ROLE OF OATP TRANSPORTERS IN STEROID UPTAKE BY PROSTATE CANCER CELLS IN VIVO

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BACKGROUND: Epidemiologic and in vitro studies suggest that SLCO-encoded organic anion transporting polypeptide (OATP) transporters influence the response of prostate cancer (PCa) to androgen deprivation by altering intratumor androgens. We have previously shown that castration-resistant metastases express multiple SLCO transporters at significantly higher levels than primary PCa, suggesting that OATP-mediated steroid transport is biologically relevant in advanced disease. However, whether OATP-mediated steroid transport can actually modify prostate tumor androgen levels in vivo has never been demonstrated.

METHODS: We sought to determine whether OATP-mediated steroid transport can measurably alter PCa androgen levels in vivo. We evaluated the uptake of dehydroepiandrosterone (DHEAS), E1S and testosterone in LNCaP cells engineered to express OATP1B1, 1B3, 2B1 or 4A1. We measured the uptake via administration of tritiated steroids to castrate mice bearing vector control or OATP1B1-, 2B1- or 4A1-expressing xenografts. We treated tumor-bearing mice with DHEAS and testosterone at physiologically relevant levels and measured intratumor accumulation of administered steroids by mass spectrometry.

RESULTS: OATP1B1- and 28-expressing xenografts each showed a threefold increase in tritiated-DHEAS uptake vs vector controls (P = 0.002 and P = 0.036, respectively). At circulating DHEAS levels similar to those in abiraterone-treated men (~15 μg dl⁻¹), OATP1B1- and 2B1-expressing xenografts showed a 3.9-fold (P = 0.057) and 1.9-fold (P = 0.048) increase in tumor accumulation of DHEAS and a 1.6-fold (P = 0.057) and 2.7-fold (P = 0.095) increase in DHEA, respectively. At the substantial circulating testosterone levels found in eugonadal men, a consistent effect of OATP1B1, 2B1 or 4A1 on testosterone uptake in vivo was not detected.

CONCLUSIONS: OATP transporters measurably alter DHEAS uptake and intratumor androgen levels in prostate tumors in vivo, even at circulating androgen levels achieved in abiraterone-treated patients. These novel data emphasize the continued need to inhibit ligand-mediated androgen receptor signaling in PCa tumors, and support prospective evaluation of studies designed to test inhibition of OATP-mediated DHEAS uptake and utilization.

**INTRODUCTION**

Androgens have a critical role in prostate cancer (PCa) progression.1 Androgen deprivation therapy remains the frontline therapy, but patients uniformly progress to castration-resistant PCa (CRPC). Residual intratumor androgens have a critical role in maintaining ligand-dependent androgen receptor (AR) activation.2 In particular, the levels of prostatic androgens after castration are capable of activating AR and maintaining androgen-regulated gene expression.3,4 and metastases from men after castration are capable of activating AR and maintaining (AR) activation.2 In particular, the levels of prostatic androgens

The source of residual tissue androgens in castrate patients reflects uptake and intracellular conversion of adrenal androgens to testosterone or dihydrotestosterone (DHT), and/or de novo synthesis of androgens from cholesterol or progesterone precursors.5,6 In particular, circulating dehydroepiandrosterone (DHEAS) levels in eugonadal men are extremely high and are not reduced by standard castration therapy.7 Moreover, although suppressed by an order of magnitude, serum DHEAS levels remain substantial in men treated with the adrenal CYP17A inhibitor abiraterone, and are likely to serve as a depot for uptake and intratumoral conversion to downstream androgens in both castrate or abiraterone-treated men.8

The organic anion transporting polypeptides (OATPs) are a superfamily of SLCO-encoded membrane transporters involved in the transport of bile acids, steroid conjugates, xenobiotics and a variety of clinically important drugs.9 Several family members mediate the uptake of steroids and steroid precursors into steroidogenic tissues such as the ovary, breast, placenta and fat; steroid substrates transported by OATP proteins include sulfated forms of pregnenolone, estrone and DHEA, all of which are potentially important substrates in PCa.9–12

