Conversion of abiraterone to D4A drives anti-tumour activity in prostate cancer

Prostate cancer resistance to castration occurs because tumours acquire the metabolic capability of converting precursor steroids to 5α-dihydrotosterone (DHT), promoting signalling by the androgen receptor and the development of castration-resistant prostate cancer1,2. Essential for resistance, DHT synthesis from adrenal precursor steroids or possibly from de novo synthesis from cholesterol commonly requires enzymatic reactions by 3β-hydroxysteroid dehydrogenase (3βHSD), steroid-5α-reductase (SRD5A) and 17β-hydroxysteroid dehydrogenase (17βHSD) iso-enzymes3,4. Abiratone, a steroidal 17α-hydroxysteroid dehydrogenase (SRD5A) and 17β-hydroxysteroid dehydrogenase (17βHSD) isoenzymes5. Abi acetate (Fig. 1b), as well as in sera from patients with CRPC who were undergoing treatment with Abi acetate (Fig. 1c, d and Extended Data Fig. 1). In the LAPC4 prostate cancer cell line, which usually has low 3βHSD activity6, conversion of Abi to D4A is detectable only if 3βHSD is overexpressed (Fig. 1e and Extended Data Fig. 2a, b). Other tissues such as the mouse adrenal (but not mouse prostate) that have robust endogenous 3βHSD enzymatic activity also convert Abi to D4A (Extended Data Fig. 2c). These results suggest that D4A is a major metabolite of Abi, requires 3βHSD for conversion and may confer effects on the tumour that are indirectly due to Abi.

D4A may impinge on multiple steps in the androgen pathway, including CYP17A1, 3βHSD, SRD5A and direct interaction with AR (Fig. 2a). Although augmented Abi drug exposure may block 3βHSD, inhibitory interactions with AR and additional steroidogenic enzymes, making the steroid A and B rings identical to testosterone, enabling conversion of abiraterone to D4A drives anti-tumour activity in prostate cancer. D4A inhibits CYP17A1, 3βHSD and SRD5A, which are required for DHT synthesis. Furthermore, competitive androgen receptor antagonism by D4A is comparable to the potent antagonist enzalutamide. D4A also has more potent anti-tumour activity against xenograft tumours than abiratone. Our findings suggest an additional explanation—conversion to a more active agent—for abiratone’s survival extension. We propose that direct treatment with D4A would be more clinically effective than abiratone treatment.

The central role and critical requirement for androgen metabolism and androgen receptor (AR) in castration-resistant prostate cancer (CRPC) are demonstrated by the clinical benefit and overall survival benefit conferred by abiratone (Abi)7, which blocks CYP17A1, an enzyme required for androgen synthesis, and enzalutamide, which potently and competitively blocks the AR8. Abi (administered in its acetate form for bioavailability) is a steroidal compound and is therefore potentially subject to conversion by steroid-metabolizing enzymes. We hypothesized that the Δ5, 3β-hydroxyl-structure of Abi, which is also present in the natural steroid substrates dehydroepiandrosterone (DHEA) and Δ5-androstenediol (Andiol), makes it susceptible to one enzyme conversion by 3βHSD isoenzymes to its Δ4, 3-keto congener (Δ4-abiraterone or D4A). This, in turn, would make the steroid A and B rings identical to testosterone, enabling inhibitory interactions with AR and additional steroidogenic enzymes, including SRD5A, which are required for DHT synthesis (Fig. 1a). Such a conversion in peripheral tissues would allow D4A to engage with multiple targets to potentiate its effects on the androgen pathway, providing an alternative explanation for the clinical efficacy of Abi therapy and thus the possibility that direct treatment might be more efficacious.

