Inhibition of androgen receptor and β-catenin activity in prostate cancer

Eugene Lee,a,b Aviv Madar,b Gregory David,a Michael J. Garabedian,d Ramanuj DasGupta,b,e,1 and Susan K. Logana,b,e,f,1

Departments of aBiochemistry and Molecular Pharmacology, bStem Cell Biology, dMicrobiology, Urology, and eNew York University Cancer Institute, New York University School of Medicine, New York, NY, 10016; and fDepartment of Biology, Center for Genomics and Systems Biology, New York University, New York, NY 10003

Edited by Charles L. Sawyers, Memorial Sloan-Kettering Cancer Center, New York, NY, and approved August 13, 2013 (received for review October 18, 2012)

Androgen receptor (AR) is the major therapeutic target in aggressive prostate cancer. However, targeting AR alone can result in drug resistance and disease recurrence. Therefore, simultaneous targeting of multiple pathways could in principle be an effective approach to treating prostate cancer. Here we provide proof-of-concept that a small-molecule inhibitor of nuclear β-catenin activity (called C3) can inhibit both the AR and β-catenin–signaling pathways that are often misregulated in prostate cancer. Treatment with C3 ablated prostate cancer cell growth by disruption of both β-catenin/T-cell factor and β-catenin/AR protein interaction, reflecting the fact that T-cell factor and AR have overlapping binding sites on β-catenin. Given that AR interacts with, and is transcriptionally regulated by, β-catenin, C3 treatment also resulted in decreased occupancy of β-catenin on the AR promoter and diminished AR and AR/β-catenin target gene expression. Interestingly, C3 treatment resulted in decreased AR binding to target genes accompanied by decreased recruitment of an AR and β-catenin cofactor, coactivator-associated arginine methyltransferase 1 (CARM1), providing insight into the recruitment of an AR and decreased AR binding to target genes accompanied by decreased re-

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1218168110/-/DCSupplemental.

The authors declare no conflict of interest.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1To whom correspondence may be addressed: E-mail: susan.logan@nyumc.org or ramanuj.dasgupta@nyumc.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1218168110/-/DCSupplemental.
higher IC50 in abl cells likely reflects the increased activity of nuclear β-catenin as seen in other androgen-independent cell lines and in a CRPC model (11, 23). Also, the higher IC50 in VCaP cells is consistent with the previous report of the androgen-repressed gene (ERG)-induced Wnt signaling (24). The IC50 needed to inhibit the AR reporter was comparable between LNCaP and abl cells (Fig. 1G, 1.44 and 1.61 μM), but higher in VCaP cells (6.51 μM), reflecting the amplification of AR in VCaP cells (22).

AR ligand competition assays were conducted to test if C3 modulates AR activity by affecting ligand binding. Samples incubated with C3 showed that C3 does not affect ligand binding (Fig. S1E). Examination of AR coactivator recruitment showed that C3 did not induce recruitment of the FxXL coactivator peptide to the AR ligand-binding domain (LBD), whereas dihydrotestosterone (DHT) and higher concentrations of the AR antagonist bicalutamide (BIC) induced dose-dependent interaction of the peptide and AR-LBD (Fig. S1F).

Fig. 1. C3 affects AR and β-catenin signaling by inhibition of protein-protein interaction. (A) Chemical structure of iCRT3 (C3). (B) Coimmunoprecipitation studies reveal that C3 inhibits β-catenin/TCF4 and β-catenin/AR interactions in LNCaP cells. Cells were treated with vehicle or 20 μM C3 for 4 h. (C) LNCaP, abl, and VCaP cells express high levels of nuclear β-catenin. Nuclear β-catenin cytoplasmic loading controls are shown. (D) C3 inhibits transcription of AR nascent mRNA. LNCaP cells were androgen-deprived for 48 h and then treated with DMSO or 20 μM C3 with or without 0.1 nM R1881 for 4 h. (E) C3 inhibits β-catenin occupancy on a TCF/LEF-binding site within the AR promoter. LNCaP cells were androgen-deprived for 3 d and then treated with vehicle or 100 nM DHT with or without 20 μM C3 for 4 h. (F) β-Catenin knockdown inhibits AR expression in abl cells. Forty-eight hours post-transfection, cells were fixed and incubated with anti-AR (red), anti-β-catenin (green) antibodies, and DAPI (blue). (Scale bar, 50 μM.) (G) Determination of the IC50 of C3 in prostate cancer cells. Cells were transfected with STF16-LUC (β-catenin luciferase reporter) or ARE-LUC (AR luciferase reporter).

As AR is transcriptionally regulated by β-catenin through TCF/lymphoid enhancer factor (LEF)-binding sites on the AR promoter, we reasoned that loss of TCF/β-catenin interaction would diminish AR mRNA levels in C3-treated cells. To test if transcription of AR is directly targeted by C3, nascent AR mRNA levels were analyzed in LNCaP cells using primers flanking exon/intron junctions. Indeed, C3 treatment decreased nascent AR mRNA levels (Fig. 1D), consistent with the decreased occupancy of β-catenin on TCF/LEF-binding sites within the AR promoter shown by chromatin immunoprecipitation (ChIP) (Fig. 1E). Depletion of β-catenin revealed that cells containing strong β-catenin levels due to incomplete knockdown (Fig. 1F, arrow) clearly have higher levels of AR than adjacent cells with lower levels of β-catenin (Fig. 1F, arrowheads), indicating that the cell-specific expression of AR is regulated by β-catenin.

