Supraphysiological androgens suppress prostate cancer growth through androgen receptor-mediated DNA damage

Payel Chatterjee, … , Samuel R. Denmeade, Peter S. Nelson

*J Clin Invest.* 2019. [https://doi.org/10.1172/JCI127613](https://doi.org/10.1172/JCI127613).

Prostate cancer (PC) is initially dependent on androgen receptor (AR) signaling for survival and growth. Therapeutics designed to suppress AR activity serve as the primary intervention for advanced disease. However, supraphysiological androgen (SPA) concentrations can produce paradoxical responses leading to PC growth inhibition. We sought to discern the mechanisms by which SPA inhibits PC and to determine if molecular context associates with anti-tumor activity. SPA produced an AR-mediated, dose-dependent induction of DNA double-strand breaks (DSBs), G0/G1 cell cycle arrest and cellular senescence. SPA repressed genes involved in DNA repair and delayed the restoration of damaged DNA which was augmented by PARP1 inhibition. SPA-induced DSBs were accentuated in *BRCA2*-deficient PCs, and combining SPA with PARP or DNA-PKcs inhibition further repressed growth. Next-generation sequencing was performed on biospecimens from PC patients receiving SPA as part of ongoing Phase II clinical trials. Patients with mutations in genes mediating homology-directed DNA repair were more likely to exhibit clinical responses to SPA. These results provide a mechanistic rationale for directing SPA therapy to PCs with AR amplification or DNA repair deficiency, and for combining SPA therapy with PARP inhibition.

Find the latest version:

[http://jci.me/127613/pdf](http://jci.me/127613/pdf)
Supraphysiological Androgens Suppress Prostate Cancer Growth Through Androgen Receptor-Mediated DNA Damage

Authors:

Affiliations:
1Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA
2Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA
3Department of Medicine, University of Washington, Seattle, WA
4Department of Urology, Johns Hopkins University, Baltimore, MD
5Department of Laboratory Medicine, University of Washington, Seattle, WA
6Department of Urology, University of Washington, Seattle, WA
7Department of Oncology, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD

# These authors contributed equally.
† These authors contributed equally.

Conflict of Interests: P.S.N. is a paid consultant/advisor to Janssen and Astellas. M.T.S. is a paid consultant/advisor to Janssen and has received research funding to his institution from Janssen, AstraZeneca, Zenith, Pfizer, and Hoffmann-La Roche. E.S.A. is a paid consultant/advisor to Janssen, Astellas, Sanofi, Dendreon, ESSA, AstraZeneca, Amgen, Clovis, and Merck; he has received research funding to his institution from Janssen, Johnson & Johnson, Sanofi, Dendreon, Genentech, Novartis, Bristol Myers-Squibb, AstraZeneca, Celgene, Clovis, and Merck. E.S.A. and J.L. are co-inventors of a biomarker technology that has been licensed to Qiagen.

*Correspondence:
Samuel R Denmeade
The Sidney Kimmel Comprehensive Cancer Center,
The Johns Hopkins University School of Medicine,
Baltimore, MD 21287, USA.
Email: denmesa@jhmi.edu

Peter S. Nelson
Division of Human Biology
Fred Hutchinson Cancer Research Center
1100 Fairview Ave N, Mailstop D4-100
Seattle, WA 98109-1024
Email: pnelson@fredhutch.org
Abstract

Prostate cancer (PC) is initially dependent on androgen receptor (AR) signaling for survival and growth. Therapeutics designed to suppress AR activity serve as the primary intervention for advanced disease. However, supraphysiological androgen (SPA) concentrations can produce paradoxical responses leading to PC growth inhibition. We sought to discern the mechanisms by which SPA inhibits PC and to determine if molecular context associates with anti-tumor activity. SPA produced an AR-mediated, dose-dependent induction of DNA double-strand breaks (DSBs), Go/G1 cell cycle arrest and cellular senescence. SPA repressed genes involved in DNA repair and delayed the restoration of damaged DNA which was augmented by PARP1 inhibition. SPA-induced DSBs were accentuated in BRCA2-deficient PCs, and combining SPA with PARP or DNA-PKcs inhibition further repressed growth. Next-generation sequencing was performed on biospecimens from PC patients receiving SPA as part of ongoing Phase II clinical trials. Patients with mutations in genes mediating homology-directed DNA repair were more likely to exhibit clinical responses to SPA. These results provide a mechanistic rationale for directing SPA therapy to PCs with AR amplification or DNA repair deficiency, and for combining SPA therapy with PARP inhibition.
Introduction

Androgens, the androgen receptor (AR) and the AR-signaling program are intimately associated with the pathogenesis of prostate cancer (PC) (1-3). Suppressing AR-signaling through androgen deprivation therapy (ADT) was determined to be an effective approach for treating advanced PC in the 1940’s, and remains a key component of current treatment regimens (4). Despite substantial initial responses, metastatic PC almost universally develops resistance to ADT leading to a clinical state termed castration-resistant prostate cancer (CRPC). The emergence of CRPC is generally accompanied by a revival of AR-signaling (5-7). Collectively, current data indicate that the AR remains a viable target for the treatment of most men with metastatic CRPC.

Though the vast majority of therapeutic strategies directed toward the AR pathway are designed to inhibit signaling, there is longstanding evidence that a subset of PCs are repressed by the administration of testosterone (T), particularly after long periods of adaptation to growth in a low androgen environment (8-11). Abundant data from preclinical models reproducibly demonstrates biphasic responses, whereby at physiological androgen concentrations proliferation is induced, but at high supraphysiological androgen (SPA) concentrations, growth is suppressed (8, 9, 12). Similar effects have been observed in breast cancers exposed to high concentrations of estrogen (13, 14). Studies of PC metastases have shown inverse relationships between AR expression and measures of cell proliferation (15), and recent prospective clinical trials of SPA have demonstrated PSA declines and tumor regression in subsets of men with CRPC (10, 11). Consequently, understanding the mechanisms underlying the growth-repressive effects of SPA may serve to determine those individuals optimally treated by this approach and identify drug combinations that could synergize with SPA to produce more substantial and durable responses.

To date, several mechanism(s) have been postulated to underlie SPA-mediated growth repression including suppressing cMyc activity, upregulating negative cell cycle regulators such as p27, and impairing DNA licensing required for cycles of DNA replication (16-18). Notably, testosterone has been shown to induce DNA damage and promote the anti-tumor effects of ionizing radiation (19, 20). This observation is
consistent with studies of regulated transcription by nuclear hormone receptors whereby receptor activation induces transient DNA double strand breaks (DSBs) at enhancer and promoter regulatory sites in order to facilitate gene expression by releasing DNA topologies that constrain RNA polymerase function (19, 21, 22).

In the context of therapeutics, preclinical studies have demonstrated a paradox: though DNA repair genes may be regulated by AR, both ADT and SPA augment the effects of radiation-induced DNA damage and improve anti-tumor responses (20, 23, 24). The AR is recruited to enhancer and promoter regions of several DNA damage repair (DDR) genes and directly enhances their transcription (23-25). Reducing AR activity by ligand deprivation or AR antagonism down-regulates the expression of these DDR genes and synergizes with ionizing radiation to induce DNA DSBs, reduce DNA repair responses, and augment cell killing (23, 24). The impairment of AR-mediated DNA repair is postulated to underlie the enhanced survival benefit observed in patients concurrently treated with localized radiation therapy and ADT.

