Prostate cancer androgen receptor activity dictates efficacy of bipolar androgen therapy through MYC

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Graphical abstract

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**Title:** Prostate cancer androgen receptor activity dictates efficacy of bipolar androgen therapy through MYC

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**Abstract:** Testosterone is the canonical growth factor of prostate cancer but can paradoxically suppress its growth when present at supraphysiological levels. We have previously demonstrated that the cyclical administration of supraphysiological androgen (SPA), termed Bipolar Androgen Therapy (BAT), can result in tumor regression and clinical benefit for patients with castration-resistant prostate cancer. However, predictors and mechanisms of response and resistance have been ill-defined. Here we show that growth inhibition of prostate cancer models by SPA required high androgen receptor (AR) activity and was driven in part by downregulation of MYC. Using matched sequential patient biopsies, we show that high pre-treatment AR activity predicted downregulation of MYC, clinical response, and prolonged progression-free and overall survival for patients on BAT. BAT induced strong downregulation of AR in all patients, which is shown to be a primary mechanism of acquired resistance to SPA. Acquired resistance could be overcome by alternating SPA with the AR inhibitor enzalutamide, which induced adaptive upregulation of AR and re-sensitized prostate cancer to SPA. This work identifies high AR activity as a predictive biomarker of response to BAT and supports a treatment paradigm for prostate cancer involving alternating between AR inhibition and activation.

**Graphical abstract:**

![Graphical abstract](image)
INTRODUCTION

Signaling through the androgen receptor (AR) is the primary oncogenic driver of prostate adenocarcinoma. Inhibition of AR signaling by androgen deprivation and AR inhibitors produces significant therapeutic and palliative benefit and constitutes the cornerstone of treatment for advanced disease. Yet this therapeutic strategy is not curative, and patients eventually progress with lethal castration-resistant prostate cancer (CRPC). Studies dating back over 30 years indicate that supraphysiological androgens (SPA) can paradoxically suppress the growth of some prostate cancer cell line and xenograft models (1–3). These studies led us to test SPA as a treatment for men with CRPC. Our approach has been to pulse intramuscular testosterone every 28 days concurrent with ongoing luteinizing hormone-releasing hormone analogue administration to result in oscillation of serum testosterone from supraphysiological to near-castrate levels. Given this oscillation of testosterone between polar extremes, we termed this treatment Bipolar Androgen Therapy (BAT) (4). We have previously described that BAT can produce clinical benefit and tumor regression for 20-30% of patients with CRPC (5–9), however predictors and mechanisms of response and resistance have been ill-defined. A better understanding of molecular mechanisms of BAT is needed to optimize patient selection and develop strategies to enhance its therapeutic efficacy.

Early studies of SPA-mediated growth inhibition of prostate cancer models suggested that this effect occurs predominantly after long-term exposure to low androgen conditions and requires treatment with supraphysiological, rather than physiological, doses of androgens (2). Thus, it seems that an adaptation of prostate cancer to inhibition of AR signaling constitutes a vulnerability to SPA. A major adaptation of prostate cancer to inhibition of AR signaling is counterbalanced enhancement of AR signaling through AR overexpression, gene amplification, activating
mutations, and expression of ligand-independent variants (10, 11). Indeed, an association between
high AR expression and growth-inhibition by SPA in prostate cancer models has been observed
(2, 12, 13), begging the question of whether high AR activity is necessary for prostate cancer
sensitivity to SPA.

We and others have recently been mapping the downstream molecular events through which SPA
results in growth inhibition of SPA-sensitive prostate cancer models. Multiple mechanisms have
been proposed, including downregulation of c-MYC (1, 13–15), AR inhibition of DNA relicensing
during mitosis (16), AR-mediated DNA damage (12, 17), and induction of ferroptosis and
immunogenic cell death (18). Notably, in vitro genome-wide positive-selection genetic screens
have failed to identify one dominant pathway mediating growth inhibition by SPA (19), suggesting
that SPA likely induces multiple parallel growth-inhibitory pathways in SPA-sensitive prostate
cancer. Yet the clinical significance of the multiple proposed mechanisms of growth inhibition by
SPA has been unclear, as no study has utilized tumor samples derived from patients treated with
SPA to date. Given that c-MYC (hereafter called MYC) is highly expressed in prostate cancer
(20, 21), is a potent driver of growth and proliferation (22), and yet has been described as
‘undruggable’ (23), we have been particularly interested in determining whether BAT can reduce
MYC expression and understanding the relative contribution of downregulation of MYC toward
growth inhibition by SPA.

Here we utilize paired sequential biopsies of metastases of patients with CRPC treated with BAT
and prostate cancer models to identify predictors and molecular mechanisms of prostate cancer
regression by BAT. We report that high AR activity is required for growth inhibition of prostate
cancer models by SPA, which occurs in part through downregulation of MYC. Similarly, in
patients, high pre-treatment AR activity predicts downregulation of MYC, tumor regression, and
progression-free and overall survival on BAT. Notably, we find that acquired resistance to SPA can be driven by downregulation of AR, but that this can be overcome by treatment with an AR inhibitor, which induced adaptive upregulation of AR.

RESULTS

Androgen receptor activity determines response to supraphysiological androgen in vitro

To assess determinants of sensitivity to SPA, we first studied human prostate cancer cell lines with varying sensitivity to SPA. SPA can be provided to cells as R1881, a potent synthetic androgen that is not metabolized in vitro, at a dose of 10nM, which is approximately 20-fold higher than the level of free testosterone in eugonadal adult men. LNCaP and VCaP cell lines are growth-inhibited by SPA, while LAPC4 and 22Rv1 cell lines exhibit primary resistance to SPA (Supplemental Figure 1). Among these cell lines, we observed that pre-treatment AR abundance and activity (as assessed by expression of the AR target prostate-specific antigen (PSA)) was higher in SPA-sensitive cell lines than SPA-resistant cell lines (Figure 1A). High baseline AR abundance and activity was required for growth inhibition by SPA, as inducible shRNA-mediated knock-down of AR in LNCaP cells (Figure 1B) resulted in resistance to SPA (Figure 1C) and rescued clonogenic survival following SPA treatment (Supplemental Figure 2). Conversely, high AR abundance was sufficient to confer sensitivity to SPA, as overexpression of AR in LAPC4 and 22Rv1 cells (Figure 1D and F) resulted in growth inhibition by SPA (Figure 1E and G). This indicates that AR abundance and activity is a major determinant of prostate cancer response to SPA in vitro.