We next tested the effect of C3 on transcriptional activities of AR and β-catenin. C3 dramatically reduced expression of both AR and Wnt/β-catenin reporter genes (Fig. S1C), and the over-expression of AR cDNA diminished the inhibitory effect of C3 on AR expression reporter (Fig. S1D), suggesting that C3 may directly influence AR activity. Dose-responsive inhibition of C3 on AR and Wnt/β-catenin transcriptional activities revealed that C3 inhibits the Wnt/β-catenin reporter with an IC50 of 0.14 μM in LNCaP, 0.89 μM in abl, and 0.97 μM in VCaP cells (Fig. 1G). The somewhat
To test if C3 inhibits prostate cell growth, LNCaP, abl, and VCaP cells were treated with 20 μM C3 for 24 h with DMSO or 20 μM C3 for 24 h. (C) Loss of protein expression of AR and β-catenin target genes in the presence of C3. LNCaP and abl cells were treated as described in A and B, and protein was extracted at the indicated time points. AR levels, normalized to tubulin levels, are presented. Four-hour vehicle-treated cells were set to 1. (D) β-Catenin knockdown decreases the expression of AR and Wnt/β-catenin target genes in LNCaP and abl cells. (E) β-Catenin knockdown decreases protein levels of AR and C-MYC in LNCaP and abl cells.

C3 Inhibits AR and β-Catenin Occupancy on Target Genes. Because β-catenin can act as an AR coactivator (28, 29) and we found that C3 inhibits AR/β-catenin protein interaction (Fig. 1), we reasoned that C3 might affect AR recruitment to target genes. To test this hypothesis, we examined AR occupancy in the presence of C3 and DHT in LNCaP and abl cells. (Fig. S4A). To circumvent the reduced levels of total AR observed in C3-treated cells in the presence of low (0.1 nM) androgen concentration (Fig. 2), we treated cells with 100 nM DHT, which stabilized AR protein and diminished the inhibitory effect of C3 on HEK 293 cells, which require Wnt ligand for activation of the pathway (27). Although higher levels of C3 modestly slow cell growth in HEK 293 cells (Fig. 3C), apoptosis was not induced (Fig. 3D). β-Catenin depletion significantly reduced growth of LNCaP, abl, and VCaP cells (Fig. 3E; Figs. S4B and S5), indicating that the proliferation of prostate cancer cells is dependent on β-catenin.

C3 Blocks Proliferation of Prostate Cancer Cells by Inducing Cell Death. To test if C3 inhibits prostate cell growth, LNCaP, abl, and VCaP cells were treated with varying concentrations of C3 or vehicle. Treatment with 20 μM C3 completely abolished growth of LNCaP, abl, and VCaP cells (Fig. 3A). C3 inhibits transcription of AR and β-catenin target genes in LNCaP (Fig. S4A) and abl (Fig. 3B) cells, as judged by quantitative PCR. LNCaP cells were androgen-deprived for 48 h and treated for 24 h with DMSO or 20 μM C3 with or without 0.1 nM R1881. abl cells were treated with DMSO or 20 μM C3 for 24 h. (A) C3 inhibits expression of AR and β-catenin target genes in LNCaP and abl cells. (B) C3 inhibits transcription of AR and β-catenin target genes in LNCaP and abl cells. (C) C3 inhibits growth of LNCaP and abl cells. (D) β-Catenin knockdown decreases the expression of AR and Wnt/β-catenin target genes in LNCaP and abl cells. (E) β-Catenin knockdown decreases protein levels of AR and C-MYC in LNCaP and abl cells.

C3 inhibits prostate cell growth, LNCaP, abl, and VCaP cells were treated with varying concentrations of C3 or vehicle. Treatment with 20 μM C3 completely abolished growth of LNCaP, abl, and VCaP cells (Fig. 3A). C3 inhibits transcription of AR and β-catenin target genes in LNCaP (Fig. S4A) and abl (Fig. 3B) cells, as judged by quantitative PCR. LNCaP cells were androgen-deprived for 48 h and treated for 24 h with DMSO or 20 μM C3 with or without 0.1 nM R1881. abl cells were treated with DMSO or 20 μM C3 for 24 h. (A) C3 inhibits expression of AR and β-catenin target genes in LNCaP and abl cells. (B) C3 inhibits transcription of AR and β-catenin target genes in LNCaP and abl cells. (C) C3 inhibits growth of LNCaP and abl cells. (D) β-Catenin knockdown decreases the expression of AR and Wnt/β-catenin target genes in LNCaP and abl cells. (E) β-Catenin knockdown decreases protein levels of AR and C-MYC in LNCaP and abl cells.

