genes with distinct functions (i.e., those involved in normal vs. cancer cell growth) share promoters with distinct response elements, and 2) compounds may differentially inhibit genes based on these distinct response elements.

Task 1. **Perform a larger screen for novel compounds using an improved protocol.**

Our screen was based on the notion that selective AR modulators might reduce side effects and slow the development of resistance. AR specificity, or selectivity, is evident at the DNA level in that certain response elements can be activated by AR but not by other steroid receptors. Genomic data confirm that AR binds a consensus 6 bp inverted repeat of 5'-TGTTCT-3' with 3 bps intervening, also recognized by glucocorticoid, progesterone and mineralocorticoid receptors, whereas a half-site can be activated by AR exclusively and not by the other receptors [2]. Ability to activate this selective ARE (sARE), in contrast to just the consensus (cARE) sequence, is requisite for complete male virilization in mice [4], and loss of the ability to recognize this sequence may lead to a more oncogenic AR [5]. Although it seems counter-intuitive for a half-site to confer more specific response, numerous examples in the literature show enhanced regulation comes from weak rather than strong binding sites [6]. Weak sites allow greater response over a broad range of inducer and with greater specificity, in large part due to cooperative interactions that can be with self (i.e., multiple clustered full- and half-sites for AR, as we characterized previously [7]) or with other factors that may be other transcription factors or non-DNA-binding factors in chromatin [8, 9]. This ability of AR, but not the other receptors, to activate from a half-site is due to AR's strong dimer interface within the DNA binding domain (**Fig. 1**). This "relaxed response element stringency" of AR relies on partners to achieve precise and strong activation.

This brief discussion clarifies the basis for our screen for compounds that differentially inhibit AR dependent on response element, and our remarkable finding that our top hit for a selective modulator is doxorubicin that acts via the response element rather than the receptor. The screen was designed to run in saturating levels of the synthetic ligand R1881 so that a compound interacting with any domain of AR would be scored, not just those binding in the hormone binding domain. In the screen not only were there high levels of AR, but also significant levels of transfected plasmid reporter DNA, so the response element itself is a plentiful target. Drugs that intercalate into DNA were hits in both the pilot and the expanded screen, suggesting they cause a structural difference between cARE and sARE sequences that could influence AR or coregulator binding and thus differentially modulate AR signaling.

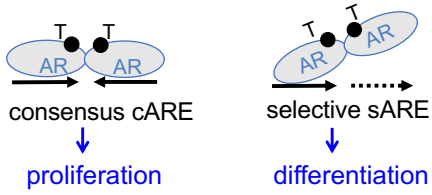


Fig. 1 – **AR dimers bind distinctly to cAREs vs. sAREs and drive distinct gene sets.** AR head-to-head dimers bind both half-sites of a cARE but only one half-site of a sARE.

Results of the larger primary screen were described in the first annual report and are summarized here. Not only were 4-times more compounds tested but the screen was optimized and efficiency enhanced using a 3-step approach rather than multiplexing as in the pilot screen. First, we screened for compounds inhibiting cARE-driven transcription following transfection into HeLa cells with high AR levels from a stably integrated expression vector. Second, these positives were retested for dose-dependence, and third, those showing dose-dependence for cARE activation were tested for effect on sARE activity. Nearly 10,000 compounds were screened from a variety of libraries available in the University of Michigan Center for Chemical Genomics, with about 2,500 FDA-approved and about 8,000 natural products and chemical compounds. Using cut-offs of greater than 70% AR inhibition and less than 50% cell toxicity, 124 primary hits were identified. Of these, 109 showed dose-dependent inhibition of the cARE reporter, and 15 of these did NOT inhibit the sARE reporter, suggesting selective action. Chemicals dubbed Compound 5 (Cpd5) and Compound 8 (Cpd8) seemed promising for selective inhibition of AR, but ultimately proved to be poor for further drug development. Yet doxorubicin reproducibly inhibited AR activation of cAREs, but not sAREs, a difference pronounced at lower concentrations.

The selective action of doxorubicin (dox) was validated in transient transfection assays with luciferase reporters in multiple cell types (**Fig. 2**). We compared differential suppression in CV-1

fibroblasts and RWPE-1 normal prostate epithelial cells, since these lines transfect and induce well but differ in hormonal response, in that sARE-driven induction is only about 25% of cARE-induction in CV-1 cells but nearly 3 times as sensitive to hormone as cARE response in RWPE-1 cells. These differences likely reflect differences in host cell-specific accessory factors. Dox repressed cARE reporters to a greater extent than sARE reporters in CV-1 but not RWPE-1 cells, and showed increased AR response at low concentrations of dox. This generally validates the differential effect of dox on cARE- vs sARE-driven gene expression and is more reproducible when examining endogenous gene expression in more relevant prostate tumor cells (see below).

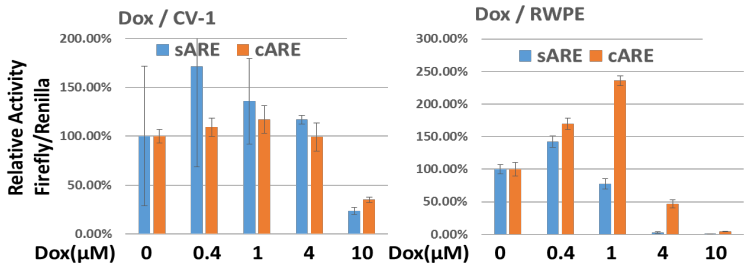


Fig. 2 – Dox suppression of cARE vs sARE activity differs in CV-1 vs RWPE-1 cells. cARE or sARE driven reporters were co-transfected with AR expression plasmids into CV-1 (left) or RWPE-1 (right) cells for Dual Luciferase Assays. Cells were starved in 2.5% charcoal-stripped serum (CSS) and then treated with 1 nM R1881 and dox or DMSO for 24 hrs.

Although we had anticipated obtaining a more classical ligand from our selective AR modulator screen, doxorubicin was a compelling hit for several reasons. First, doxorubicin has been used in cancer chemotherapy since the 1950's and is still used in late stages of prostate cancer, although the mechanism is not well understood. Moreover, it is already FDA approved and further study could allow its repurposing, with different applications dependent on stage of disease (see below). This anthracycline (also known as Adriamycin) intercalates into DNA, disrupting base pairing, which disrupts Topoisomerase II action leading to double strand breaks and activating the DNA damage response (DDR) [10]. Recent studies show that AR regulates some genes involved in DDR [11], and promotes radio-resistance in prostate cancer therapy. Furthermore, Topo2α cooperates with AR to contribute to prostate cancer progression [12]. Therefore, a hormone-DNA repair signaling network makes doxorubicin a plausible AR antagonist in prostate cancer [13]. Given results discussed below, low doses of doxorubicin that accentuate the differential between cARE- and sARE-driven genes may be especially beneficial in watchful waiting or early stages of prostate cancer, since this treatment may favor AR-dependent normal or differentiative behavior rather than proliferative and tumorigenic functions of AR.

Task 2. Probe modulatory effect of doxorubicin on AR control and cell phenotype.

To determine the extent to which the differential effect of doxorubicin on multimerized response elements also occurs on natural promoters, and whether this would distinguish genes involved in differentiation or survival from those involved in oncogenic growth, we examined endogenous gene expression in multiple prostate cell lines. In order to avoid complications of overexpression in transfection, analysis was restricted to cells with endogenous AR, thus excluding RWPE-1 cells. We contrasted expression in LNCaP, LAPC-4 and C4-2B cell lines, representing early, mid and late-stage prostate cancer. The first two cell lines are dependent on androgen for growth, whereas C4-2B is androgen-independent, in that AR acts regardless of hormone to direct a distinct gene expression program, similar to that of castrate resistant prostate cancer (CRPC) [14]. Expression of genes were tested that were known to be upregulated by AR, repressed by AR, or suspected of interaction with AR signaling in DNA damage response or DNA repair. In **Fig. 3** are shown results from LNCaP and C4-2B cells, stimulated for 24 hrs with R1881 +/- doxorubicin.