We found that D4A is detectable in the sera of mice administered Abi acetate (Fig. 1b), as well as in sera from patients with CRPC who

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**Figure 1** | Structural consequences of the conversion from Abi to D4A that occurs both in mice and in patients, and requires 3βHSD. a, Schematic of Abi conversion to D4A. 1 Double bond and C3-position for substrates and products of 3βHSD. b, Abi is converted to D4A in vivo. Abi acetate was injected intraperitoneally in five mice. Blood was collected 2 h and 4 h after injection. Serum concentrations of Abi and D4A were quantified by mass spectrometry and are represented as the percentage of the sum total of Abi + D4A. c, D4A is detectable in all patients (n = 12) with CRPC treated with Abi acetate. d, Representative mass spectrometry tracing of D4A and Abi from the serum of a patient treated with Abi acetate. e, 3βHSD1 is capable of converting Abi to D4A. LAPC4 cells overexpressing 3βHSD1 were treated with 10 μM Abi (or [3H]DHEA) for 24 h. Abi, D4A (as a percentage of Abi + D4A), [3H]DHEA and [3H]DHEA metabolites (labelled metabolites) were separated by HPLC. Results are shown as mean (n = 3) ± s.d. with biological replicates. The experiment was repeated independently at least three times.

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normal dosing probably does not. On the other hand, D4A is approximately tenfold more potent than Abi at blocking the conversion of $[^3H]$DHEA by 3βHSD to Δ5-androstanediol (AD) in LNCaP and VCaP cells, as assessed by thin-layer chromatography (TLC; Extended Data Fig. 3a) and high-performance liquid chromatography (HPLC; Fig. 2b and Extended Data Fig. 3b). D4A inhibits both human isoenzymes, 3βHSD1 and 3βHSD2, with mixed inhibition kinetics (Fig. 2c). CYP17A1 inhibition is the major direct mechanism of action for Abi11. Structural studies of modified steroidal azoles suggest that the A-ring conformation of D4A does not significantly perturb binding to CYP17A1 (ref. 12). D4A and Abi similarly block conversion of $[^3H]$pregnenolone by CYP17A1 to DHEA (percentage conversion to DHEA after 3 h incubation for vehicle, 1 nM D4A and 1 nM Abi was 70.1%, 4.2% and 2.6%, respectively) by HPLC. D4A but not Abi or enzalutamide inhibits 5α-reductase activity. LAPC4 cells were treated with $[^3H]$AD and the indicated drug concentrations. Steroids were quantified by HPLC. Arrows denote 5α-reduced androgens. SRD5A1 (ref. 13). D4A (10 μM) nearly completely blocked conversion at 3 h.

Abi has been reported to have modest affinity for AR, particularly in the presence of mutations in the ligand-binding domain14. Conversion...
Comparisons of D4A with enzalutamide on DHT-induced endogenous PSA expression demonstrate that D4A is equivalent to enzalutamide against mutant and wild-type AR (Fig. 3e, f and Extended Data Fig. 5c, d). Downstream of androgen-responsive gene expression, effects of D4A and enzalutamide on DHT-stimulated cell growth are equivalent (Fig. 3g), both of which are more potent than Abi.

To determine whether the observed effects of D4A on inhibition of steroid synthesis demonstrated in tissue culture also occur in tumours, effects in two prostate cancer xenograft models with robust 3βHSD enzymatic activity were assessed. Subcutaneous mouse xenograft tumours of VCaP and LNCaP cells, which both harbour a mutant gene encoding a missense in 3βHSD1 that effectively increases enzyme activity, were grown in male NSG mice. Fresh tumours were harvested, minced and incubated with [3H]DHEA plus Abi or D4A (0.1–10 μM). Similar to effects shown in Fig. 2, D4A is tenfold more potent than Abi in blocking conversion from DHEA by 3βHSD to AD in LNCaP (Fig. 4a) and VCaP xenografts (Fig. 4b). For example, 0.1 μM D4A is equivalent to 1 μM Abi for blocking AD accumulation at 48 h in both LNCaP and VCaP xenografts. To test whether the combined effects of D4A on inhibition of steroid synthesis and direct blockade of AR lead to augmented anti-tumour activity compared with Abi, VCaP xenografts were grown subcutaneously in orchietomized mice with DHEA pellet implantation (to mimic human adrenal physiology; Fig. 4c). Time from initiation of treatment with D4A, Abi acetate or vehicle to tumour progression (>20% increase in tumour volume) was assessed by generating Kaplan–Meier survival curves.