Downregulation of MYC contributes to growth inhibition by supraphysiological androgen

AR activation has previously been shown to downregulate MYC in normal prostate epithelial cells (24–26) and models of prostate cancer (1, 13–15). We observed that SPA downregulates MYC,
but only in cells lines with high AR abundance and activity (i.e., SPA-sensitive) and not in SPA-resistant cell lines (Figures 1A and 2A). High pre-treatment AR abundance was required for downregulation of MYC by SPA, as inducible shRNA-mediated knock-down of AR in LNCaP cells disabled MYC downregulation by SPA (Figure 2B). Moreover, high pre-treatment AR abundance was sufficient to induce downregulation of MYC by SPA given that AR overexpression resulted in MYC downregulation in LAPC4 and 22Rv1 cells (Figures 1D and F). MYC downregulation contributed to growth inhibition by SPA as constitutive expression of MYC partially rescued growth inhibition of LNCaP and LAPC4-AR cells treated with SPA (Figure 2C-F), as previously shown (2, 13). Notably, downregulation of MYC is not a general feature of prostate cancer growth arrest, as the highly active chemotherapy agent docetaxel induced growth arrest of LNCaP cells but did not result in downregulation of MYC (Supplementary Figures 3A-B). Thus, downregulation of MYC is a specific feature of SPA treatment. Altogether, these data suggest that SPA inhibits growth of prostate cancer with high AR abundance in part through downregulation of MYC.

**Androgen receptor activity determines response to Bipolar Androgen Therapy**

To assess molecular mechanisms of BAT in patients with metastatic CRPC (mCRPC), we evaluated clinical samples from a prospective clinical trial (NCT03554317) that included on-study sequential paired biopsies of soft tissues metastases before (“pre-BAT”) and after 3 cycles of BAT (“on-BAT”) (Figure 3A). Twenty-four patients had tumor samples collected at both time points that were adequate for analysis by immunohistochemistry (IHC), and 15 of these patients had paired samples adequate for RNA sequencing analysis. Ten of 24 patients were considered to be responders, based on the presence of a decline in the serum PSA by at least 50% or tumor volume by at least 30% on cycle 4 day 1 of BAT. Characteristics of these patients are listed in
Supplementary Table 1. We first quantified AR protein abundance by immunohistochemistry after performing serial dilutions of the AR antibody to ensure staining in the linear range. To separately measure AR in nucleus and cytoplasm, as well as total cellular AR protein, we developed an iterative multiplex assay using AR, as well as keratin 8, which was used to help train a random forest classifier to segment total tumor cellular area. In this manner, by image analysis we were able to obtain optical density (OD) measurements as a continuous variable separately for the nuclei, cytoplasm and whole cell (see methods). In pre-BAT samples, responders did not exhibit higher total cellular AR protein abundance than non-responders (Figure 3B), which directly correlated with AR protein abundance in cytoplasm and nucleus, as well as AR mRNA abundance (Supplementary Figure 4A-C). Prior to BAT, the nuclear-to-cytoplasmic ratio of AR was greater than one in all patients, indicating that the majority of AR resides in the nucleus in advanced mCRPC, which was not significantly different between responders and non-responders (Supplementary Figure 4D). To examine whether there was greater variation in pre-treatment AR activity between responders and non-responders, we generated an AR activity score using Mann-Whitney ranking of expression of 10 canonical AR target genes (AR_{MW} score) (see Supplemental Methods). Notably, responders had significantly higher pre-BAT AR_{MW} scores than non-responders (p=0.011) (Figure 3C). The AR_{MW} score was not driven by expression of one dominant gene (Supplementary Figure 5A), the included gene transcripts did not exhibit significant co-linearity (Supplementary Figure 5B), and the score did not correlate with AR protein abundance (Supplementary Figure 5C), indicating each included gene contributed unique data to the score, which overall was distinct from AR abundance. This demonstrates that AR activity is controlled by factors beyond protein abundance in advanced CRPC, which likely includes activating or inactivating gene mutations in AR and activity and abundance of AR co-factors and regulators (27,
Stratifying patients by a cut-off ARAMW score of 0.6 (selected due to its ability to stratify patients with distinct outcomes), we observed that patients with high (>0.6) ARAMW scores exhibited greater PSA responses (p=0.010) (Figure 3D), greater decrease in tumor volume (p=0.005) (Figure 3E), a trend toward longer radiographic progression-free survival (p=0.058) (Figure 3F), and longer overall survival (p=0.002) (Figure 3G) on BAT. Given that serum PSA concentration is in part dependent on cancer cell AR activity, we assessed whether there was an association between pre-BAT serum PSA and response to BAT. There was a trend toward higher pre-BAT serum PSA among responders compared with non-responders (p=0.064) (Figure 3H). Together these data indicate that pre-treatment AR activity is a major determinant of CRPC response to BAT, and the ARAMW score may constitute a valuable predictive biomarker for this therapy.

Strengths of the ARAMW score are that it does not require a reference expression vector and is independent of differences in sequencing depth and processing. Therefore we applied ARAMW scoring to an independent cohort of 266 patients with mCRPC who were not exposed to BAT (SU2C/PCF cohort) (29). Among these patients, the prevalence of the biomarker (score >0.6) was 36.5% (Supplementary Figure 6A). Analysis of the SU2C/PCF cohort indicated that high AR activity is not independently prognostic (i.e., ARAMW score >0.6 does not predict favorable outcomes independent of BAT treatment) (Figure 3I) and is not clearly associated with particular genomic alterations or other patient factors (Supplementary Figure 6B-D).

