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Abiraterone acetate induces CREB1 phosphorylation and enhances the function of the CBP-p300 complex, leading to resistance in prostate cancer cells

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Abstract

Purpose: Abiraterone acetate (AA), an inhibitor of CYP17A1, is an FDA-approved drug for advanced prostate cancer. However, not all patients respond to AA, and AA resistance ultimately develops in patients who initially respond. We aimed to identify AA resistance mechanisms in prostate cancer cells.

Experimental Design: We established several AA-resistant cell lines and performed a comprehensive study on mechanisms involved in AA resistance development. RNA-seq and phospho-kinase array screenings were performed to discover that the cAMP response element–CRE binding protein 1 (CREB1) was a critical molecule in AA resistance development.

Results: The drug-resistant cell lines are phenotypically stable without drug selections, and exhibit permanent global gene expression changes. The phosphorylated CREB1 (pCREB1) is increased in AA-resistant cell lines and is critical in controlling global gene expression. Upregulation of pCREB1 desensitized prostate cancer cells to AA, while blocking CREB1 phosphorylation re-sensitized AA-resistant cells to AA. AA treatment increases intracellular cAMP levels, induces kinases activity, and leads to the phosphorylation of CREB1, which may subsequently augment the essential role of the CBP/p300 complex in AA-resistant cells, since AA-resistant cells exhibit a relatively higher sensitivity to CBP/p300 inhibitors. Further

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pharmacokinetic studies demonstrated that AA significantly synergizes with CBP/p300 inhibitors in limiting the growth of prostate cancer cells.

Conclusions: Our studies suggest that AA treatment upregulates pCREB1 which enhances CBP/p300 activity, leading to global gene expression alterations, subsequently resulting in drug resistance development. Combining AA with therapies targeting resistance mechanisms may provide a more effective treatment strategy.

Keywords

Abiraterone acetate; prostate cancer; resistance; CREB1; CBP/p300 complex

Introduction

Androgens and the androgen receptor (AR) play crucial roles in prostate cancer development and progression (1–3). Androgen deprivation therapy (ADT) is the most effective and widely used treatment for patients with hormone sensitive prostate cancer (HSPC) (4). Almost all patients with HSPC respond to ADT initially before progressing to castration resistant prostate cancer (CRPC)(4–6). Despite progression to castration resistance, the AR remains a key target since CRPC can be sustained by circulating adrenal androgens and *de novo* synthesis of intratumoral androgens (5). Recognition of continued AR dependence has spurred development of agents that further disrupt AR signaling for treating CRPC (4,7,8). Several second-generation AR-targeted drugs were developed and became the standard of care for patients with CRPC. These FDA approved second-generation antiandrogens include Abiraterone acetate (AA), enzalutamide, and more recently, apalutamide and darolutamide (9,10). While second-generation AR targeted drugs have initial responses and prolong the life of some patients with advanced prostate cancer, ~25% of patients do not respond to these drugs, and among patients who respond, resistance will ultimately occur (11,12,13).

AA is an orally administered specific inhibitor of the cytochrome P450 17 α -hydroxylase/17, 20 lyase (CYP17A1), a key enzyme in the synthesis of sex steroids (14). AA has been shown to reduce intraprostatic androgens, as demonstrated in a randomized phase 2 neoadjuvant study in localized high-risk HSPC (15). AA can block androgen production and AR signaling by inhibiting CYP17A1 as well as through other mechanisms. AA might compete with androgen precursors for occupancy of cell membrane transporter proteins to reduce androgen levels within cells. Meanwhile, AA can also be metabolized *in vivo* into the more active 4-abiraterone, which blocks multiple steroidogenic enzymes and antagonizes AR (16,17). Currently, AA is considered to be a standard of care treatment for advanced prostate cancer patients (13). In the context of metastatic CRPC (mCRPC), AA effectively causes tumor regression and prolongs median overall survival by an average of 4–5 months, and median time to PSA progression by 1.9 months (13). Even though AA prolongs overall survival, not all patients respond to AA, and AA resistance ultimately develops in those patients who do initially respond (18). Uncovering mechanisms underlying resistance to AR-targeted therapies like AA is critical to enhancing treatment efficacy, identifying new therapeutic strategies, and improving clinical outcomes in prostate cancer.

Resistance to AR-targeted therapies is a major clinical challenge in prostate cancer. Due to the heterogeneity of prostate tumors, it is possible that some rare cells with intrinsic resistance may exist prior to treatment due to pre-existing altered genetic programs, and these cells may relapse following therapeutic challenges. However, treatments frequently induce acquired resistance via *de novo* genetic or epigenetic alterations, resulting in adaptations to the drug target itself or recruitment of other survival pathways. Several putative mechanisms contributing to AA resistance have been described, including ligand-dependent and independent mechanisms. Upregulation of CYP17A1 provided a growth advantage for castration resistant VCaP xenografts treated with AA (19). The selection and expression of 3 β HSD1 (367T), a gain-of-function mutation in the steroidogenic machinery, increases the conversion of adrenal-derived dehydroepiandrosterone to dihydrotestosterone (DHT) in a LAPC-4 xenograft model of AA resistance (20). A ligand-independent mechanism was described wherein induction of AR splice variants (AR-v7; ARs without the ligand binding domain) conferred ligand-independent AR transactivation, leading to AA resistance in xenografted LNCaP cells (21,22). Additional studies indicated the association of AR-v7 in circulating tumor cells from CRPC patients with AA resistance (23). Cancer cells of different genetic backgrounds may develop AA resistance via different mechanisms. Other cell-dependent mechanisms or tumor microenvironment-related mechanisms may also contribute to AA resistance. Enhancing treatment efficacy of AA and reducing the probability of resistance development in patients with prostate cancer remain critical clinical challenges. In this study, we specifically address potential cell-dependent AA resistance mechanisms and apply genome-wide approaches to interrogate mechanisms involved in the development and maintenance of AA resistance in prostate cancer cell lines. To this end, we established several AA-resistant prostate cancer cell lines from genetically distinct parental cell lines. The resistant subclones are phenotypically stable in the absence of drug selection, exhibit permanent global gene expression changes, and demonstrate a significantly compromised AR signaling pathway. Our comprehensive study of these AA-resistant cell lines detailed distinct molecular factors driving AA resistance in different prostate cancer cell lines and revealed potential novel therapeutic strategies for combinational AA treatments.