C3 inhibits prostate cell growth, LNCaP, abl, and VCaP cells were treated with varying concentrations of C3 or vehicle. Treatment with 20 μM C3 completely abolished growth of LNCaP, abl, and VCaP cells (Fig. 3A). C3 inhibits transcription of AR and β-catenin target genes in LNCaP (Fig. S4A) and abl (Fig. 3B) cells, as judged by quantitative PCR. LNCaP cells were androgen-deprived for 48 h and treated for 24 h with DMSO or 20 μM C3 with or without 0.1 nM R1881. abl cells were treated with DMSO or 20 μM C3 for 24 h. (A) C3 inhibits expression of AR and β-catenin target genes in LNCaP and abl cells. (B) C3 inhibits transcription of AR and β-catenin target genes in LNCaP and abl cells. (C) C3 inhibits growth of LNCaP and abl cells. (D) β-Catenin knockdown decreases the expression of AR and Wnt/β-catenin target genes in LNCaP and abl cells. (E) β-Catenin knockdown decreases protein levels of AR and C-MYC in LNCaP and abl cells.
C3 inhibits self-renewal ability of prostate cancer cells that express higher levels of stem cell markers. The in vitro sphere-forming assay has been widely used to study prostate stem/cancer stem-cell biology (43). Human prostate stem-forming cells have also been demonstrated to self-renew and regenerate prostate tissue in vivo (44). When we generated spheres with prostate cancer cells, the spheres were maintained through serial passaging (Fig. S3A), and the expression of the stem-cell markers Oct4, Nanog, and Sox2 was increased in spheres compared with adherent cells (Fig. S4). When we compared the sphere formation efficiency in cells treated with vehicle or C3, a single dose of C3 on day 0 significantly decreased the number of spheres in LNCaP, abl, and VCaP cells (Fig. 5A and Fig. S4C). To test if C3 inhibits sphere-forming abilities of a heterogeneous group of cells obtained from mouse primary prostate cancer cells, we used the mouse model of prostate-specific Pten deletion. In this mouse model, Ptenf/f;PB-Cre, Cre-mediated Pten homozygous deletion is directed by the prostate-specific probasin (PB) promoter (45). As shown in Fig. S5C, C3 treatment decreased sphere formation efficiency of the primary cells isolated from two Ptenf/f;PB-Cre mice. We also explored the idea that prostate cancer stem cells are resistant to the conventional AR antagonist BIC. LNCaP cells were treated with vehicle, BIC, or C3, and the proliferation of adherent cells as well as the number of spheres in determined if C3 affected occupancy of commonly acknowledged coactivators of AR and β-catenin including p300, glutamate receptor-interacting protein 1 (GRIP1), coactivator-associated arginine methyltransferase 1 (CARM1), and BRG1 on AR target genes. These coactivators were recruited to both AR and β-catenin/TGF-binding sites (33,36), and the physical interaction between some of the coactivators and AR or β-catenin has been shown (9, 28, 37, 38). GRIP1 and p300 are histone acetyltransferases (39, 40), and BRG1 is a core component of the SWI/SNF chromatin-remodeling complex (41). CARM1 is a histone methyltransferase that methylates arginine residues such as arginine 17 of histone H3 (H3R17), which is associated with transcriptional activation (42). The protein levels of these coactivators were not affected by C3 treatment (Fig. S6A). When we tested the occupancy of p300, GRIP1, CARM1, and BRG1 on PSA4 enhancer in the absence and presence of C3, no differences were observed (Fig. S6B). However, similar to the AR and β-catenin ChIP results (Fig. 4C and D), C3 inhibited CARM1 occupancy on PSA4 and UBE2C enhancers (Fig. 4E) accompanied by decreased levels of dimethyl-H3R17 (Fig. 4F). These results suggest that β-catenin might mediate CARM1 recruitment to AR and that the reduced levels of CARM1 and dimethyl-H3R17 in PSA4 and UBE2C enhancers in the presence of C3 impaired the open chromatin structure of androgen response elements (AREs) required for AR binding. Supporting this idea, CARM1 inhibitor treatment recapitulated the inhibitory effect of C3 on PSA4 and UBE2C (but not C-MYC) transcription as well as AR recruitment on PSA4 and UBE2C (Fig. S7).

Fig. 3. C3 induces growth arrest and apoptosis in LNCaP and abl cells. (A) Growth curve assays and cell-cycle profile of LNCaP and abl cells in the presence of C3. (Upper) Growth of cells treated with DMSO or indicated concentration of C3 daily. (Lower) Cell-cycle analysis of cells. (B) C3 treatment induces apoptosis in LNCaP and abl cells. (C and D) C3 does not induce significant growth arrest or apoptosis in HEK293 cells. HEK293 cells were treated as in A and B. (E) β-Catenin knockdown inhibits growth of LNCaP and abl cells. Cells were stably infected with lentiviral vectors encoding control small hairpin RNA (shRNA) (sh-control) or β-catenin shRNA (sh–β-catenin) and subjected to proliferation assay. Cell were treated with DMSO or 20 μM C3 unless otherwise indicated.

Fig. 4. C3 inhibits AR and β-catenin occupancy on target genes. (A) C3 does not inhibit expression of AR, β-catenin, and CARM1 when treated with 100 nM DHT in LNCaP cells. (B) C3 decreases expression of PSA, UBE2C, and C-MYC when treated with 100 nM DHT in LNCaP cells. (C–F) C3 decreases occupancy of AR, β-catenin, CARM1, and dimethyl-H3R17 on AR-binding sites. Cells were androgen-deprived for 3 d and then treated with vehicle or 20 μM C3 and 100 nM DHT for 16 h (A and C–F) or 24 h (B).
that the cell-free areas observed in C3-treated tumors represent necrotic regions. Because AR is a C3 target as well as a master regulator of prostate tumor growth, we also tested the expression level of AR in C3-treated tumors. Consistent with results presented in prostate cancer cell lines (Fig. 2), the HIC of AR showed a marked decrease in AR expression in C3-treated tumors compared with vehicle-treated tumors (Fig. 6B).

