Therapeutic targeting of the CBP/p300 bromodomain blocks the growth of castration-resistant prostate cancer

Lingyan Jin\textsuperscript{1}, Jesse Garcia\textsuperscript{2}, Emily Chan\textsuperscript{2}, Cecile de la Cruz\textsuperscript{2}, Ehud Segal\textsuperscript{2}, Mark Merchant\textsuperscript{2}, Samir Kharbanda\textsuperscript{3}, Ryan Raisner\textsuperscript{1}, Peter M Haverty\textsuperscript{4}, Zora Modrusan\textsuperscript{5}, Justin Ly\textsuperscript{6}, Edna Choo\textsuperscript{6}, Susan Kaufman\textsuperscript{7}, Maureen H Beresini\textsuperscript{7}, F. Anthony Romero\textsuperscript{8}, Steven Magnuson\textsuperscript{9}, & Karen E Gascoigne\textsuperscript{1,10}

\textsuperscript{1}Department of Discovery Oncology, Genentech, Inc., South San Francisco, California, USA.
\textsuperscript{2}Department of Translational Oncology, Genentech, Inc., South San Francisco, California, USA.
\textsuperscript{3}Calico Labs, South San Francisco, California, USA.
\textsuperscript{4}Department of Bioinformatics, Genentech, Inc., South San Francisco, California, USA.
\textsuperscript{5}Department of Molecular Biology, Genentech, Inc., South San Francisco, California, USA.
\textsuperscript{6}Department of DMPK, Genentech, Inc., South San Francisco, California, USA.
\textsuperscript{7}Department of Biochemical and Cellular Pharmacology, Genentech, Inc., South San Francisco, California, USA.
\textsuperscript{8}Unity Biotechnology, Brisbane, California, USA.
\textsuperscript{9}Department of Discovery Chemistry, Genentech, Inc., South San Francisco, California, USA.
\textsuperscript{10}Correspondence: Karen Gascoigne, Genentech, 1 DNA way, South San Francisco, 94080.

Email: gascoigne.karen@gene.com, tel: 617 460 5168

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Corresponding author: Karen Gascoigne, Genentech, 1 DNA way, South San Francisco, 94080. Email: gascoigne.karen@gene.com, tel: 617 460 5168, fax: 650 742 5179

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Abstract

Resistance invariably develops to anti-androgen therapies used to treat newly diagnosed prostate cancers, but effective treatments for castration-resistant disease remain elusive. Here we report that the transcriptional co-activator CBP/p300 is required to maintain the growth of castration-resistant prostate cancer. To exploit this vulnerability, we developed a novel small-molecule inhibitor of the CBP/p300 bromodomain that blocks prostate cancer growth in vitro and in vivo. Molecular dissection of the consequences of drug treatment revealed a critical role for CBP/p300 in histone acetylation required for the transcriptional activity of the androgen receptor and its target gene expression. Our findings offer a preclinical proof of concept for small molecule therapies to target the CBP/p300 bromodomain as a strategy to treat castration-resistant prostate cancer.

Introduction

Prostate cancer (PC) cell growth is driven by activity of the Androgen Receptor (AR), and therapies that target the androgen-signaling axis have been highly successful in treatment of the disease (1,2). Despite the success of these agents in slowing disease progression, prostate cancer remains one of the leading causes of cancer deaths in men, with approximately 26,000 deaths in 2016 (3). The majority of patients who progress on anti-androgen therapies (a state termed Castration Resistant Prostate Cancer (CRPC)) retain activity of the AR signaling pathway, as evidenced by maintained AR and Prostate-Specific Antigen (PSA) levels (4-8). Preclinical models have identified a number of mechanisms by which AR activity can be maintained in the presence of therapy. These include alterations to androgen synthesis pathways, AR amplification, point mutations in the AR ligand binding domain (LBDmut), and expression of AR
splice variants lacking the LBD (ΔLBD) (7,9-12). Many of these alterations have also been observed in clinical samples (13,14).

Current PC therapies that target the androgen-signaling axis focus on inhibition of AR or its ligand. Thus, resistance mechanisms commonly center on re-establishment of AR activity, via the mechanisms described above. However, in order to co-ordinate gene expression, AR must act in concert with numerous co-activator proteins (15). The formation of AR-co-activator complexes promotes the opening of chromatin at AR binding sites, and the recruitment of transcriptional machinery to target genes. We hypothesized that this dependence on co-activator proteins for the functional output of AR activation may present a potential point of intervention in CRPC. The highly homologous histone acetyltransferases CBP (cAMP response element binding protein (CREB) binding protein) and P300 are known co-activators of AR, and have been implicated in enhancing the response to androgen (16-18). Consistent with this, CBP/P300 have been proposed to play an oncogenic role in prostate cancer, and up-regulation of both proteins has been observed in tumors (19-21).

In the current study we use a novel small-molecule inhibitor of the CBP/P300 bromodomain to demonstrate a key role for this domain in the co-activator functions of CBP and P300. We describe significant dose-dependent inhibition of AR signaling and PC proliferation in vitro and in vivo. Taken together these data strongly support CBP/P300 bromodomain inhibition as a therapeutic strategy in CRPC.

Materials and Methods

Inhibitor Characterization and Use
Enzalutamide and JQ1 were obtained from Selleckchem, dissolved in DMSO and used at the indicated concentrations. See Supplementary Note 1 for the detailed experimental synthesis procedures for GNE-049. For cellular experiments GNE-049 was dissolved in DMSO and used at the indicated concentrations.

GNE-049 potency and selectivity was evaluated in a panel of biochemical bromodomain binding assays. All biochemical assay protocols were carried out as previously described (22-24).