Materials and Methods

Cell lines and cell culture

The prostate cancer cell lines LNCaP and PC-3 were obtained from ATCC (American Type Culture Collection); LNCaP-abl was provided by Dr. Myles Brown's laboratory. LAPC-4 cell line was originally acquired from University of California Los Angeles. LNCaP, PC-3, and LAPC-4 were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin-streptomycin. LNCaP-abl was cultured in phenol red-free RPMI 1640 medium supplemented with 5% charcoal striped FBS (cFBS) medium (Gibco) and 1% penicillin and streptomycin. All cells were grown at 37 °C in a humidified atmosphere at 5% CO₂. Drug-resistant prostate cancer cell lines were generated from parental LNCaP, LAPC-4, and LNCaP-abl cells by long-term culturing in the presence of increasing amounts of AA; established AA resistant cell lines, denoted LNCaP-AAr, LAPC4-AAr, and LNCaP-abl-AAr respectively, were routinely maintained in medium

containing 5 μ M AA. All cell lines are authenticated and routinely screened for contamination of mycoplasma.

Drugs, activators, and inhibitors

Abiraterone acetate, GSK126, and SGC-CBP30 were obtained from Selleck; forskolin, 666–15, and C646 from Sigma; Enzalutamide (MDV3100) from Moltarget. The doses and durations of treatments are as described in the Figures.

Gene overexpression

The CREB Dominant-Negative Vector Set (Takara Bio (valid) # 631925) was obtained for overexpressing wild type CREB1 and mutated CREB. Cells were harvested 48 or 72 h after transfection by Lipofectamine 2000 (Invitrogen) for protein and mRNA analysis.

Reverse transcription and quantitative PCR (RT-qPCR)

Total RNA was extracted from cells by using TRIzol Reagent (Life Technology). The RNA concentration and purity were measured by NanoDrop. 2–3 μ g of total RNA was used to generate cDNA using the iScript R Transcription Supermix (Bio-Rad). Real time qPCR was performed using SsoFast EvaGreen Supermix in CFX96 Thermal Cycler (Bio-Rad). PCR-based amplification was performed using the following primers: AR F: 5'-CCTGGCTTCCGCAACTTACAC-3'; AR R: 5'-GGACTTGTGCATGCGGTACTCA-3'; AR-V7 F: 5'-CGTCTTCGGAAATGTTATGAAGC-3'; AR-V7 R: 5'-GAATGAG-GCAAGTCAGCCTTTCT-3'; PSA F: 5'-AGGCCTTCCCTGTACACCAA-3', PSA R: 5'-GTCTTGGCCTGGTCATTTC-3', CREB1 F: 5'-GGAGCTTGTAACACCGGTAA-3'; CREB1 R: 5'-GCATCTCCACTCTGCTGGTT-3'; GAPDH F: 5'-GAAGGTGAAGGTCTGGAGTC-3', GAPDH R: 5'-GAAGATGGTGATGGGATTTC-3'. All relative expression levels were calculated by normalizing with the expression level of GAPDH. The expression levels were calculated according to the comparative CT method (2^{-CT}).

Western blotting analysis

Cells were washed in ice-cold PBS and lysed in 2x Laemmli Sample Buffer (Bio-Rad) with 1 mM PMSF. Protein concentrations were determined using Pierce BCA protein assay kit (Thermo Scientific). The samples were then separated by SDS-PAGE and transferred to PVDF membrane (Bio-Rad). The membrane was blocked with 5% skimmed milk in TBST for 1 h at room temperature, followed by incubation with a primary antibody overnight at 4 °C. After washing the membrane 3 times by TBST, the membrane was incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The signals on blots were then detected by ECL Western Blotting Substrate (Bio-Rad) and exposed on Autoradiography Films (Bioexpress) or ChemiDoc MP Imaging System (Bio-RAD).

Viability assays

Cells were plated at 2.5×10^3 per well in 96-well plates in complete media one day before drug treatment. Cells were either treated with DMSO or with indicated drugs for specific time periods. Then, cells were incubated with a 1:10 dilution of cell proliferation Reagent

WST-1 (Roche Molecular Biochemicals) in serum-free medium for 1 h at 37 °C. The absorbance at 450 nm was measured using a BioTek plate reader.

Colony formation assay

8000 cells were seeded per well in 6-well plates in triplicate. At the indicated time points, the medium was aspirated and cells were fixed and stained with a crystal violet staining solution [0.4% crystal violet (MilliporeSigma), 50% methanol] for 20 min at room temperature. After removing the staining solution, plates were washed twice with PBS. The plates were then left to dry overnight and the number of colonies (a colony defined as 50 cells) was counted.

RNA-seq and pathway analysis.