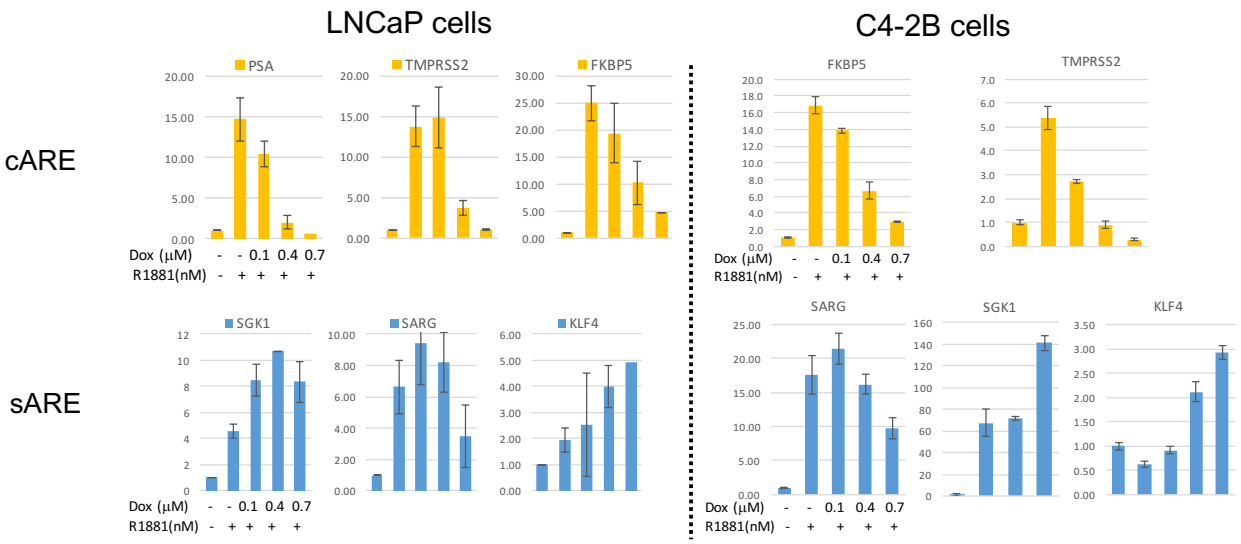


Fig. 3 – Doxorubicin differentially affects AR target gene expression in hormone-sensitive LNCaP cells and in hormone-resistant C4-2B cells. Cells were plated in 6-well plates in RPMI 1640 medium with 10% FBS for 2 days, and then hormone-starved in 2.5% CSS for 24 hrs. Cells were then treated with 10 nM R1881 alone or with varying dox concentrations for 24 hrs. Total RNA was extracted and qRT-PCR performed to quantify gene expression. Genes representing the cARE pattern of response are PSA, TMPRSS2 and FKBP5; genes indicative of sARE response are SGK1 and SARG. KLF4 is included since it is induced by dox but is likely an indirect AR target.

PSA, TMPRSS2 and FKBP5 are classic AR targets in the prostate that are known to be dependent on consensus AREs for induction. (PSA is not shown for C4-2B cells since its response to hormone in this line is atypical.). SGK1 and SARG are prototypical sARE-driven genes, identified by their altered expression in mice bearing a mutated AR that can recognize cAREs but not sAREs [2, 4]. A function for SARG (Specifically Androgen-Regulated Gene), also known as C1orf116, has not yet been determined. KLF4 is shown with the sARE target genes although AR response elements for this gene have not been determined; KLF4 is moderately responsive to R1881 in LNCaP cells but less so in C4-2B cells. KLF4 fits the pattern of potentially driving differentiation rather than proliferation since it is a known tumor suppressor gene, acting in part through P21-dependent cell cycle arrest [15], and in our studies shows pronounced upregulation by doxorubicin.

In these early studies, AR target genes with characterized cAREs consistently showed dose-dependent inhibition by increasing concentrations of dox. We tested numerous AR targets, including genes involved in DNA damage response, to find genes that showed induction above hormone-induced levels with low doses of dox but only SARG, SGK-1 and KLF4 demonstrated this effect consistently. This in part suggests that relatively few AR targets show this behavior, perhaps not surprising given that relatively few genes have been identified as dependent on sAREs in the AR mutant mice. Potential candidate genes were identified more readily by taking the approach of RNA-seq to characterize global response at the transcriptome level (see below).

The modulatory effect of doxorubicin on AR-driven cell phenotype was determined by assaying cell proliferation. In both LNCaP and LAPC4 cells (shown here for LNCaP cells, **Fig. 4**), low dose dox selectively slowed AR-induced proliferation, whereas at higher concentrations dox slowed cell proliferation regardless of R1881 with evident cell death. Given substantial literature on dox-induced toxicity (apoptosis, senescence, etc.), we more directly studied effects on tumor growth in mouse xenografts (Task 4). Combined with results on target gene expression, a major conclusion from

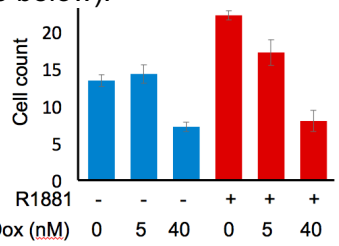


Fig. 4 – Low dose dox slows AR-dependent growth. LNCaP cells were treated with R1881 and dox for 5 days, followed by cell counting (x 10^3).

experiments of Task 2 is that the selective effect of doxorubicin of inhibiting cARE-driven expression to a much greater extent than sARE expression occurs at low drug doses.

Task 3. **Examine mechanism of selective effect of doxorubicin on AR action.**

To explore the mechanism underlying the differential effect of dox on AR action, we first tested whether dox bound differentially to cARE or sARE sequences or whether dox binding differentially affected subsequent AR binding. This was tested by the classic approach of Electrophoretic Mobility Shift Assays (**Fig. 5**). The specificity of the AR-ARE shift was confirmed by supershift with specific antibody. The shift on the autoradiogram appears weaker for the sARE than the cARE probe in the absence of dox, which is expected due to the overall weaker affinity of AR for sAREs than for cAREs [16]. As the dox concentration is increased, binding of AR to the cARE decreases more than to the sARE, indicating greater sensitivity of the AR-cARE complex to dox intrusion. This is somewhat surprising given that there are two contact sites for AR monomers in the cARE compared to only one in the sARE (see Fig. 1) and may reflect differential conformational change of DNA induced by dox. This difference in DNA structure may vary with the precise sequence and may impose structural constraints that affect AR binding in a more stringent manner for the cARE whereas contact of one monomer to the sARE may be more flexible in nature.

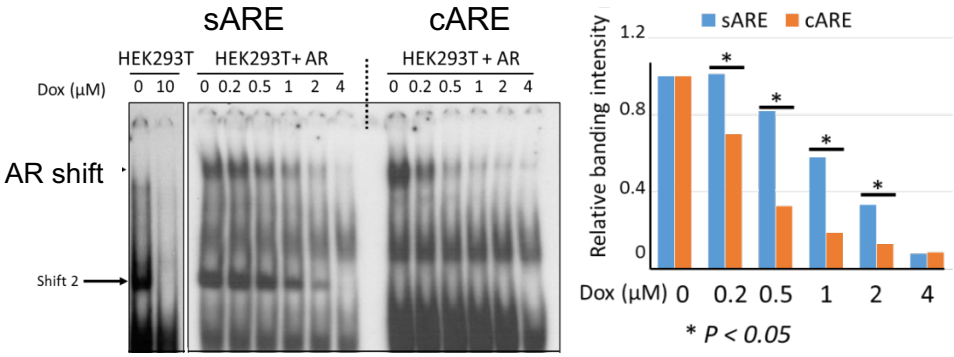


Fig. 5 – Dox disrupts AR binding to cAREs more readily than to sAREs. Protein-DNA interaction was determined by mobility shift assays, performed with 5 μg nuclear extracts from AR-transfected HEK293T cells and 1 ng ^{32}P-cARE or-sARE oligo probes, mixed on ice for 10 min with varied dox concentrations and then complexes separated by electrophoresis. Left - the specific AR-ARE shift is denoted by an arrowhead; shift 2 is due to non-AR factors. Right – histogram indicating image density from scans of 3 independent experiments.

The EMSA result intriguingly demonstrated a distinct difference conferred by doxorubicin *in vitro* in AR binding to cAREs vs. sAREs. To determine to what extent dox affects AR binding to DNA *in vivo* in the context of chromatin, chromatin immunoprecipitation (ChIP) assays were performed for specific individual genes (conventional ChIP) (**Fig. 6**). The ChIP profile mirrored gene expression - the cARE-driven genes PSA and TMPRSS2 showed reduced AR present in chromatin along with decreased gene expression as the dox dose increased. In contrast, for the sARE-driven genes, SGK1 and SARG, more AR was evident at low concentrations

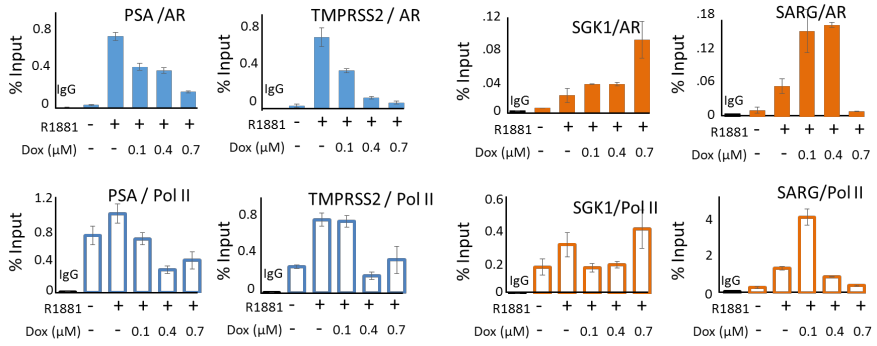


Fig. 6 - Dox differentially affects AR recruitment to chromatin. LNCaP cells were starved in 2% CSS for 48 hrs, then stimulated with 10 nM R1881 +/- dox for 24 hrs. Cells were crosslinked with formaldehyde and lysed; chromatin was sonicated to 300-1000 bps. ChIP assays used AR (top) and PolII (below) antibodies, with IgG control; purified DNA was quantified by qRT- PCR. Left (blue) - qRT-PCR targeting promoter regions of cARE-driven PSA and TMPRSS2. Right (red)- qRT-PCR targeting sAREs of SGK1 (promoter) and SARG (intron).

of dox, in accord with increased gene expression. PolII binding was similar to that of AR, indicating the effect of dox directly impacts gene expression, in both positive and negative directions.