Binding of biotinylated small-molecule ligands to recombinant His-tagged bromodomains was assessed by time-resolved fluorescence resonance energy transfer (TR-FRET). Test compounds that compete with the biotinylated ligand for bromodomain binding reduce the TR-FRET signal. A Bioluminescence Resonance Energy Transfer (BRET) assay was used to measure cellular engagement of CBP by GNE-049 through disruption of the interaction between a tagged histone H3 construct and a CBP-luciferase construct in transfected HEK293 cells. The assay was carried out as previously described (22). To determine the inhibition of MYC expression, MV-4-11 cells were plated at 10,000 cells per well in 96-well plates in RPMI1640 media supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Test compounds diluted in DMSO were transferred to the cell plates, keeping final DMSO concentration constant at 0.1%, and incubated for 4 h at 37 °C. Lysis and analysis for MYC expression were carried out using QuantiGene 2.0 reagents (Affymetrix/eBioscience, probe set cat # SA-50182) and following the manufacturer’s instructions. Luminescence was read using an EnVision plate reader (PerkinElmer) and EC$_{50}$s generated in Genedata Screener using a 4-parameter non-linear regression fit.

Pharmacokinetic properties of GNE-049 were determined in mice at WuXi AppTech in Shanghai, China. Three SCID.Beige mice were obtained from SLK Laboratory Animal Co., Ltd., Shanghai,
P.R. China. All animals were female, 6-9 weeks old at the time of study and weighed between 15 and 25 g. The mice were dosed with GNE-049 30 mg/kg p.o. (suspended in 0.5% w/v methylcellulose, 0.2% w/v Tween 80). Food and water were available ad libitum to all animals. Serial blood samples (15 µL) were collected by tail nick at 0.083, 0.25, 0.5, 1, 3, 8, and 24 h after the oral administration. All blood samples were diluted with 60 µL water containing 1.7 mg/mL EDTA and kept at -80 °C until analysis. All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and were approved by the Institution's Animal Care and Use Committee.

Concentrations of GNE-049 were determined by a non-GLP LC-MS/MS assay. The diluted blood samples were prepared for analysis by placing a 20 µl aliquot into a 96-well plate followed by the addition of 200 µl of acetonitrile containing an internal standard mixture (0.1 µg/mL diclofenac). The samples were vortexed and centrifuged at 4000 rpm for 20 minutes at 4 °C; 70 µL of the supernatant was diluted with 140 µL of 0.1% formic acid (FA) in water and 10 µL of the solution was injected onto an analytical column. An ACQUITY UPLC System (Waters) coupled with an API 4000 mass spectrometer (AB Sciex, Foster City, CA) was used for sample analysis. The mobile phases were 0.3% FA and 2 mM NH₄OAc in water/ACN (v:v, 95:5) (A) and 0.3% FA & 5 mM NH₄OAc in ACN/water (v:v, 95:5) (B). The gradient was started at 20% B and increased to 90% B for 1.2 minutes, maintained at 90% B for 0.4 minutes, then decreased to 20% B within 0.1 minute. The total flow rate was 0.55 ml/min, and samples were injected onto an ACQUITY BEH C8 (100 × 2.1 mm, 1.7 µm) analytical column with a total run time of 1.7 minutes. Data were acquired using multiple reactions monitoring (MRM) in positive ion electrospray mode with an operating source temperature of 550 °C. The MRM transition was m/z 511.2 → 471.2 for GNE-
049 and 296.0 → 214.0 for diclofenac. The lower and upper limits of quantitation of the assay for GNE-049 were 0.005 and 10 µM, respectively.

**Cell Culture and Proliferation Assays**

All prostate cancer cell lines were obtained from ATCC, authenticated by STR and tested to ensure free from mycoplasma within 3 months of use. All lines were cultured in RPMI1640 media supplemented with 10% fetal bovine serum and 2 mM glutamine, and used for experimentation within 1 month of thawing from frozen stock. Androgen deprivation was achieved by culturing cells in RPMI media supplemented with 10% charcoal stripped serum (CCS) (Sigma Aldrich) for at least 5 days. Androgen stimulation was then achieved by addition of 0.1 nM Methyltrienolone (R1881) (Sigma-Aldrich) for at least 24h. Cell proliferation in 2D was evaluated in a 384-well format using CellTiter-Glo® reagent (Promega) according to manufacturer’s instructions. 3D growth was assessed by culture in soft agar in a 96 well format. Growth was assessed by colony number and area counts using an optical plate reader.

For assessment of drug combination effects the Bliss independence model was used (25). The Bliss expectation was determined with the equation (A+B)-A*B, in which A and B are the fractional growth inhibitions induced by agent A and B at a given dose. The difference between the Bliss expectation and the observed growth inhibition with agents A and B used at the same dose is determined to be the Bliss excess. A Bliss sum score was calculated by summing the bliss excess values of all dose combinations. To determine if positive Bliss sum scores represented robust synergy, we ranked our data compared to previous data sets generated in the same manner (26,27).
Stable Cell Line Generation

Stable LNCaP cell lines over-expressing wild type or mutant AR were generated by Lentiviral delivery of a plasmid encoding N-terminally FLAG tagged AR. Briefly, lentivirus was generated by co-transfection in 293T cells with plasmid Lv102-AR (GeneCopoeia, Inc.) (WT or F877L introduced by site directed mutagenesis (QuickChange II Agilent), envelop plasmid VSVG and packaging plasmid delta8.9 at a molar ratio of 1:2.3:0.2. 48 hours after transfection, lentiviral supernatant was collected, filtered through a 0.45 μM syringe, and added to LNCaP cells. Cells were centrifuged at 1800 rpm for 45 minutes at room temperature and incubate at 37 °C for 3 days before adding puromycin at a final concentration of 2 ug/ml. 3 weeks after puromycin selection, LNCaP cells stably expressing wild type or mutant AR were used for subsequent analysis.