**Discussion**

The physical, functional, and genetic interaction of AR and β-catenin has been described in a number of reports (22, 23, 37, 38). However, targeting this interaction to develop proof-of-concept small-molecule inhibitors in prostate cancer as potential therapeutics in prostate cancer has not yet been shown. Here we demonstrate that a small-molecule inhibitor of the Wnt/β-catenin pathway, C3, can also potently disrupt the AR pathway in prostate cancer by simultaneously disrupting two important protein–protein interactions, TCF/β-catenin and AR/β-catenin. Additionally, disruption of TCF/β-catenin has an additive inhibitory effect on AR signaling by the concomitant down-regulation of AR expression, a transcriptional target of TCF/β-catenin, thereby exacerbating the effect of C3 on both AR and Wnt/β-catenin signaling activity in prostate cancer. Importantly, C3 inhibited cell growth in a xenograft model of CRPC in vivo, demonstrating proof-of-concept for this therapeutic approach.

Many molecular mechanisms have been suggested to explain the sustained AR signaling in CRPC (5, 46). In particular, it was suggested that tumor cells adapt to the low androgen environment by activating AR transcriptional activity through alternative signals. In line with this model, the enhanced crosstalk between AR and Wnt/β-catenin in aggressive disease models has been reported repeatedly (11, 23). In addition to this “adaptation” mechanism, the other hypothesis is that a small subset of tumor cells becomes resistant to androgen ablation therapy and responsible for recurrent disease (46). These CSCs are believed to drive tumorigenesis by self-renewal and differentiation (13). Although the role of Wnt/β-catenin in embryonic stem-cell self-renewal is controversial (47), the studies conducted on multiple tumor types have consistently shown that Wnt/β-catenin signaling is highly activated in CSCs (16–18), probably due to the effect of the tumor microenvironment. Our data support the idea that prostate CSCs are tolerant to conventional anti-androgen treatment, as the in vitro sphere formation of LNCaP cells was not affected by BIC treatment (Fig. 5D). However, the sphere-forming efficiency of both LNCaP and abl cells was significantly reduced by C3 treatment (Fig. 5B and D), suggesting that the Wnt/β-catenin–signaling pathway can be a good candidate for CSC-targeted drug development.

In conclusion, we show that a small-molecule inhibitor of nuclear β-catenin disrupts both Wnt/β-catenin and AR-signaling activities to induce cell death in prostate cancer cells. Structure and medicinal chemistry-based lead optimization of C3 and scaffold molecules with similar activities may be ideal candidates for Wnt/AR-specific drug development.

**Materials and Methods**

LNCaP and abl cells were cultured in RPMI 1640 (Cellgro) supplemented with 10% (vol/vol) FBS (HyClone) and 10% (vol/vol) charcoal-stripped FBS (CFBS), containing 1% streptomycin, 1% penicillin, and 1% fungizone. For quantitative RT-PCR, immortalized prostate epithelial cells (NCM460, NCI-H292, and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% (vol/vol) FBS and 1% penicillin-streptomycin. For quantitative RT-PCR, immunoblot, biochemical fractionation, immunoprecipitation, and ChIP assays, LNCaP and VCaP cells were hormone-deprived in 5% (vol/vol) charcoal-stripped FBS (CFBS), and BIC was added to the media. Absorption at 570 nm was measured twice weekly. Animal studies were performed at the Memorial Sloan-Kettering Cancer Center (MSKCC). The animal research was approved by the MSKCC Institutional Animal Care and Use Committee protocol number 04-03-009. Primer sequences used in the study are provided in Tables S1 and S2. Additional methods are available in SI Materials and Methods.
Tissue sections were stained with H&E and antibodies against cleaved caspase-3, Ki67, and AR. (Lee et al. PNAS [DasGupta lab] for help with RNA-sequencing data analysis; Dr. Upal BasuRoy for technical advice and Susan Ha for the helpful comments; and Paul Levine for help with the Androgen Receptor Competitor and Co-activator assay. This research was supported by New York State Stem Cell Science Grant CD26880 (to E.L.), American Cancer Society Grant RSG-11-108-01-CCD (to R.D.), National Cancer Center Institute Grant CA85305, Cancer Center Grant SP30CA16087-31, and National Center for Advancing Translational Sciences National Institutes of Health Grant UL1 TR000038 (to S.K.L.).) Representative H&E staining and IHC. Xenograft tumors were extracted at the end of treatment. (Insets) Higher magnification of each image. (Scale bar, 100 μM.)

ACKNOWLEDGMENTS. We thank Elisa de Stanchina from Memorial Sloan-Kettering where the xenograft experiments were done; Dr. Sujash S. Chatterjee (Dana-Farber for help with BNA-sequencing data analysis; Dr. Upal BasuRoy for technical advice and Susan Ha for the helpful comments; and Paul Levine for help with the Androgen Receptor Competitor and Co-activator assay. This research was supported by New York State Stem Cell Science Grant CD26880 (to E.L.), American Cancer Society Grant RSG-11-108-01-CCD (to R.D.), National Cancer Center Institute Grant CA85305, Cancer Center Grant SP30CA16087-31, and National Center for Advancing Translational Sciences National Institutes of Health Grant UL1 TR000038 (to S.K.L.).) Representative H&E staining and IHC. Xenograft tumors were extracted at the end of treatment. (Insets) Higher magnification of each image. (Scale bar, 100 μM.)