Data thus far on differential effects of low dose doxorubicin show strong accord between AR recruitment to chromatin and transcriptional effects but only for a small set of genes. Discovery of additional affected genes was hampered by lack of genes characterized for both AR-specific response and sARE-like response elements. Effects of dox on genomic activity in general are known with regard to active vs. inactive genes [17, 18], but gene-specific effects have not been studied and might be informative for heterogeneity in drug response. Identification of the set of differentially affected AR-responsive genes would support our overall hypothesis in several ways: the mechanism (is this effect mediated by "selective" AREs?); the biology (do these genes direct different growth behavior in tumors and by what pathways?); what are long-term consequences of low dose dox treatment (is low dox appropriate for particular stages of prostate cancer?). We thus embarked on ChIP-seq studies as a means to identify genome-wide targets of AR plus dox action.

As detailed last year, we optimized conditions for large-scale ChIP assay, with respect to cell growth (LNCaP hormonal induction was better in 10 than 15 cm dishes, likely due to hypoxia in the larger plates), induction timing (12 hrs for optimal AR binding in chromatin), and antibodies (the AR antibody PG-21 was preferable to N-20). The assays were performed using the HighCell ChIP kit from Diagenode, on sonicated fragment sizes of 200 bp. Following overnight antibody incubation, indexed adapters were ligated onto purified DNA fragments in several steps using the Sciclone NGS Workstation, to produce bar-coded libraries appropriate for multiplexing and paired-end sequencing on the Illumina platform. Additional details can be found in Asangani et al. [19]. All libraries passed QC and were sequenced on the Illumina HiSeq 2000 Sequencer, giving 100 nucleotide read lengths and read depths of at least 30M per sample.

As a control for AR binding, TBP was used to mark active genes more reliably than PolII, which also binds inactive enhancers and paused promoters [20]. As shown in the read peak heat maps (**Fig. 7**), TBP demonstrates the dox effect is specific for AR and not all transcription factors.

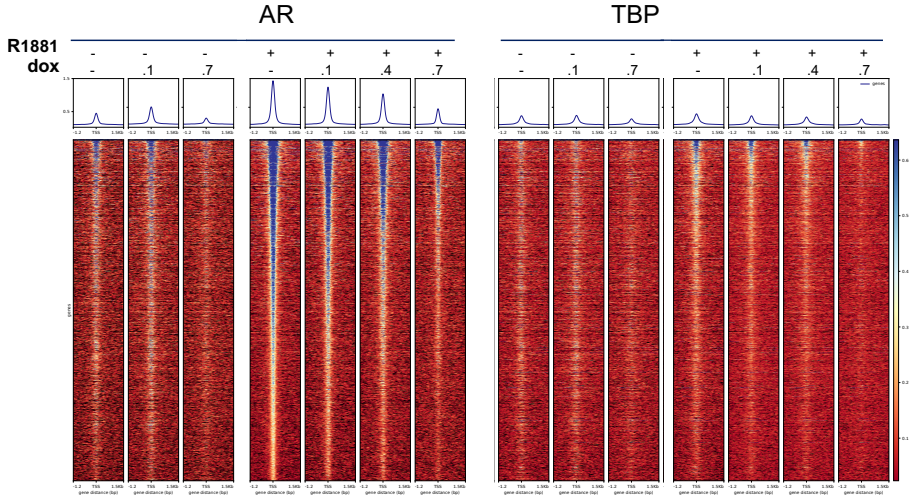


Fig. 7 – Global AR, but not TBP, binding declines as dox concentration increases. Heat map representation of AR or TBP binding peaks for different treatment groups are shown. Peaks were called using MACS with all default settings, with a moderately stringent significance cut-off. Genomic target sites are rank-ordered based on the level of factor enrichment at each binding site within -1.2 to + 1.5 kb of the transcription start site.

The majority of TBP sites do not depend on androgen and are resistant to the effects of dox, unlike the AR binding sites, which show a dose-dependent decrease as dox concentration increases.

An overview of dox disruption of AR binding comes from principal component analysis (**Fig. 8**, upper left). Samples clustered in the center have negligible differences in AR peaks – these are the uninduced cells and the high dox sample where transcription is rapidly declining and cells are dying. In the three R1881-treated samples, active AR drives major differences. Examination of

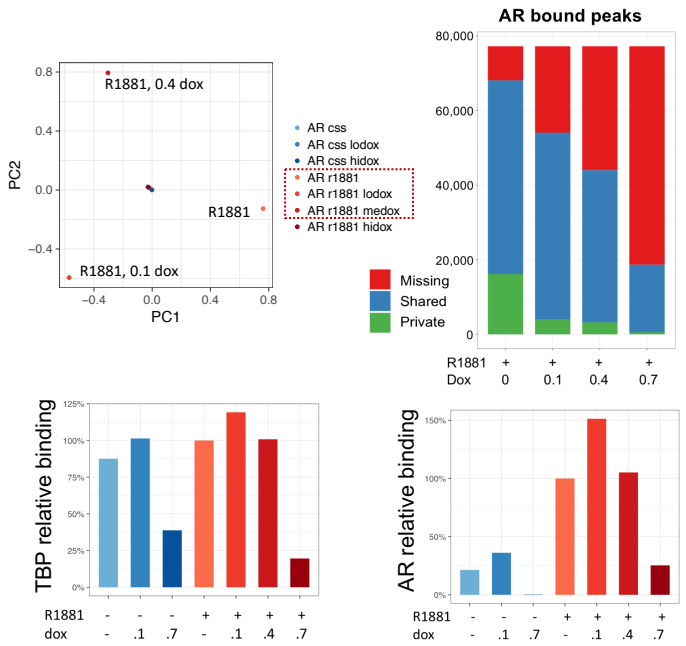


Fig. 8 – Dox disrupts global AR binding in a dose-dependent manner. ChIP enrichment levels within a peak or site were calculated after sequence alignment to the HG37 reference genome using Bowtie, sorted by NovoSort and duplicates removed with Samtools; additional bioinformatics details are in Asangani et al. [18]. Upper left shows PCA analysis, with dramatic differences between R1881-induced samples. Lower left shows TBP relative binding. Upper right shows AR bound peaks per treatment, for 77,000 unique AR sites sorted to "missing" peaks (red - peaks found in other conditions but not self), "shared" peaks (blue - in common with other conditions), and "private" peaks (green - unique to that treatment – these are likely background). The histogram in the lower right shows AR binding relative to +R1881 but without dox. In sum these data indicate that low dox increases AR binding at a few sites but reduces binding for most.

TBP relative binding (**Fig. 8**, lower left) confirms little significant effect of dox on TBP except at high levels, which reflects toxicity. More broadly this suggests that the effect of low dose dox is AR-mediated and not on global transcription per se. The histogram to the upper right of Fig. 8 shows the 77,000 unique AR sites in this dataset divided by their occurrence in different samples – the blue segment of the bar indicates the dose-dependent loss of AR binding sites with increasing dox. That the green portion does not increase between treatments indicates that low dox is not altering AR binding sites or causing new AR binding sites to appear but rather may be redistributing AR to increase binding at existing sites. This also is seen in the histogram of AR relative binding in Fig. 8, lower right, that, combined with the other data, shows remarkably greater AR binding to a small number of genes. As will be seen below, this redistribution to existing sites likely relies on cooperativity with nearby factors that are binding partners of AR.