However, OATP proteins mediate transport of numerous other drugs and endogenous substrates of potential relevance to PCa biology, including statins, cardiac glycosides, glitazones, metformin and green tea catechins (and even taxanes), all with known or postulated impacts on prostate carcinogenesis and/or progression.13–17 Moreover, while the uptake of steroids by OATP transporters is a plausible hypothesis for associations of SLCO genotype with PCa outcomes, the impact of OATP expression on steroid uptake specifically in PCa models has been limited to evaluation of DHEAS by OATP2B1 and OATP1A2.18,19 Furthermore, whether OATP-mediated steroid transport can actually modify PCa
androgen levels in vivo has never been demonstrated. We evaluated the uptake of DHEAS, E1S and testosterone in vitro and in vivo in LNCaP cells engineered to express OATP1B1, 1B3, 2B1 or 4A1, the four SLCO genes we found to be most highly overexpressed in CRPC metastases.20

MATERIALS AND METHODS

Generation of stable cell lines

PCR was used to amplify open-reading frames for SLCO1B1, 2B1, 1B3 and 4A1 from p-CMV6-XL4 expression vectors (Origene, Rockville, MD, USA). Clones were verified by restriction digest and DNA sequencing (using T7-fwd/-rev and internal primers to sequence the entire open-reading frames) and subcloned into pLenti7.3/V5-DEST vector (in which CMV drives SLCO expression and SV40 drives GFP expression; Supplementary Figure 1) using the Gateway system (Invitrogen, Carlsbad, CA, USA). Virus was generated using the ViraPower System (Invitrogen/Thermo Fisher, Waltham, MA, USA). LNCaP cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and transduced at passages 25–30. Cell line authentication of stable lines was performed by STR profiling (DDC Medical, Fairfield, OH, USA). OATP-overexpressing LNCaP cells were maintained in 10% fetal bovine serum in RPMI-1640 and were not subjected to further subcloning before use.

Deglycosylation of OATP protein extracts and immunoblotting

Protein extracts were deglycosylated using Protein Deglycosylation Mix (New England Biolabs, Ipswich, MA, USA) following the manufacturer’s instructions. Briefly, whole-cell lysates (30 μg) were made using RIPA buffer containing protease and phosphatase inhibitors (Roche Applied Sciences, Penzberg, Germany). Deglycosylation was performed for 5 h at 37 °C. Lysates were electrophoresed on 4–12% Bis-Tris gels (Invitrogen/Thermo Fisher) with MES (2-(N-morpholino)ethanesulfonic acid) buffer, transferred to nitrocellulose, blocked with 5% bovine serum albumin in phosphate-buffered saline/0.1% Tween-20 and probed with anti-V5 antibody (1:200; Invitrogen/Thermo Fisher) or anti-β-actin (1:500; Santa Cruz, Dallas, TX, USA). Proteins were visualized using Supersignal West Femto Chemiluminescent Substrate (Thermo Fisher, Waltham, MA, USA).

Immunofluorescence

Cells were grown on sterile 4-well Lab-Tek II chamber slides (Thermo Fisher) as described above, fixed for 15 min at room temperature in 1% paraformaldehyde and permeabilized with 0.2% Triton-X (Bio-Rad, Hercules, CA, USA) for 5 min. Cells were blocked with 1% bovine serum albumin in phosphate-buffered saline for 30 min in room temperature and incubated with anti-V5 antibody (1:200; Invitrogen) for 1 h, followed by 30 min incubation with Alexa Fluor 594-conjugated anti-mouse secondary antibody (1:2000; Thermo Fisher). Cells were mounted using Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA, USA), and visualized by immunofluorescence microscopy.

Accumulation of 3H-DHEAS, 3H-E1S and 3H-testosterone into OATP-overexpressing LNCaP cells in vitro

Accumulation of tritiated (3H) steroids was conducted on OATP-overexpressing LNCaP cells seeded on poly-L-lysine coated 24-well plates. Cells were pretreated with uptake buffer (135 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 0.6 mM MgSO4, 6 mM d-glucose and 10 mM HEPES, pH 7.4) for 20 min, and then incubated for 10 min in uptake buffer supplemented with 10 nM 3H-labeled DHEAS, E1S or testosterone (Perkin-Elmer, Waltham, MA, USA). Cells were washed 3 x in ice-cold phosphate-buffered saline, solubilized in 0.2% sodium dodecyl sulfate and mixed with scintillation fluid (National Diagnostics, Atlanta, GA, USA). The radioactivity (counts per minute) was measured via a liquid scintillation counting (LS 6000IC Scintillation Counter; Beckman Coulter, Brea, CA, USA); the radioactivity values (counts per minute) were normalized to total protein concentration. Each experiment was conducted in duplicate and repeated a minimum of three times.