30. Ou CY, et al. (2011) A coactivator role of CARM1 in the dysregulation of j-catenin ac
catenin to promote target gene activation. EMBO J 20(7):4935–4943.
tional coactivators p300 and CBP are histone acetyltransferases. Cell 87(5):953–959.

Lee et al. PNAS | September 24, 2013 | vol. 110 | no. 39 | 15715
For androgen receptor (AR) reporter assays, nuclear and cytoplasmic lysates were prepared using Lipofectamine 2000 reagent (Invitrogen). After transfection, cells were hormone-deprived in 5% (vol/vol) charcoal-stripped FBS (CFBS) media for 24 h and then treated with R1881 (Perkin-Elmer) with or without 20 μM C3 for an additional 24 h before being lysed in 1× Reporter Lysis buffer (Promega). Cell extracts were analyzed for luciferase activity, and the values were normalized to β-galactosidase activity.

Cell Proliferation Assay. The neutral red growth assay was performed by plating 2.5 × 10^5 cells in 24-well plates. The following day, LNCaP and VCaP cells were hormone-deprived in 5% (vol/vol) CFBS media for 24 h and then treated with 0.1 mM R1881 with or without C3, whereas HEK293 and abl cells were treated only with or without C3. C3 was added daily with more media, and an equal amount of DMSO was added daily to the control group. Neutral red dye was added directly to the media to obtain a 2% (vol/vol) final concentration. After 1 h, cells were washed in PBS and the dye was extracted with lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM DTT, 1% Triton X-100, 1% acetic acid). The absorbance of the solution was then measured at 540 nm.

Time-Resolved Fluorescence Resonance Energy Transfer. Lanthascreen Time-Resolved Fluorescence Resonance Energy Transfer Androgen Receptor Coactivator Assay (Invitrogen) was used according to the manufacturer’s instructions. Samples were prepared in triplicate on 384-well plates and incubated at room temperature (RT) for 4 h before data collection. The polarization values were obtained using a Tecan Ultra plate reader with the excitation at 530 nM and emission at 590 nM.

For immunofluorescence staining, cells were fixed in 4% (vol/vol) paraformaldehyde in PBS for 20 min, washed with PBS three times, permeabilized with 0.2% Triton-X in PBS for 20 min, blocked with 5% (vol/vol) normal goat and 5% (vol/vol) normal horse serum in PBS for 1 h, and incubated with anti-AR (441; Santa Cruz) and anti-β-catenin (for nuclear staining, ABC, Milipore; for cytoplasmic and membrane staining, Sigma) antibodies diluted in blocking buffer overnight at 4 °C. The following day, cells were washed with PBS three times and then incubated with secondary antibodies conjugated with FITC and Texas red (Vector Lab) at RT for 1 h. Subsequently, cells were washed in PBS three times and mounted with DAPI-mounting solution (Vector Lab). For immunohistochemistry, xenograft tumors were extracted at the end of treatment and fixed in 4% (vol/vol) paraformaldehyde, embedded in paraffin, and sectioned. Tissue sections were stained as previously described (1) using antibodies against AR (N-20, Santa Cruz Biotechnology), Ki67, and cleaved caspase-3 (Cell Signaling).

Biochemical Fractionation. Nuclear and cytoplasmic lysates were prepared as described previously (2). Cells were collected by centrifugation, swelled in a hypotonic buffer, and lysed by extruding the cells through a 25-gauge hypodermic needle. This homogenate was then centrifuged at 14,000 × g for 5 min to pellet the nuclei, and the supernatant was saved as the cytoplasmic fraction. The proteins were extracted by resuspension of the nuclear pellet in a high-salt buffer and separation of debris by centrifugation. The resulting supernatant was retained as the nuclear fraction.

Quantitative RT-PCR. Total RNA was isolated using the RNeasy kit (Qiagen, Inc.) and then reverse-transcribed at 55 °C for 1 h using SuperScript III reverse transcriptase and oligo(dT) 20 primers (Invitrogen). Real-time PCR was performed using gene-specific primers and 2XSYBR green Taq-ready mix (Sigma-Aldrich). To analyze nascent AR mRNA, RNA was extracted, treated with DNase, subjected to cDNA synthesis, and analyzed by quantitative PCR (qPCR) using primers flanking exon/intron. Data were analyzed by the delta delta CT method using RPL19 as a control gene and normalized to control samples, which were arbitrarily set to 1. Primer sequences are provided in Table S1.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as previously described (3). Proteins were double cross-linked with dithiobis(succinimidyl propionate) (DSP) (Pierce) for 20 min and 1% formalin for 10 min. Cells were lysed, nuclei collected and lysed in sonication buffer, and sonicated for

Lee et al. www.pnas.org/cgi/content/short/1218168110

1 of 9
12 min (30 s on, 30 s off) in a Bioruptor sonicator (Diagenode, model XL). Sonicated lysates were precleared for 2 h with Protein A/G agarose beads blocked with salmon sperm DNA (Millipore). Supernatants were then incubated overnight with the following antibodies: a mixture AR (441) and AR (N-20), β-catenin (H-102), p300 (C-20), GRIP1 (M-343, Santa Cruz Biotechnology); CARM1 (Bethyl Lab); total H3, H3R17me2, BRG1 (Abcam); and H3K4me2 (Upstate). Control ChIP was performed with normal mouse IgG and normal rabbit IgG sera. Immunocomplexes were then washed and cross-linking reversed. DNA was isolated with the Qiagen PCR purification kit and qPCR was performed using 1–5 μL of DNA. Relative enrichment was calculated as a percentage of 4% input normalized to IgG. Primer sequences are provided in Table S2.