**Bipolar Androgen Therapy downregulates MYC in responding patients**

We next assessed molecular changes induced by BAT in the paired sequential tumor biopsies. BAT increased the AR nuclear-to-cytoplasmic ratio in most patients (Figure 4A), but notably to a greater degree in responders (Figure 4B). This may suggest that nuclear recruitment/retention
and/or cytoplasmic clearance of AR is related to a clinical response to BAT. We also examined MYC expression by quantitative image analysis of MYC immunohistochemistry. Most patients had high expression of MYC protein in the pre-BAT tumor sample (Figure 4C). BAT decreased the median MYC H-score (Figure 4C), with responders exhibiting a greater decrease in the MYC H-score than non-responders (Figure 4D) and a subset of patients having a near-complete ablation of MYC expression (Figure 4E). The change in MYC protein expression directly correlated with change in MYC mRNA expression (Figure 4F), suggesting that BAT suppressed MYC at the level of transcription and/or mRNA stability. BAT also decreased the median Ki-67 H-score (Figure 4G), with responders exhibiting a trend toward greater decrease in the Ki-67 H-score than non-responders (Figure 4H), and some patients showing almost complete loss of Ki-67 (Figure 4I). The change in MYC protein expression directly correlated with the change in Ki-67 expression (Figure 4K) and the change in tumor volume on CT scan (Figure 4J). Notably, only patients with pre-BAT ARAMW scores greater than 0.6 exhibited significant decrease in MYC protein expression (Figure 4L), supporting the concept that high pre-BAT AR activity is required for downregulation of MYC and tumor regression by SPA.

The mechanism by which AR activation suppresses MYC in prostate cancer was recently suggested to occur through AR-mediated sequestration of cofactors and decreased activity of distal super-enhancers (SE) near PCAT1 that regulate the MYC promoter, as well as those of neighboring transcripts embedded in the topologically associated domain (TAD) on 8q24 (13). We noted that transcripts of genes located within the 8q24 TAD, PCAT1 and PVT1, had similar change in expression as MYC on BAT (r=0.87, p<0.0001, and r=0.66, p=0.007, respectively) (Figure 4M), which supports an idea that BAT reduces MYC mRNA expression via disruption of distal SE activity.
By principal component analysis of the RNA sequencing data, the differences in gene expression profiles between patients was generally much greater than the differences in gene expression induced by BAT (Supplementary Figure 7A). This inter-patient heterogeneity of gene expression and the relatively small number of patients studied limited some analyses of the data. Notably, only 5 genes were identified to be statistically significantly altered by BAT, including downregulation of AR, ANKRD30A, and LINCO0993, and upregulation of RERGL and PARM1 (Supplementary Figure 7B). In contrast, numerous genes were differentially expressed between responders and non-responders (Supplementary Figure 7C), however all of these genes were expressed at low levels (log₂TPM less than 3) so the importance of these differences is uncertain.

Previous studies have suggested that SPA can induce DNA damage and downregulation of homologous recombination repair (HRR) gene expression in prostate cancer cell lines (12) and patients with HRR genomic alterations may have heightened clinical responses to BAT (12, 30). In this dataset, BAT did not significantly alter expression of a panel of HRR genes, nor was the change in expression of these genes different between responders and non-responders (Supplementary Figure 8A), and there was no difference in clinical outcome based on the presence of a genomic HRR alteration by clinical testing among these patients (Supplementary Figures 8C-F).

**Downregulation of androgen receptor drives acquired resistance to supraphysiological androgen**

 Clinically we have observed that most patients with CRPC who initially respond to BAT acquire secondary resistance after approximately 6-12 months of therapy (9). Similarly, the SPA-sensitive cell line LNCaP, which was initially cell cycle-arrested in G0-G1 after 5 days of SPA, re-entered the cell cycle following 12-19 days of continuous SPA exposure (Figure 5A). These cells were
verified to have acquired resistance to SPA, as re-treatment with increasing doses of R1881 resulted in no change to clonogenic survival (Figure 5B). The transcriptional and chromatin accessibility profiles of LNCaP with acquired resistance to SPA (LNCaP-SPAR) appeared most similar to VEH-treated cells (Supplementary Figure 9A-B), suggesting these cells revert to a pre-treatment phenotype. Resistance did not appear to be driven by complete failure of SPA to activate AR, as Hallmark Androgen Response genes remained induced in both SPA-sensitive and resistant cells (Supplementary Figure 9C). Instead, development of resistance to SPA was associated with decreased expression of AR mRNA and protein, decreased AR activity assessed by decreased KLK3 (encodes PSA) and PSA expression, and loss of suppression of MYC (Figure 5C-F). The AR promoter had reduced accessibility as early as 5 days of SPA, which persisted at 26 days (Figure 5G), consistent with prior reports indicating that ligand-bound AR exhibits negative autoregulation at the level of AR gene transcription (31). MYC target gene sets were globally reactivated following development of resistance to SPA (Supplementary Figure 9D), and MYC re-expression was associated with re-expression of PCAT1 and PVT1 (Supplementary Figure 10A) and reorganization of 8q24 SE accessibility (Supplementary Figure 10B). Dual inhibition of MYC by SPA and the bromodomain inhibitor JQ1 resulted in greater suppression of MYC mRNA expression and a longer duration of growth arrest of LNCaP cells than treatment with SPA alone (Supplementary Figure 11A-B), suggesting that loss of suppression of MYC drives acquired resistance to SPA. To determine whether downregulation of AR was driving this loss of suppression of MYC and development of acquired resistance, we constitutively expressed AR in LNCaP and LN95 cells (Figure 5H and Supplementary Figure 12A). LNCaP-AR and LN95-AR cells exhibited enhanced suppression of MYC by SPA (Figure 5H and Supplementary Figure 12A), followed by extensive vacuolization (Supplementary Figure 13) and cell death, not
resistance (Figure 5I and Supplementary Figure 12B). This indicates that downregulation of AR is a mechanism of acquired resistance to SPA in vitro.