Cell Transfection and RNA interference

Knock-down of AR, CBP and P300 was accomplished by RNA interference. For each, a pool of 4 commercially available siRNAs was used (Dharmacon - SMARTpool ON-TARGETplus. AR: L-003400-00-0005, CREBBP: LU-003477-00-0002, EP300: L-003486-00-0005). 100 nmol of siRNA was transfected using Liopfectamine® RNAiMAX (Invitrogen) according to the manufacturer’s instruction. Gene expression was evaluated 72 h after siRNA transfection, and cell viability was assessed 5 days after transfection.

Western blot and immuno-precipitation

Cells were lysed in RIPA buffer and separated on a denaturing poly-acrylamide gel (Invitrogen) according to manufacturer’s instructions. Proteins were transferred to nitrocellulose membrane
using the IBLOT® system (Invitrogen) and blocked for 2 h in 5 % non-fat milk solution. Membranes were incubated overnight with the following antibodies: AR (Santa Cruz Biotechnology) CBP (Santa Cruz Biotechnology), P300 (Bethyl Labs), Tubulin (DM1a Abcam). Signal was detected using fluorescently labeled secondary antibodies (IRDye® Li-COR) and the Odyssey CL-x imager (Li-COR). FLAG tagged AR was immune-precipitated from LNCaP nuclear extract, obtained by sequential extraction with hypotonic buffer (10mM HEPES pH 7.9, 10mM KCl, 1.5mM MgCl2, 0.34M sucrose, 10% glycerol, Triton X 0.1%, 1mM DTT and protease inhibitor) and nuclear lysis buffer (20nM Tris pH8, 10% glycerol, 1% NP40, 2mM EDTA, 400mM NaCl, 1mM DTT and protease inhibitor). Extracts were incubated with anti-FLAG antibody (Sigma-Aldrich) overnight, before precipitation with protein G magnetic beads (Dynabeads, Thermo). Immune precipitates were washed three times with PBS + 1% NP40, once with PBS, and boiled in SDS sample buffer.

**Gene expression analysis**

RNA was purified from cells using the RNeasy kit (Qiagen) according to manufacturer’s instructions. Quantitative RT-PCR was performed using Taqman assay (ThermoFisher Scientific, Inc.) on ABI QuantStudio 7 Flex real-time PCR system. For whole transcriptome RNA-sequencing RNA libraries were made using TruSeq RNA Sample Preparation Kit v2 (Illumina). Size of the libraries was confirmed using Fragment Analyzer (Advanced Analytical Technologies) and their concentration was determined by a qPCR based method using Library Quantification Kit (KAPA). The libraries were multiplexed and then sequenced on Illumina HiSeq2500 (Illumina) to generate 30M of single-end 50 base-pair reads. Data has been made publically available at the European Nucleotide Archive (ENA), project ID PRJEB20821. Gene-
set enrichment analysis (GSEA) was performed using Broad Institute software (http://software.broadinstitute.org/gsea/index.jsp)\textsuperscript{27}. For total mRNA quantification polyadenylated mRNA was isolated from total RNA using the Oligotex mini kit (Qiagen) according to manufacturers instructions, and quantitated using a NanoDrop spectrophotometer (Thermo Scientific).

**Chromatin immuno-precipitation and sequencing**

After appropriate drug treatment, cells were cross-linked by 15 minute incubation in 1% formaldehyde, then quenched for 5 minutes in 125 mM glycine. Subsequent steps were performed at Active Motif. Cells were lysed and chromatin sheared by sonication to an average length of 300-500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield. 30 μg of chromatin was precleared with protein A agarose beads (Invitrogen). Immunoprecipitation was then carried out using the following antibodies: anti-H3K27Ac (Active motif), anti-AR (Santa Cruz Biotechnology), anti-P300 (Santa Cruz Biotechnology). Reactions were spiked with *Drosophila* chromatin (Active Motif 53083) and a *Drosophila* specific antibody (Active Motif 61686) as a mechanism for normalization. Complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65 °C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation.
ChIP and Input DNAs were prepared for amplification by converting overhangs into phosphorylated blunt ends and adding an adenine to the 3’ ends. Illumina genomic adapters were ligated and the sample was size-fractionated (200-300 bp) on an agarose gel. After a final PCR amplification step (15 cycles), the resulting DNA libraries were quantified and sequenced on Illumina NextSeq 500 (75 nt reads, single end).

Reads were aligned to the human genome (GRCh37) using the GSNAP algorithm (version 2013-10-10) with the following settings: "-M 2 -n 10 -B 2 -i 1 --pairmax-dna=1000 --terminal-threshold=1000 --gmap-mode=none --clip-overlap". Fragment length was determined by the strand cross-correlation method. Reads were extended to this fragment length before coverage was calculated at a per-nucleotide level using uniquely mapping reads.

These coverage values were normalized using the coverage from uniquely mapping reads for the Drosophila melanogaster genome as follows. All reads were also aligned to the Drosophila melanogaster genome (version BDGP5.25.64 from Ensembl) using the above GSNAP settings and version. The GRCh37 uniquely mapped coverage values (H) were normalized by the Drosophila uniquely mapped coverage values (D) to produce scaled coverage values (Hs) with the following formula: Hs = (H/D) * 1e6. MACS (PMID: 22936215 version 2.0.10) was used to identify coverage peaks using the default settings. Data has been made publicly available at the ENA, project ID PRJEB20981.