Figure 3 | D4A binds to AR, inhibits AR chromatin occupancy, expression of AR-responsive genes and cell growth. a, b, D4A potently binds to both mutant and wild-type AR. D4A, Abi and enzalutamide (Enz) (0.001–10 μM) were used to compete with 0.1 nM [3H]R1881 for mutated AR (LNCaP) or wild-type AR (LAPC4). Intracellular radioactivity was normalized to protein concentration. c, Dose-dependence of D4A versus Enz for inhibition of AR chromatin occupancy. LNCaP cells were treated with the indicated concentrations of DHT, D4A and Enz for 3 h. AR chromatin occupancy for PSA, TMPRSS2 and FKBP5 was detected with ChIP. AR ChIP was normalized to untreated control for each gene. d, D4A inhibits PSA, FKBP5 and TMPRSS2 expression. LNCaP cells were treated with DHT (0.5 nM), DHEA (40 nM) or R1881 (0.1 nM) with or without Abi or D4A (1 μM) for 24 h. Gene expression was detected by quantitative PCR (qPCR) and normalized to RPLP0. e, f, D4A inhibition of DHT induced PSA expression is comparable to Enz in LNCaP and LAPC4. g, D4A inhibits DHT (0.5 nM) induced cell growth in LNCaP. Cells were quantified at the indicated time points by assaying DNA content after 2, 4 and 6 days of treatment. Experiments in a, b and g were performed with biological replicates; c-f were performed with technical replicates. All experiments were repeated independently three times. All results are shown as mean (n = 5 for g; n = 3 for all other experiments) ± s.d.
and comparing treatment groups with the log-rank test. Progression was significantly delayed in the D4A group compared with the Abi acetate group ($P = 0.011$). Fold-change in tumour volume is shown for each treatment group in Extended Data Fig. 6. We also compared xenograft growth using the same method with the C4-2 cell line model.

D4A treatment increased progression-free survival compared with Abi acetate and enzalutamide (Fig. 4d). In serum collected from D4A-treated mice at the end of the xenograft study there was no detectable increase in deoxycorticosterone, which is the mineralocorticoid that is most highly elevated in patients treated with Abi acetate, causing hypertension and hyperkalaemia (Extended Data Fig. 7)\textsuperscript{15,16}.

Figure 4e depicts the multiple points in the androgen pathway at which conversion of Abi by 3βHSD to D4A in patients impinges on AR signalling and prostate cancer progression, and the relative potencies of D4A, Abi and enzalutamide.

The next-generation hormonal therapies, Abi and enzalutamide, each have a single predominant target (CYP17A1 and AR, respectively). These drugs have clinically validated that androgen synthesis and AR stimulation are both essential components required to spur the development and progression of CRPC. After oral administration, Abi acetate is hydrolysed and thereby converted to Abi, which is thought to be the major active agent by way of blocking CYP17A1. The major recognized metabolites of Abi result from hepatic CYP3A4 and SULT2A1 processing, forming the N-oxide of Abi and Abi sulfate, respectively. Neither of these modifications affects the $\Delta^5$, 3β-hydroxyl-structure of the steroid backbone. In contrast, conversion to D4A modifies the steroidal structure to one that more robustly engages with AR, SRD5A and 3βHSD, thereby blocking androgen signalling at all these steps, while retaining CYP17A1 inhibition. The clinical significance of conversion of Abi to D4A and effects on individual components of the androgen pathway in patients must be viewed in light of pharmacokinetic studies that show a maximum serum concentration ($C_{\text{max}}$) of approximately 1 $\mu$M and also wide inter-patient variability\textsuperscript{17}. Furthermore, our findings suggest that D4A also has much more potent anti-tumour activity against CRPC when directly compared with Abi.