Returning to the patient samples, we saw that BAT induced strong downregulation of AR protein in most patients (Figure 6A-B). The change in AR protein correlated with the change in AR mRNA on BAT (Figure 6C), suggesting that ligand-bound AR inhibits AR gene transcription in patients, as previously described in vitro (31). Higher pre-BAT AR predicted greater decrease in AR by BAT (Figure 6D). This might be explained by a threshold effect, meaning that BAT decreases AR to a threshold minimum level below which AR is not further suppressed by BAT. These data demonstrate that adaptive downregulation of AR occurs in patients and might lead to acquired resistance to BAT over time. This mechanism is consistent with an emerging conceptual idea that acquired resistance to cancer therapy is often driven by plastic (i.e. reversible) cellular alterations, rather than gene mutation (32). Overall these data suggest that low AR expression and activity is a mechanism of primary and acquired resistance to BAT (Figure 6E).

AR inhibition re-sensitizes prostate cancer to supraphysiological androgen

We have previously reported that patients who have progressed on BAT appear to have enhanced clinical responses to subsequent AR inhibition (6–9). For example, in the TRANSFORMER study, patients with mCRPC who had not received prior BAT exhibited a PSA_{50} response rate of 25% and median response duration of 3.8 months to the AR inhibitor enzalutamide, while patients who had progressed on BAT exhibited a PSA_{50} response rate of 78% and median duration of response of 10.9 months to enzalutamide (9). Similarly, LNCaP-SPAR cells were more growth-inhibited by enzalutamide compared to parental LNCaP (Figure 7A-B). This may be due to reduction of AR abundance by prior treatment with SPA (Figure 5F) that can sensitize cells to AR inhibition. Notably, enzalutamide treatment resulted in adaptive upregulation of AR in both cell lines (Figure
7C) and enhanced downregulation of MYC (Figure 7D) and growth inhibition by subsequent treatment with SPA (Figure 7E). This indicates that response to SPA can be restored after development of acquired resistance through use of AR inhibitors like enzalutamide to induce adaptive upregulation of AR.

While BAT was originally designed to cycle testosterone levels to minimize adaptation to high or low levels of androgens, these data suggest that there may be clinical benefit to more extreme oscillation of AR activity by alternating the use of SPA with an AR inhibitor. To test this therapeutic strategy, we used a patient-derived xenograft (PDX) model derived from a metastasis of a patient with CRPC that was adapted to grow in a castrated mouse (SkCaP-1R) (33). This PDX, which expresses high AR and the AR splice-variant AR-V7 and is resistant to the second generation androgen signaling inhibitors abiraterone and enzalutamide (33), initially regressed in response to SPA, but acquired resistance after about 5 months (Figure 7F). Regression was associated with an almost complete loss of MYC expression, while acquired resistance was associated with a decrease in AR and return of MYC expression (Figure 7G-H). SPA suppressed mRNA expression of AR and AR-V7 as early as 21-30 days (Figure 7G-I). Notably, when SPA was alternated every 21 days with enzalutamide (SPA-ENZA), this PDX did not acquire resistance after 160 days of observation (Figure 7F). Subcutaneous tissues from the animals that received SPA-ENZA for 160 days were analyzed histologically and nests of cancer cells were observed, but these cells lacked significant staining for the proliferation marker Ki-67 (Figure 7H). These data suggest that repeat cycling of SPA and AR inhibition may prevent the development of acquired resistance and lead to more durable growth inhibition of prostate adenocarcinoma.
DISCUSSION

Previous clinical studies have demonstrated that BAT can be an effective therapy for some patients with mCRPC. This study assessed the molecular mechanisms that drive efficacy of BAT. Our results indicate that high prostate cancer AR activity is required for tumor regression by BAT, which occurs in part through downregulation of MYC. High AR expression was required for downregulation of MYC and growth inhibition by SPA in vitro, as knock-down of AR in SPA-sensitive LNCaP cells rescued MYC downregulation and cell cycle arrest following treatment with SPA. Knock-down of AR in these cells achieved AR levels comparable to LAPC4 and 22RV1 cell lines, which exhibit primary resistance to SPA. In patient samples, high AR activity was similarly required for anti-tumor activity of BAT, as there was no patient with a low ARA\textsubscript{MW} score (defined by our cut-off as less than 0.6) who exhibited significant downregulation of MYC and/or clinical evidence of tumor regression by BAT. Moreover, the prevalence of this biomarker (ARA\textsubscript{MW} score greater than 0.6) among patients with mCRPC in the SU2C/PCF data accurately estimates prior measurements of BAT efficacy in larger clinical trials. If the biomarker is required for a PSA\textsubscript{50} response to BAT and the PSA\textsubscript{50} response rate among biomarker-positive patients is 70%, then the predicted PSA response rate to BAT among patients with mCRPC is 25.6% (i.e., 36.5% x 70%). This is consistent with the measured PSA\textsubscript{50} response rate to BAT of 24.3% among 173 patients with mCRPC across two clinical trials (7–9), and provides further plausibility that the ARA\textsubscript{MW} score can predict response to BAT. Together, our results suggest that an ARA\textsubscript{MW} score greater than 0.6 is required for clinical benefit from BAT, and this gene expression score could function as a biomarker for patient selection if validated. A limitation to use of this biomarker is that it requires fresh tumor tissue, which can be difficult to obtain in some patients. Therefore,
investigation into surrogates for this biomarker using molecular imaging or circulating tumor markers may be clinically useful.

We also observed that primary resistance to SPA can be overcome by forced overexpression of AR in LAPC4 and 22Rv1 cells in vitro. Similarly, Litvinov et al. previously demonstrated that forced overexpression of AR in PC3 cells confers sensitivity to growth inhibition by SPA (34). It should be noted, however, that high AR activity may not be universally sufficient to confer sensitivity of prostate cancer to SPA, as DU145 cells remain resistant to growth inhibition by SPA, even with high AR overexpression (34). We observed that 3 of 15 patients in our cohort had ARA_MW scores greater than 0.6, but did not obtain a PSA_{50} or objective response to BAT. While these 3 patients did not meet guideline definitions for a clinical response to BAT, two of these patients experienced prolonged overall survival following BAT, most similar to patients who did exhibit a clinical response. This suggests that biochemical and objective responses may not predict overall benefit from BAT for some patients. Nonetheless, it seems that while high AR activity is required for prostate cancer growth inhibition by BAT, it may not be sufficient, and future studies should assess additional factors that are required to confer sensitivity to BAT.