RNA-seq was performed at the Center for Cancer Computational Biology at Dana-Farber Cancer Institute (DFCI). Differential expressed gene analysis between AA-resistance and parental cell line was performed using DESeq2 (24). Enriched Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were annotated using clusterProfiler R package(25). Enrichment of other molecular signatures were performed with Gene Set Enrichment Analysis (GSEA) program (<https://software.broadinstitute.org/gsea/index.jsp>) (26).

Phospho-kinase screen

We used Proteome Profiler Human Phospho-Kinase Array (R&D Systems) following the manufacturer's protocol. Cells were lysed in Lysis Buffer 6 from the array kit. Total cellular proteins (300 µg) were hybridized to the array membranes overnight. The arrays were washed and incubated with the detection antibody cocktails for 2 h, followed by IRDye 800CW Streptavidin for 30 min. Signals on membranes were detected using ImageQuant LAS 4000 (GE Healthcare) imaging system.

Cyclic AMP (cAMP) measurement

The concentration of cAMP was determined using a complete ELISA kit (Abcam, Cat. Ab133051) according to the manufacturer's instruction. Briefly, cells were extracted in 0.1 M HCl and after neutralization, cell extracts were mixed with anti-cAMP antibody and alkaline phosphatase for colorimetric detection in microplate reader.

Evaluation of Synergistic effects

Synergism can be calculated with different methods using effect-based strategies such as the Bliss independence or the HSA (Highest Single Agent) model, or by dose-effect-based strategies such as the Loewe additivity model (27). Here, we applied two different tools to assess synergy: the Chou-Talalay method based on Loewe additivity (28) and Synergyfinder (29), scoring synergism using the 4 main models, HSA, Loewe, BLISS, and ZIP (Zero Interaction Potency). When different doses of drugs were combined, clear synergistic effects were indicated by CI (combination index) values < 1 calculated by the Chou-Talalay method. To confirm the synergistic combinations observed with the Chou-Talalay method, we used Synergyfinder, and synergy scores > 1 indicated synergism.

Statistical analysis.

Results are reported as mean $\pm$ SE unless otherwise noted. Comparisons between two groups were performed using an unpaired two-sided Student's t test ($P < 0.05$ was considered significant). Bar graphs were generated using GraphPad Prism software (version 7.0 GraphPad Software, Inc, La Jolla, CA).

Results

Various types of cancer cell lines are sensitive to AA.

AA exhibits broad activity across diverse types of cancer cell lines based on data from the PRISM (Profiling Relative Inhibition Simultaneously in Mixtures) multiplexed cell line profiling database (30). Examining the data for AA (BRD: BRD-K24048528-001-02-5) which contains the results of pooled-cell line chemical-perturbation viability screens of 514 total cell lines from 22 primary diseases, 66.3% of the cell lines (341/514) are sensitive to AA (Log2 fold change drug sensitivity < 0). This includes all three prostate cancer cell lines including AR positive 22RV1, LNCaP clone FGC and AR negative DU145, which respectively exhibited 44%, 41% and 4% lower cell counts, indicating that 22RV1 and LNCaP are relatively more sensitive to AA than DU145 (Supplementary Fig. S1A). Interestingly, many cell lines of other cancer types are also highly sensitive to AA, for example the pancreatic cancer cell line T3M4 (Log2 fold change = -1.539), breast cancer cell line MCF7 (Log2 fold change = -1.434), and the urinary tract cancer cell line UMUC1 (Log2 fold change = -1.374).

We further investigated the relationship between AA sensitivity and the expression and dependency of AR and CYP17A1 based on expression profiling and RNAi screen data in the pan cancer DepMap database of cell lines (<https://depmap.org/portal/>). No correlation was observed between AA sensitivity and the expression and dependency of AR or CYP17A1 when compared among all tested cancer cell lines (Supplementary Fig. S1B,C). We further tested the AA sensitivity of additional prostate cancer cell lines of distinct genetic backgrounds, including the AR positive, androgen dependent cell lines LNCaP and LAPC-4; androgen independent, AR positive LNCaP-abl; and AR negative PC-3 (Fig. 1A). As demonstrated, these 4 cell lines exhibited similar levels of AA sensitivity, despite their distinct genetic backgrounds. The growth of cell lines can be completely inhibited with 10 μ M AA and the IC₅₀ at day 6 is ~ 2.5 μ M, similar to what is seen in patients. This result indicated that the presence of AR may not be a prerequisite for the sensitivity to AA in prostate cancer cell lines.

Together with the data from the PRISM analysis of pan-cancer cell lines, our observations open up a broad range of questions relating to alternative targets of AA in different types of cancer cells, new potential applications of AA in treatment of other types of cancer, and potential diverse mechanisms leading to AA resistance. We focused on uncovering mechanisms underlying resistance to AR-targeted therapies, which is key to enhancing treatment efficacy and identifying new therapeutic strategies to improve clinical outcomes in prostate cancer. To reach this goal, we established several AA resistant prostate cancer cell

lines from genetically distinct parental cell lines and performed comprehensive analysis of potential mechanisms involved in the development of AA resistance in prostate cancer cells.

Development of AA-resistant prostate cancer cell lines.