The potential clinical utility of treating patients with CRPC directly with D4A is dependent on the underlying mechanisms of resistance to Abi, which have not been fully elucidated, and the clinical settings in which the benefit from Abi is exhausted. The evidence suggests that sustained AR signalling characterizes at least a subset of Abi resistance cases. For example, increased AR copy number in CRPC is associated with absence of clinical response to Abi\textsuperscript{18}, and increased AR protein expression appears to occur upon the development of acquired clinical resistance to Abi\textsuperscript{14}. Although Abi is a potent CYP17A1 inhibitor, studies of urinary steroid metabolites in patients demonstrate that androgen synthesis inhibition is incomplete, raising the possibility of sustained AR signalling characterizes at least a subset of Abi resistance. In contrast to hepatic Abi metabolites, it is probable that conversion of Abi to D4A in peripheral tissues leads to D4A concentrations that are higher in peripheral tissues (such as CRPC) than are present in serum. The effects of D4A on androgen signalling in CRPC, in particular distal steps in DHT synthesis and activity as an AR antagonist, may therefore be underestimated on the basis of serum concentrations alone. Nonetheless, D4A levels in peripheral tissues and the precise contribution of D4A to the clinical activity of Abi have yet to be determined.

Finally, our results raise the possibility that there may be a previously unappreciated class effect of steroidal versus non-steroidal CYP17A1 inhibitors. In contrast to Abi acetate, TAK-700, a non-steroidal CYP17A1 inhibitor, failed to prolong survival in metastatic CRPC\textsuperscript{12}. It is possible that the absence of active downstream steroid metabolites may have contributed to these findings. This issue should be considered as other steroidal and non-steroidal CYP17A1 inhibitors undergo further clinical investigation.
In conclusion, we have identified a novel Abi metabolite that is present in patients with CRPC treated with Abi acetate and has more potent anti-tumour activity than the parent drug. Conversion to D4A may be responsible for some of the clinical activity observed with the use of Abi. We suggest that treatment with D4A is likely to result in a greater clinical benefit than Abi.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

**Received 1 October 2014; accepted 10 March 2015.**

**Published online 1 June 2015.**

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**Acknowledgements** This work has been supported in part by funding from a Howard Hughes Physician-Scientist Early Career Award (to N.S.), by the Prostate Cancer Foundation (to N.S.), by an American Cancer Society Research Scholar Award (12-038-01-CCE; to N.S.), grants from the US Army Medical Research and Materiel Command (PC080193 to N.S. and PC121382 to Z.L.), and additional grants from the National Cancer Institute (R01CA168899, R01CA172382, and R01CA190289; to N.S.).

**Author Contributions** Z.L. performed gene expression, metabolism, chromatin immunoprecipitation (ChIP) and mouse xenograft studies. A.B., M.A. and D.B. performed mass spectrometry studies. J.A.G. and R.D. participated in clinical studies. J.L. and S.K.U. performed enzymology studies, and S.K.U. also performed chemical syntheses. Z.L., R.J.A. and N.S. designed the studies and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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No statistical methods were used to predict determine sample size.

**Cell lines.** LNCaP and VCaP cells were purchased from the American Type Culture Collection and maintained in RPMI-1640 with 10% FBS. LAPC4 cells were provided by L. Chung and maintained in RPMI-1640 with 10% FBS. All experiments using LNCaP and VCaP were performed in plates coated with poly-DM-ornithine (Sigma-Aldrich). A 293-cell line stably expressing human CYP17A1 was generated by transfection with pcDNA3.1/CYP17A1 (gift from W. Miller) and selection with G418 as described previously\(^\text{23}\). Cell lines were authenticated by DDC Medical and determined to be negative for mycoplasma with primers 5'-ACACATGGGAGCTGTAA-3' and 5'-GTTCATCGACTTTAGCCAAGCGG-3'.

**Chemicals.** Abi acetate was purchased from Medkoo Biosciences. Abi and D4A were authenticated by DDC Medical and determined to be negative for myco-

**Cell lineic metabolism.** Cell lines were seeded and incubated in 12-well plates with 0.2 million cells per well for 24 h and then incubated with a mixture of radioactive (\(^3\)H-labelled) and non-radioactive androgens (final concentration, 100 nM; \(\sim 1,000,000\) counts per minute (cpm); well/PerkinElmer) at 37 °C. Aliquots of medium were collected at the indicated times. Collected medium was treated with 1,000 units of \(\beta\)-glucuronidase (Helix pomatia; Sigma-Aldrich) at 65 °C for 2 h, extracted with 860 \(\mu\)l ethyl acetateisoctane (1:1), and concentrated under nitrogen gas.