A subtlety of our findings is that tumor AR activity, but not AR abundance, predicted clinical outcomes on BAT. We developed a quantitative method using immunohistochemistry to measure AR abundance in tissue. AR protein abundance highly correlated with AR mRNA abundance by RNA sequencing, but not with AR activity. This supports prior work that shows factors beyond AR protein abundance determine AR activity (28, 35). These factors may include AR gene mutations that alter protein activity and/or abundance of coregulators that can amplify or diminish AR function. A better understanding of regulation of AR activity may allow for development of strategies to boost and sustain AR activity to enhance sensitivity to BAT.
A key question to understanding BAT is: what are the molecular events that result in regression of tumors with high AR activity? Our results suggest that SPA/BAT can significantly downregulate MYC expression, and this occurs only in prostate cancer with high AR activity. This is in accordance with prior reports that demonstrate the ability of SPA to downregulate MYC in prostate cancer cell lines (1, 13–15), but is the first evidence that this phenomenon occurs in patients. Guo et al. recently showed that SPA can result in repression of MYC transcription in prostate cancer cell lines with high AR expression by altering superenhancer (SE) activity on 8q24 (13). In support of this mechanism, we show that in patient samples, the change in MYC expression following BAT directly correlates with the change in expression of genes co-regulated by SEs on 8q24. Interestingly, Guo et al. also demonstrate that repression of MYC by SPA occurs through a process independent of AR DNA binding (13). This may suggest that highly active ligand-bound AR can bind and sequester or ‘squelch’ factors required for transcription of MYC. Identification of these factors that become limiting for MYC transcription following BAT may lead to testing of combination therapy strategies to concurrently target these factors with BAT to achieve greater therapeutic efficacy. Notably we find that forced constitutive expression of MYC in SPA-sensitive cell lines only achieves a partial rescue of growth inhibition by SPA. This suggests that downregulation of MYC contributes to, but is not the sole mechanism of, growth inhibition by SPA/BAT. We anticipate that MYC-independent maladaptive effects of SPA may also contribute to growth inhibition, such as AR inhibition of DNA relicensing during mitosis (16), AR-mediated DNA damage (12, 17), and induction of ferroptosis and immunogenic cell death (18).

Acquired resistance is a significant limitation to use of BAT. We show that acquired resistance to SPA can be driven by downregulation of AR in vitro. BAT induced downregulation of AR mRNA and protein in most patients in our study, consistent with prior reports indicating that ligand-bound
AR exhibits negative autoregulation at the level of AR gene transcription (31). We found that acquired resistance could be prevented in the SPA-sensitive cell line LNCaP through constitutive AR expression, which is not subject to negative autoregulation. Therefore, methods to prevent AR negative autoregulation have potential to prevent acquired resistance of BAT. To our knowledge, no such methods currently exist, however AR-axis inhibitors are widely known to induce adaptive upregulation of AR in patients with CRPC (10, 11). Thus, we tested sequential treatment of prostate cancer models with SPA followed by the AR inhibitor enzalutamide. Our results indicate that acquired resistance to SPA can be overcome by treatment with enzalutamide, which induced upregulation of AR and increased susceptibility to SPA.

This study indicates that prostate cancer engages in classical endocrine negative feedback loops to titrate AR to abundance of ligand. Our results suggest that these feedback loops generate vulnerabilities that might be exploited therapeutically. To test this clinically, we have initiated a clinical trial in which patients are repeatedly switched from BAT to enzalutamide to BAT, entitled, “Sequential Testosterone and Enzalutamide Prevents Unfavorable Progression (STEP-UP)” (NCT04363164). By shifting the treatment strategy to anticipate resistance, this dynamic protocol is an attempt to use game theory (36) to minimize resistance and maximize physician control of prostate cancer growth.

Limitations of our study are that it included a relatively small number of patients and was restricted to patients and models of prostate adenocarcinoma. While some have postulated that BAT may reverse transdifferentiation of prostate adenocarcinoma to neuroendocrine prostate cancer, which can occur as mechanism of resistance to AR-axis inhibitors (37), we did not assess this possibility in the current study. We anticipate that prostate cancer of diverse histology with low AR activity will not be growth-inhibited by SPA/BAT. However, beyond a direct inhibition of cellular
proliferation, it is possible that BAT can alter other cellular behaviors that promote cancer fitness such as invasion, metastasis, and evasion of the immune system, which may or may not be dependent on cancer cell AR activity. Our study did not directly measure these other processes, although they may be indirectly captured by patient overall survival, which implies that overall benefit from BAT is mainly restricted to tumors with high AR activity. Future studies should assess these effects directly to more fully understand potential utility of BAT.

Overall this study may enable rational use of BAT for treatment of mCRPC. We identify a subgroup of patients most likely to benefit from BAT and a strategy to limit acquired resistance to this therapy, which should be validated in clinical trials. Moreover, this work promotes a new paradigm for treatment of advanced prostate cancer. While the status quo as it pertains to treatment of advanced prostate cancer is persistent and potent AR inhibition, this work provides rationale to alternate between AR inhibition and activation with BAT to prolong the lives of patients with this disease.