Establishment of OATP-expressing LNCaP xenografts in vivo

All animal work was performed in accordance with protocols approved by the Fred Hutchinson Center Institutional Animal Care Use Committee (file 50751). OATP-expressing or vector control LNCaP cells (2 million cells mixed 1:1 in the Matrigel) were subcutaneously injected into flanks of eugonadal CB-17 SCID (8-week-old) male mice (Taconic, Hudson, NJ, USA). When tumors reached 300 mm3, animals were castrated, and 10–14 days later, mice were randomized using a random number generator in a non-blinded manner to the indicated treatment arms.

Figure 1. Stable overexpression of V5-tagged OATP1B1, 1B3, 2B1 and 4A1 in LNCaP cells. (a) Immunoblot of OATP proteins in LNCaP cells transduced with the pLenti7.3/V5-DEST plasmid expressing V5-tagged OATP1B1, 1B3, 2B1 or 4A using antibody against V5-tag. The effect of enzymatic deglycosylation on the apparent molecular mass vs the glycosylated protein is shown (first four lanes vs second four lanes). β-Actin was used as a loading control.50 The variable molecular weights of native OATP proteins reflect post-translational N-linked glycosylation, whereas deglycosylated OATPs have a predicted a molecular weight of ~60–70 kDa.51 (b) Immunofluorescence staining with anti-V5 antibody (red color) of cells overexpressing the indicated OATP construct or vector control. Hoechst (light blue) stain visualizes the nuclei. Figures are shown at ×40 magnification. OATP, organic anion transporting polypeptide.
Uptake of $^3$H-labeled steroids in OATP-expressing LnCaP xenografts in vivo

After anesthetization, mice were coinjected intravenously with 1 000 000 d.p.m. (disintegrations per minute) C14-sucrose (Perkin-Elmer) and 1 000 000 d.p.m. of $^3$H-labeled DHEAS (specific activity (SA): 60–100 Ci mmol$^{-1}$), E1S (SA: 40–60 Ci mmol$^{-1}$) or testosterone (SA: 85–105 Ci mmol$^{-1}$) in 0.2 ml of lactated Ringer’s solution. After 5 min blood was collected, animals were killed and tumors resected. Triplicate tumor pieces (0.1 g each) and serum (50 µl) were placed into vials with 0.75 ml Solvable (Perkin-Elmer) at room temperature for 3–5 days with daily swirling. After tissues were dissolved, 7.5 ml of Opti-Fluor (Perkin-Elmer) was added, equilibrated overnight and radioactivity (d.p.m.) was measured via liquid scintillation counting. Results were expressed as the ratio of radioactivity in the tumor/serum (d.p.m. g$^{-1}$)/(d.p.m. µl$^{-1}$) of the 3H-labeled steroid corrected for the tumor/serum ratio (d.p.m. g$^{-1}$)/(d.p.m. µl$^{-1}$) of the C14-sucrose vascular marker.

Steroid accumulation in OATP-expressing LnCaP xenografts in vivo

Tumor-bearing mice were randomized to vehicle (corn oil, 100 µl daily via intraperitoneal injection), DHEAS (10 mg kg$^{-1}$ per day daily intraperitoneally) or testosterone (2.5 mg kg$^{-1}$ per day daily intraperitoneally). After 14 days, mice were killed and both serum and tumor tissues were snap frozen for the measurement of steroids by mass spectrometry as we have published previously.$^2^2$1

Statistical analysis

$P$-values for in vitro uptake studies were calculated from one-sample two-sided $t$-tests vs a hypothetical value representing a mean fold change of one. Owing to the greater variance between groups that characterize in vivo studies, $P$-values were calculated from nonparametric Mann–Whitney rank tests vs control. $P$-values < 0.05 were considered significant, with $P$-values $\leq$ 0.15 trending toward significance. Outlier observations (predetermined as ± 3 s.d.) were excluded from the analysis. The variation within each group and definition of center values and error bars are provided in each figure. There was no adjustment for multiple comparisons. The methods for measurement of $^3$H-steroid uptake give coefficients of variation in control tissues of ~ 30%. With $n = 10$ per group, this would provide 90-95% power to detect an approximately 50% increase in uptake by tumor tissues. We expect the steroid uptake by tumor tissues to be several fold greater than that in vector controls (consistent with in vitro findings) and therefore within the power of our study.