**HPLC analysis.** HPLC analysis was performed on a Waters 717 Plus HPLC or an Agilent 1260 HPLC. Dried samples were reconstituted in 50% methanol and injected into the HPLC. Steroids and drug metabolites were separated on a Kinetex 100 mm x 2.6 mm, 2.6 \(\mu\)m particle size C\(_4\) reverse-phase column (Phenomenex) using a methanol/water gradient at 30 °C. The column effluent was analysed using a dual-wavelength ultraviolet–visible detector at 254 nm or \(\beta\)-RAM model 3 in-line radioactivity detector (IN/US Systems) using Liquisint scintillation cocktail (National Diagnostics). Alternatively, dried samples were applied to plastic-backed silica gel plates and separated by TLC using a mobile phase of 3:1 chloroform:ethyl acetate, followed by exposure of the plates to a phosphorimager screen backed silica gel plates and separated by TLC using a mobile phase of 3:1 chloroform:ethyl acetate, followed by exposure of the plates to a phosphorimager screen.

**Mass spectrometry.** Mass spectrometry analysis was performed by the Cleveland Clinic Biological Resources Unit facility. All mouse studies were Institutional Review Board-approved protocol (case 7813). Blood was collected between 2 and 14 h after the 1,000 mg daily dose in ethylenediaminetetraacetic acid (EDTA). Mice were killed at treatment day 15 or when the tumour size was twofold greater than baseline. The signature of all the experiments was determined on the basis of DNA content as detected by Hoechst stain and a Wallac Victor2 1420 Multilabel counter (PerkinElmer).

**Cell proliferation assay.** LNCaP cells (0.1 million cells per millilitre) were seeded in 96-well plates and cultured in phenol-red-free RPMI-1640 mediums plus 10% charcoal stripped FBS with androgens and/or drugs. Medium was changed every other day. After 2, 4 or 6 days, cells were collected and lysed after treatment. Growth was then determined on the basis of DNA content as detected with Hoechst stain and a Wallac Victor2 1420 Multilabel counter (PerkinElmer). A two-tailed Student’s \(t\)-test was used to determine significance.

**Animal care and use committee.** Sample size was determined on the basis of our prior studies of steroidogenesis inhibition in xenograft models of CRPC\(^{13}\). Criteria for progression were determined on the basis of similar criteria used for clinical progression. Mice were surgically orchietomized and implanted with a 5 mg 90-day sustained-release DHEA pellet (Innovative Research of America) to mimic CRPC in the context of human adrenal physiology. Two days later, \(10^6\) VCaP or C4-2 cells were injected subcutaneously with matrigel. Once tumours reached 300 mm\(^3\) (length x width x height x 0.52), mice were arbitrarily (but not strictly randomized) assigned to vehicle (n = 9 or 10 mice for VCaP and C4-2 respectively), Abi acetate (n = 10 mice for both cell lines), DAA (n = 10 mice for both cell lines) or enzalutamide (n = 11 for C4-2) treatment groups. Abi acetate and DAA (0.5 mmol per kg per day in 0.1 ml 5% benzyl alcohol and 95% safflower oil solution) were administered via 5 ml per kg intraperitoneal injection every day for up to 15 days. Control groups were administered 0.1 ml 5% benzyl alcohol and 95% safflower oil solution via intraperitoneal injection every day. Mice in the enzalutamide group were fed with enzalutamide in chow every day (10 mg per kg per day)\(^{12}\). Treatment was not blinded to the investigator. Tumour volume was measured daily, and time to increase in tumour volume by 20% was determined. Mice were killed at treatment day 15 or when the tumour size was twofold greater than baseline. The significance of the difference between treatment groups was assessed by Kaplan–Meier survival analysis using a log-rank test in SigmaStat 3.5. A two-tailed Student’s \(t\)-test was used to determine significance in Extended Data Fig. 6.