Enhancers regions were identified by processing the H3K27Ac ChIP sequence reads and associated MACS 2.0 peaks with the ROSE program (PMID:23582322) using the settings "-g HG19 --t 0". For the comparison of enhancer and Transcription Start Sites (TSS), these enhancers were scaled to a common width of 5kb. TSS regions were centered on the TSS of...
each NCBI RefSeq transcript (release 53) and set to a common width of 5kb. Coverage, normalized to sample library size, was counted in these regions, for each sample, using 25 bins.

**In vivo studies**

All procedures were approved by and conformed to the guidelines and principles set by the Institutional Animal Care and Use Committee of Genentech and were carried out in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. Prostate cancer Patient-Derived Xenograft tumors (PDXs) were established by passage of tumor fragments (~1 mm³) subcutaneously (s.c.) into 6 to 8 week old recipient mice. One to three days prior to inoculation with TM00298, female NSG mice (Jackson Laboratories, Sacramento, CA) were inoculated s.c. with 12.5 mg 90-day slow release dihydrotestosterone (DHT) pellets (Innovative Research of America, Sarasota, FLA). TM00298 primary tumor fragments (between passages 2-9) were implanted s.c. in the hind flank into DHT-pellet inoculated NSG mice. For the LuCAP-77 male NSG mice (Jackson Laboratories, Sacramento, CA) aged 6 to 7 weeks old were transplanted in the right flank subcutaneously with tumor fragments without addition of DHT pellets. For LuCAP-96.1 tumors male C.B-17 SCID.bg mice were inoculated as above. For the LuCAP-37V model castrated male C.B-17 SCID.bg mice were inoculated as above, without addition of DHT pellets. Tumors were monitored until they reached a mean tumor volume of ~150-350 mm³ and then animals were randomized into one of four treatment groups (PD analyses (n=5-9 per group); efficacy studies (n=10/group for LUCAP-96.1 and LuCAP-77, 5/group for LuCAP35V): [1] Vehicle controls (0.5% methylcellulose/0.2% Tween-80 in water and 1% carboxymethylcellose/0.1% Tween-80/5%DMSO in water (100 µL total), dosed twice daily (BID) via oral gavage (PO)); [2] GNE-049 (25 mg kg⁻¹ for PD study, 30 mg kg⁻¹
for efficacy studies (100 µL total), dosed twice daily (BID), PO; [3] enzalutamide (10 mg kg⁻¹ (100 µL total) PO, QD), and [4] GNE-049 plus enzalutamide at these same doses and schedules. For PD studies, animals were dosed for 7 days with tumors being collected 3 to 4 hr post-last dose.

Tumor size and body weight measurements were made twice per week. Tumor volumes were measured in two dimensions (length and width) using Ultra Cal-IV calipers (model 54 – 10 – 111; Fred V. Fowler Co.; Newton, MA) and analyzed using Excel, version 11.2 (Microsoft Corporation; Redmond WA) or Prism 6 (GraphPad Software Inc., La Jolla, CA). The tumor volume was calculated with the following formula: Tumor size (mm³) = (longer measurement × shorter measurement²) × 0.5. Animal body weights were measured using an Adventura Pro AV812 scale (Ohaus Corporation; Pine Brook, NJ). Percent weight change was calculated using the following formula: Group percent weight change = (new weight – initial weight)/ initial weight) × 100. To analyze the repeated measurement of tumor volumes from the same animals over time, a mixed-modeling approach was used (28). This approach addresses both repeated measurements and modest dropouts due to any non-treatment-related removal of animals before the end of study. Cubic regression splines were used to fit a non-linear profile to the time courses of log₂ tumor volume at each dose level. The non-linear profiles were then related to dose within the mixed model. Tumor growth inhibition (TGI) as a percentage of vehicle was calculated as the percentage of the area under the fitted tumor volume–time curve (AUC) per day for each dose group in relation to the vehicle, using the following formula:

% TGI = 100 × [1 – (AUCdose per day ÷ AUCvehicle per day)].
Results

AR-Driven Prostate Cancer Cell Lines are Sensitive to CBP/P300 Bromodomain Inhibition

To investigate the role of CBP/P300 in prostate cancer biology, we selected a panel of cell line models in which to evaluate their contribution to cell proliferation (Fig. 1A). We identified 6 cell lines with varying AR expression – LNCaP and MDA-PCa2b (AR WT), VCaP (AR overexpressing), 22RV1 (AR splice variant lacking LBD (ΔLBD) (29)), PC3 and DU145 (AR absent). We confirmed that AR-expressing cell lines were dependent on AR for proliferation by siRNA knockdown, using an N-terminally targeted siRNA that down-regulated both full-length and ΔLBD AR (Fig. 1B and Supplementary Fig. S1A). We also confirmed that cell lines were dependent on CBP/P300 for proliferation by siRNA knockdown (Fig. 1C and Supplementary Fig. S1B) (30). CBP and P300 co-depletion inhibited proliferation even in cell lines lacking AR, consistent with a core role for these co-activators in transcriptional regulation.

To probe the potential for therapeutic targeting of CBP/P300 in prostate cancer, we generated a small-molecule inhibitor (GNE-049), targeting the chemically tractable bromodomain of CBP/P300 (Fig. 1D). We and others have previously shown CBP/P300 bromodomain inhibition to have anti-tumor activity in hematological cancer models (31-33). GNE-049 is a potent inhibitor of both the CBP (IC$_{50}$ = 1.1 nM) and P300 (IC$_{50}$ = 2.3 nM) bromodomains in a biochemical bromodomain-binding assay (Supplementary Fig. S1C). This compound displayed excellent potency (12 nM) in a bioluminescence resonance energy transfer (BRET) cellular assay and inhibited the expression of MYC with an EC$_{50}$ of 14 nM in MV-4-11 cells (Supplementary Fig. S1D,E). Importantly, this compound is exquisitely selective for CBP/P300 in general, and remarkably selective (3,850-fold) for CBP/P300 over BRD4 (IC$_{50}$ = 4,240 nM) (Supplementary
Table S1). The compound afforded a good balance of cell potency, selectivity and favorable PK properties (Supplementary Fig. S1F), and was therefore selected for additional studies.