We established several stable AA-resistant prostate cancer cell lines by adapting parental cells to continuously increasing AA concentrations up to 10 μ M, and the surviving cells were maintained in 5 μ M AA for a period longer than 5 weeks. The control parental cells were treated with an equal amount of ethanol (solvent for AA, Supplementary Fig. S2A). AA-resistant cell lines were generated from LNCaP, LAPC-4, and LNCaP-abl, and referred to as LNCaP-AAr, LAPC4-AAr, and LNCaP-abl-AAr, respectively. As shown in Fig. 1B, 5 μ M AA efficiently reduced LNCaP cell proliferation by ~80% after 6 days of treatment, while LNCaP-AAr cells in the presence or absence of 5 μ M AA exhibited an equal growth efficiency to that of parental LNCaP cells in the absence of AA; LAPC4-AAr (Fig. 1C) and LNCaP-abl-AAr (Supplementary Fig. S2B) showed similar cell proliferation characteristics. To determine the aggressiveness of AA-resistant cell lines, a transwell invasion assay was conducted and compared between the AA-resistant cell lines and their corresponding parental cell lines (Fig. 1D). The results indicated that LNCaP-AAr and LAPC4-AAr exhibited an increased invasive potential compared to LNCaP and LAPC-4, respectively (Fig. 1D). Additional experiments demonstrated that these AAr cell lines do not revert in the absence of AA selection, indicating that the established AA-resistant cell lines are phenotypically stable. Even though LNCaP-AAr and LAPC4-AAr survive in the presence of AA, they do not survive in medium containing charcoal stripped FBS. The level of AR mRNA is slightly increased in LNCaP-AAr compared to that in LNCaP (Fig. 1E), however, expression of PSA was dramatically reduced in LNCaP-AAr. Importantly, even though DHT can modestly induce PSA expression in LNCaP-AAr, the amount of DHT-induced PSA expression in LNCaP-AAr was very limited, suggesting that AR function in LNCaP-AAr is compromised (Fig. 1E). Similar events were seen in LAPC-4 and LAPC4-AAr (Supplementary Fig. S2C). These results were further confirmed by Western blot analysis of protein expression levels (Supplementary Fig. S2D). Additionally, AA-resistant cell lines also exhibited a relative resistance to enzalutamide, another AR targeted drug: Enzalutamide IC_{50} =8.814 μ M in LNCaP compared to IC_{50} =15.1 μ M in LNCaP-AAr. A similar trend was seen in LAPC-4 (IC_{50} = 10.95 μ M) and LAPC4-AAr (IC_{50} = 20.19 μ M) (Fig. 1F).

Molecular genetic characterization of AA-resistant cell lines.

To characterize our AA-resistant cell lines and to identify potential mechanisms involved in the development of AA resistance, we performed gene expression profiling by RNA-seq in all AA-resistant and corresponding parental AA-sensitive cell lines. Comparison of RNAseq datasets revealed global gene expression pattern changes in AA-resistant cells. The volcano plots (Fig. 2A) demonstrate the up- or downregulated genes between the parental and AA-resistant cell lines for LNCaP, LAPC-4, and LNCaP-abl. Using criteria of 2.0-fold change of expression level and p value <0.05, hundreds of differentially expressed genes between each pair of cell lines were identified (Supplementary Fig. S3A). Different pairs of cell lines exhibited drastically disparate differentially-expressed genes and only a few genes overlapped between cell lines (Fig. 2B), including 4 common upregulated genes *KCNGL1*, *HEPH*, *RARRES3*, and *GBP3*, as well as 12 common downregulated genes *LPAR3*,

HOMER2, NKX3-1, IGF1R, SLC45A3, UGT2B11, PMEP1, KIF5C, BEND4, and UGT2B28 in AA-resistant cell lines. The expression of 4 upregulated genes are androgen independent, while expression levels of 11 of the 12 downregulated genes are androgen dependent. The absence of significant overlap among differentially expressed genes suggested the possibility that different molecular determinants may be involved in the development of AA resistance in cell lines of different genetic backgrounds.

Among the differentially expressed genes, androgen regulated genes account for 28.2%, 23.7%, and 30.6% of the total differentially changed genes in LNCaP, LNCaP-abl and LAPC-4, respectively, indicating the primary influence of AA on AR signaling (Supplementary Fig. S3B, C). We further addressed the significance of the documented putative etiologies causing AA resistance in our stable AA-resistant cell lines, such as the involvement of *CYP17A1*, AR splice variant *AR-v7*, glucocorticoid receptor (GR), and *SGK1* (19,21,22,31,32). First, we did not find a significant change in the *CYP17A1* expression level in LNCaP-AAr, LAPC4-AAr, or LNCaP-abl-AAr (Fig. 2C). However, a VCaP-AAr derived from VCaP (data not shown) exhibited a 3-fold increase in *CYP17A1* expression, which is consistent with a previously reported study in VCaP xenografts (19). Secondly, it has been reported that the level of *AR-v7* transcript is associated with the efficacy of AA and enzalutamide treatment in men with CRPC (32). However, *AR-v7* levels varied in different AA-resistant cell lines. *AR-v7* expression was increased in LNCaP-AAr vs. LNCaP, while it was decreased in LAPC4-AAr compared to that in LAPC4. LNCaP-abl and LNCaP-abl-AAr did not have a significant difference in *AR-v7* expression levels (Fig. 2C). Thirdly, we investigated the potential role of GR in AA resistance, since upregulation of *GR* was noted as a potential mechanism for enzalutamide resistance (31). We examined the expression level of *GR* and *SGK1*, a gene regulated both by AR and GR (Fig. 2C). It appeared that LNCaP had a much lower expression level of *GR* than that in LNCaP-abl. However, *GR* expression was significantly increased in LNCaP-AAr (23-fold) but only slightly increased in LNCaP-abl-AAr. *SGK1* expression in LNCaP and LNCaP-abl does not respond to DHT induction, and *SGK1* expression levels in LNCaP-AAr and LNCaP-abl-AAr are higher than those in the LNCaP and LNCaP-abl by 5.6- and 2.6-fold, respectively, presumably via increased GR activity. In contrast, LAPC-4 and LAPC4-AAr displayed no difference in *GR* expression, and *SGK1* expression strongly responds to DHT induction in LAPC-4 (14-fold). This result indicated that *SGK1* in LAPC-4 remains AR-regulated and its expression in LAPC4-AAr was reduced. Thus, no evidence supported the activation of GR in LAPC4-AAr. In summary, we did not find that any of the previously reported etiologies as a consistently shared mechanism for the development of AA resistance across these prostate cancer cell lines.