METHODS

Cell culture and reagents

LNCaP and VCaP cell lines were obtained from American Type Culture Collection (ATCC). LAPC4 and 22Rv1 cell lines were a gift from J Isaacs (Johns Hopkins, Baltimore). LNCaP, LAPC4, and 22Rv1 were grown in RPMI 1640 (Gibco; 11835-055) supplemented with 10% fetal bovine serum (Corning), sodium lactate 1.6mM, sodium pyruvate 0.5mM, L-alanine 0.43mM, 1% pen-strep (Gibco). VCaP was grown in DMEM (ATCC 30-2002) supplemented with 10% fetal bovine serum (Corning) and 1% pen-strep (Gibco). Full-length AR cDNA was cloned into the BamH1/Sal1 site of pLenti-CMV-GFP-Puro (Addgene; 17448, E Campeau and P Kaufman laboratories) and empty vector control was generated through excision of GFP of this plasmid.
These vectors, along with pCDH-puro-cMyc vector (Addgene; 46970, J Wang laboratory), pCDH-
EF1-FHC empty vector control (Addgene, 64874, R Wood laboratory), piSMARTvector-PGK-
TurboGFP-TRE3G-shAR vectors (Horizon; V3IHSPGG_8216343 and V3IHSPGG_7292640) were transfectioned into 293T cells (ATCC) along with pMD2.G (Addgene; 12259) and psPAX2 (Addgene; 12260) packaging vectors using lipofectamine (Invitrogen) to produce AR-puro, cMyc-puro, control-puro, and Tet-On-shAR-puro lentivirus particles. Two days after transduction with indicated virus, vector-expressing cells were selected with puromycin 1ug/ml for 72 hours. To constitutively express MYC in LAPC4-AR cells, LAPC4-AR cells were transfectioned with pCDNA3-HA-HA-humanCMYC vector (Addgene; 74164, M Roussel laboratory) using lipofectamine (Invitrogen) then selected for stable expression with G418 (Sigma) for 7 days. Cells were maintained at 37 °C in 5% CO2. They regularly tested negative for mycoplasma contamination using MycSensor PCR Assay kit (Agilent Technologies). R1881 was obtained from Sigma, enzalutamide from Selleck Chem, and testosterone cypionate from Steraloids.

**Proliferation, cell viability, clonogenic survival, and cell cycle analyses of cell lines**

For proliferation and cell viability assays, cells were plated in triplicate on 6-well (0.1 x 10^6 cells/well) or 12-well (0.05 x 10^6 cells/well) plates and incubated with R1881, enzalutamide, or vehicle control for indicated duration. Cells were counted using a hemocytometer with viability assessed by trypan blue exclusion. For clonogenic survival assessment, cells were plated on 6-well plates at low density (1500 cells/well) in 1 ml fresh media and 250 ul conditioned media obtained from a confluent flask of the parental cell line. Colonies were stained with crystal violet (Sigma; 0.5% in 20% methanol) after 10-20 days. For cell cycle analysis, cell pellets were resuspended in cold 70% ethanol at least overnight, then subsequently washed with PBS, and resuspended in 50
ug/ml propidium iodide (Sigma) and 100 ug/ml RNAse (Sigma) and run on the BD FACSCelesta flow cytometer with analysis using FlowJo software 10.4.2.

**Western blot analyses**

Cells or tissues were lysed with 1x denaturing lysis buffer (Cell Signaling technology) containing protease and phosphatase inhibitors (Roche). Protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Scientific), and 5-20 ug of lysate was resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk for 1 hour, then incubated with primary antibody overnight. Primary antibodies used were: anti-cMYC (Abcam, clone Y69, 1:1000 dilution), anti-AR (Santa Cruz, clone sc7305, 1:1000 dilution), anti-PSA (Dako, clone A0562, 1:1000 dilution), anti-vinculin (Millipore, clone V284, 1:2000 dilution). Anti-rabbit IgG, HRP-linked Antibody (CST, clone 7074S, 1:5000 dilution) and Anti-mouse IgG, HRP-linked Antibody (CST, clone 7076S, 1:5000 dilution) were used as secondary antibodies.

**Quantitative real-time polymerase chain reactions**

RNA was extracted using the RNeasy kit (Qiagen) and cDNA generated using the high-capacity cDNA reverse transcription kit (ThermoFisher). RT-PCR were performed in triplicate using 500ug cDNA, 10ul TaqMan Gene Expression Master Mix (ThermoFisher), and 1ul 20x TaqMan Gene Expression Assay probe mix for MYC (Hs00153408_m1), AR (Hs00907244_m1), KLK3 (Hs02576345_m1), and ACTB (Hs01060665_g1) (ThermoFisher) on an ABI7500 RT-PCR System (ThermoFisher). Relative gene expression was determined by delta-delta CT.

**Clinical trial design and procedures**
The COMbination of Bipolar Androgen Therapy and Nivolumab (COMBAT-CRPC; NCT03554317) clinical trial was a single-arm, multicenter, open-label phase II study of BAT in combination with the anti-PD1 agent nivolumab for patients with metastatic CRPC that had progressed on at least one novel androgen receptor-target therapy. The inclusion and exclusion criteria and pre-specified study end points were previously described (38). Patients were required to have soft tissue metastases amenable to biopsy to participate. Patients were treated with 3 cycles of BAT (testosterone cypionate 400 mg intramuscular every 28 days) followed by concurrent BAT and nivolumab 480 mg intravenously every 28 days until progression. Paired core-needle tumor biopsies were performed prior to treatment and after 3 cycles of BAT monotherapy. Response was assessed with PSA at each cycle, and CT chest, abdomen, and pelvis and technetium-99 bone scan every 3 cycles.

**Mouse models and tumor studies**

Male NSG mice aged 8-12 weeks were obtained from the Sidney Kimmel Comprehensive Cancer Center (SKCCC) Animal Core Facility and surgically castrated. SKCaP patient-derived xenograft tissue was minced, mixed with Matrigel (BD Biosciences) and implanted subcutaneously on the flank. Testosterone was administered by implantation of a slow-release subcutaneous pellet in the opposite flank. Pellets were assembled using 25 mm sections of Silastic Laboratory tubing, filled with 30 mg testosterone cypionate (Steraloids INC A6960-000), sealed on both ends with Silastic Medical Adhesive Type A, then sterilized. Enzalutamide (Selleck Chem) was administered by oral gavage 10 mg/kg/day in 200 ul 1% carboxymethyl cellulose, 0.1% Tween-80, 5% DMSO. Tumors were measured twice weekly using microcalipers, and tumor volume was calculated using the following formula: 0.5236 x L x W x H. At study completion, mice were euthanized and tumors were extracted. Tumors were flash frozen for subsequent lysis for immunoblot analyses and
formalin-fixed for subsequent immunohistochemistry analyses. All mice were housed in the Johns Hopkins animal facility.

**Data Availability**

The sequencing data described have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE213444.