In the present study we sought to determine the mechanisms and the context by which SPA optimally exerts anti-tumor effects. We confirmed previous findings demonstrating that SPA induces DNA DSBs in the setting of AR expression, and found that elevated AR levels, as observed in a subset of men treated with ADT and progressing to CRPC, potentiate SPA-induced DSBs and rates of apoptosis. Further, PC cell lines or PDXs deficient in BRCA2 exhibit elevated DNA damage and cell death when exposed to SPA. The pharmacological inhibition of PARP1 or DNA-PKcs augmented these SPA effects. In support of these observations, metastatic CRPC patients with mutations in genes mediating homology-directed DNA repair were more likely to exhibit clinical responses to SPA. These results support specific clinical strategies designed to optimize the use of SPA for the treatment of men with CRPC.
Results

Supraphysiological concentrations of androgens induce DNA damage in prostate cancers expressing the androgen receptor

To determine the relationships between the AR, androgens, and the induction of DNA damage, specifically double-strand breaks (DSBs), we evaluated the effects of SPA on PC cell lines with or without native AR activity; LNCaP and PC3, respectively, or cells engineered to express high levels of AR; LNCaP\textsuperscript{AR} and PC3\textsuperscript{AR} (Fig 1A). We treated these cells with a range of androgen concentrations and measured DNA damage, senescence, and apoptotic responses. Normal eugonadal serum concentrations of testosterone (T) and the high-affinity T metabolite 5-\(\alpha\)-dihydrotestosterone (DHT) are \(~17\) nM (500 ng/dL) and \(~1\) nM respectively for men aged 60 years, with DHT approximately 5-fold more potent than T with respect to activating the AR (26, 27). In men treated with surgical or medical ADT, serum concentrations of T and DHT are \(~1\) nM and 0.1-0.5 nM, respectively (27). We used DHT, or the synthetic androgen methyltrienolone/R1881 as it exhibits AR binding affinities equivalent to DHT and unlike DHT is non-aromatizable. We exposed PC cells to charcoal-stripped growth medium depleted of androgens (CS-FBS), to reflect castrate conditions, 1 nM DHT/R1881 to reflect eugonadal concentrations, or 10 nM DHT/R1881 to reflect SPA concentrations. We quantified DNA DSBs by comet assays and by confocal immunofluorescence staining for \(\gamma\)H2AX and 53BP1.

Treatment with R1881 or DHT resulted in concentration-dependent increases in \(\gamma\)H2AX and 53BP1 foci in AR expressing cells (Fig 1B,C and Fig S1A-E). Very rare foci were observed in AR-null PC3 cells (Fig 1C and Fig S1B,D,E). LNCaP cells engineered to express AR \(~50\)% above the endogenous levels exhibited substantially higher numbers of DSBs at 1 nM R1881: 16 foci/cell compared to 2 foci/cell (p<0.01), which increased further with higher R1881 concentrations (p<0.01) (Fig 1B,C). AR-positive VCaP cells also showed increases in \(\gamma\)H2AX foci following SPA treatment (Fig S2A,D). The introduction of AR into PC3 cells resulted in substantial increases in \(\gamma\)H2AX and 53BP1 following exposure to androgens and the foci numbers increased with higher androgen concentrations (Fig 1C; Fig S1B,D,E). Comet
assays demonstrated evidence of DNA damage in both LNCaP and LNCaP^{AR} cells following SPA treatment: the tail moment and length both were significantly increased at 6 h compared to control, and persisted up to 24h in LNCaP^{AR} cells (Fig S1I,J). We also evaluated AR-null DU145 cells and LNCaP cells engineered to eliminate AR expression (LNCaP^{APPC}) (28). No increase in γH2AX foci were observed in either line after exposure to R1881 (Fig S2B,C), confirming that the AR is required for the androgen-induced generation of DNA DSBs.

The level of AR also influenced the kinetics of DSB resolution. The number of γH2AX foci as well as comet tail length and moment peaked at 6 hours after exposure to R1881 or DHT in parental LNCaP cells with endogenous AR expression, and the foci declined to near baseline levels by 24h (Fig 1D; Fig S1F,I,J). In LNCaP^{AR} cells, the number of foci further increased at 12h and persisted at 24h. Similar results were observed in PC3^{AR} and with DHT (Fig S1G,H). The time difference in foci persistence after R1881 versus DHT treatment could be explained by the rapid metabolism of DHT compared to R1881 (29, 30).

We determined that T exposure, in contrast to DHT and R1881 induced γH2AX and 53BP1 foci primarily in AR overexpressing LNCaP^{AR} and PC3^{AR} cells, and at an earlier 3 h time point (Fig S3A-F). Previous studies reported that T dissociates from the AR four times faster than DHT, and in agreement with these kinetics, by 6h damage foci were largely resolved (31).

As DNA damage can occur in the context of cell division with DNA replication, and AR activation can promote cell proliferation, we sought to determine if SPA could induce DNA damage in growth-arrested cells. We used isoleucine deprived medium (IDM) to arrest cells in G1 (Fig S4A), an approach previously shown not to influence AR expression (32). We observed no increase in DNA damage in LNCaP or LNCaP^{AR} cells grown in IDM. The addition of SPA to G1-arrested LNCaP and LNCaP^{AR} cells significantly increased DNA damage as measured by γH2AX and 53BP1 foci (Fig S4B-E).

**SPA increases nuclear AR, Serine 81 AR phosphorylation and AR transcriptional output**

Previous studies have determined that AR occupancy is saturated by ligand concentrations of ~2 nM
(Kd ~0.1-2 nM) (33-37), and consequently the mechanisms by which higher pharmacological concentrations of androgens exert biologic effects in PC remain to be established. Notably, the activity of the AR in prostate epithelium is influenced by mass action physiology involving the number of receptors, ligand concentrations, and the affinity and on/off rates of AR-ligand interactions. Though AR binding may be saturated in the eugonadal state, the type of androgen, for example T versus DHT, can substantially influence AR activity by altering dissociation rates. Prior studies have demonstrated that higher T concentrations can overcome more rapid off-rates relative to DHT, and overcome the potency deficit by mass action: T at concentrations above the levels required to saturate AR binding exert effects equivalent to DHT, potentially by stabilizing receptor-hormone complexes (38). Further, high DHT levels and supraphysiological T concentrations (20 nM) have been shown to increase cellular AR protein levels by enhancing rates of synthesis and reducing turnover (39).

To determine if SPA increases AR nuclear localization we quantitated AR and serine-81 phosphorylated AR (S81-AR) in PC cells. Phosphorylation of AR at serine-81 is regulated via CDK1 and CDK9 and is vital for ligand-stimulated AR binding to chromatin and for recruiting coactivators needed for transcription (40-42). Compared to PC cells exposed to eugonadal androgen concentrations in standard growth medium, SPA concentrations of 10 nM R1881 or DHT significantly increased nuclear S81-AR levels. PC cells engineered to overexpress AR also exhibited higher nuclear S81-AR and these levels were further increased by R1881 or DHT (Fig 1E,F and Fig S5A-E).

In PC, the liganded-AR regulates a diverse gene expression program that includes a subset of repressed genes and a larger number of genes where AR binding activates transcription, including those encoding well-characterized secreted proteins such as PSA/KLK3 (43). We measured transcript levels of KLK3, NKX3.1 and TMPRSS2 by qRT-PCR 8, 24, and 48 h following exposure to increasing concentrations of R1881 and DHT. Most notably at 48 h, each 10-fold increase in R1881 was accompanied by further increases in KLK3 and NKX3.1 and TMPRSS2 transcript abundance (Fig 1G). This association was magnified in LNCaP^AR cells overexpressing AR (Fig 1G). For DHT, transcript levels peaked at 24h and at a lower concentration (10 nM) (Fig S5F). Collectively, these results indicate that exposure to SPA can increase AR
nuclear localization and AR transcriptional output beyond that observed at eugonadal ligand concentrations reported to saturate AR binding.