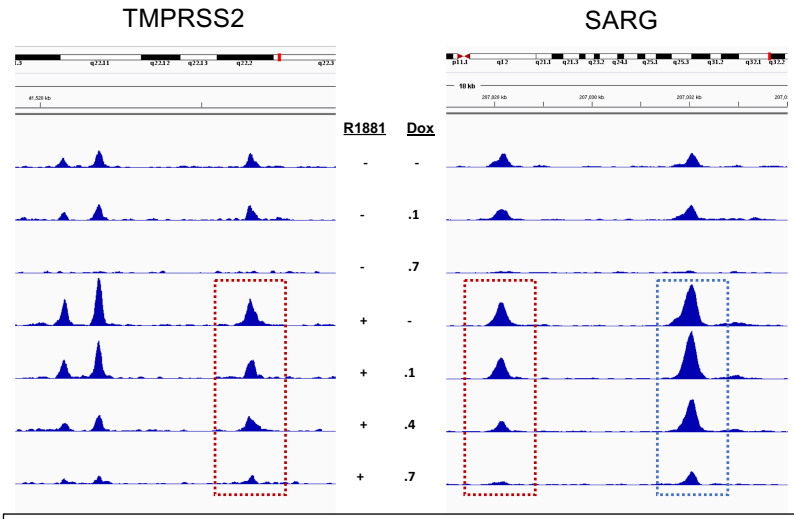


Fig. 9 – AR binding to enhancers of TMPRSS2 and SARG. Shown aligned for each sample are genome browser views for the TMPRSS2 enhancer about 10 kb 5' of the gene and the SARG (C1orf116) enhancer within intron 1. The dotted red boxes encircle peaks of mapped sequence reads that decrease in accord with dox dose, indicating underlying cAREs, whereas blue dots encircle the SARG peak that only declines at higher dox doses, indicating the sARE.

AR binding peaks were individually examined via genome browser representations for genes such as TMPRSS2 and SARG (**Fig. 9**). Sequence reads were aligned to the HG37 reference genome using Bowtie with all default settings, aligned reads sorted using NovoSort and exact duplicates removed using Samtools [19]. For each peak, overlapping reads were counted and the number divided by the length of the site; to correct for differences in sequencing depth and coverage, values were further normalized by the number of aligned reads per million. The area of each peak on the ChIP-seq gene track therefore correlates with the number of

sequence reads. In comparing the peak produced by R1881 alone to that with 0.1 μM doxorubicin (3rd and 4th scans up from bottom), low dose dox is seen to decrease the TMPRSS2 peak (boxed in red) by about 50% whereas the SARG peak on the right, boxed in blue, is actually increased. The red-boxed SARG peak on the left behaves more like the TMPRSS2 peak. This reflects that genes may be affected by multiple AREs, some consensus and some nonconsensus. These regulatory sequences may be somewhat redundant but may also differ in role dependent on time- and tissue-specific circumstances. This may in part account for variability in response to low dose doxorubicin encountered in some experiments.

A compelling result of the ChIP-seq studies is that AR binding motifs vary with doxorubicin treatment, confirming that dox differentially affects a subset of AR target genes (**Fig. 10**). In the

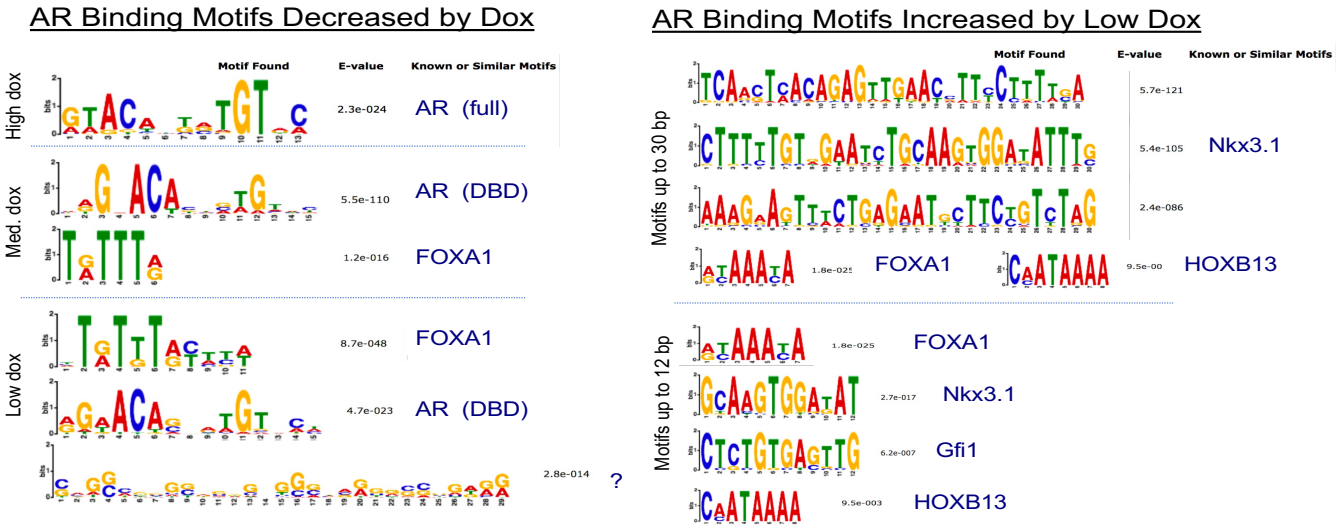


Fig. 10 – AR binding motifs in ChIP-seq vary with doxorubicin treatment. Transcriptional motifs were discovered by MEME or DREME for ungapped sequence motifs up to 30 bp in length. Motifs shown are for the top 1000 peaks changed by dox. Left - motifs that decreased with high, medium or low dox; Right - motifs that increased with low dox. E-values are indicated, as are factors with known or similar motifs from transcription factor databases.

LNCaP cells treated with high concentrations of doxorubicin (0.7 μM), the AR binding sites that are most changed (decreased relative to the control treated with R1881 but not dox) are those with a consensus ARE, which is the only motif that is found with significance. This corroborates the effect of high dox to effectively obliterate the androgen-induced transcription program. At medium levels of dox (0.4 μM), most affected sites are AREs but FOXA1 sites are also affected. At low dox (0.1 μM), the most abundant motifs are for FOXA1, followed by AREs (and an intriguing G-rich tract of unknown significance). FOXA1 is the well-described pioneer factor for AR binding, in that FOXA1 sites in prostate cancer cells genome-wide are frequently near or overlapping AREs [21]. FOXA1 binds first to these shared sites, prior to hormone-induced receptor binding. A half-site ARE motif was not evident in this approach, in agreement with our earlier studies that suggest the half-site ARE (sARE) is most resistant to dox. Overall, the motifs decreased by dox treatment confirm that binding to AREs is lost with dox in a dose-dependent manner.

Of greater interest are the AR binding motifs that are increased by low dose dox (**Fig. 10,** right). In addition to sites that are increased are those that are maintained, representing peaks most resistant to dox treatment. Remarkably, neither cAREs nor sAREs are apparent in these motifs but rather a number of key prostate-specific AR-interacting factors, such as NKX3.1 and HOXB13, as well as FOXA1. Motifs for Gfi1 are intriguing since this factor represses cell cycle progression, adding to the notion that these sites mark genes involved in differentiation rather than

proliferation. Overall, these motifs that are increased by low dose dox treatment suggest that AR binding, presumably to half-sites, is dependent on key prostate-specific interacting proteins.

The ChIP-seq data confirms that the differential dox effect is based on differences in AR binding sites, but does not in itself identify the actual genes that are differentially regulated. To identify affected genes directly, via their product mRNAs, we employed RNA-seq to assess transcriptomes from LNCaP cells that were starved in CSS, treated with 1 nM R1881 for 24 hrs, or treated with R1881 plus 0.1, 0.4 0r 0.7 μM doxorubicin, as before. RNA-seq polyA+ libraries were prepared using Illumina's TruSeq RNA preparation kit and sequenced in paired end mode using the Illumina HiSeq 2000, as previously described. For each gene, a rank list was generated by ordering each gene in the differential expression analysis by the DESeq2 log fold change value (log2foldchange) [22]. These rank lists were used in a weighted, pre-ranked Gene Set Enrichment Analysis (GSEA) against MSigDBv5. Significant associations were determined for any gene set having an FWER p-value below 0.01. This computational analysis determines whether a given gene set, corresponding to a biological process or pathway, is significantly coordinately regulated, thereby shedding light on the underlying mechanism. The addition of R1881 produced the expected upregulation (or downregulation) of AR target genes, for a well-characterized set called "AR signaling targets up-regulated by AR" [19] (**Fig. 11**). The gene expression profile with 0.1 μM dox was almost indistinguishable from that produced by R1881 alone - as the dox concentration increased, the profile showed dramatic downregulation of AR targets.

Fig. 11 – The AR target gene signature is progressively diminished by increasing concentrations of dox. Gene Set Enrichment Analysis (GSEA) was performed on RNA-seq data, using MSigDB as a resource for annotated gene sets. The enrichment score is shown by the green line, with genes ranked by expression as black lines.