**Treatment of Cells with siRNAs.** For transient knockdown of β-catenin, siGENOME SMARTpool siRNAs against β-catenin and control siRNA were purchased from Dharmacon. A total of 1.2 × 10^6 cells were plated in 6-cm plates and transfected for 4 h with siRNAs and Lipofectamine 2000. After transfection, cells were hormone-deprived in 5% (vol/vol) CFBS media for 24 h, then treated with 0.1 nM R1881 for an additional 24 h (LNCaP) or cultured in 10% (vol/vol) CFBS media for 48 h (abl), and used for the indicated experiments.

The cell lines stably depleted with β-catenin were generated with lentiviral pGIPZ small hairpin RNA (shRNA) against β-catenin (sh-β-catenin; Open Biosystems, RHS4430-98912789) or a control shRNA (Open Biosystems, RHS4743). After infection, cells were plated at a very low density and selected for 10 d with 1 mg/mL puromycin (Sigma-Aldrich). Each resistant cell colony was collected and expanded in the selection media to 10 d with 1 mg/mL puromycin (Sigma-Aldrich). The number of spheres was counted at the end of culture. To compare mRNA levels of stem-cell marker genes, cells were cultured in the same media as described above under adherent or sphere-forming conditions. To harvest spheres for RNA extraction, the media-containing spheres were passed through a 40-μm cell strainer (BD Biosciences), and the spheres on top of the strainer were collected by inverting the strainer and washing with PBS.

**Gene Set Enrichment Analysis.** For RNA-sequencing, abl cells were either treated with vehicle or 20 μM C3 or transfected with siGENOME SMARTpool siRNAs against AR, β-catenin, or control as described above. Cells were harvested 24 h after vehicle or C3 treatment or 48 h after siRNA treatment and subjected to RNA-sequencing analysis (LC Sciences). To generate the Gene Set Enrichment Analysis (GSEA) data, we used a modified R version of the original GSEA R code (Broad institute). We assayed for the enrichment of differentially expressed genes (ranked by absolute log twofold change of treatment vs. control) with two gene sets, β-catenin target genes (www. stanford.edu/group/nusselab/cgi-bin/wnt/target_genes) and AR target genes (5). Results are depicted by plotting the entire GSEA enrichment curves for each comparison of treatment vs. control and for a reference nonsense comparison of control vs. control (over different replicates).

**Statistics.** For comparison of pooled data between two different groups, unpaired t tests were used to determine significance. For comparison of data among three groups, one-way ANOVA was used to determine significance.

**Fig. S1.** C3 treatment does not affect β-catenin expression at membrane, does not inhibit ligand binding to AR, and does not result in recruitment of AR coactivators. 

(A) LNCaP cells show strong nuclear staining of active β-catenin (unphosphorylated at serine 37 and threonine 41). Cells were fixed and incubated with anti-active β-catenin (green) antibody and DAPI solution (blue). (Scale bar, 50 μM.)

(B) C3 does not affect β-catenin expression at the membrane. LNCaP cells were treated with vehicle or 20 μM C3 for 24 h and fixed. Cells were then incubated with anti-β-catenin (green) antibody and DAPI solution (blue). (Scale bar, 50 μM.)

(C) C3 inhibits Wnt/β-catenin and AR reporter gene activity in LNCaP cells. Cells were transfected with the indicated luciferase reporter constructs: STF16-LUC (β-catenin reporter) and ARE-LUC (AR reporter). Cells were androgen-deprived for 24 h after transfection, and either 0.1 nM ethanol or 10 nM R1881 was added with or without 20 μM C3 for 24 h. (D) AR overexpression circumvents C3 repression of AR-responsive transcription. LNCaP cells were transfected with the AR-responsive reporter construct ARE-LUC, together with 0, 0.05, 0.1, and 0.2 μg of an AR expression vector. Cells were androgen-deprived 24 h after transfection, and 0.1 nM R1881 was added with or without 3 μM C3 for 24 h. (E) Fluorescence resonance energy transfer (FRET)-based assay shows that C3 treatment does not result in recruitment of FxxLF coactivator peptide to AR LBD. Increasing concentrations of DHT, bicalutamide (BIC), or C3 were incubated with purified AR ligand-binding domain (LBD) protein for 4 h, and the polarization values were measured. (F) Fluorescence polarization-based AR ligand competition assay shows that C3 does not affect ligand binding to AR. Vehicle, 1 μM DHT, and 1 μM or 20 μM C3 were incubated with purified AR ligand-binding domain (LBD) protein for 4 h, and the emission values were measured.
Fig. S2. C3 inhibits expression of AR and β-catenin target genes. (A) LNCaP cells were androgen-deprived for 48 h and treated with DMSO or 20 μM C3 with or without 0.1 nM R1881 for 4 or 12 h. (B) LNCaP cells were androgen-deprived for 48 h and treated with DMSO or 20 μM C3 with or without 0.1 nM R1881 for 24 or 48 h. abl cells were treated with DMSO or 20 μM C3 for 24 or 48 h. (C) VCaP cells were androgen-deprived for 48 h and treated for 24 h with DMSO or 20 μM C3 with or without 0.1 nM R1881. (D) Protein from VCaP cells treated as described in C was extracted at 24 and 48 h after the initial treatment and immunoblotted with indicated the antibodies.
Fig. S3. GSEA for gene lists ranked by absolute log twofold change, capturing expression patterns from abl cells treated with inhibitor of β-catenin responsive transcription 3 (C3), si-β-cat, si-AR, or control (ctrl, si-control, and vehicle). (A and A′) Represent a list of 124 β-catenin target genes. (B and B′) Represent 175 annotated AR target genes. (A) C3/ctrl and si-β-cat/ctrl have the highest maximal enrichment score (ES) of 0.57 and 0.52, followed by si-AR (0.41). Shaded area for each comparison reflects the ES values that lie within the interquartile range (within the 25th and 75th percentiles). The interquartile range was determined from the GSEA curves of every possible pair-wise comparison of a treatment (two independent replicates) to a control experiment (vehicle and si-ctrl, two replicates each). For reference, the gray-shaded area shows the interquartile range of comparing each control to the additional three controls (i.e., a GSEA curve that may be expected by factors other than the treatments assayed in this work). There is a clear separation between the GSEA graphs for C3/ctrl and