We evaluated the effect of CBP/P300 bromodomain inhibition by GNE-049 on prostate cancer growth in both a two-dimensional (2D) setting with growth of cells on plastic, and a three-dimensional setting (3D) setting with growth in soft agar. Proliferation of AR-expressing PC cell lines was significantly impacted by GNE-049 treatment, to an extent greater than enzalutamide, in all models. This effect was observable under both 2D and 3D growth conditions (Fig. 1E). However, viability of AR negative PC3 and DU145 cells was unaffected by GNE-049 treatment under any conditions.

The CBP/P300 Bromodomain is Required for AR Target Gene Expression

To investigate in more detail the selective effect of GNE-049 only on AR-expressing PC cells, we evaluated the impact of CBP/P300 knockdown and GNE-049 on expression of select AR target genes (Fig. 2A, B). While GNE-049 did not impact AR levels (Supplementary Fig. S2A), it repressed AR target-gene expression in a dose-dependent manner in all three AR-expressing cell lines (Fig. 2B), as well as Myc expression in a subset of lines (Supplementary Fig. S2B). Consistent with modulation of the AR signaling axis by GNE-049, enzalutamide was not strongly synergistic with GNE-049 in LNCaP cells, in both gene-expression and cell-viability assays (Supplementary Fig. S2C, D). In a cell-viability assay GNE-049 was moderately synergistic with the BET inhibitor JQ1, as reported previously (32) (Supplementary Fig. S2E). In all cell lines the extent of AR target gene repression with GNE-049 was greater than that with enzalutamide. In particular, we noted significant repression of AR target-genomes in 22RV1 cells, which remain insensitive to enzalutamide due to expression of ARΔLBD. This result suggests that even in the
absence of ligand dependence, the co-activator function of CBP/P300 is required for AR-driven gene expression. Consistent with published data, this was recapitulated by CBP and P300 co-depletion by siRNA, which also significantly repressed AR target-gene expression (Fig. 2A) (30). To confirm that GNE-049 impacted the full repertoire of AR-regulated genes, we conducted whole transcriptome RNA-sequencing (RNA-seq) of cell lines after 24 h treatment with 1 μM GNE-049 (Fig 2C). While we observed no global repression of transcription upon drug treatment (Supplementary Fig S2F), Gene-Set Enrichment Analysis (GSEA) indicated that a hallmark set of AR target genes was down-regulated by GNE-049 treatment, implying a significant impact on the transcriptional output of AR (Fig. 2C) (34).

**The CBP/P300 Bromodomain is Required for the Response to Androgen**

The observation that GNE-049 treatment reduced expression of known AR target genes suggests that GNE-049 may directly impact the ability of PC cells to respond to androgen stimulation. To test this, we starved cells of androgen by growth in charcoal-stripped serum (CSS) for 5 days, and then assessed gene expression via RNA-seq after 24 h of stimulation with 0.1 nM R1881 (synthetic androgen). We identified a set of 292 genes induced by androgen in LNCaP cells (Fig. 3A and Supplementary Table S2). GSEA analysis confirmed that this gene set significantly overlapped with previously identified AR target gene signatures (35) (Fig. 3B). Co-treatment with 1 μM of GNE-049 in parallel with R1881 stimulation prevented induction of this gene set, in a manner similar to enzalutamide co-treatment (Fig. 3A). Consistent with this analysis, RT-PCR of the *KLK3* gene (encoding PSA) indicated that GNE-049 could block R1881-induced *KLK3* expression in a dose-dependent manner in LNCaP cells (Fig. 3C). GNE-049 treatment also blocked androgen-induced proliferation when cells were stimulated with R1881 in
the presence of 1 μM GNE-049 for 6 days (Fig. 3D). In particular, we noted significant reduction in proliferation of R1881-stimulated 22RV1 cells in the presence of GNE-049, even though these cells remained unresponsive to enzalutamide in this assay. Taken together, these data suggest that the CBP/P300 bromodomain is required for prostate cancer response to androgen, and imply a direct role for the bromodomain of CBP/P300 in mediating the co-activator function for AR.

Inhibition of the CBP/P300 bromodomain Prevents AR Co-activator Function

We sought to better understand the mechanism by which CBP/P300 functionally regulate the AR transcriptional response by investigating AR and CBP/P300 co-association and localization on chromatin. Consistent with an AR co-activator function, P300 could be co-immunoprecipitated with AR in nuclear extract from LNCaP cells expressing FLAG-tagged AR (Fig. 4A). We also confirmed the co-localization of these proteins on chromatin by chromatin-immunoprecipitation and sequencing (ChIP-seq) analysis of LNCaP cells after 24h stimulation with R1881 (Fig. 4B,C). Using the set of 292 androgen-induced genes identified by RNA-seq (Fig. 3a), we defined a set of direct AR target genes, as those R1881-induced genes with AR peaks located at their promoters or nearby Androgen Responses Element (AREs). This represented 117 of the 292 R1881-induced genes (Fig. 4B). Of these genes, 98 (83%) also had overlapping P300 peaks located at their promoters or AREs (Fig. 4B,C). These data confirm that AR and P300 function together on chromatin.