Identification of potential pathways involved in the development of AA resistance.

To identify potential cellular processes and transcriptional programs related to AA resistance, we performed GSEA based on Gene Ontology (GO) pathways of differentially expressed genes between AA-resistant cell lines and their corresponding parental cell lines. Even though most of the differentially expressed genes vary among the three pairs of cell lines, it appeared that the differentially expressed genes showed enrichment in certain pathways including androgen response, JNK, cAMP and VEGF (Fig. 2D). Additionally, the

EIF4e regulated pathway is enriched in the LAPC4-AAr cell line. Interferon gamma response, cAMP and CEBP1 are enriched in the LNCaP-abl-AAr cell line (Supplementary Fig. S4, Supplementary Table S1,2,3). The GO pathways (Supplementary Fig. S5) additionally showed that the upregulated differentially expressed genes are enriched in cellular uronic acid and glucuronate metabolic process, while the downregulated differentially expressed genes are enriched in flavonoid and xenobiotic metabolic process, cellular glucuronidation, and mesoderm development. This analysis indicated that differentially expressed target genes in different AA-resistant cell lines may be different, but they are associated with similar molecular pathways and metabolic processes. Thus, their altered expression may potentially be mediated via similar signaling pathways.

We further analyzed key transcription factors that regulate the differentially expressed genes by MetaCore of GeneGo (<https://portal.genego.com/>) (Fig. 2E). As anticipated, AR and GCR-alpha (sharing a similar DNA binding site with AR) are on the top of the list. In addition, CREB1, c-Myc, ESR1, and p53 were identified. Surprisingly, CREB1, the cAMP response element-CRE binding protein 1, ranked as the top hit for all comparisons, indicating that the CREB1 signaling pathway may play an important role in regulating global gene expression changes in AA-resistant cell lines and suggested the potential involvement of kinases and the CREB1 signaling pathway in the development of AA resistance. Following this observation, we investigated the activation of CREB1 in AA-resistant cell lines and explored the possibility that activation of CREB1 may desensitize prostate cancer cells to AA.

CREB1 phosphorylation is upregulated in AA-resistant cell lines.

Since mRNA levels of CREB1 were not different in parental and AA-resistant cell lines, up regulation of CREB1 in AA-resistant cell lines most likely relies on post-transcriptional events. CREB1 is activated by phosphorylation of Serine 133 by various kinases, including PKA and Ca/calmodulin-dependent protein kinases. To confirm the activation of CREB1 in AA-resistant cell lines, we compared phosphorylation profiles of kinases and their substrates in LNCaP-AAr and LAPC4-AAr with those in LNCaP and LAPC-4, using a Phospho-Kinase Array Kit containing 43 kinase phosphorylation targets (Fig. 2F). Phosphorylated CREB1 (pCREB1), c-Jun (p-cJun), and WNK1 (p-WNK1) were all upregulated in both LNCaP-AAr and LAPC4-AAr cell lines. Further western blot analyses confirmed that levels of pCREB1 and p-cJun were significantly upregulated in both LNCaP-AAr and LAPC4-AAr, though the level of pWNK1 was only mildly increased compared to the parental cell lines (Fig. 2G). We noted that other than CREB1, cJun phosphorylation is significantly increased in AA-resistant cell lines. For this current study, we chose to focus on the study of CREB1 in details; the role of cJun in AA-resistance development will be presented separately.

Phosphorylation of CREB1 could occur due to an increase of intracellular cAMP levels and subsequent activation of associated protein kinases (33). We further compared the intracellular cAMP levels in LNCaP-AAr and LAPC4-AAr with their corresponding parental cell lines (Fig. 3A). LNCaP-AAr exhibited ~2.7 fold higher level of cAMP than in LNCaP, and LAPC4-AAr had ~1.5 fold higher than in LAPC-4. Significantly increased

cAMP was also detected in LNCaP and LAPC-4 after short-term treatment with AA (Fig. 3B). cAMP levels in LNCaP gradually increased with AA treatment from day 0 to day 8. Detailed mechanisms leading to increased cAMP levels in AA-resistant cell lines are currently not fully determined. We postulated that activation of adenylate cyclase or inhibition of phosphodiesterase might occur upon AA treatment in prostate cancer cells; however, from the RNAseq data, we noted that the mRNA level of the alpha-2A adrenergic receptor, (*ADRA2A*) gene, which inhibits adenylate cyclase, is significantly reduced in LNCaP-AAr and LAPC4-AAr compared to those in LNCaP and LAPC-4 (Supplementary Fig. S6A). It is possible that the reduction of *ADRA2A* in AA-resistant cell lines may have served to augment the activity of adenylate cyclase, which in turn increased the level of cAMP and facilitated phosphorylation of CREB1.