**SPA inhibits prostate cancer cell growth and induces apoptotic and senescence responses in AR-overexpressing prostate cancer cells**

A consequence of cellular DNA damage is the engagement of potent growth arrest programs that serve to pause the cell cycle to allow repair mechanisms to correct damage, or to induce senescence or apoptosis if the extent of damage exceeds repair capacity. To evaluate the effects of SPA on these parameters, we seeded LNCaP, LNCaP\(^{AR}\), PC3 and PC3\(^{AR}\) cells in equal numbers and treated them with androgen levels approximating physiological (0.1-1.0 nM R1881) or SPA (10 nM R1881) concentrations. After 3 days, physiological equivalent androgen levels increased LNCaP cell growth 24% (p<0.01) whereas SPA (10 nM R1881) repressed growth 21% (p=0.04), and the growth repressive effects were accentuated in LNCaP\(^{AR}\) cells expressing high AR levels: 38% reduction by SPA versus a 26% increase in growth with 0.1 nM R1881 (p<0.01) (**Fig 2A**). The growth of AR-null PC3 and DU145 cells was unaffected by R1881 exposure whereas SPA repressed the growth of AR\(^+\) VCaP cells by 32% and PC3\(^{AR}\) cells by 31% (p<0.01)(**Fig S6A,B**). In accordance with these findings, SPA significantly reduced the fraction of LNCaP, LNCaP\(^{AR}\) and PC3\(^{AR}\) cells in the S phase of the cell cycle (**Fig 2B; Fig S6C**).

SPA exposure increased apoptosis by 45% (p<0.01) in PC3\(^{AR}\) cells compared to isogenic wild type PC3 cells (**Fig S6D**), though SPA did not increase apoptosis in VCaP, LNCaP or LNCaP\(^{AR}\) (**Fig S6E and data not shown**). However, SPA induced a more robust senescence response as measured by the expression of senescence-associated \(\beta\)-galactosidase (SA\(\beta\)Gal): in physiological androgen concentrations, 5% of LNCaP cells were SA\(\beta\)Gal positive whereas 30% of LNCaP cells stained for SA\(\beta\)Gal following SPA treatment (p <0.01) (**Fig 2C**). A significant increase in senescent cells was also measured in PC3\(^{AR}\) cells exposed to SPA concentrations (**Fig S6F**).
SPA attenuates DNA repair through homology-mediated and non-homologous mechanisms

Previous studies have demonstrated that the AR regulates a spectrum of genes involved in repairing DNA damage (23, 24). Repressing AR function with ligand depletion or AR antagonists reduces the expression of DNA repair genes and augments the effects of ionizing radiation including the potentiation of DNA DSBs (20, 23). These findings provide a mechanism explaining the clinical benefit of combining ADT with radiotherapy for localized PC (44, 45). We confirmed the previous findings by quantitating transcripts encoding genes involved in homology directed repair (HDR)- and non-homologous end-joining (NHEJ)-repair in LNCaP cells exposed to charcoal-stripped growth medium (CSS), analogous to ADT. ADT modestly reduced the expression of several genes comprising these pathways (Fig 2D). We also evaluated a panel of DNA repair genes demonstrated to be directly AR regulated by virtue of AR binding to regulatory androgen-response elements (ARE) (23). ADT modestly reduced the expression of a subset of these genes in LNCaP cells (Fig 2D). Notably, exposure to SPA significantly and substantially reduced the expression of DNA repair genes, most predominantly those involved in HDR and those previously determined to be direct AR targets (Fig 2D). For example, compared to LNCaP cells assessed in normal growth medium, cells assayed in CSS expressed significantly lower levels of BRCA2 transcripts (1.68 fold; p<0.010), whereas exposure to SPA levels (10 nM R1881) reduced BRCA2 transcripts 4-fold (p<0.001). These results indicate that in addition to inducing DNA damage, SPA represses the genes capable of mediating repair, a finding that may underlie the senescence responses observed in PC cells exposed to high androgen concentrations. We also evaluated the expression of genes involved in other DNA repair pathways such as mismatch repair (MMR), base excision repair (BER) and nucleotide excision repair (NER). Most of the changes were not significant and not consistent in LNCaP and VCaP cells which both exhibit DNA damage following SPA (Fig S7A,B).

To determine if SPA treatment results in a functional impairment of DNA repair, we assessed HR pathway proficiency by measuring RAD51 foci and NHEJ pathway activity by DNA end-ligation assays (46). Compared to control, LNCaP and LNCaP^AR cells exposed to 10 nM R1881 had no induction of Rad51 foci, indicating compromised HR repair (Fig S8A). NHEJ repair was also attenuated by SPA treatment.
Nuclear extracts from LNCaP, LNCaP^{AR}, PC3 and PC3^{AR} were able to efficiently re-ligate a linearized plasmid (Fig S8B). In contrast, nuclear extracts from LNCaP, LNCaP^{AR} and PC3^{AR} cells exposed to SPA for 6 h or 24 h failed to ligate DNA. SPA did not impact DNA end-ligation in wildtype AR-null PC3 cells (Fig S8B).

**PARP inhibition augments SPA-induced DNA damage and induces cellular senescence**

Having established that androgens consistently induce DNA damage in PC cells through mechanisms that are sensitive to both AR levels and ligand concentrations, we next sought to determine if inhibiting DNA repair mechanisms would further augment SPA-induced DNA damage and promote apoptosis or senescence. We first targeted PARP1, a multifunctional enzyme involved in the repair of DNA strand breaks (47). Treatment with the PARP1 inhibitor olaparib (OLA) slightly increased the number of γH2AX foci from ~1 foci per cell (FPC) in vehicle treated LNCaP cells to 4 FPC in OLA treated cells (p<0.01)(Fig 2E,F). A similar response was observed in PC3 cells (Fig 2G). Overexpression of the AR in both lines increased OLA-induced DSBs (p<0.001). Further, OLA exposure substantially increased both γH2AX and 53BP1 foci in LNCaP, LNCaP^{AR}, and PC3^{AR} cells when given concurrently with SPA, and the extent of DSBs was associated with AR expression: the number of γH2AX foci/cell increased from a baseline of 1 in vehicle-treated LNCaP to 16 in LNCaP cells treated with both OLA and 10 nM R1881 (p<0.01) to 49 FPC in LNCaP^{AR} cells treated with both OLA and 10 nM R1881 (p<0.01) (Fig 2E,F). OLA treatment alone modestly reduced the growth of LNCaP or LNCaP^{AR} cells by 10% and 17.5%, respectively (Fig 2H). The addition of OLA to SPA further augmented the growth-repressive effects of SPA alone, from 38% to 45% in LNCaP^{AR} cells, though OLA did not further augment effects of SPA on PC3^{AR} growth (Fig 2H; Fig S6H). The combination of OLA and SPA did not increase apoptosis rates (Fig S6D), but the number of senescent cells was substantially increased: the addition of OLA to SPA increased SA-βGal positive cells from 30% to 50% (p<0.01) and 20% to 35% (p<0.01) in LNCaP^{AR} and PC3^{AR} cells, respectively (Fig 2I; Fig S6G).
DNA-PKcs inhibition attenuates SPA-induced DNA damage and promotes cell survival

The mechanisms promoting the repair of DNA DSBs exhibit cell cycle dependency with homology-directed repair occurring in S/G2 and NHEJ occurring throughout the cell cycle phases where it is the predominant mechanism of repair in G1 (48). As previously reported, SPA induces PC cell growth arrest in G1 (Fig 2B) (49, 50), therefore instead of HR, AR/SPA-induced DSBs should primarily undergo repair by NHEJ. To evaluate NHEJ activity we focused on a key regulatory component of NHEJ, DNA dependent protein kinase, catalytic subunit, DNA-PKcs. Following DNA DSBs, DNA-PKcs is recruited by the DNA end-binding Ku70/80 heterodimer and undergoes rapid autophosphorylation at S2056 (pS2056) (46, 51, 52). Subsequent phosphorylation on T2609 (pT2609; in the ABCDE cluster) by ATM or ATR leads to its dissociation from the DNA ends, which is required for the progression of NHEJ repair (52-54).