To test the hypothesis that GNE-049 directly inhibits the AR co-activator function of CBP/P300, we evaluated the impact of drug treatment on acetylation of histone 3 (H3) at lysine 27 (H3K27Ac). H3K27Ac is directly mediated by CBP/P300, and modification of this residue...
promotes an open chromatin structure, allowing for active gene transcription (36). Consistent with a block in co-activator function, GNE-049 treatment markedly reduced H3K27Ac at AR/P300 overlapping peaks (Fig. 4C, D). In addition to changes at specific AR target genes, a reduction in H3K27Ac levels could also be observed globally at enhancers upon GNE-049 treatment (Supplementary Fig. S3A,B), indicating a potential impact on other co-activator functions of CBP/P300 in addition to AR. Surprisingly, GNE-049 did not disrupt the interaction between AR and P300 (Fig. 4A), nor did it impact AR or P300 recruitment to chromatin by androgen stimulation (Fig. 4C, D). These data suggest a model wherein GNE-049 inhibits the AR co-activator function of P300, preventing histone acetylation and activation of gene transcription, but not disruption of AR/co-activator complex formation. This implies that the bromodomain, while dispensable for chromatin binding, plays a critical role in the AR co-activator function of CBP/P300.

**Endocrine Therapy Resistance Models Remain Sensitive to CBP/P300 Bromodomain Inhibition**

The observation that GNE-049 can significantly inhibit the proliferation of prostate cancer cells suggests such inhibitors may represent a novel therapeutic strategy in this disease. As CRPC remains a major unmet medical need, we wished to further evaluate the effectiveness of GNE-049 treatment in this context. To better model the progression from Castration–Sensitive (CS) to Castration-Resistant (CR) disease, we sought to generate cell line models that recapitulate some of the common mechanisms of androgen therapy resistance, in the same background as a CS model. To this end, we generated LNCaP cell lines over-expressing either WT AR, or F877L mutant AR (ARmut), a mutation identified in patients and known to confer resistance to
enzalutamide (Supplementary Fig. S4A) (11,37,38). As expected, expression of ARmut in LNCaP cells decreased sensitivity to enzalutamide (Fig. 5A). However, ARmut protein still co-immunoprecipitated with P300, suggesting a continued requirement for P300 co-activator activity (Supplementary Fig. S4B). Consistent with this, ARmut expressing cells remained highly sensitive to GNE-049, with significant inhibition of cell proliferation and AR target gene expression (Fig. 5A, B). These data suggest GNE-049 could have significant therapeutic impact in the context androgen therapy resistant disease.

CBP/P300 Bromodomain Inhibitors Show Anti-tumor Activity In Vivo

While cell line models provide an essential tool to dissect the role of CBP/P300 in prostate cancer, their long-term propagation in vitro can lead to adaptation and alterations that may no longer fully recapitulate the clinical disease. To better model the clinical utility of CBP/P300 inhibitors for the treatment of PC, we examined GNE-049 activity in vivo in Patient Derived Xenograft (PDX) models of prostate cancer. These models have been continually propagated in vivo and may therefore better reflect the human disease state (39). Quantitative PCR (qPCR) analysis of tumors after 7 days of treatment with GNE-049, enzalutamide, or the combination of both drugs indicated significant suppression of AR target genes KLK3 (PSA), TMPRSS2, FKBP5 and MYC, in the AR expressing TM00298 PDX model (Fig. 6A). Consistent with these findings, GNE-049 treatment significantly impaired the growth of TM00298 tumors, resulting in 55% tumor growth inhibition (TGI), relative to 21% TGI with enzalutamide after 18 days (Fig. 6B). The utility of GNE-049 was further validated in three additional AR positive PC PDX models. Activity was observed in LuCaP77, LuCaP96.1 and LuCAP35V, both as single agents (86, 75% and 91% TGI, respectively) and when combined with enzalutamide (106%, 118% and 105% TGI, respectively).
respectively) over 21 days (Fig. 6C, D,E). As enzalutamide demonstrated some single-agent activity in the LuCaP77 and LuCaP96.1 models (53% and 39% TGI, respectively), the combination anti-tumor activity was deemed to be additive, consistent with in vitro data (Fig. 6C, D). LuCAP77 and LuCAP35V are well-documented castration resistant models (40,41), confirming the activity of GNE-049 in the CRPC setting. Treatments were tolerated over the course of the studies, although tumor-related cachexia contributed to progressive animal body weight loss in all groups (Fig. 6B-E). Taken together these data indicate a significant role for the CBP/P300 bromodomain in vivo in the response to androgen, and demonstrate that pharmacological inhibition of the CBP/P300 bromodomain can inhibit prostate cancer tumor growth.

**Discussion**

While androgen-directed therapies have successfully extended the lifespan of PC patients, many ultimately still succumb to their disease, and CRPC remains a significant unmet medical need. The common reemergence of AR signaling in CRPC patients highlights a critical dependence on this pathway, and the need for additional strategies for its inhibition. Reemergence of AR signaling leads to restoration of transcriptional output, and increased expression of AR target genes, including cell cycle regulators responsible for driving proliferation. This transcriptional activity of AR requires the interaction with essential co-factors, which act to open chromatin and recruit the basal transcription machinery. As such, AR co-factors represent a potential point of intervention in prostate cancer. Co-factor inhibitors would have the hypothesized advantage that as they are ‘downstream’ of AR, resistance cannot easily be brought about by up-regulation of AR or its ligand, making the case for co-factor inhibitor use up front in combination with traditional
anti-androgens to enhance efficacy and block potential resistance mechanisms. Advances in technologies to interrogate chromatin have resulted in the identification of a number of essential AR co-activators and their proposal as potential drug targets (42-46). Historically, such co-factors and epigenetic regulators have proved notoriously difficult targets for the design of small-molecule inhibitors, hindering progress in this area. However, recent advances in the design of small-molecule bromodomain inhibitors have provided the opportunity to evaluate the potential of AR co-activator inhibitors (47).