Upregulation of pCREB1 promotes resistance to AA

To determine the impact of pCREB1 on AA sensitivity, we compared the relative proliferation efficiency in cells responding to AA treatment after the CREB1 activity is up- or downregulated, respectively. Overexpression of wild type CREB1 rendered the wild type cells resistant to AA, while overexpressing mutated CREB1 (S133A, an essential kinase site) did not significantly affect sensitivity to AA (Fig. 3C). Treatment with Forskolin, which raises levels of cAMP, significantly increased pCREB1 and the growth efficiency of LNCaP and LAPC-4 in the presence of AA (Fig. 3D). Blocking pCREB1 with a low dose (50 nM) of the CREB inhibitor 666-15 (at a concentration insufficiently affecting cell growth) significantly re-sensitized the response of LNCaP-AAr and LAPC4-AAr to AA (Fig. 3E). We found that knocking down CREB1 significantly reduced growth efficiency (Supplementary Fig. S6B). We did not knockdown CREB1 by siCREB/or shCREB to evaluate the impact of downregulation of CREB1 on the AA sensitivity due to its growth essentiality, which may potentially bias the impact of AA treatment. In summary, our data confirmed the role of CREB1 in AA resistance in prostate cancer cells and that CREB inhibitors can restore sensitivity to AA in resistant cells.

Assessment of the impact of CBP/P300 on the efficacy of AA

Upon phosphorylation at Ser133, pCREB1 can activate transcription regulation through its interaction with CBP/p300, which is a transcription co-regulator containing a histone acetyltransferase and a bromodomain (34,35). A considerable amount of evidence has demonstrated that CBP/P300 works as an interactor with CREB1. Based on the BioGRID database (<https://thebiogrid.org/>), within the CREB1 interactor network, CBP (CREBBP) and p300 (EP300) have been shown to be the likeliest to physically interact with CREB1 (Fig. 4A). It is possible that upregulation of pCREB1 may have augmented the role of CBP/p300 in AA-resistant cell lines for survival, consequently sensitizing them to treatment with CBP/p300 inhibitors. We tested this hypothesis in LNCaP and LNCaP-AAr by evaluating the efficacy of two CBP/P300 inhibitors: SGC-CBP30, an inhibitor selectively binding to the bromodomain; and C646, an inhibitor of histone acetyltransferase activity. The dose-response curves were generated by assessing cell viability and IC₅₀ values for LNCaP were calculated. Indeed, IC₅₀ of SGC-CBP30 for LNCaP-AAr (IC₅₀=2.86 μM) were lower than those for LNCaP (IC₅₀=4.87 μM). A similar trend was also observed using C646 (Fig. 4B), and the same trend was observed in LAPC-4. (Supplementary Fig. S6C) Thus, we

hypothesized that the transcriptional regulation program coordinated by pCREB1 and CBP/P300 may have been one of the major pathways leading to genome wide transcriptome alteration in some AA-resistant cell lines, subsequently promoting resistance and sustaining cell growth in the presence of AA. We further tested whether inhibition of CBP/p300 can resensitize LNCaP-AAr to treatment with AA. Fig. 4C showed that addition of a low dose of C646 (1.25 μ M) or SGC-CBP30 (1 μ M) significantly increased the sensitivity of LNCaP-AAr to AA. In the presence of a low dose of CBP/p300, 5 μ M AA treatment reduced growth of LNCaP-AAr by half, and even treatment with 1.25 μ M AA exhibited modest growth reduction. This data supports the important role of CBP/p300 in sustaining the AA resistance in AA-resistant cell lines.

Since phosphorylation of CREB1 and the transcriptional regulation of CBP/p300 are two essential steps in the same signaling pathway, we investigated the possibility that the AA may synergize with the efficacy of CBP/P300 inhibitors and vice versa. Synergistic effects of AA with the CBP/P300 inhibitors, SGC-CBP30 and C646, were studied and scored in LNCaP. All CI (combination index, calculated by Chou-Talalay method) values between AA and SGC-CBP30 or C646, except for the highest concentrations of AA and C646 were <1 indicating a synergistic effect (Fig. 5A). Synergy scores (calculated by Synergyfinder) of the combination of AA vs. SGC-CBP30 or AA vs. C646 were 15.26 and 12.35, respectively, further confirming the synergism (Fig. 5B). Concentrations of drugs exhibiting regions of the highest synergy were generally in the lower micromolar ranges *in vitro* in cell culture systems. The best synergistic scores of 32.54 and 24.76, respectively, were observed when 2 μ M of AA/ 2.5 μ M of SGC-CBP30 or 2 μ M of AA/ 3 μ M of C646 were applied. This result suggested that combinations of low doses of multiple drugs targeted to different proteins in the same signaling pathway may substantially increase the efficacy of the treatment strategy based around AA. Fig. 5C sums up our overall findings on the involvement of CREB1 in the AA resistance development in prostate cancer cells. AA treatment can activate PKA or other kinases, leading to the phosphorylation of CREB1 and subsequently, pCREB1 may enhance the activity of the CBP/p300 complex, thus promoting AA resistance in prostate cancer cells.

Discussion

We demonstrated here that AA treatment could induce acquired resistance in cell culture systems by increasing intracellular cAMP levels and CREB1 phosphorylation, causing subsequent activation of the CBP/p300 pathway. Augmented CBP/p300 function could facilitate *de novo* genetic or epigenetic alterations leading to global transcriptome changes and resulting in adaptations to the drug target itself via recruitment of other survival pathways. Even though the putative etiologies including the involvement of CYP17A1, AR splice variant AR-v7, and GR contributed to the development of AA resistance in a portion of prostate cancer cells, these mechanisms are not sufficient to explain the inevitable tumor progression and development of resistance in all patients treated with AA. Our data indicated that AA treatment effectively reduces cell growth, presumably via impairing AR signaling, though it also simultaneously induces potential survival mechanisms leading to inevitable development of acquired resistance. The AA-resistant cell lines also exhibit cross-resistance to enzalutamide, as observed in the clinical setting (36). However, AA-resistant