Treatment of LNCaP cells with 10 nM R1881 produced significant increases in pS2056 DNA-PKcs foci after 6h (from 1 to 11 foci/cell; p=0.05) (Fig 3A,C), indicating hyperactivation of DNA-PKcs, followed by foci resolution after 24h (Fig 3C). LNCaP\textsuperscript{AR} and PC3\textsuperscript{AR} cells showed augmented and sustained pS2056 DNA-PKcs foci: at 6 h, the number of foci per cell in LNCaP and LNCaP\textsuperscript{AR} cells measured 10 and 19, respectively (p<0.01) and at 24 h, the number of FPC in LNCaP and LNCaP\textsuperscript{AR} cells measured 4 and 29, respectively (p<0.01) (Fig 3B,C). As S2056 phosphorylation is dose-dependent, increased DNA damage will induce more pS2056 foci, which is concordant with more damage foci observed in LNCaP\textsuperscript{AR} and PC3\textsuperscript{AR} cells compared to the parental cells. AR\textsuperscript{+} VCaP cells also demonstrated pS2056 DNA-PKcs foci following SPA treatment whereas in AR-null PC3 and LNCaP\textsuperscript{APiPC} cells, no pS2056 DNA-PKcs foci were observed (Fig 3D; Fig S8C,D).

We next sought to determine the effects of PARP inhibition with respect to DNA-PKcs activity following SPA-induced DNA damage. OLA treatment alone had insignificant effects on either S2056 or T2609 DNA-PKcs phosphorylation (Fig 3A-D), consistent with the minimal induction of DNA damage resulting from single agent OLA (Fig 2E-G). In contrast, the addition of OLA to SPA significantly increased pS2506 foci in AR-expressing PC cells (Fig 3B,C,D). These foci peaked at 6h and largely resolved in parental
LNCaP cells 24h after treatment, but further increased in the LNCaP\textsuperscript{AR} and PC3\textsuperscript{AR} cells expressing high levels of AR (Fig 3C,D). Collectively, these findings may reflect the PARP1 trapping effects of OLA that delay PARP dissociation and impair DNA repair (55).

Previous reports determined direct interactions between AR and DNA-PKcs, with DNA-PKcs serving as a co-activator of AR transcriptional function (24). Regulated transcription by nuclear hormone receptors involve DNA DSBs that are mediated by TOP2B and a complex that includes PARP1, Ku70/80 and DNA-PKcs (21, 22). We hypothesized that AR over-expression and enhancement of AR activity via SPA, may serve to maintain this regulatory complex on DNA and impair dissociation. In order to determine the status of DNA-PKcs dissociation we examined T2609 phosphorylation, which is a necessary event for subsequent DNA-PKcs displacement required for the remaining steps in NHEJ repair (54). Parental LNCaP cells treated with SPA exhibited the expected auto-phosphorylation event at S2056 followed by the resolution of these foci at 24h and the gain of pT2609 foci at 24h (Fig 3C,E,F). SPA further increased pS2056 foci (24 hrs) in LNCaP\textsuperscript{AR} cells overexpressing AR without measurable changes in pT2609 foci (Fig 3E,F), indicating that DNA-PKcs is hyperactivated and persists on chromatin in AR over-expressing cells leading to defective dissociation and potentially impaired DNA repair (Fig S8E). We also did not observe substantial changes in pT2609 foci in PC3\textsuperscript{AR} cells exposed to SPA (Fig 3G). OLA treatment increased pT2609 DNA-PKcs foci in LNCaP cells, but not in LNCaP\textsuperscript{AR} or PC3\textsuperscript{AR} cells where these foci were not observed (Fig 3F,G).

We next sought to determine whether inhibition of DNA-PKcs would reduce or augment DNA damage induced by SPA. Whereas SPA exposure induced DNA DSBs in LNCaP cells, with further increases in AR over-expressing LNCaP\textsuperscript{AR} and PC3\textsuperscript{AR} cells, treatment with the DNA-PKcs inhibitor Nu7441 eliminated the induction of γH2AX foci (Fig 4A,B). Treatment of LNCaP and LNCaP\textsuperscript{AR} cells with Nu7441 nearly abolished the DNA-PKcs pS2056 foci resulting from SPA exposure (Fig 4C). Treatment with Nu7441 also substantially reduced nuclear S81-AR foci and S81-AR phosphorylation, supporting previous studies demonstrating a co-regulatory role for DNA-PKcs enhancing AR activity (Fig 4D, Fig S8F) (24). The abrogation of measurable DNA damage by DNA-PKcs inhibition translated to the loss of PC growth
repression without the induction of apoptosis and senescence following SPA treatment. Individually, SPA but not Nu7441 reduced cell viability by 21% relative to vehicle in LNCaP cells. However, the addition of Nu7441 to SPA enhanced the growth of LNCaP, LNCaP\textsuperscript{AR} and PC3\textsuperscript{AR} cells relative to SPA alone (Fig 4E,F). The growth of AR-null PC3 cells was not affected by either single agent or combination drug treatment (Fig 4F).

Prostate cancers deficient in BRCA2 exhibit enhanced sensitivity to SPA, PARP antagonists and DNA-PKcs inhibition

Recent molecular profiling studies have determined that ~20% of metastatic PCs exhibit aberrations in genes mediating homology directed DNA repair (HDR), most notably BRCA2 (56). In HDR defective cells, most of the DNA damage repair occurs by NHEJ. We hypothesized that in the absence of HDR, SPA treatment would induce more substantial DNA damage and consequent impairment of cell survival. To evaluate this possibility, we created a tetracycline inducible shRNA BRCA2 cell line (LNCaP\textsuperscript{shBRCA2}) and separately genetically modified LNCaP cells by CRISPR/Cas9 to generate a line of BRCA2 deficient LNCaP cells (LNCaP\textsuperscript{BRCA2}) (Fig 5A and Fig S9A). Compared to parental LNCaP, SPA induced substantially greater γH2AX foci in LNCaP\textsuperscript{shBRCA2}, 5 FPC versus 33 FPC (p<0.001)(Fig 5B,C). Similar effects were observed in LNCaP\textsuperscript{BPRC2} cells (Fig S9B,D). The enhanced effects of SPA in LNCaP\textsuperscript{shBRCA2} were further augmented by OLA with 33 γH2AX foci produced by SPA, 23 foci by OLA, and 50 foci produced by SPA plus OLA (Fig 5B,C). Similar effects were observed in LNCaP\textsuperscript{BRCA2} cells exposed to SPA and OLA (Fig S9B,D). SPA also induced a greater number of pS2056 DNA PKcs foci which was further increased by OLA (Fig 5D,E and Fig S9C,E). Treatment of LNCaP\textsuperscript{shBRCA2} cells with SPA, the DNA-PKcs inhibitor Nu7441, or OLA increased caspase activity by 15% (p<0.01), 22% (p<0.01) and 13% (p<0.05), respectively (Fig 5F) with similar effects observed in the LNCaP\textsuperscript{BRCA2} cells (Fig S9F). Combining Nu7441 with SPA further augmented caspase activity and growth repressive effects of SPA: 15% growth inhibition with SPA alone versus 37% with the combination of SPA and Nu7441 (p<0.01)(Fig 5G, Fig S9G). Exposure of
LNCaP cells to doxycycline did not enhance DNA damage, trigger caspase activity or reduce growth significantly (Fig S9H-J).