Legend continued on following page.
si-β-catenin/ctrl treatments compared with si-AR treatment. (B) Identical GSEA analysis as in A, but with AR target genes. There is a clear separation between the GSEA graphs of si-AR and si-β-catenin compared with controls, but it is not as strong as for the β-catenin target gene set, shown in A. Nonetheless, the GSEA analysis indicates an enrichment for a set of commonly regulated AR target genes by both AR and β-catenin, whereas a substantial number of target genes seem to be regulated solely by AR and independently of β-catenin. (A′ and B′) Cluster analysis and treview of β-catenin (A′) or AR target genes (B′). Average linkage clustering was performed based on log twofold change with respect to control by using the uncentered correlation metric with Cluster 3.0 for Mac. The Cluster analysis depicts the strong overlap/correlation between differentially expressed genes between C3 treatment and si-β-catenin, compared with si-AR, suggesting that most genes that are modulated by β-catenin can also be regulated by C3. Taken together, the GSEA and clustering analyses suggest that the gene signature profile of C3 is very similar to that of si-β-catenin, suggesting minimal off-target effects. (C) Overlap of genes down-regulated by si-β-catenin with si-AR or C3. For each treatment and for each of the eight resulting down-regulated gene lists, we then iteratively evaluated the overlap between the most down-regulated genes (starting at the 50 most down-regulated genes) and the 1,000 gene-wide standard list (e.g., if out of the first 200 C3 down-regulated genes, 100 were also found in the standard list, then the overlap at that point is 50%). Because some of the off-target effects (OTEs) observed are due to sources of variability other than the treatment (e.g., DMSO vehicle for C3 vs. si-ctl for si-β-catenin, and si-AR, incomplete knockdown for siRNA experiments, etc.), we calculated the FC values of each treatment for the four controls. The observed overlap between the genes down-regulated by each treatment based on the eight distinct FC lists and the standard list of genes is represented: si-β-catenin (green), si-AR (red), and C3 (purple). For all treatments, the observed overlap is much higher than the percentage of overlap that is expected by chance alone (gray). In all cases, we shaded the 25–75 percentiles around the mean line as a measure of error. In the absence of variability, the green line would be set at 100% at all points. The amount of overlap observed for C3 targets is comparable to the overlap observed with si-AR. (D) Normalized overlap of genes down-regulated by si-β-catenin with si-AR and C3 with si-β-catenin targets. The normalized overlap is calculated at each point 50, 100, 150, . . . , 1,000 as (% overlap C3 or AR)/(% overlap si-β-catenin) × 100 from the mean values depicted in C (i.e., dividing the purple or red lines in C by the green line in C). The normalized overlaps range between 48 and 74% for si-AR and 58 and 67% for C3 and are largely comparable. For the top 150 down-regulated genes, si-AR has greater overlap with si-β-catenin than C3, but that trend reverses and C3 has greater overlap with si-β-catenin over the top 1,000 genes. This puts the percentage of OTEs for C3 treatment at 33–42%. However, we note that much of this OTE effect is likely due to factors other than C3 selectivity, as the si-AR down-regulated targets give very similar results to C3, and si-AR as a direct target of β-catenin is expected to have very high overlap with si-β-catenin targets.

**Fig. S4.** Effect of C3 treatment and β-catenin knockdown on VCaP cells. (A) C3 inhibits growth of VCaP cells. Cells were treated with DMSO or 20 μM C3 every day. (B) β-Catenin knockdown inhibits growth of VCaP cells. (Left) Cells were transfected with control siRNA (si-control) or β-catenin siRNA (si-β-catenin) and subjected to proliferation assays. (Right) VCaP cells transfected with si-β-catenin show decreased levels of β-catenin. (C) C3 inhibits sphere formation in VCaP cells. Cells were cultured under sphere-forming conditions. Vehicle or 20 μM C3 was added at a single time on day 0, and the number of spheres was counted on day 14.
Fig. S5. LNCaP and abl cells infected with shRNA against β-catenin show decreased levels of β-catenin. Cells were stably infected with lentiviral vectors encoding control shRNA (sh-control) or β-catenin shRNA (sh-β-catenin).