RNA-seq data was displayed by "volcano plots" to examine the effects of dox treatment on individual genes. These plots show significance on the y axis and fold change on the x axis; in this analysis, adjusted p-values were <0.05 for all genes. **Fig. 12** shows that R1881 massively induces numerous genes compared to growth in CSS (left panel), and many of these are canonical AR targets (e.g., TMPRSS2, KLK2/3, FKBP5 circled in red). The middle panel reveals that the addition of 0.1 μM dox to R1881 differs only subtly when compared to unstimulated (CSS) growth, since the major effect is AR stimulation, but some AR targets show reduced fold changes. However, when the effect of R1881 + 0.1 μM dox is compared to R1881-induced cells (right panel), down-regulation of many genes becomes apparent, and many of these genes are involved in cell cycle control and down-regulation of proliferation (e.g., CENP, MCM, BUB, TOP2A, in blue). Some genes are upregulated, in particular those involved in signaling by the tumor suppressor p53 (e.g., MDM2, CDKN1A, BTG, in green). Although no major differentiative signatures were apparent, it was striking that cell cycle arrest genes were turned back on by low dose dox, indicating a strong anti-proliferative effect.

As the dox concentration was increased (**Fig. 13**), androgen target genes decreased, cell cycle genes were further down-regulated and DNA damage response (DDR) genes were induced, in a dose-dependent manner. At the medium concentration of 0.4 μM dox, pathway analysis

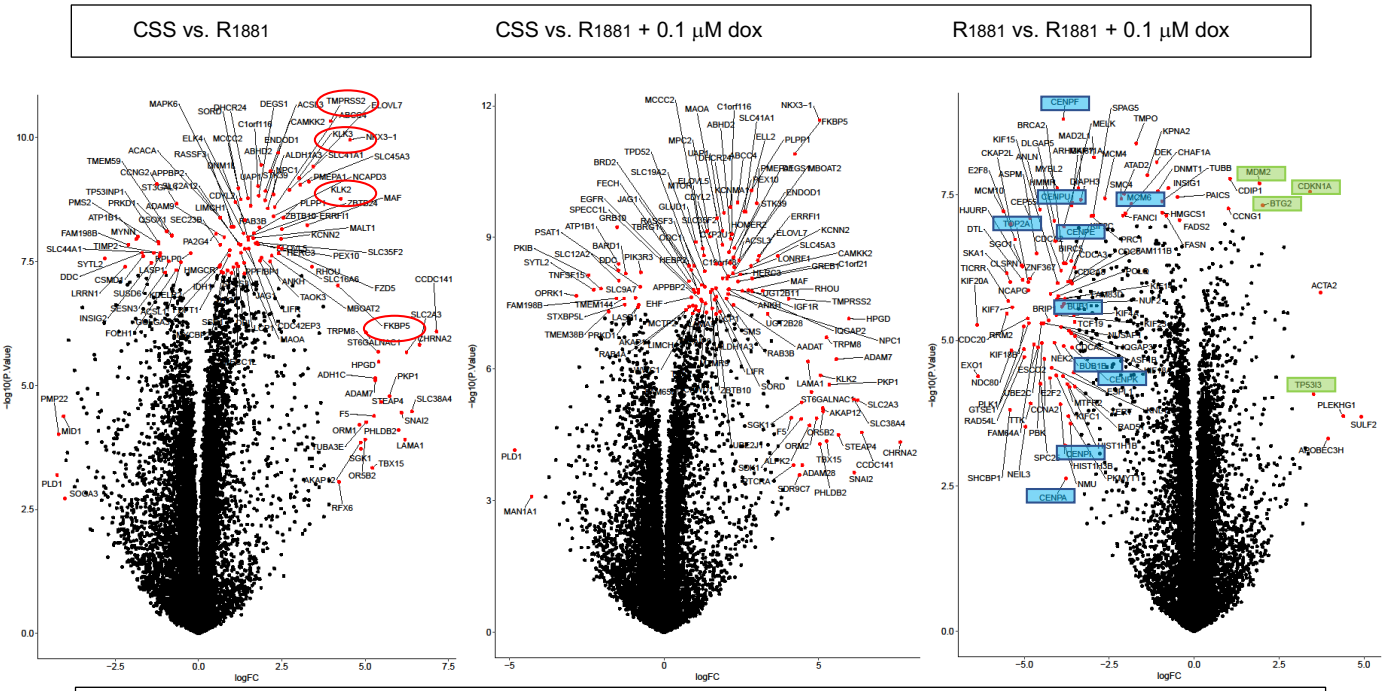


Fig. 12 – RNA-seq data confirms the anti-proliferative effect of low dose dox. Differentially expressed genes from RNA-seq data were visualized by volcano plots (P-value on the y axis, log fold change on the x), for conditions noted above. Genes marked by red dots were most significant (P<0.05) with fold changes above the cut-off. Some classic AR targets are circled (left); the effect of dox is subtle in the presence of R1881 (middle). The effect of low dox is compared to hormone alone (right), with key downregulated genes boxed in blue and upregulated genes boxed in green.

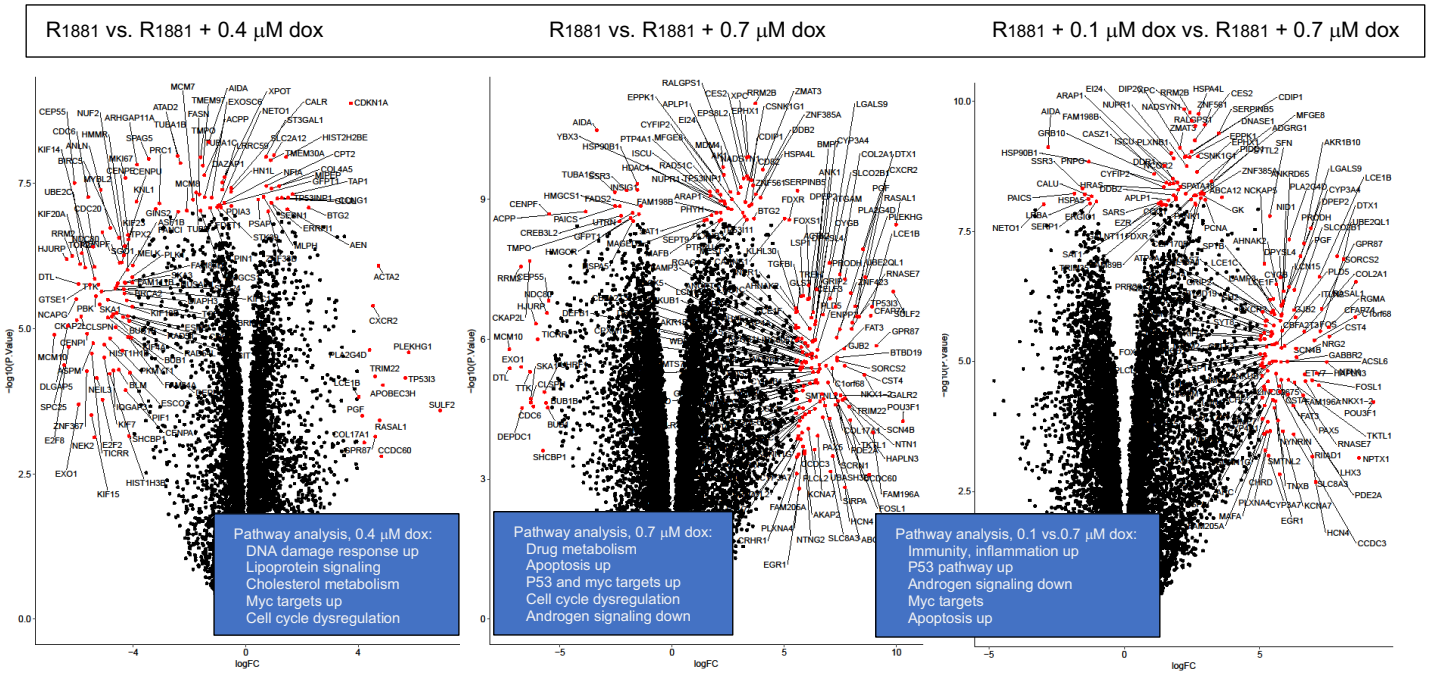


Fig. 13 – Cell cycle control and DNA damage response vary with dox concentration. Differentially expressed genes from RNA-seq data were analyzed as in Fig. 12. Shown in blue boxes are the major pathways most significantly affected in Pathway Analysis. P-values, adjusted p-values, enrichment scores and normalized enrichment scores were all highly significant.

revealed major differential expression of genes involved in DDR, lipoprotein signaling, and cholesterol metabolism, with myc targets upregulated and cell cycle genes dysregulated. At the high concentration of 0.7 μM dox, there was further differential expression for genes involved in drug metabolism, dysregulation of cell cycle genes, and upregulation of apoptosis, myc targets and p53 signaling. Perhaps of most biological significance is the near complete reduction of androgen-regulated expression. This analysis clearly demonstrates a dose response in gene expression to dox, reflecting a pause in cell growth at 0.1 μM, a switch to increasing oncogenic growth at 0.4 μM, and a strong picture of cancer pathways and cell toxicity by 0.7 μM dox. While no hidden surprises were revealed in this data, strong confirmation is again provided of the differential effect of dox at low dose on distinct sets of targets. Moreover, the genes most affected restore more "normal" androgen-regulated growth in this cancer cell line in which AR does not drive proliferation but rather causes cells to exit the cell cycle, as a prerequisite possibly to further differentiation.