**Enzyme assays.** To test DAA as an inhibitor of 3βHSD, enzyme assays were performed as described previously\(^{27}\). Briefly, incubations were prepared with recombinant human 3βHSD1 or 3βHSD2 (in yeast microsomes, 45 ± 2.5 \(\mu\)g protein per incubation, respectively), \(^3\)H[progesterone (100,000 c.p.m., 1–20 \(\mu\)M], and DAA (5–20 \(\mu\)M) or ethanol vehicle in 0.5 ml of potassium phosphate buffer (pH 7.4). After a pre-incubation at 37 °C for 1–3 min, NAD+ (1 mM) was added, and the incubation was conducted at 37 °C for 20 min. The reaction was stopped by addition of 1 ml ethyl acetateisoctane (1:1), and the steroids were then extracted into the organic phase and dried. The steroids in the dried extracts were resolved by HPLC and quantitated by in-line scintillation counting.

**AR competition assay.** Cells were cultured in serum-free medium for 48 h and then treated with \[^3\]H[R1881 and the indicated concentrations of DAA, Abi, enza-

**Mass spectrometry.** Patient serum collection and drug extraction. Twelve patients with mCRPC undergoing treatment with Abi acetate were recruited under an Institutional Review Board-approved protocol (case 7813). Blood was collected using Vacutainer Plus serum blood collection tubes (BD367814, Becton Dickinson). Blood was collected between 2 and 14 h after the 1,000 mg daily dose of Abi acetate was administered. Blood was allowed to clot and tubes were centri-

**Mouse xenograft study.** Mouse serum was collected for steroid analysis. Twenty microlitres of serum and internal standard (d\(_4\)-deoxycorticosterone) was deriva-

**Stable isotope dilution liquid chromatography–mass spectrometry analysis.** Mouse serum samples. Samples were analysed on a Thermo TSQ Quantiva-Prelude SPLC system (Thermo Scientific) with Aria MX 2.1 and Tracefinder 3.3.260.22 software for instrument controls and quantitation. Analyte separation was achieved with an Accucore 50 mm x 3 mm, 2.6 \(\mu\)m C\(_18\) column (Thermo Scientific) using a mobile phase of liquid chromatography–mass spectrometry grade methanol and water (Thermo Scientific), a gradient of 25–100% methanol and a flow rate of 0.6 ml min\(^{-1}\). Steroids were ionized by electrospray ionization and in positive ion mode. Multiple reaction monitoring was used to follow mass...
transitions for the oximes of deoxycorticosterone ($m/z$: 361.2/124.1) and $d_7$-deoxycorticosterone ($m/z$: 369.4/128.1) (Steraloids). Concentrations were determined using stable isotope dilution analysis.

Patient serum analysis. Blood was obtained from prostate cancer patients with consent under a protocol approved by the Cleveland Clinic Institutional Review Board. Samples were analysed on an ultra-HPLC station (Shimadzu) with a DGU-20A3R degasser, two LC-30AD pumps, a SIL-30AC autosampler, a CTO-10A column oven and a CBM-20A system controller in tandem with a QTRAP 5500 mass spectrometer (AB Sciex). The mobile phase consisted of liquid chromatography–mass spectrometry grade (Fisher) methanol:acetonitrile:water (44:36:20). Separation of drug metabolites was achieved using a Zorbax Eclipse plus 150 mm $\times$ 2.1 mm, 3.5 $\mu$m C18 column (Agilent) at a flow rate of 0.2 ml min$^{-1}$. Drug metabolites were ionized using electrospray ionization in positive ion mode. Multiple reaction monitoring was used to follow mass transitions for D4A ($m/z$: 348.2/156.3), abiraterone ($m/z$: 350.3/156.1) and $d_4$-cortisol ($m/z$: 367.1/121.1). Standard curves were generated using human serum spiked with known concentrations of each metabolite to enable determination of unknown concentrations in patient samples.
Extended Data Figure 1 | D4A is detectable in patients with prostate cancer treated with Abi acetate. a, Mass spectrometry tracings of Abi and D4A in the serum of 12 patients treated with Abi acetate. Blood was drawn between 2 and 14 h after the administration of the 1,000 mg daily dose. b, Duration of Abi acetate therapy and time from last dose to blood draw for individual patients.
Extended Data Figure 2 | 3βHSD converts Abi to D4A.  