**Statistics**

Results are displayed as mean ± standard deviation, unless otherwise indicated. Statistical comparison between groups was performed using a two-tailed Student’s t test using GraphPad Prism v8.0 software. Statistical comparison of proportions was performed using a Chi-squared test. Statistical comparison of time-to-event data was performed using Kaplan-Meier analysis with log-rank test using GraphPad Prism v8.0. Analysis of correlation was performed using Pearson’s correlation calculation. $p$ values less than 0.05 were considered statistically significant.

**Study Approval**

The COMbination of Bipolar Androgen Therapy and Nivolumab (COMBAT-CRPC; NCT03554317) clinical trial was approved by the Institutional Review Board at Johns Hopkins, and all accrued patients provided written informed consent. For animal studies, we have complied with all relevant ethical regulations in accordance with Johns Hopkins Institutional Animal Care and Use Committee.

**AUTHOR CONTRIBUTIONS**

Conceptualization: LAS, ESA, AMD, JTI, MCM, SRD
Methodology: LAS, RK, DES, ESA, AMD, JTI, MCM, SRD
Investigation: LAS, RK, DES, EAT, DMR, SLD, LA, YY, CGA, JLH, TJ, KAB, JNE, JM, AG, AS
Visualization: LAS, DES
Funding acquisition: ESA, SKK, SRD
Supervision: SY, JL, WNB, SKK, ESA, AMD, JTI, MCM, SRD
Writing – original draft: LAS
Writing – review & editing: All authors
The order of co-first authors was determined based on their contributions to the manuscript.

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REFERENCES


7. Markowski MC et al. A multicohort open-label phase II trial of bipolar androgen therapy in men with metastatic castration-resistant prostate cancer (RESTORE): A comparison of post-


15. Lam H-M et al. Durable response of enzalutamide-resistant prostate cancer to supraphysiological testosterone is associated with a multifaceted growth suppression and


35. Lee E et al. GREB1 amplifies androgen receptor output in human prostate cancer and contributes to antiandrogen resistance [Internet]. *Elife* 2019;8. doi:10.7554/eLife.41913