cell lines display a higher sensitivity to CBP/p300 inhibitors as shown by their reduced IC₅₀, suggesting that the biological importance of CBP/p300 may have been augmented in AA-resistant cell lines. Based on the RNAseq data, we did not observe a significant difference of p300 (EP300) mRNA levels between resistant and sensitive cell lines, though CBP (CREBBP) mRNA levels were slightly increased (by ~1.3 fold) in AA-resistant cell lines (Supplementary Fig. S7C). Both the increased level of pCREB1 and/or the slightly increased expression of CREBBP could contribute to the increased activity of CBP/p300 complex in AA-resistant cell lines. Inhibition of the function of CBP/p300 resensitized AA-resistant cells to AA, suggesting that the augmented role of CBP/p300 in AA-resistant cells fosters development of resistance. The functional impact of AA on the coordinated signaling pathway of CREB and CBP/p300 provided a molecular rationale explaining the synergistic effects between AA and CBP/p300 inhibitors, that we observed *in vitro* in cell culture systems. A Phase 2 clinical trial of CCS1477 (<https://clinicaltrials.gov/ct2/show/NCT03568656>), a small molecule inhibitor of the bromodomain of the CBP/p300 administered as an oral capsule, is ongoing in patients with advanced prostate cancer. We present here a rationally-designed combination for two clinically-relevant drugs providing greater efficacy than the sum of the two single agents for future treatment considerations for patients with advanced prostate cancer.

Accumulating evidence shows that CREB1 is overexpressed in numerous human cancers, including astrocytoma (37), breast cancer (38), gastric cancer (39), glioma (40), HCC (41), mesothelioma (42), non-small cell lung cancer (NSCLC) (43), ovarian cancer (44), and prostate cancer (45). Remarkably, phosphorylated CREB1 was significantly elevated in bone metastatic prostate cancer specimens (46). It has been noted that ADT can induce the activation of CREB and promote neuroendocrine development of prostate cancer cells (47). The ADT-activated CREB can enhance EZH2 activity and downregulate a downstream target TSP1 (thrombospondin-1, an anti-angiogenesis factor), which in turn promotes neuroendocrine differentiation of some androgen independent prostate cancer cell lines (47). However, we did not observe an obvious neuroendocrine phenotype characterized by loss of AR expression or elevated expression levels of neuroendocrine markers, including enolase 2 and chromogranin A/B. The possibility of EZH2 involvement in AA resistance in some of the cell lines is not completely excluded; however, our AA resistant cell lines only showed a mild reduction in sensitivity to the EZH2 inhibitor GSK126, and we did not observe a significant change of EZH2 mRNA and protein levels in AA-resistant cell lines (Supplementary Fig. S8). The study by Zhang et al. (47) showed that enzalutamide is also able to promote the CREB-EZH2-TSP1 pathway. This study together with our data suggested that the clinically observed AA resistance post enzalutamide treatment could be due to the activation of CREB.

Activation of CREB1 phosphorylation was observed in all three AA-resistant cell lines developed from different parental cell lines. Only a few overlapping differentially expressed genes could be identified, and none of these belong to the pool of CREB1 target genes. It is possible that due to their genetic differences, different CREB1 downstream targets may be involved in the maintenance of resistance and cell growth in different AA-resistant cell lines. There is a very limited amount of genome-wide data on CREB and/or CBP/p300 transcriptome and ChIP-Seq in prostate cancer cells. Comparing the expression level of the

CREB target gene data set developed in the TRANSFAC database on human transcriptional regulation (48,49), we found that differentially expressed CREB target genes are very different among the three pairs of prostate cancer cell lines (Supplementary Fig. S7). Few genes overlapped between each pair and none among all three pairs. We think that different AA-resistant cell lines may exhibit different sets of differentially expressed CREB or CBP/p300 target genes and some of the differentially expressed target genes may have potential clinical benefit in future therapeutic applications. To better assess CREB-CBP/p300 targets of importance for improving the efficacy of AR targeted drugs, we plan to develop datasets of CREB and/or CBP/p300 transcriptome and CHIP-Seq in our AA-sensitive and -resistant prostate cancer cell lines for a comprehensive investigation of target genes.

In summary, our study indicates that AA treatment itself induces adaptive mechanisms for cancer cell survival and growth. Even though patients initially respond to treatment, eventually their cancer progresses and becomes resistant to treatment. Thus, rationally designed combination therapies targeting known resistance mechanisms may significantly improve efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance:

Resistance to androgen receptor-targeted therapies is a major clinical challenge in prostate cancer. Our study demonstrates a new acquired AA resistance mechanism which involves the activation of CREB1 and the CBP/p300 complex, following AA treatment. Our data also demonstrated that low doses of AA significantly synergize with CBP/p300 inhibitors in limiting the growth of prostate cancer cells. This result indicated that combining AA with therapies targeting resistance mechanisms may provide a more effective treatment strategy. We present here a rationally-designed combination for two clinically-relevant drugs, providing a greater efficacy than the sum of the two single agents, for future treatment considerations for patients with advanced prostate cancer.

Figure 1. AA sensitivity in prostate cancer cell lines and characterization of AA resistant cell lines.