A further indication that low dose doxorubicin treatment leads to differential biology and is not just simply sub-toxic is seen by displaying genes that are differentially affected across treatment groups as a heat map (**Fig. 14**). The low and medium doses are most similar (indicated by lines at top of figure), in between the mostly downregulated genes of high dose dox and the upregulated genes of androgen regulation without dox treatment. Some of the genes that maintain expression in low dox similar to that with androgen treatment are enlarged to the right of the map, along with indication of their functional roles (e.g., NCAPD3 and CENPN involved in chromosome separation; ENDOD1 – an endonuclease involved in prostate tumor suppression). Many but not all of these genes follow predictions for increased or decreased expression with dox concentration. Some of these genes suggest interesting candidates to target in order to restore the ability of AR to maintain normal function, for instance in being anti-proliferative.

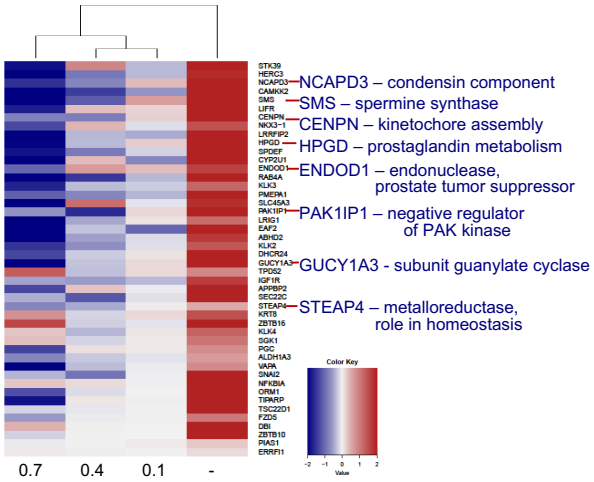
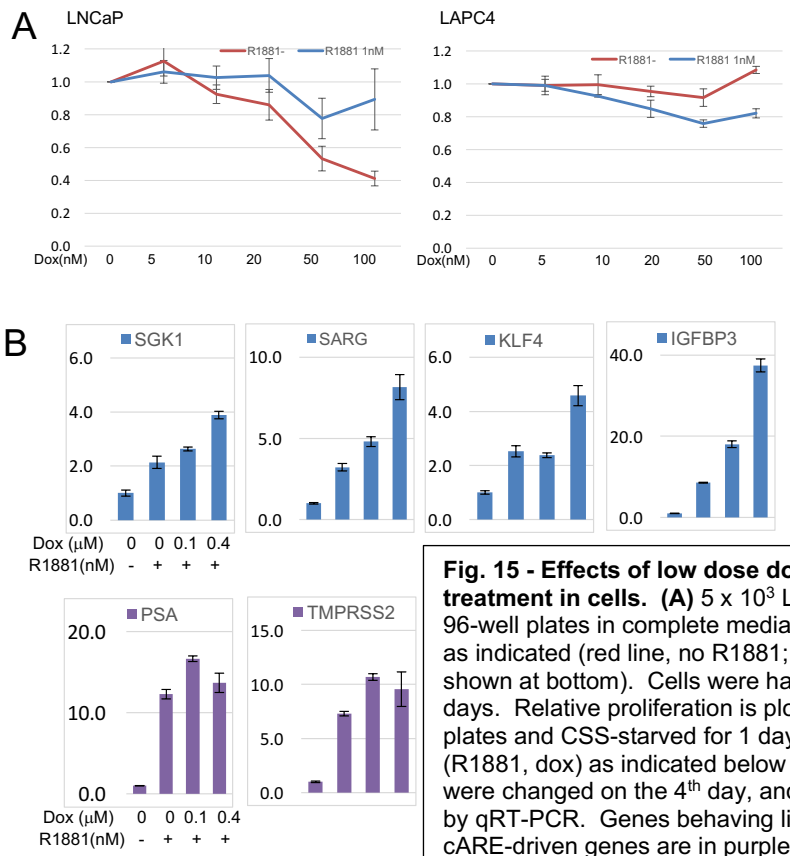


Fig. 14 – Heat map with nonbiased clustering comparing the most differentially expressed genes from RNA-seq data across treatment groups of LNCaP cells. Dox dose is shown at bottom in μM, with a color key indicating value of log fold change to right. Selected gene names and functions are enlarged to right as examples.

Task 4. **Determine differential antitumor effects of low vs. high dose doxorubicin in mice.**

The differential actions of doxorubicin on AR binding to DNA and on expression of gene sets with distinct functions are intriguing, and match predictions of our overall hypothesis, but for translational relevance, we sought to determine to what extent these effects in tissue culture affected actual tumor growth. First we determined long-term effects of dox treatment in cultured cells. While higher concentrations of dox become quickly toxic, and toxicity varies with cell line, both LNCaP and LAPC4 cell lines could withstand low dose dox treatment for at least one week (**Fig. 15A**), suggesting effects might be discernible in mouse xenograft tumor growth. By 7 days of treatment, 100 nM (0.1 μM) dox inhibited proliferation in LNCaP cells in the absence of R1881, but this is likely more a reflection of the absence of androgen, which these cells depend on for growth, than any toxicity of such low levels of dox; the dox effect in the presence of R1881 was somewhat variable but minimal overall. In LAPC4 cells, dox was modestly more inhibitory in the presence than absence of androgen but still not significantly toxic to cell growth. We next evaluated effects on gene expression with 7 days of treatment for sARE-driven genes (SGK1, SARG, KLF4, IGFBP3) and cARE-driven genes (PSA, TMPRSS2) (**Fig. 15B**). With longer treatments,



enhancement of AR induction by dox at low doses is still evident for genes with known sAREs (SGK1, SARG) and genes suspected of being under similar regulation. The inhibitory effect on cARE-driven genes is less pronounced, in part because these experiments were conducted with 1 rather than 10 nM R1881, in accord with greater physiological relevance. Results in LAPC4 cells were similar. Thus differential dox regulation is evident for at least one week of treatment. Cell line differences could be due to downstream or secondary effects.

Fig. 15 - Effects of low dose doxorubicin are sustained over 1 week of treatment in cells. (A) 5 x 10^3 LNCaP or LAPC4 cells per well were seeded in 96-well plates in complete media, then starved 2 days in CSS prior to treatment as indicated (red line, no R1881; blue line, 1 nM R1881; dox concentration is shown at bottom). Cells were harvested and MTT assays performed after 7 days. Relative proliferation is plotted. **B)** LNCaP cells were plated in 6-well plates and CSS-starved for 1 day, followed by treatment with compounds (R1881, dox) as indicated below the histograms. Fresh media and compounds were changed on the 4th day, and RNA was harvested after 7 days for assay by qRT-PCR. Genes behaving like sARE-driven genes are above in blue, cARE-driven genes are in purple below.

To more closely mimic conditions of human prostate cancer, particularly early stage, mouse tumor hosts (CB17 scid male mice) were clamped to a constant level of testosterone that kept serum levels close to those of human [23]. This was done by surgical castration and concurrent implantation of silastic tubing implants with 25 mg testosterone for sustained release. After 1-1.5 weeks to allow hormone levels to equilibrate, 1 x 10^6 LAPC4 cells in Matrigel (1:1) were injected subcutaneously into each hind flank. Tumor growth was followed by caliper measurements twice weekly after tumor detection by palpation. There are few reports in the literature of doxorubicin treatment of mice, either with respect to dose or administration method. We chose intraperitoneal injection as most effective, and doses of 0, 0.5, 1.7 and 5 mg/kg, made up in 0.9% sterile saline at a concentration such that 100 μl delivered the appropriate amount, given twice weekly. Doses for human treatment are upwards of 5 mg/kg. This dose is apparently even more toxic in mouse than man (known cardiotoxicity) since many mice in this group died after less than 2 weeks of treatment; remaining mice were switched to the medium dose but few of these were rescued by decreasing the dose at that stage. Each group initially had 10-12 mice; some mice succumbed during the course of the experiment for various reasons. We planned to initiate treatment when tumors reached 200 mm^3 in size, but some tumors grew unexpectedly rapidly and so for many mice treatment began later than desirable. Some mice were treated prior to tumor detection and in some of these tumors became evident during treatment or upon sacrifice. Those that attained 3 weeks of treatment, or had tumors greater than 2 cm^3, were euthanized to retrieve the samples. Resected tumors were dissected into portions for histology (10% formalin), RNA analysis (in RNA*later*) or flash frozen.