Fig. S6. C3 does not affect the occupancy of p300, GRIP1, BRG1, or dimethyl-H3K4 on PSA enhancer. (A) C3 does not inhibit expression of p300, GRIP1, and BRG1. LNCaP cells were androgen-deprived for 3 d and then treated with vehicle or 20 μM C3 in the presence of 100 nM DHT for 16 h. (B) LNCaP cells were treated as described in A and cross-linked at 16 h posttreatment.

Fig. S7. Inhibition of CARM1 activity recapitulates the effect of C3 on target gene expression and AR recruitment to AR-binding sites. (A) CARM1 inhibitor (Calbiochem) decreases expression of AR and Wnt/β-catenin target genes PSA and UBE2C, but not C-MYC in LNCaP cells. Cells were androgen-deprived for 3 d and then treated with vehicle or 5 μM CARM1 inhibitor in the presence of 100 nM DHT for 24 h. (B) C3 decreases occupancy of AR on PSA and UBE2C. LNCaP cells were treated as described in A and cross-linked at 16 h posttreatment.
Fig. S8. Effect of C3 treatment and β-catenin knockdown on sphere formation efficiency of prostate cancer cells. (A) LNCaP and abl spheres are maintained through serial passaging. Cells were cultured in sphere-forming conditions, and the 1st spheres were dissociated and replated to generate 2nd and 3rd spheres. The number of spheres was counted at each generation to measure the efficiency. (B) β-Catenin depletion decreased sphere formation efficiency in LNCaP and abl. Cells were stably infected with lentiviral vectors encoding control shRNA (sh-control) or β-catenin shRNA (sh-β-catenin). Cells were cultured under sphere-forming conditions and imaged at the end of the culture. (Scale bar, 100 μM.) (C) C3 inhibits sphere formation in primary prostate cells harvested from the mouse model of prostate-specific Pten deletion. Cells were cultured under sphere-forming conditions. Vehicle or 20 μM C3 was added at a single time on day 0, and the number of spheres was counted on day 14. One-way ANOVA, *P < 0.05.
Table S1. RT-qPCR primer sequences used in this study

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL19</td>
<td>CACAAGCTGAAGGCAGACAA</td>
<td>GCGTGCTCCCTTGTTCTTAG</td>
</tr>
<tr>
<td>AR (nascent)</td>
<td>TGGTGCCATACCTCTGTCGA</td>
<td>GGCGCAGAGTACCTCTGT</td>
</tr>
<tr>
<td>AR (mature)</td>
<td>TACCAGCTCACAAGCTCCT</td>
<td>GAACGTAGCAGCTTCTC</td>
</tr>
<tr>
<td>PSA</td>
<td>CCAAGTTCTAGCTGCTGTGTC</td>
<td>GCAACATTACAGAAAGTGG</td>
</tr>
<tr>
<td>NKX3-1</td>
<td>AGAAAAGGACTTGGGGTCTT</td>
<td>TTTGGAAGCTCTTGAA</td>
</tr>
<tr>
<td>C-MYC</td>
<td>TCGGAAGGACTATCTCGTG</td>
<td>GTGTGTTGCCCCTCTGAC</td>
</tr>
<tr>
<td>CDC20</td>
<td>CCTCTGGTCTCCCAATTAC</td>
<td>ATGTGACCTTTGAGTTC</td>
</tr>
<tr>
<td>CDK1</td>
<td>CCTAGTACTGCAATCTGGGAAATT</td>
<td>CCTGGAATCTGCTAAGCAC</td>
</tr>
<tr>
<td>UBE2C</td>
<td>TGGTCTGCCCCTGATGATGT</td>
<td>AAAAGCTGTTGGGGTTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCTCAACGACCCATTGTC</td>
<td>CCCCTGTGCTGAGCAAAAT</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>GATTTGATGGAGTTGGACATG</td>
<td>CCAGGTAACGTGCTGCCAG</td>
</tr>
<tr>
<td>OCT4</td>
<td>CGAAAGAGAAAGGAGATGGGC</td>
<td>GCCGGTACAGACACAC</td>
</tr>
<tr>
<td>NANOG</td>
<td>CTCCACACATTGCTGAACCTCAG</td>
<td>CTGCACACATGCTATTTCC</td>
</tr>
<tr>
<td>SOX2</td>
<td>AACCCCAAGATGCAACACTC</td>
<td>CAGGGGCGTAGTTATTTAA</td>
</tr>
</tbody>
</table>

Table S2. ChIP primer sequences used in this study

<table>
<thead>
<tr>
<th>Genomic site</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCF-binding site on AR promoter</td>
<td>CAAAATTGAGGGCTCTATGTG</td>
<td>TTGCTTGAAGACCCTTCA</td>
</tr>
<tr>
<td>PSA enhancer</td>
<td>TGGGACAACTTGGAAAACCTG</td>
<td>CCAGGAGATGCTGTTTTCA</td>
</tr>
<tr>
<td>UBE2C enhancer</td>
<td>TGCCCTGAGATGGAGGAGAGAAGATG</td>
<td>TGTGGTTTTCATGAGGAG</td>
</tr>
<tr>
<td>C-MYC promoter</td>
<td>GCTCTCCACTTGGCCCTTTTA</td>
<td>GTGCCAAATTTCCTAGGC</td>
</tr>
</tbody>
</table>