In this study we describe a CBP/P300 bromodomain inhibitor with properties allowing direct in vivo assessment of the role of these co-activators in PC biology. Using the potent and selective inhibitor GNE-049, we demonstrate the requirement for CBP/P300 bromodomain activity for the response to androgen in PC, and for the growth of PC models both in vitro and in vivo. Specifically, we show that inhibition of the CBP/P300 bromodomain prevents its co-activator function at AR by preventing the acetylation of H3K27 normally associated with androgen-induced recruitment of AR to chromatin. Since we observe no reduction in P300 or AR recruitment to chromatin in response to drug inhibition, the exact mechanism by which the CBP/P300 bromodomain contributes to histone acetylation remains an exciting open question, and a point for further study. Using GNE-049 to probe the role of this domain in PC biology, we demonstrate significant inhibition of AR target gene as well as Myc expression in several CRPC models, leading to a block in proliferation. These models include those with clinically relevant mechanisms of resistance to current therapies.

Based on our findings we propose a model where in CBP/P300 bromodomain inhibition is sufficient to prevent AR activity at chromatin, thereby blocking expression of key proliferative
gene signatures and tumor growth, and for the first time demonstrate the potential for therapeutic inhibition of CBP/P300 for the treatment of prostate cancer.

Acknowledgments

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References


8. Taplin M-E, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, et al. Mutation of


Figure Legends

Figure 1. AR-expressing prostate cancer cell lines are sensitive to CBP/p300 bromodomain inhibition. A) Western blot showing AR expression levels in prostate cancer (PC) cell lines. B) PC cell line viability measured by CellTiter-Glo® 6 days after transfection with AR targeted siRNAs or non-targeted control (NTC) siRNAs. Data represent mean ± standard
deviation (SD). N=4. C) PC cell line viability measured by CellTiter-Glo® 6 days after transfection with CBP or P300 targeted siRNAs. Data represent mean ± standard deviation (SD). N=4. D) Chemical structure of the CBP / P300 bromodomain inhibitor (GNE-049). E) PC cell lines were cultured under 2D or 3D (soft agar) growth conditions, and treated with the indicated concentrations of compound. For 2D growth conditions viability was measured by CellTiter-Glo® after 6 days of treatment. For 3D growth conditions colony size was measured after 21 days of treatment. Data represent mean ± SD. N=4.

Figure 2. The CBP/P300 bromodomain is required for AR target gene expression. A) RNA levels of the indicated AR target genes were determined by RT-PCR 3 days after LNCaP cells were treated with CBP & P300 targeted siRNAs. B) LNCaP, VCaP and 22RV1 cells were treated for 24 h with a two-fold dilution series of the indicated compounds, starting at 1μM. Expression of the indicated AR target genes was then measured by RT-PCR. Data represent the mean ± SD. N= 3. C) GSEA analysis of RNA-seq data, after the indicated cell lines were treated for 24 h with 1 μM GNE-049 or DMSO control. N=3.

Figure 3. The CBP/p300 bromodomain is required for response to androgen. A) Z-score representation of gene expression determined by RNA-seq, after LNCaP cells were deprived of androgen for 5 days, and then stimulated with 0.1 nM R1881 for 24 h ± 1 μM enzalutamide or GNE-049, N=3. Heatmap shows all genes (292) up-regulated 1.5-fold or more by R1881 treatment. B) GSEA analysis of the Hallmark AR target gene signature in RNA-seq data, after LNCaP cells were deprived of androgen for 5 days, and then stimulated with 0.1 nM R1881 for 24 h. C) LNCaP cells were deprived of androgen for 5 days, before 24h stimulation with 0.1 nM
R1881 in the presence of a two-fold dilution series of the indicated compounds, starting at 1 μM. *KLK3* (PSA) expression was then measured by RT-PCR. Data represent the mean ± SD. N= 3.

D) PC cells were deprived of androgen for 5 days, before 48h stimulation with 0.1nM R1881 in the presence of 600 nM of the indicated compounds. Data represent CellTiter-Glo® readout 48 h after stimulation, mean ± SD. N=4.

**Figure 4. Inhibition of the CBP/P300 bromodomain prevents its AR co-activator function**

A) LNCaP cells stably-expressing FLAG tagged AR were treated with 1 μM GNE-049 for 24 h. FLAG-tagged AR was then was immunoprecipitated (IP’d) from nuclear extracts with anti-FLAG, or control IgG. IPs were probed for the presence for P300 using anti-P300 antibodies. 4% total extract is shown as an input control. B) Chromatin IP sequencing with anti AR, P300 and histone H3K27Ac antibodies was performed on LNCaP cells after 24 h stimulation with 0.1 nM R1881. Diagram indicated the overlap between AR, P300 and H3K27Ac peaks at genes induced by R1881 treatment (as determined in Figure 3A). C) Genome browser representation of ChIP-seq tracks at the *KLK3* (PSA) and *ALDH1A3* genes. Chromatin IPs with anti-AR, anti-P300 and anti-histone H3K27Ac are shown, after 24 h 0.1 nM R1881 stimulation ± 1 μM GNE-049. Y-axis scale shows normalized reads per million per base pairs. D) Summary plots showing P300 and H3K27Ac ChIP-seq peak size, 24 h after stimulation with R1881 ± GNE-049, for peaks overlapping with AR peaks induced by R1881 treatment (as determined in Figure 3A). Axes show normalized reads per million per base pairs in each peak.