To provide further evidence supporting the hypothesis that HR-deficient PCs exhibit enhanced sensitivity to SPA-induced DNA damage, we evaluated the acute in vitro effects of SPA in three patient derived xenograft (PDX) lines: LuCaP96CR with BRCA2 loss, and LuCaP35 and LuCaP70, without known HDR defects (Fig S10A,B) (57). Cells from PDX tumors were established as short-term in vitro cultures, and effects following acute exposure to SPA (18h) or longer treatment (72h) were measured either by DNA damage markers or by growth and caspase assays. At a time point 18 hours after SPA treatment, LuCaP96CR cultures exposed to 10 nM R1881 increased γH2AX foci from a steady-state level of 3 foci/cell to 36 foci/cell (p<0.01)(Fig 5H,I). OLA treatment also increased γH2AX foci from 2-3 foci/cell to 20 foci/cell and the combination of SPA and olaparib increased the foci number to 50 foci/cell (p<0.01) (Fig 5H,I). SPA also increased DNA-PKcs pS2056 foci from 3 in vehicle treated cells to 34 foci/cell with SPA, and the combination of SPA and OLA further increased pS2056 foci/cell from 30 to 45 (p<0.01) (Fig 10C).

In contrast to the marked DNA damage effects observed in LuCaP96CR cells, tumor cells from LuCaP35 and LuCaP70 PDX lines exhibited modest increases in γH2AX foci/cell following SPA treatment (2 to 11 and 2 to 22, respectively) measured 4h after SPA treatment or the combination of SPA and OLA (Fig 5I). The damage was largely resolved in these cells by 18h whereas significantly greater damage foci persisted in LuCaP96CR cells (Fig 5I).

We confirmed these findings ex-vivo using tissue slice cultures of castration-resistant LuCaP35CR and LuCaP96 tumors to maintain the 3D architecture of tumor cells and microenvironment constituents. After 3 days of exposure to SPA, LuCaP96 tumors exhibited persistent DNA damage in the form of γH2AX foci (Fig S10D,E). The combination of OLA and SPA further induced damage foci (p<0.01) compared to SPA or OLA alone. While acute exposure to SPA and/or OLA increased γH2AX foci in LuCaP35CR cells which have intact BRCA2, the foci did not persist and were equivalent to pretreatment levels 3 days after treatment (Fig S10D,E).
Prostate cancer patients with DNA repair deficiency exhibit enhanced clinical responses to SPA

Given that SPA is able to induce DNA damage in PC preclinical models, we hypothesized that mutations in genes involved in DNA damage repair would associate with improved responses to Bipolar Androgen Therapy (BAT) in patients with CRPC (10). To test this, we obtained biospecimens from CRPC patients enrolled in ongoing clinical trials testing BAT, a form of intermittent high-dose testosterone designed to produce rapid fluctuations in serum testosterone from the near castrate to supraphysiological (>1500 ng/dl) range over the course of one month (10). We performed germline or somatic next-generation sequencing on biospecimens that included plasma (i.e. cell-free DNA) (N=79), tumor tissue (N=21) and saliva (N=10). Several clinical grade NGS platforms were used. Given the concern for false negatives, cases were excluded if plasma NGS did not reveal any somatic alteration. Absence of a germline alteration was not assumed to indicate absence of somatic alterations.

Most patients received BAT following one or more next-generation AR pathway antagonists, while 6 received BAT as first-line CRPC therapy. Of 65 cases where a germline or somatic pathogenic alteration in any gene was detected, 29 (45%) had evidence of homology directed repair deficiency (HRD), with mutations found in BRCA2 (N=10), ATM (N=8), CHEK2 (N=5), PALB2 (N=3), CDK12 (N=3), CHD1 (N=2), FANCA (N=1), FANCD2 (N=1) and BRCA1 (N=1). Mutations in HR genes associated with increased PSA$_{50}$ responses (i.e. ≥50% decline in PSA from baseline): 15/29 (52%) patients with HRD demonstrated a PSA$_{50}$ response compared to only 6/33 (18%) patients without HDR (Chi$^2$ $P = 0.005$) (Fig 6, Table S1).
Discussion

Despite compelling preclinical evidence demonstrating that exposure to high concentrations of androgens can retard PC growth, particularly after adaptation to ADT, clinical outcomes have not been consistent. Four small contemporary trials of testosterone treatment in CRPC have been reported, with two trials of continuous administration that did not achieve supraphysiological levels showing limited clinical responses (58, 59), and two trials using an intermittent ‘bipolar’ treatment regimen achieving transient supraphysiological levels reporting more robust responses with PSA declines (>50%; PSA_{50}) or radiographic responses in 30-50% of men (10, 11). Our results indicate that high SPA concentrations combined with high AR levels produce the most substantial and sustained DNA damage, with attendant growth arrest and cellular senescence. These results may explain divergent clinical responses observed in patients treated with different testosterone regimens.

The AR positively regulates a spectrum of DNA repair genes, and repressing AR activity is documented to attenuate DNA repair and promote radiation-induced cytotoxicity (23, 24). However, prior studies have determined that the expression of several DNA repair genes (e.g. BRCA2, ATM, others) in CRPC is inversely related to AR activity (60), and the AR has been shown to directly repress genes involved in DNA replication including several with repair functions (25). We confirmed that several genes encoding DNA damage/repair proteins are down-regulated by ADT in vitro, notably those involved in homology-directed repair, but we also determined that SPA exposure repressed these genes to a significantly greater extent. These findings indicate that SPA may synergize with PARP inhibitors (PARPi) or DNA damaging therapeutics (20). We determined that SPA-induced DNA damage occurs within hours, and the extent of DNA damage is correlated with AR overexpression and higher ligand levels, which in combination suppressed growth and induced senescence and apoptosis. These results are compatible with a mechanism of growth repression via transcription-associated AR-programmed double-strand DNA breaks (19, 21, 61).

Our findings support a critical role for DNA-PKcs, a key kinase involved in NHEJ-mediated DNA repair, as a modulator of SPA induced AR mediated DNA damage. In AR over-expressing cells, we
observed enhanced and persistent DNA-PKcs phosphorylation foci at S2056, which is important for DNA-PKcs activation and chromatin binding. SPA prolonged S2056 DNA-PKcs phosphorylation, and impeded Thr2609 phosphorylation, an event which is ultimately required for DNA-PKcs dissociation from chromatin. These effects were amplified by co-treatment with the PARP inhibitor olaparib. Our data suggest that retention of DNA-PKcs on chromatin in AR over-expressing cells hinders completing the DNA repair process confirmed by an impaired DNA end-ligation assay, contributing to the elevated and persistent DNA damage induced by SPA. Notably, a previous study determined that AR splice variants (ARVs) could promote the repair of DNA damage induced by radiation via binding of ARVs to the catalytic subunit of DNA-PK (62). In contrast to ADT which increases the expression of ARVs, SPA has been shown to repress the expression of ARVs (10), a result expected to compromise ARV-mediated DNA repair and promote growth arrest.

To further investigate the clinical context where SPA may be most efficacious, we evaluated tumors with BRCA2 loss and consequent HRD. We observed enhanced responses to SPA including accentuated DNA damage and apoptosis in such tumors. These findings are congruent with our clinical data showing associations between HR gene mutations and favorable responses to BAT. These findings support clinical studies of SPA in combination with PARP inhibitors and trials evaluating this approach have recently been initiated [clinicaltrials.gov: NCT03516812]. This work also supports clinical trials testing SPA in combination with DNA-PKcs inhibitors and in PC patients with AR amplification or that exhibit HR gene mutations.
Materials and Methods

Study design. The overall objective of this study was to determine the mechanism(s) by which supraphysiological concentrations of androgens (SPA) repress prostate cancer growth. We used multiple prostate cancer cell lines and preclinical models with variable expression (de novo or engineered) of the androgen receptor (AR) and quantitated cellular responses across concentration ranges of androgens. We evaluated the effects of combining SPA with pharmacological inhibition of DNA repair pathway components including PARP and DNA-PKcs. We engineered cells to inactivate BRCA2 and quantitated phenotypic responses to SPA relative to intact BRCA2. Standard methods and measurements were used to assess proliferation, apoptosis, senescence, DNA damage, and gene expression. A minimum of 3 biological replicates for each in vitro assay were obtained and are shown in the text and/or figure legends. Clinical samples were obtained from patients enrolled on studies of SPA and clinical grade NextGen sequencing assays were used to determine germline or somatic mutations in genes known to mediate DNA repair. Clinical responses were determined by study personnel blinded to the results of DNA repair gene mutations.