Figure 1. High pre-treatment AR activity is required and sufficient for growth inhibition by SPA. (A) AR, PSA, and MYC protein expression by western blot of prostate cancer cell lines treated with VEH or SPA for 72 hours. Representative blot of n=3 independent experiments. (B) AR protein expression by western blot of LNCaP expressing doxycycline-inducible shRNA against AR pretreated with indicated concentration of doxycycline (doxy) for 72 hours. Representative blot of n=2 experiments. (C) Viable cell counts of LNCaP-shAR pretreated with indicated concentration of doxycycline for 72 hours then VEH or SPA for 96 hours (n=3 independent experiments). (D) AR and MYC protein expression by western blot of LAPC4 expressing empty vector (EV) or AR treated with VEH or SPA for 7 days. Representative blot of n=3 independent experiments. (E) Viable cell counts of LAPC4-EV and LAPC4-AR cell lines treated with VEH or SPA for 7 days (n=3 independent experiments). (F) AR and MYC protein expression by western blot of 22Rv1 expressing empty vector (EV) or AR treated with VEH or SPA for 4 days. Representative blot of n=3 independent experiments. (G) Viable cell counts of 22Rv1-EV and 22Rv1-AR cell lines treated with VEH or SPA for 7 days (n=3 independent experiments). VEH, vehicle control EtOH 0.01%. SPA, R1881 10nM. (C, E, G) p value by unpaired two-tailed t-test comparing final cell counts. Biological replicates indicated in gray with mean of each independent experiment in color. i-shAR, inducible-short hairpin RNA against AR. For western blots, vinculin was used as a loading control.
**Figure 2. High pre-treatment AR is required for downregulation of MYC by SPA, which contributes to growth inhibition.** (A) MYC mRNA expression by quantitative PCR (qPCR) of prostate cancer cell lines treated with VEH or SPA for 72 hours (n=3 independent experiments). Ct-value was first normalized to ACTB for each sample, then to VEH for each cell line, and expressed as mean ± standard deviation with p values by unpaired two-tailed t-test with Welch correction for unequal variances. (B) MYC protein expression by western blot of LNCaP expressing doxycycline-inducible shRNA against AR pretreated with indicated concentration of doxycycline (doxy) for 72 hours then VEH or SPA for 96 hours. Representative blot of n=2 experiments. (C) AR and MYC protein expression by western blot of LNCaP-empty vector (LNCaP-EV) and LNCaP-MYC cell lines treated with VEH or SPA for 72 hours. Representative blot of n=3 independent experiments. (D) Viable cell counts of LNCaP-EV and LNCaP-MYC cell lines treated with VEH or SPA for 7 days (n=3 independent experiments). p value by unpaired two-tailed t-test. Biological replicates indicated in gray with mean of each independent experiment in color. (E) AR and MYC expression by western blot of LAPC4-EV, LAPC4-AR, and LAPC4-AR-MYC cell lines treated with VEH or SPA for 7 days. Representative blot of n=3 independent experiments. (F) Viable cell counts of LAPC4-EV, LAPC4-AR, and LAPC4-AR-MYC cell lines treated with VEH or SPA for 7 days (n=3 independent experiments). p value by unpaired two-tailed t-test. Biological replicates indicated in gray with mean of each independent experiment in color. VEH, vehicle control, EtOH 0.01%. SPA, R1881 10nM. For western blots, vinculin was used as a loading control.
Figure 3. High pre-treatment AR activity predicts clinical benefit from BAT. (A) Clinical trial design. CRPC, castration-resistant prostate cancer. BAT, Bipolar Androgen Therapy. T, testosterone. (B) Pre-BAT total AR optical density (OD) by image analysis among non-responders (NR) and responders (R) with median indicated by line (n=24). Responders are those with a PSA\textsubscript{50} response or objective response on C4D1. p value by unpaired two-tailed t-test. (C) Pre-BAT ARA\textsubscript{MW} score among NR and R with median indicated by line (n = 15). p value by unpaired two-tailed t-test. Percent change in PSA on C4D1 color-coded by ARA\textsubscript{MW} score. PSA\textsubscript{50} response indicated by dashed line. p value by Chi-squared comparison of proportions. (E) Percent change in tumor volume on C4D1 by ARA\textsubscript{MW} score. p value by unpaired two-tailed t-test. (F) Radiographic progression-free survival on BAT stratified by ARA\textsubscript{MW} score. p value by log-rank. (G) Overall survival on BAT stratified by ARA\textsubscript{MW} score. p value by log-rank. (H) Pre-BAT serum PSA among NR and R with median indicated by line (n=24). p value by unpaired two-tailed t-test. (I) Overall survival of patients in the SU2C/PCF cohort (n=81) stratified by ARA\textsubscript{MW} score. p value by log-rank.
**Figure 4.** BAT downregulates MYC in responding patients. (A) AR nuclear-to-cytoplasmic ratio (AR N:C) in paired tumor biopsies (n=24). (B) Percent change in AR N:C among non-responders (NR) and responders (R) with median indicated by line. (C) MYC H-score in paired tumor biopsies (n=24). (D) Percent change in MYC H-score among NR and R with median indicated by line. (E) Example of immunohistochemistry for MYC in paired biopsy samples from a responding patient. (F) Correlation of MYC RNA change from C1D1 to C4D1 with MYC protein change from C1D1 to C4D1 (n = 15). (G) Ki-67 H-score in paired tumor biopsies (n=24). (H) Percent change in Ki-67 H-score among NR and R with median indicated by line. (I) Example of immunohistochemistry for Ki-67 in paired biopsy samples from a responding patient. (J) Correlation of percent change in tumor volume from C1D1 to C4D1 with MYC protein change from C1D1 to C4D1 (n = 23; 1 patient excluded for lack of measurable disease). (K) Correlation of percent change in Ki-67 H-score with percent change in MYC H-score (n=24). (L) Percent change in MYC H-score stratified by ARASW score (n = 15) with median indicated by line. (M) Correlation of change in expression of genes within the 8q24 topologically associated domain (TAD) with change in MYC expression (n = 15). (A, C, G) p value by paired two-tailed t-test. (B, D, H) p value by unpaired two-tailed t-test. (F, J, K, M) r and p values by Pearson’s correlation calculation.
Figure 5. Downregulation of AR drives acquired resistance to SPA. (A) Cell cycle analysis by propidium iodide staining of LNCaP cells treated with VEH or SPA. Average values of n=2 independent experiments. (B) Clonogenic survival of LNCaP cells treated for 26 days with VEH or SPA, followed by 7 days rest without treatment, followed by treatment with dose of R1881 as indicated. Representative photograph of n=2 independent experiments. (C-E) AR, KLK3, MYC mRNA expression by qPCR of LNCaP cells treated with VEH or SPA (n=3 independent experiments). Ct-values were normalized to ACTB for each sample, then to VEH x 5 days, and expressed as median ± standard deviation with p values by unpaired two-tailed t-test. (F) AR, PSA, and MYC protein expression by western blot of LNCaP cells treated with VEH or SPA for 5 or 26 days. Representative blot of n=3 independent experiments. (G) Chromatin accessibility by ATAC-seq of the AR promoter (dotted box) of LNCaP cells treated with VEH or SPA for 5 or 26 days (performed in duplicate). (H) AR and MYC protein expression by western blot of LNCAP-EV and LNCAP-AR cells treated with VEH or SPA for 72 hours. Representative blot of n=3 independent experiments. (I) Cell cycle analysis by propidium iodide staining of LNCaP-EV and LNCaP-AR cells treated with VEH or SPA. Average values of n=3 independent experiments. VEH, vehicle control, EtOH 0.01%. SPA, R1881 10nM, LNCaP-SPAR, LNCaP with acquired resistance to SPA. For western blots, vinculin was used as a loading control.
Figure 6. BAT downregulates AR. (A) Total AR optical density (OD) by image analysis in paired tumor biopsies (n=24) color-coded by response. p value by paired two-tailed t-test. (B) Example of immunohistochemistry for AR (1:10,000 antibody dilution) in paired biopsy samples from a responding patient. (C) Correlation of AR RNA change from C1D1 to C4D1 with AR protein change from C1D1 to C4D1 (n = 15). r and p values by Pearson’s correlation calculation. (D) Correlation of AR protein change with pre-BAT total AR OD. r and p values by Pearson’s correlation calculation. (E) Schematic model of primary and acquired resistance to BAT.
Figure 7. Acquired resistance to SPA can be overcome by alternating between AR activation and inhibition. (A) Experimental design schematic. LNCaP-SPAR are LNCaP with acquired resistance to SPA. (B) Viable cell number of LNCaP and LNCaP-SPAR cells following treatment with VEH, SPA, or ENZA for 5 days (n=3 independent experiments). Biological replicates indicated in gray with mean of each independent experiment in color. Percent of VEH is indicated for ENZA-treated cells. (C) AR protein expression by western blot of cells treated as per a. Representative blot of n = 3 independent experiments. (D) MYC protein expression by western blot of LNCaP and LNCaP-SPAR cells following treatment with VEH or ENZA for 5 days followed by VEH or SPA for 5 days. Representative blot of n=3 independent experiments. (E) Viable cell number of cells treated as per d (n=4 independent experiments). p values by unpaired two-tailed t-test. Biological replicates indicated in gray with mean of each independent experiment in color. (F) Tumor size of SKCaP-1R patient-derived xenograft (PDX) following no treatment (Control; n=4 mice), continuous testosterone (SPA; n=4 mice), or SPA alternating with enzalutamide every 3 weeks (SPA-ENZA; n=3 mice). p value by unpaired two-tailed t-test comparing final measurements. (G) AR, and MYC protein expression by western blot of SKCaP-1R untreated (control) or treated with SPA. (H) Hematoxylin and eosin (H&E) staining and immunohistochemistry for MYC and Ki-67 of SKCaP-1R following no treatment (Control), continuous SPA for 7 or 160 days, or SPA-ENZA for 160 days. Representative photograph of n = 3 mice per group. (I) RNA in situ hybridization for AR and AR-V7 in tumors of SKCaP-1R untreated (control) or treated with SPA for 30 days. Representative photograph of n=3 mice per group. For western blots, vinculin used as a loading control.