The xenograft experiment bears repeating, largely because tumor growth was very heterogeneous and most tumors were treated at a later stage than optimal. From initial histological

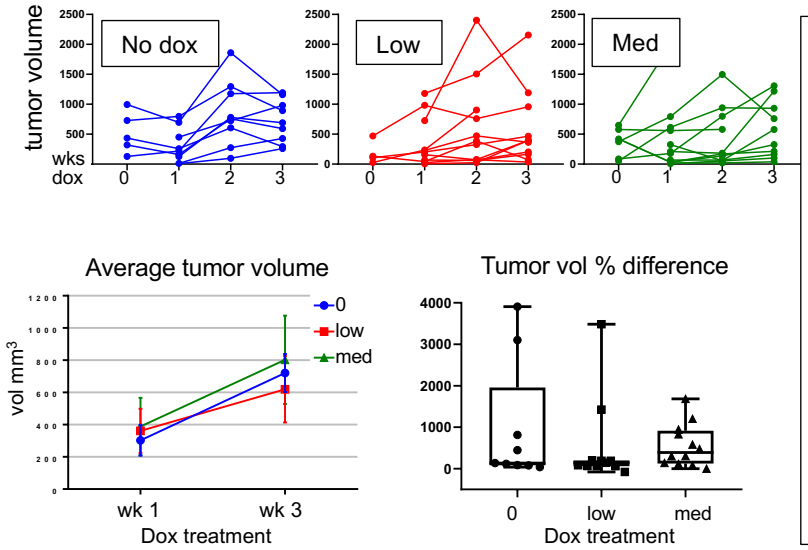


Fig. 16 – Mouse xenograft tumor growth is affected by dox treatment. $1x10^6$ LAPC4 cells were inoculated into flanks of castrated mice bearing testosterone pellets (to keep T levels close to human), and followed by palpation, with volume calculated as (length x width^2)/2. Graphs at the top represent tumor growth in mice treated for at least 2 weeks with no, low or medium dox doses (0, 0.5, 1.7 mg/kg). Growth was heterogeneous, but as shown on the lower left, trended to slower growth for the low dox dose, compared to untreated or treated with higher dox doses. This is also suggested by the box plot representation on the lower right.

examination, most tissue sections represented late stage cancer, with large areas of necrosis. Despite the great variability in tumor growth rates (**Fig. 16**), found within groups as well as within the two tumors of one mouse, there is a trend that suggests tumors in mice treated with low dox doses grew more slowly than tumors in either untreated mice or mice treated with higher dox doses. This suggests these tumors may be less proliferative or show more differentiated growth.

Remarkably, despite the tumor heterogeneity, gene expression in these samples strongly upheld the pattern of sARE-marked genes being induced at the low dox dose, unlike cARE-driven genes that were inhibited in a more linear fashion dependent on dose (**Fig. 17**). Pools of RNA from 10 tumor samples per group were subjected to qRT-PCR and assayed in triplicate; fold-changes were normalized to actin and are shown relative to the untreated control. The striking reproducibility of the gene expression data is indicated in part by the difficulty in seeing the error bars that are in fact on the figure; the bar for SEM is barely visible above the bar for the low dose level of NKX3.1 in the figure at left.

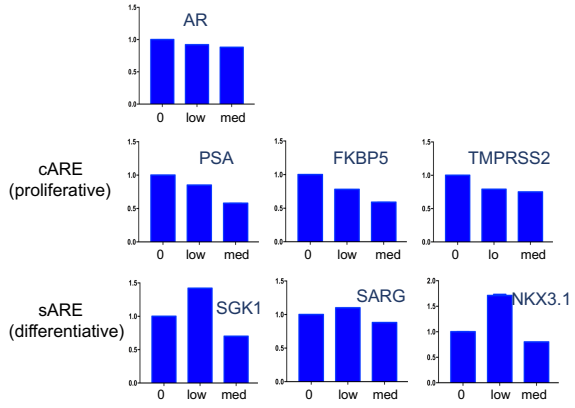


Fig. 17 – Differential gene expression is evident in xenograft tumors from dox-treated mice. RNA was purified from tumor tissue using the RNeasy Mini Kit (Qiagen), converted to cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo) and qRT-PCR performed with SYBR green reagents on a StepOnePlus RealTime PCR system. Each bar represents 10 tumor samples, assays were run in triplicate with differences highly statistically significant – bars for SEM can be seen on the graph at high magnification. AR is shown at top, showing little change; cARE-driven genes show steady decline whereas sARE-driven genes show the expected increase at low dox.

In sum, the experimental results of this project strongly support our initial hypothesis that selective AR modulators (SARMs) may prevent expression of genes involved in cancer cell proliferation but allow expression of genes for normal cell growth and differentiation. That this property is conferred by doxorubicin, a clinically used chemotherapeutic, may allow its rapid repurposing for more specific anti-cancer treatments. Derivatives of dox may be found that are also less toxic. In addition, some of our findings have relevance to basic studies of DNA response elements and gene regulation in general, and provide further evidence that "weak" response elements underlie selectivity and specificity of gene control.

KEY RESEARCH ACCOMPLISHMENTS:

- In high-throughput screening of ~10,000 compounds, doxorubicin consistently validated in eliciting differential activation of consensus vs. selective AREs by AR, for transfected and endogenous AR targets. The extent of this effect varies with gene and cell-type.
- Doxorubicin acts by intercalating into DNA, which disrupts AR binding to cAREs to a greater extent than to sAREs. This mechanism was demonstrated *in vitro* by EMSA and *in vivo* by conventional ChIP assays that show differential AR recruitment to chromatin.
- The differential action of doxorubicin is pronounced at low concentrations, when effects on cell proliferation and growth are minimal and is evident prior to cell toxicity.
- In ChIP-seq analysis, the differential dox effect was shown to be specific to AR and not to transcription in general, and showed that low dose dox does not target a new set of AR binding sites but substantially increases AR binding to a small set of pre-existing AREs. Further, the sites where AR binding increases are context-dependent, relying on adjacent prostate-specific factors with sites presumably overlapping sARE half-sites.
- In RNA-seq analysis, the differential effect of dox at low dose restores normal AR action to slow proliferation and sustain cell survival, demonstrated by downregulation of cell cycle genes at low dose and upregulation of DNA damage response at higher doses.
- In mice bearing xenograft tumors, low dox treatment slowed growth relative to no or higher dose treatment. Despite heterogeneity in tumor growth for all treatments, tumor gene expression was remarkably consistent in showing downregulation of cARE-type AR targets but the characteristic induction at low dose for genes characterized as sARE-dependent.
- These accomplishments sum to support doxorubicin as a selective modulator of AR action, suppressing activation of cAREs while maintaining or enhancing activation of sAREs. The cARE elements are more associated with oncogenic actions while sARE elements are associated with genes necessary for differentiation or more normal cell growth.
- This proof of our hypothesis provides a basis for further study of whether doxorubicin could be leveraged in prostate cancer treatment to delay the tipping point from tumor suppression to oncogenesis. Low dose dox may prove to be useful in watchful waiting or in early treatment of early stage disease, alone or in combination with androgen ablation, to reduce side effects and delay resistance.

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Opportunities for Training and Professional Development

During the course of this project, one postdoctoral fellow received training. Dr. Shihan He was in the lab for 2.5 years, leaving in June of 2017 for a position in a small bioinformatics company in North Carolina. He is more suited to the environment of industry versus academia.

Dissemination of Results

In this last year, an abstract of this work was selected for an oral presentation at ENDO, the yearly symposium of the Endocrine Society, and was presented 3/17/18, in Chicago, IL. The abstract for this talk is attached in the Appendix.

I was invited as the plenary speaker to present this work at the Great Lakes Nuclear Receptor Conference, on 10/18/18, in Minneapolis, MN.

I presented a poster at the 11th Multi-Insititutional Prostate SPORE meeting in Ft. Lauderdale, March 4-6, 2018. The abstract is attached in the Appendix.

By way of outreach, I have presented some of these results in a manner more accessible to nonscientists, as a talk entitled "*The Science, Medicine and Politics of Prostate Cancer*". In June of 2016, I presented this talk to a group of retired individuals interested in current biomedical research – the group was more engaged than an average class of graduate students. In September of 2017, I gave a similar presentation to the Michigan Society of Fellows, a group of outstanding postdoctoral scholars representing nearly all academic fields. Again the interest level and questions generated were inspiring.