Cell culture and treatment: PC cell lines PC3, LNCaP, VCaP and DU145 were obtained from American Type Culture Collection (ATCC, Manassas, VA). The creation of PC3<sup>AR</sup> and LNCaP<sup>APIPC</sup> have previously been described (15, 31). LNCaP<sup>AR</sup> were kindly provided by Charles Sawyers. All cells were cultured as recommended by their suppliers and genotyped for authenticity by STR analysis. All cells were confirmed negative for mycoplasma. DHT (Cayman Chemical: Ann Arbor, MI), R1881 (Perkin Elmer: Waltham, MA), testosterone (T) (Sigma: St. Louis, MO), olaparib (Sellek Chemical: Houston, TX), and the DNA-PKcs inhibitor Nu 7441 (Tocris Bioscience: Bristol, UK) were used in this study. Cells were treated with DHT, T or R1881 and olaparib simultaneously for 24h for confocal studies and 72h for cell death and survival assays. The derivation and propagation of the LuCaP PDX models were described previously (57). For ex vivo studies, LuCaP 35, 70 and 96CR PDX tumors were dissociated using Miltenyi Biotec’s tumor dissociation kit (Bergisch Gladbach, Germany), plated in 6 well dishes and treated with R1881, olaparib or the combination.
Confocal microscopy: Cells were plated on coverslips in 6 well dishes except for LuCaP 96CR, where coverslips were not used. After treatment, cells were fixed with 3.0% paraformaldehyde for 20 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked in 3% fetal bovine serum in PBS containing 0.1% Triton X-100 for 1 h. The coverslips were immunostained using γH2AX (Millipore: Burlington, MA), 53BP1, S2056 DNA PKcs, Thr2609 DNA-PKcs (Abcam, Cambridge, UK) and RAD51 (Santa Cruz Biotechnology: Dallas, TX) primary antibodies, followed by fluorescently-conjugated secondary antibodies (Invitrogen: Carlsbad, CA). When possible, cells were co-stained with multiple antibodies. Mounting and staining of the nuclei were performed using Vectashield containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories: Burlingame, CA). The number of foci from 70-100 cells were manually counted across multiple microscope fields. For each field the average number of foci was determined per cell. The average of the fields was then plotted. Images were analyzed by Image J (National Institute of Health: Bethesda, MD).

Western blot: Cells or fragments of LuCaP xenografts were washed once in 1x PBS before lysis in 1% SDS, 1% NP-40, 2% Tween-20, 1.5M urea in PBS with protease inhibitors and phosphatase inhibitors obtained from Pierce Biotechnology (Waltham, MA). Lysates were collected with a cell scraper and boiled for 2 min. DNA was sheared by sonication the lysates were subjected to immunoblotting as described (63) and probed with antibodies against S81AR (Santa Cruz Biotechnology: Dallas, TX), BRCA2 (Millipore Sigma: Burlington, MA), β-Tubulin (Sigma, St. Louis, MO), AR, and GAPDH (both from Genetex, Irvine, CA).

Growth Assays: Cell growth was assayed by plating 5000 cells per well in a TC-treated 96-well black-sided, clear bottom plate (Corning: Corning, NY) and allowed to adhere for 24 hours then treated with R1881 or T, DHT, olaparib and/or Nu 7441 for 72 hours and assayed for apoptosis and viability using ApoLive Glo (Promega: Madison, WI) following the manufactures instructions.

Cell cycle Analysis: Cell-cycle distribution was determined by flow cytometry for cells treated with R1881 and olaparib for 72 hours. Cells were fixed in 70% ethanol and incubated in a solution containing propidium iodide (PI; 50 mg/mL), RNase A (0.1 mg/mL), Triton X (0.05%), and analyzed on a Canton 2-2 flow
cytometer (Beckton Dickinson: Franklin Lakes, NJ). The raw data obtained were analyzed by Flowjo version 10 software (Ashland, OR). The results were normalized to control cells.

**Cell synchronization by isoleucine-deprivation:** Isoleucine-deprivation was performed by allowing the cells to become sub-confluent and then replacing the media with isoleucine-depleted RPMI as previously described (32).

**Comet Assay:** The comet assay silver staining kit (Trevigen, Gaithersburg, MD) was used following the manufacturer’s protocol as previously described (46). Image analysis and quantification were conducted via Image J (NIH: Bethesda, MD). Tail moment (TM) and tail length (TL) were measured to quantify DNA damage. TM= % of DNA in the tail X TL; where % of DNA in the tail = tail area (TA) X tail average density (TAI) X100/(TA X TAI) +[head area (HA) X head area intensity (HAI)]

**DNA end-ligation assay:** One microgram of EcoRI-digested pUC19 DNA (as a surrogate for DSBs) was treated with nuclear extracts of LNCaP, LNCaP\textsuperscript{AR}, PC3 and PC3\textsuperscript{AR} cells (either untreated or treated with R1881 for 6 h and 24 h) in reaction buffer (46). The end-ligation mixtures were incubated at 37C for 30 min-1 h and separated by electrophoresis on 0.6% agarose gels. Linearized EcoRI-digested pUC19 DNA with or without protein extract was used as negative control, and linearized pUC19 DNA treated with T4 DNA ligase was used as a positive control.

**Senescence Assay:** The senescence assay was performed by using a senescence β-galactosidase staining kit (Cell Signaling: Danvers, MA) following the manufacturer’s instructions. Cells were plated in 6 well plates one day before treatment with R1881 and or olaparib. After three days, cells were fixed and stained with staining solution containing X-gal. The percentage of β-galactosidase-positive cells was determined by counting five different fields (~70 cells/sample).

**Whole transcriptome sequencing (RNAseq) and analysis.** Biological replicate cultures of LNCaP cells grown in phenol-red free RPMI-1640 supplemented with 10% FBS were treated for 24 hours with media containing either 10% FBS, 5% charcoal-stripped serum (ADT), or 5% charcoal-stripped serum plus 100 nM R1881 (SPA). RNA was isolated, sequenced, aligned, and analyzed as previously described (28). RNA
sequencing data are deposited in the Gene Expression Omnibus database under the accession number GSE119598.

**Ex-vivo tissue culture and assays of treatment responses:** We used gelatin or collagen sponges (Vetspon; Sigma: St. Louise, MO) as a scaffold for culturing LuCaP 35CR and LuCaP 96 tumors that were dissected into 1–2 mm³ slices following published methods (64). After 24h, tissue slices were treated for 3 days continuously with 50 nM R1881 or 25 µM olaparib or the combination. As indicated in the protocol, drug concentrations were 5-fold greater than standard tissue culture concentrations. After 3 days the tumor slices were placed in OCT (Fisher Healthcare: Hampton, NH) and flash frozen in liquid nitrogen. Frozen sections (0.8 micron) were cut for staining. Confocal microscopy was performed by fixing tissue sections with paraformaldehyde after removing the OCT.

**RNA collection and quantitative real-time PCR.** Total RNA was isolated from 6-well cell culture plates using an RNEasy kit (Qiagen: Hilden, Germany) following the manufacturer’s protocol. An Applied Biosystems 7900 sequence detector with SYBR Green PCR master mix (Invitrogen: Carlsbad, CA) was used for qRT-PCR. PrimerQuest (IDT: San Jose, CA) was used to design primers, and reactions were normalized to the expression of the housekeeping gene RPL13A. A water negative control did not produce significant amplification products. Primer sequences were as follows: AR 5’-GAATGAGGCACCTCTCTCAAG-3’, 5’-CAGCCCATCCACTGGAATAA-3’; KLK3 5’-GCATGGGATGGGGATGAAGTAAG-3’, 5’-CATCAAAATCTGAGGTTGTCTGGA-3’; RPL13a 5’-CCTGGAGGAGAGGAAGGAAAAGA-3’, 5’-TTGAGGACCTCTGTGTATTTG-3’; NKX3.1 5’-ACTAATGAGGTACCGTCAAGGC-3’, 5’-TGGCCAACCTTCACTAATTATATGAG-3’; TMPRSS2 5’-CATGATCTGGCCGGCTTCCTGCAGG-3’, 5’-CTTGATATCCCTATCGCCACCAGATA-3’.