(A) Growth curves of different prostate cancer cell lines. AR positive, androgen dependent LNCaP and LAPC-4, and androgen independent LNCaP-abl and PC-3 were treated with different concentrations of AA; (B) Growth curves and colony formation assays of LNCaP and LNCaP-AAr. Solid and dotted lines represent growth in the absence or presence of 5 μ M AA, respectively; (C) Growth curves and colony formation assays of LAPC-4 and LAPC4-AAr. Solid and dotted lines represent growth in the absence or presence of 5 μ M AA, respectively; (D) Transwell assay, comparing LNCaP and LNCaP-AAr, and LAPC-4 and

LAPC4-AAr. The bar graph on the right shows the quantitative comparison; (E) Comparison of AR and PSA expression levels in LNCaP and LNCaP-AAr. RT-PCR was performed. For DHT induction, cells were maintained in C-FBS for 48 h and then were treated with 10 nM DHT for 16 h; (F) Comparison of Enzalutamide sensitivity. Cell growth in the presence of different concentrations of Enzalutamide was measured in LNCaP/LNCaP-AAr and LAPC-4/LAPC4-AAr. IC₅₀ values indicated by red lines. Figure values represent the mean $\pm$ SE of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 2. Transcriptomic analysis revealed mechanisms involved in AA resistance development. (A) Volcano plots of differentially expressed genes comparing AA resistant and parental cell lines. LNCaP-AAr, LAPC4-AAr and LNCaP-abl-AAr, were compared with their corresponding parental cell lines LNCaP, LAPC-4 and LNCaP-abl, respectively. Red denotes significant variables; green indicates non-significant variables; (B) Venn diagrams showing the overlap of differentially expressed genes (Left side: up-regulated genes; right side: down-regulated genes); (C) Comparison of expression levels of *CYP17A1*, *AR-v7*, *GR* and *SGK1*. mRNA levels were determined by RT-PCR in LNCaP, LNCaP-abl, and LAPC-4 and their

corresponding AA resistant cell lines. inserted is the fold change of expression level of SGK1 after 16 h DHT induction in cFBS. Expression levels were compared by Student's t test, n.s not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; (D) GSEA analysis of the differentially expressed genes; (E) Metacore analysis of key transcription factors that regulate the differentially expressed genes identified by RNAseq between parental and resistant cell lines (blue, LNCaP-AAr VS LNCaP; purple, LNCaP-abl-AAr VS LNCaP-abl; orange, LAPC4-AAr vs. LAPC-4); (F) Phospho-kinase array screening. Experiments were performed in LNCaP and LAPC4 and their corresponding AA resistant cell lines. Differentially expressed phosphor-proteins are labeled by red frames including C3/C4 (p-CREB1@S133), C15/C16 (c-Jun@S63) and F13/F14 (WNK1@T60); (G) Validation of differentially expressed phosphor-proteins by Western blot analysis.

Figure 3. CREB1 activation desensitizes AA sensitivity in prostate cancer cell lines.

(A) Comparison of cAMP levels in LNCaP-AAr and LAPC4-AAr with their parental LNCaP and LAPC-4, respectively; (B) AA treatment increased cAMP levels. LNCaP (Left) and LAPC-4 (right) were treated with 5 μM AA for different days; (C) Over-expression of wild type CREB1 enhanced AA resistance in LNCaP and LAPC-4. LNCaP and LAPC-4 were transfected with the wild type CREB1 construct (CREB), the CREB1 mutant construct, which contained a mutation at S133A (CREB-133), or vehicle. 48 h after transfection, cells were either treated with 5 μM AA or an equivalent amount of DMSO. Left side shows the

comparison of cell growth. Right side shows Western blot analyses of the protein levels of CREB1 and p-CREB1 in cells before and after transfection; (D) The effect of Forskolin on AA sensitivity in LNCaP (left) and LAPC-4 (middle). Western blot analyses on the right showed that Forskolin treatment increased the pCREB1 level. (E) The impact of the CREB1 inhibitor, 666–15, on AA sensitivity in LNCaP-AAr (left) and LAPC4-AAr (middle). Western blot (right) demonstrated the effect of 666–15 on the level of CREB1 and pCREB1. All values represent the mean $\pm$ SE of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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Figure 4. AA treatment affects the sensitivity of prostate cancer cells to CBP/p300 inhibitors. (A) CREB1 interactor network in *Homo sapiens* based on the data from BioGRID database; (B) LNCaP-AAr exhibited a relatively higher sensitivity to CBP/P300 inhibitors, compared to LNCaP. Cell growth was measured in the presence of different concentrations of CBP/P300 inhibitors, SGC-CBP30 and C646. IC₅₀ values are indicated by red lines; (C) Inhibition of CBP/p300 re-sensitized LNCaP-AAr to AA treatment. Cell proliferations were measured at 72 h after treatment with different drugs as indicated. All experiments were completed with three biological replicates.

Figure 5. AA treatment synergizes the effect of CBP/P300 inhibitors.

(A) AA treatment synergizes the effect of CBP/P300 inhibitors in LNCaP. Cells were treated for 72 h with CBP/P300 inhibitor, C646 (left) or SGC-CBP30 (right), alone or in combination with AA and cell viability was determined. A dose-effect analysis of drug combinations to determine synergism/antagonism, based on the Chou-Talalay method, was performed using the Compusyn software. Combination index (CI) values shown above the bars were mostly < 1 , indicating a synergistic effect of both drugs at specific concentrations; (B) Synergy scores were calculated using the Synergyfinder software. ZIP Synergy scores $>$

0 indicate synergism (red regions) and scores < 0 indicate antagonism (green regions). Concentrations marked with green boxes on the x and y-axis indicate concentrations encompassing the region of the highest synergy (indicated by the white rectangle). The value in the white box represents the averaged synergy score for the region of the highest synergy. 3D graphs shown as well; (C) Diagram illustrating proposed model of CREB1 mediated AA resistance.