Next Reporting Period – Nothing to Report

4. IMPACT

Results of this project have a two-fold impact. First, regarding translation of these results to the clinic, our data provide proof of concept that activity of the androgen receptor may be modulated to inhibit pathways driving cancer but promote those for normal differentiative growth. The agent of this modulation was identified in our high-throughput screen and is a known drug, doxorubicin, that targets the DNA response elements bound by AR rather than the receptor itself. The DNA elements vary between different genes, with a consensus response element (cARE) driving most genes associated with proliferation and cancer, but a more selective response element (sARE) utilized by genes involved in differentiation. Doxorubicin at high doses is used clinically in late stage prostate cancer, but the selective effects we have found are at low doses, well before the occurrence of toxic side effects. This modulation of gene expression at low dose may be most readily produced in early stages of cancer, or in watchful waiting, before disease progresses to "oncogenic rage". This would in effect constitute a repurposing of the already approved doxorubicin. Chemical derivatives of dox exist that are less toxic but have not yet been tested for differential effects on gene expression. A next step would be to enlist clinical colleagues for a trial in which liquid biopsies (blood, urine) could be obtained to assess whether gene expression changes in cells or RNA could be detected that mirror differential effects found in tissue culture and in preclinical mouse models. A clinical trial of efficacy in early stage prostate cancer or watchful waiting would be an ultimate goal.

Additional impact applies to basic studies of gene regulation. Current treatments for a broad host of diseases, including cancer, could be developed around targeting of genes regulated by transcription factors in key pathways. However, targeting transcription factors has been difficult - steroid receptors are an exception in having a ligand binding domain that permits small molecules to regulate their activity. Drugs that target DNA binding domains and impact

specific gene expression have not had much success. Moreover, DNA response elements have been increasingly categorized via huge bioinformatic data sets, but this has not had the predictive value anticipated. Thus the selectivity of dox gives a unique window into mechanisms of selectivity, and how gene sets may be recognized by DNA binding elements, both for a cognate factor and for its DNA binding partners. Further understanding of the basic science underlying selectivity is likely to impact human health, in predictable and unpredictable ways.

5. CHANGES/PROBLEMS

There have been no significant changes in this last year. Although work went slowly due to reduced personnel, I am satisfied that the final results were worth the wait.

6. PRODUCTS

After replication and expansion of some experiments (especially the xenograft experiment) results will be published, probably in one large comprehensive manuscript.

The meeting abstracts for ENDO and the Multi-institutional SPORE meeting are below. There was no abstract for the GLNRC plenary address.

Robins DM, Bagamasbad PD, He S, Cieslik M, Chinnaiyan A, Kregel S: *Promoter-selective androgen receptor modulation for prostate cancer therapy*. Endo Soc 2018.

Robins DM, Bagamasbad PD, Cieslik M, Chinnaiyan A, Kregel S, He S: *Promoter-selective androgen receptor modulation for prostate cancer therapy*.

7. PARTICIPANTS

Name: Diane M. Robins, Ph.D.
Project Role: P.I.
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Person Mo.: 1
Contribution: Dr. Robins conceived and oversaw the experiments and reported the results
Funding: 10% from this award (decreased to 8% in the last year), 2.5% from the SPORE

Name: Michele Brogley
Project Role: Res. Assistant
Research ID: n/a
Person Mo.: 5
Contribution: Ms. Brogley assisted with cell culture, molecular assays, mouse experiments
Funding: Ms. Brogley's effort on this project was at 40%

Name: Shihan He, Ph.D.
Project Role: Research Associate
Research ID (ORCID): 0000-0001-5806-8583
Person Mo.: 12, until 06/09/17 when he moved to a new position
Contribution: Dr. He performed cell gene expression, cell growth and ChIP assays
Funding: Dr. He was funded by this DOD grant until 06/09/17

8. SPECIAL REPORTING REQUIREMENTS – N/A

9. APPENDICES

Abstract for the Multi-Institutional Prostate SPORE meeting, 3/6/2018 (poster)

Abstract for the Endocrine Society Symposium, ENDO, 3/17/2018 (selected talk)

11th Annual Multi-Institutional Prostate Cancer Program Retreat
March 4-6, 2018

Title: Promoter-selective Androgen Receptor Modulation for Prostate Cancer Therapy

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Body:

The androgen receptor (AR) is a key therapeutic target in prostate cancer, yet despite potent new drugs to block hormone synthesis and AR function, resistance arises and AR remains active. To combat resistance, we sought selective AR modulators (SARMs) to inhibit AR target genes that drive cancer but not normal cell growth. Our hypothesis is that these genes differ in androgen response elements (AREs), with genes driving proliferation relying on consensus inverted repeats (cAREs) and genes promoting differentiation relying on AR-selective half-sites (sAREs). From a high-throughput screen for compounds that elicit differential AR regulation, doxorubicin (dox) had the greatest selective effects on AR-driven gene expression. Dox, one of the first chemotherapeutics, intercalates into DNA and elicits DNA damage response, a pathway also influenced by AR. In prostate cancer cells, low concentration dox treatment (100 nM) remarkably increased expression of sARE-driven genes, whereas cARE-driven targets were inhibited. Cell proliferation was unaffected at low dox concentration. We showed by protein-DNA interaction studies that AR binding was more resistant to disruption by dox for binding to sAREs than for binding to cAREs. Further, AR was differentially recruited to chromatin in ChIP assays, with reduced AR binding to cARE- driven genes such as TMPRSS2, but enhanced binding at low concentration to sARE-driven genes such as SARG (C1orf116).

To define genome-wide the set of genes sensitive to low concentration dox treatment, we performed ChIP-Seq for AR, and as a control TATA-box binding protein (TBP), in androgen-dependent LNCaP cells treated for 12 hrs with R1881 (1 nM), dox (0.1, 0.4 or 0.7 μM) or both. Compared to control androgen-stimulated conditions, few novel AR binding peaks were induced by dox treatment at any concentration. However, low dose dox increased the strength of AR binding (indicated by reads per peak) on average by nearly 50%, while the total number of AR peaks decreased with dox in a dose-dependent manner. This illustrates that a very small subset of genes showed enhanced AR binding at pre-existing sites with low dose dox, suggesting greater relative AR binding strength, in contrast to most genes that showed decreased binding of AR. This effect was not seen with TBP, which showed no effect of dox or R1881 on binding, indicating the effect is not influencing global transcription but rather is AR-mediated. We are currently examining expression of this subset of dox-induced AR-dependent genes in existing cancer databases and testing the effect of dox concentration on tumor progression in a mouse xenograft model. Together, these data suggest there may be a doxorubicin “sweet spot” that, dependent on cancer stage and in combination with other therapeutics, might slow tumor growth, reduce side effects and/or delay resistance.

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Promoter-Selective Androgen Receptor Modulation for Prostate Cancer Therapy

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Abstract:
The androgen receptor (AR) is a key therapeutic target in prostate cancer, yet despite potent new drugs to block hormone synthesis and AR function, resistance arises and AR remains active. To combat resistance, we sought selective AR modulators (SARMs) to inhibit AR target genes that drive cancer but not normal cell growth. Our hypothesis is that these genes differ in androgen response elements (AREs), with genes driving proliferation relying on **c**onsensus inverted repeats (**c**AREs) and genes promoting differentiation relying on AR-**s**elective half-sites (**s**AREs). From a high-throughput screen for compounds that elicit differential AR regulation, doxorubicin (dox) had the greatest selective effects on AR-driven gene expression. Dox, one of the first chemotherapeutics, intercalates into DNA and elicits DNA damage response, a pathway also influenced by AR. In prostate cancer cells, low concentration dox treatment (100 nM) remarkably increased expression of sARE-driven genes, whereas cARE-driven targets were inhibited. Proliferation was unaffected at low dox concentration. We showed by protein-DNA interaction studies that AR binding was more resistant to disruption by dox for binding to sAREs than for binding to cAREs. Further, AR was differentially recruited to chromatin in ChIP assays, with reduced AR binding to cARE-driven genes such as TMPRSS2, but enhanced binding at low concentration to sARE-driven genes such as SARG (C1orf116).
To define genome-wide the set of genes sensitive to low concentration dox treatment, we performed ChIP-Seq for AR and, as a control, TATA-box binding protein (TBP), in androgen-dependent LNCaP cells treated for 12 hrs with R1881 (1 nM), dox (0.1, 0.4 or 0.7 uM) or both. Compared to control androgen-stimulated conditions, few novel AR binding peaks were induced by dox treatment at any concentration. However, low dose dox increased the strength of AR binding (indicated by reads/peak) on average by nearly 50%, while the total number of AR peaks decreased with dox in a dose-dependent manner. This illustrates that a very small subset of genes showed enhanced AR binding at pre-existing sites with low dose dox, suggesting greater relative AR binding strength, in contrast to most genes that showed decreased binding of AR. This effect was not seen with TBP, which showed no effect of dox or R1881 on binding, indicating the effect is not influencing global transcription but rather is AR-mediated. We are currently examining the expression pattern of this subset of dox-induced AR-dependent genes in existing cancer databases and testing effect of dox concentration on tumor progression in a mouse xenograft model. Together, these data suggest there may be a doxorubicin “sweet spot” that, dependent on cancer stage and in combination with other therapeutics, might slow tumor growth, reduce side effects and delay resistance.
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