**Establishment of BRCA2 deficient LNCaP cell lines.** We established two LNCaP cell line models with BRCA2 deficiency. We constructed an inducible BRCA2 knock-down model using a doxycycline/tetracycline system. A Tet-shBRCA2 construct was generated by cloning the following sequence (GGGAAACACTCAGATTAAA_TGACTAGT_TTTAATCTGAGTGTTTCCC_TTTTTT)(65) into the EZ-Tet-pLKO-Hygro vector (Addgene plasmid 85972), as previously described (66). LNCaP cells were
infected with lentivirus and selected with 500ug/mL hygromycin. Knockdown was induced by addition of 100ng/mL doxycycline to media for at least 96 hours. Samples were lysed with RIPA lysis buffer and normalized by BCA assay (Pierce). Lysates were run on 3-8% tris acetate SDS gels (Invitrogen) and transferred onto Immoblin-FL PVDF membrane (Millipore). Primary antibodies include: BRCA2 (Cell Signaling Technologies, 10741, 1:2000) and hFab-Rhodamine-Tubulin (BioRad, 12004165, 1:5,000). Secondary antibody includes: StarBright520-goat-anti-rabbit (BioRad, 12005869, 1:5,000). Antibodies were diluted in blocking buffer (5% BSA/TBST). Images were acquired on a BioRad ChemiDocMP fluorescence imaging system.

We used a CRISPR/Cas9 approach to delete BRCA2 in LNCaP cells. To create the sgRNA targeting BRCA2, an sgRNA protospacer of GAAACCATCTTATAATCAGC was cloned into the ESP3I enzyme (Thermofisher: Cambridge, MA) sites of the lentivirus expression vector lentiCRISPRv2 (Plasmid #52961, www.addgene.org) (from PMID 25075903) using annealed oligos + BRCA2_sgRNA+: caccgGAAAC-CATCTTATAATCAGC BRCA2_sgRNA: aaacGCTGATTATAAGATGGTTTCc. LNCaPs were transduced with the BRCA2 sgRNA CRISPR vector and selected for 5 days with puromycin. They were then plated 1 cell/well in 96 well plates. Resulting colonies were screened by western blot for loss of BRCA2 expression using an antibody from Millipore (Burlington, MA) and a particular isolate (no. B11) was chosen for the experiment. Further analyses of isolate B11, designated here as LNCaPBRCA2, demonstrated a mixed-heterozygous population comprising cells with wild-type sequence and cells with 102 or 21 bp deletions detected.

Clinical Study Design. The primary goal of the clinical study was to evaluate the genomic discriminators of response/resistance to SPA using an approach administering monthly high-dose testosterone, termed Bipolar Androgen Therapy (BAT), in men with CRPC. Supraphysiological testosterone alternate with low or near-castrate levels. We obtained biospecimens from CRPC patients enrolled into two ongoing clinical trials testing BAT. The clinical studies were approved by institutional review boards (IRBs) of the Johns Hopkins University and the University of Washington/Fred Hutchinson Cancer Research Center. Informed consent was obtained after the nature and possible consequences of the studies were explained.
The first study is a Phase II trial testing BAT following progression on either enzalutamide (Cohort 1), abiraterone (Cohort 2), or medical/surgical castration alone (Cohort 3) [clinicaltrials.gov: NCT02090114] (11). Following progression on BAT, patients were re-challenged with the agent they received prior to the enrolling (i.e. enzalutamide, abiraterone or ADT). The co-primary endpoint was PSA50 response (i.e. ≥50% decline in PSA from baseline) following treatment with BAT and following re-challenge with enzalutamide (Cohort 1), abiraterone (Cohort 2) or ADT (Cohort 3) after progressing on BAT. The second study is a Phase II randomized trial testing BAT vs. enzalutamide in patients who had previously progressed on abiraterone [clinicaltrials.gov: NCT02286921]. The primary endpoint of this study is clinical/radiographic progression. Both trials were approved by the Johns Hopkins Institutional Review Board (IRB). Additional biospecimens were obtained at the University of Washington per separate blood/tissue collection protocols approved by the Fred Hutchinson Cancer Research Center/University of Washington Cancer Consortium IRB.

In both clinical trials, BAT was administered as an intramuscular injection of either testosterone cypionate or enanthate 400 mg every 28 days. Subjects were also maintained on ADT in order to suppress endogenous gonadal androgen synthesis. Testosterone cypionate and enanthate have identical pharmacokinetics and have been shown to produce supraphysiologic testosterone levels (>1500 ng/dl) within a few days of the injection followed by a decline in testosterone levels to the near-castrate range by day 28 in patients maintained on ADT (10, 67). Samples underwent germline or somatic next-generation sequencing, and a variety of clinical grade NGS platforms were used, including UW-OncoPlex (N=75), PlasmaSELECT (Personal Genome Diagnostics, Inc) (N=12), FoundationOne CDX (Foundation Medicine, Inc) (N=11), Color (Color Genomics) (N=10) and Guardant360 (Guardant Health, Inc) (N=1) (68). One additional case was sequenced as part of the SU2C/PCF International Dream Team study as previously described (56). Given the concern for false negatives, cases were excluded if plasma-based next-generation sequencing did not reveal a somatic alteration. Absence of a germline alteration was not assumed to indicate absence of somatic alterations. Associations between PSA50 response to BAT and mutations in genes of interest (i.e. DNA repair genes) were evaluated and differences were sought using a chi-squared test.
**Statistics.** Two-tailed Student’s T-test or a two-way ANOVA followed by Tukey’s multiple comparison test were used to compare significance between grouped quantitative data sets (e.g qRTPCR, foci number, growth, and caspase activity data) using GraphPad Prism 8.0 software (La Jolla, CA). At least 3 replicates were used for each experimental group. Each experiment was analyzed in total and the presented statistical significance in the graphs represents this analysis. Differences were considered significant if $p \leq 0.05$.

**Study Approval.** The clinical studies were approved by institutional review boards (IRBs) of the Johns Hopkins University and the University of Washington/Fred Hutchinson Cancer Research Center. Informed consent was obtained after the nature and possible consequences of the studies were explained.
Author Contributions

P.C., M.T.S., J.M.L., S.R.D., and P.S.N. designed the research; P.C., J.M.L., I.C., M.D.N., S.B.F, R.T., E.M., J.L., C.C.P., H.M.L., E.C., and E.S.A. performed the research; P.C., M.T.S., J.M.L, and I.C. analyzed the data; P.C. and P.S.N. wrote the paper and all authors edited and approved the final manuscript. The order of equal contributing co-first authors was determined by drawing lots.

Acknowledgements

We are grateful to the patients who participated in these studies. We acknowledge Fred Hutch Scientific Imaging Shared Resource for assistance with imaging experiments. We thank Holly Nguyen, Lisha Brown, and Lisa Ang for their technical support involving LuCaP tumor studies.

Funding: We gratefully acknowledge research support from the Cancer Center Support Grant P30CA015704-40, NIH P50CA97186, R21CA194798, P01CA163227. PC was supported by a CDMRP post-doctoral fellowship award W81XWH-15-1-0535. MDN was supported by a CDMRP post-doctoral fellowship award W81XWH-16-1-0206. MTS was supported by a Prostate Cancer Foundation Young Investigator Award and DOD Award W81XWH-16-1-0484. JL is supported by NCI CA185297 and DOD CDMRP W81XWH-15-2-0050. ESA is partially supported by NIH grants P30CA006973 and R01 CA185297, and CDMRP grant W81XWH-16-PCRP-CCRSA. CCP was supported by DOD awards PC170510, PC170503P2, and PC141019.
REFERENCES


SUPPLEMENTARY MATERIALS

Table S1. DNA repair gene mutations and PSA responses in men with CRPC enrolled on clinical trials of supraphysiological androgen therapy.