**a.** 3βHSD1 expression permits catalysis of DHEA to AD. LAPC4 cells were transfected with 3βHSD1 or vector and treated with [3H]DHEA. Medium was collected 24 h later and androgens were separated and quantified by HPLC.  

**b.** 3βHSD1 expression allows conversion of Abi to D4A. LAPC4 cells were transfected with 3βHSD1 or vector and then treated with Abi. Medium was collected after 24 h and D4A and Abi were separated by HPLC.  

**c.** 3βHSD enzymatic activity present in the mouse adrenal gland but not prostate gland converts Abi to D4A. Mouse adrenal and prostate glands were harvested and minced before culturing in the presence of media containing Abi. Medium was collected after 24 h for separation and quantitation of D4A and Abi by HPLC.
Extended Data Figure 3 | D4A inhibits 3βHSD1 activity. a, D4A inhibits 3βHSD1 activity in LNCaP. Cells were treated with [3H]DHEA (DHEA: 100nM; [3H]DHEA, 1,000,000 c.p.m. per well) with 0.1, 1 or 10 μM D4A and Abi, for 9 and 24 h. DHEA and AD were separated and quantified by TLC. ImageJ was used to quantify steroids. For ease of comparison, arrows denote AD percentage for 0.1 μM D4A and 1 μM Abi treatment groups. b, VCaP cells were treated with [3H]DHEA and the indicated concentrations of D4A and Abi. The percentages of DHEA and AD were determined by HPLC. Experiments were performed with biological replicates (n = 3) and results are shown as mean ± s.d.
Extended Data Figure 4 | D4A has a higher affinity for both mutant-type AR (LNCaP cells) and wild-type AR (LAPC4 cells) than abiraterone (Abi) and bicalutamide (Bic) and inhibits AR chromatin occupancy better than Abi. a, Competition plots for D4A, Abi and Bic. b, Competition plots for unlabelled R1881 and D4A. Displacement of \([{}^{3}H]\)R1881 is described in Methods. Experiments were performed with biological replicates \((n = 3)\) and results are shown as mean ± s.d. c, D4A inhibition of AR chromatin occupancy is superior to Abi. LNCaP cells were treated with the indicated concentrations of DHT, D4A, Abi and enzalutamide (Enz) for 3 h. AR chromatin occupancy for PSA, TMPRSS2 and FKBP5 was detected with ChIP. Experiments were performed with technical replicates \((n = 3)\) and results are shown as mean ± s.d. All experiments were repeated independently at least three times.
Extended Data Figure 5 | D4A inhibits expression of androgen-responsive genes. a, D4A inhibits PSA expression in LAPC4 cells. Cells were treated with DHT (0.5 nM), DHEA (40 nM) or R1881 (0.1 nM) with or without Abi or D4A (1 μM) for 24 h. b, D4A inhibits PSA expression in LAPC4 in a dose-dependent manner. c, D4A inhibits AR target gene expression in C4-2 cells. Cells were treated with vehicle control (Ctrl), DHT (0.5 nM) or DHEA (40 nM) with or without Abi (1 μM), D4A (1 μM) or Enz (1 μM) for 24 h. d, D4A is comparable to Enz in inhibiting DHT-induced target gene expression in VCaP cells. Gene expression was assessed in triplicate, detected by qPCR and normalized to RPLP0. Experiments were performed with technical replicates (n = 3) and results are shown as mean ± s.d. All experiments were repeated independently at least three times.
**Extended Data Figure 6 | D4A impedes VCaP xenograft growth.** *P* < 0.05 and **P** < 0.01 for the difference between D4A (n = 10 mice) and AA (n = 10 mice) treatment groups. *N* = 9 mice for the control group.
Extended Data Figure 7 | D4A does not increase deoxycorticosterone concentrations. Serum of mice undergoing long-term treatment with D4A was collected and deoxycorticosterone concentrations were determined by liquid chromatography–mass spectrometry. Compared with control mice injected with vehicle, D4A does not increase deoxycorticosterone concentrations. For both D4A and control groups, \( n = 9 \) biological replicates (mice).