**Figure 5. Cell line models of clinical endocrine therapy resistance remain sensitive to CBP/P300 bromodomain inhibition**

A) LNCaP cells overexpressing WT AR (AR) or F877L LBD
mutant AR (mut) were treated with the indicated concentrations of compound. After 6 days of treatment viability was measured by CellTiter-Glo®. Data represent mean ± SD. N=4. B) Cell lines were treated for 24 h with a two-fold dilution series of the indicated compounds, starting at 1 μM. Expression of the indicated AR target genes was then measured by RT-PCR. Data represent the mean ± SD. N= 3.

Figure 6. CBP/P300 bromodomain inhibitors show anti-tumor activity in vivo A) TM00298 PDX prostate tumor-bearing mice were randomized into four groups. Mice were treated by oral gavage (PO) with vehicle or the indicated compounds twice (BID) (GNE-049) or once daily (QD) (enzalutamide). After 7 days tumors were harvested 4 h after the last dose and expression of the indicated AR target genes assessed by RT-PCR. Each point represents data from an individual mouse. B) TM00298 PDX prostate tumor-bearing mice were randomized into four groups, 9 or 10 mice per group. Mice were treated PO with vehicle or the indicated compounds BID (GNE-049) or QD (enzalutamide). Tumor volume (left panel) and body weight (right panel) were monitored over 18 days of continuous treatment with the indicated compounds. Data represent mean ± SD. C) LuCaP-96 PDX prostate tumor-bearing mice were randomized into four groups, 9 or 10 mice per group. Mice were treated PO with vehicle or the indicated compounds BID (GNE-049) or QD (enzalutamide). Tumor volume (top panel) and body weight (bottom panel) were monitored over 21 days of continuous treatment with the indicated compounds. Data represent mean ± SD. D) LuCaP-77 PDX prostate tumor-bearing mice were randomized into four groups, 9 or 10 mice per group. Mice were treated PO with vehicle or the indicated compounds BID (GNE-049) or QD (enzalutamide). Tumor volume (top panel) and body weight (bottom panel) monitored over 21 days of continuous treatment with the indicated compounds. Data represent mean ± SD.
E) LuCaP-35V PDX prostate tumor-bearing mice were randomized into four groups, 5 mice per group. Mice were treated PO with vehicle or the indicated compounds BID (GNE-049) or QD (enazalutamide). Tumor volume (top panel) and body weight (bottom panel) monitored over 21 days of continuous treatment with the indicated compounds. Data represent mean ± SD.
Figure 1: AR expressing prostate cancer cell lines are sensitive to CBP/P300 bromodomain inhibition.

A) Western blot showing AR and Tubulin levels in prostate cancer cell lines.

B) Bar graph showing % Viability for different siRNA treatments in prostate cancer cell lines.

C) Bar graph showing % Viability for different siRNA treatments in prostate cancer cell lines.

D) Chemical structure of Enzalutamide.

E) Graphs showing % Viability and Colony size (%) for different compound concentrations in AR positive and AR negative prostate cancer cell lines.

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Figure 2: The CBP/P300 bromodomain is required for AR target gene expression.

A

KLK3 (PSA)

TMPRSS2

LNCaP

VCaP

22RV1

B

KLK3 (PSA)

TMPRSS2

LNCaP

VCaP

22RV1

C

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 NES = -2.2  FDR <0.0001  
 NES = -2.0  FDR <0.0001  
 NES = -1.8  FDR <0.0001
Figure 3: The CBP/P300 bromodomain is required for response to androgen.
Figure 4: Inhibition of the CBP/P300 bromodomain prevents its AR co-activator function.

A) Western blot analysis showing the inhibition of the CBP/P300 bromodomain by GNE-049 in FLAG-AR and P300 proteins.

B) Venn diagram illustrating the overlap of AR, P300, and H3K27Ac peaks in stimulated cells compared to starved cells with and without GNE-049 treatment.

C) Heatmap analysis of ALDH1A3 and KLK3 genes showing the induction of R1881 in AR, P300, and H3K27Ac peaks in stimulated cells compared to starved cells with and without GNE-049 treatment.

D) Scatter plot showing the peaks of AR, P300, and H3K27Ac in DMSO and GNE-049 treatments in stimulated and starved cells.

KLK3 gene (encoding PSA)
Chr.19: 51,351,619-51,366,090

ALDH1A3 gene
Chr.15: 101,418,009-101,458,831

GNE-049

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Figure 5. Cell line models of clinical endocrine therapy resistance remain sensitive to CBP/P300 bromodomain inhibition

A LNCaP-parental LNCaP-AR LNCaP-ARmut

B LNCaP-AR LNCaP-ARMut

PSA TMPRSS2 Myc

DMSO Enzalutamide GNE-049

1 µM 1 µM 1 µM 1 µM 1 µM 1 µM

% Viability Colony size (%)
Compound conc. [M] Compound conc. [M]

% RNA (%) % RNA (%) % RNA (%)

PSA RNA (%) TMPRSS2 RNA (%) Myc RNA (%)

DMSO Enzalutamide GNE-049 DMSO Enzalutamide GNE-049 DMSO Enzalutamide GNE-049

1 µM 1 µM 1 µM 1 µM 1 µM 1 µM

% RNA (%) % RNA (%) % RNA (%)

PSA RNA (%) TMPRSS2 RNA (%) Myc RNA (%)

DMSO Enzalutamide GNE-049 DMSO Enzalutamide GNE-049 DMSO Enzalutamide GNE-049

1 µM 1 µM 1 µM 1 µM 1 µM 1 µM
Figure 6: CBP/P300 bromodomain inhibitors show anti-tumor activity in vivo.
Therapeutic targeting of the CBP/p300 bromodomain blocks the growth of castration-resistant prostate cancer

Lingyan Jin, Jesse Garcia, Emily Chan, et al.

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