Figure S1. Androgen induced DNA DSBs are dependent on AR

Figure S2. Assessments of DNA damage in AR negative and positive cells following SPA and olaparib treatment

Figure S3. Kinetics of testosterone induced DNA DSBs.

Figure S4. SPA induces DNA damage in quiescent prostate cancer cells.

Figure S5. Effects of SPA on AR localization and function.

Figure S6. Effects of SPA and PARP inhibition on prostate cancer cell proliferation, apoptosis and senescence

Figure S7. Involvement of other repair pathways: BER, NER and MMR along with polymerases, in LNCaP cells following SPA treatment.

Figure S8. Assessments of HR- and NHEJ-DNA repair pathways following SPA treatment in prostate cancer cells.

Figure S9. Evaluating the effects of SPA and olaparib in a LNCaP BRCA2 CRISPR model.

Figure S10. Assessment of the effects of SPA and olaparib in LuCaP PDX models with and without BRCA2 loss.
Figure 1. Supraphysiological androgen concentrations promote DNA damage and enhance AR transcriptional output. 

A. qRT-PCR quantitation of AR transcript levels (n=5).

B. Confocal immunostaining of γH2AX in LNCaP and LNCaP\textsuperscript{AR} cells in normal growth medium, Ct, or 24 h after treatment with 1 nM or 10 nM R1881.

C. Quantitation of γH2AX foci in all cell lines exposed to 1 and 10 nM R1881; Average number of foci were plotted by calculating the mean foci/cell from different fields.

D. Quantitation of γH2AX foci measured in LNCaP and LNCaP\textsuperscript{AR} cells at time intervals following exposure to 10 nM R1881.

E. Immunofluorescence analysis of AR S81 phosphorylation in LNCaP cells in control medium (ct) or 10 nM 1881.

F. Quantitation of AR S81 foci in LNCaP and LNCaP\textsuperscript{AR} cells.

G. Transcript levels of KLK3, NKX3.1 and TMPRSS2 in LNCaP cells following exposure to R881 concentrations 0.01 to 100 nM. In C,
D and F, data represent the mean ± SD; n=3 replicates per experiment; p≤0.05 (*), p<0.01 (**) by two-way ANOVA.
Figure 2. Supraphysiological androgen concentrations influence the growth of prostate cancer cells, alter the expression of DNA repair genes, and augment the effects of PARP inhibition.

A. Quantitation of prostate cancer cell growth 72 hours after treatment with concentration ranges of R1881.
B. Assessment of cell cycle phase by flow cytometry 72 hours following treatment with R1881.
C. Assessment of cellular senescence by quantitation of β-galactosidase staining 72 hours after androgen treatment.
D. Transcript levels determined by RNAseq analysis in LNCaP cells in standard growth medium (FBS) or in androgen depleted medium, ADT (CS-FBS), or androgen depleted medium supplemented with 10 nM R1881, SPA.
E. Confocal immunostaining assay for γH2AX foci in prostate cancer cells in control medium.
alone (Ct) or supplemented with olaparib (Ola) alone or with R1881. F. Quantitation of γH2AX foci per LNCaP cell. G. Quantitation of γH2AX foci per PC3 cell. H. Quantitation of prostate cancer cell growth after treatment with olaparib and/or R1881. I. Assessment of cellular senescence by quantitation of β-galactosidase staining. In A, C, and F-I, data represent the mean ± SD; n=4 replicates per experiment; p≤0.05 (*), p<0.01 (**) by two-way ANOVA.
Figure 3. Supraphysiological androgens alter DNA-PKcs phosphorylation. Confocal immunostaining of DNA-PKcs S2056 phosphorylation in LNCaP (A) and LNCaP\textsuperscript{AR} (B), 24 hours after treatment with R1881 10 nM and olaparib (Ola). C,D. Quantitation of S2056 foci after 6h or 24h of R1881 and Ola treatment. E. Confocal immunostaining of DNA-PKcs T2609 phosphorylation foci in LNCaP, LNCaP\textsuperscript{AR} and PC3\textsuperscript{AR} 24h after treatment with R1881 10 nM and/or Ola. F,G. Quantitation of T2609 foci in cell lines 24h after R1881 and Ola treatment. In C, D, F, and G data represent the mean ± SD; n=4 replicates per experiment; p≤0.05 (*), p<0.01 (**) by two-way ANOVA.
Figure 4. Inhibition of DNA-PKcs attenuates SPA-induced DNA damage and prostate cancer growth repression. A. Confocal immunostaining of γH2AX foci LNCaP and LNCaP<sup>AR</sup> cells. B. Quantitation of γH2AX foci LNCaP and LNCaP<sup>AR</sup> cells (top) and PC3 and PC3<sup>AR</sup> cells (bottom) with DNA-PKcs inhibition and exposure to supraphysiological androgens. C. Confocal immunostaining of DNA-PKcs S2056 foci and D. AR S81 foci respectively in LNCaP and LNCaP<sup>AR</sup> cells after 24h treatment with SPA with 1h pre-treatment with DNA-PKcs inhibitor Nu7441. E. and F. Quantitation of LNCaP, LNCaP<sup>AR</sup> and PC3 and PC3<sup>AR</sup> cell growth following 3 days of treatment with the DNA-PKcs inhibitor Nu7441 and/or 10 nM R1881. In B, E, and F, data represent the mean ± SD; n=4 replicates per experiment; p≤0.05 (*), p<0.01 (**) by two-way ANOVA.
Figure 5. SPA-induced DNA damage and repression of prostate cancer growth are enhanced by BRCA2 loss and PARP inhibition. A. Western immunoblot of BRCA2 in protein extracts from LNCaP-shBRCA2 cells in the presence or absence of DOX. B. Confocal immunostaining and C. quantitation of γH2AX in LNCaP-shBRCA2 cells following 10 nM R1881 and/or Olaparib treatment for 24h in the presence of DOX. D. Confocal immunostaining and E. quantitation of DNA PKcs S2056 foci in DOX treated LNCaP-shBRCA2 cells following 10 nM R1881 and/or olaparib treatment for 24h. F. Quantitation of apoptosis by caspase activity, and G. growth of LNCaP-shBRCA2 cells in the presence or absence of DOX after 3 days treatment with R1881, Nu7441 or olaparib. H. Confocal immunostaining of γH2AX and DNA-PKcs S2056 foci in
dissociated cells from the prostate cancer \textit{BRCA2-/-} LuCaP96 PDX line, exogenously treated with 10 nM R1881 with or without olaparib for 18h. I. Quantitation of $\gamma$H2AX foci in dissociated cells from HR-intact LuCaP35, LuCaP70 and HR-deficient LuCaP96CR cells following 4h and 18h of R1881 or olaparib treatment. In C, E, F, G, and I data represent mean ± SD; n=4 replicates per experiment; p≤0.05 (*), p<0.01 (**) by two-way ANOVA.
Figure 6. Clinical Response to supraphysiological testosterone treatment is associated with mutations in homologous recombination DNA repair genes. PSA waterfall plot for patients receiving BAT as part of two ongoing Phase II trials. Data is presented for patients with and without pathogenic germline or somatic mutations in HR DNA repair pathway genes (i.e. HRD). PSA declines of ≥50% (PSA\textsubscript{50} response) were more frequent in patients with HRD compared to those without (PSA\textsubscript{50} response: 15/29 [52%] vs. 6/33 [18%], \textit{Chi}^2 P=0.005). *Percent change in PSA truncated at 100%.