RESULTS

Overexpression of OATPs in LNCaP cells

We have previously shown increased expression of SLCO genes in CRPC metastases compared with primary PCa, most significantly for transcripts encoding SLCO1B1 (13.8-fold, $P = 0.01$), SLCO1B3 (3.6-fold, $P = 0.05$), SLCO2B1 (5.4-fold, $P = 0.003$) and SLCO4A1 (30-fold, $P = 0.001$).$^{25}$ To determine the impact of these transporters on tumor steroids in vivo, we stably overexpressed them in LNCaP cells, which generally have low SLCO gene expression (Supplementary Table 1). Immunoblot of glycosylated and deglycosylated proteins revealed the expected bands at ~ 80 and 60 kDa, respectively,$^{22,24}$ including splicing variants for OATP2B1 and 4A1 (Figure 1a).$^{25}$ OATP-expressing lines showed prominent cell membrane staining for the V5-tag (Figure 1b). To verify functionality of OATP proteins generated in this manner, we took advantage of Fluo-3 uptake reported for OATP3B1 and tested Fluo-3 uptake in our OATP1B3-overexpressing cells.$^{26}$ We observed a 50% increase in the uptake of Fluo-3 in the OATP1B3-overexpressing cells vs vector control, suggesting OATP1B3 produced in our overexpressing cells is functional.

Steroid hormone accumulation in OATP-overexpressing LNCaP cells in vitro

As OATPs are primary transporters for DHEAS and E1S (and these are both relevant to PCa biology), we tested the uptake of these steroids in vitro, as well as uptake of testosterone, which was previously demonstrated in OATP1B3-transfected COS-7 cells.$^{27,29}$

The mean increase of DHEAS in cells overexpressing OATP1B1, 1B3, 2B1 and 4A1 was ~ 4-fold (range 1.3–5.8; $P = 0.02$), 2-fold (range 1.1–4.7; $P = 0.085$), 2.1-fold (range 1.1–2.6 $P = 0.039$) and 1.7-fold (range 0.9–2.1; $P = 0.032$) vs control cells (Figure 2a and Table 1). These fold changes are similar to DHEAS uptake observed

![Figure 2. Mean steroid hormone accumulation in OATP-overexpressing LNCaP cells in vitro. Cells were incubated at 37 °C for 10 min with 10 nm $^3$H-labeled (a) DHEAS, (b) E1S and (c) testosterone and counts were measured by scintillation counting. Each assay was normalized to protein amount and is presented as the fold uptake over the vector control (represented by the dotted horizontal line). Individual values for replicate experiments of each OATP-expressing cell line are shown as dots within box-and-whisker plots, where horizontal lines indicate median values; white boxes denote the 75th (upper margin) and 25th percentiles (lower margin), and upper and lower bars indicate minimum and maximum values, respectively. $P$-values were calculated using a one-sample $t$-test vs a hypothetical value representing a mean fold change of one ($P$-values $\leq 0.05$ were considered significant; all $P$-values $\leq 0.15$ are shown). DHEAS, dehydroepiandrosterone; OATP, organic anion transporting polypeptide.](image-url)
whereas DHEAS uptake by OATP4A1 has not been reported—expressing 1B1 (3-fold), whereas DHEAS uptake by OATP1B3 in our studies was limited owing to poor engraftment of this tumor line.)

Uptake of 3H-labeled steroids in OATP-expressing LnCaP cell lines. OATP2B1-expressing LnCaP cells. 18 consistent with the twofold increase previously reported for OATP2B1-expressing xenografts.

The mean increase in E1S in cells overexpressing OATP1B1 and 2B1 was 30-fold (range 22–39; P = 0.001) and 7-fold (range 2.2–12.2; P = 0.032), and was not significantly increased in cells expressing 1B3 (mean 1.4, range 0.6–3.7; P = NS) or 4A1 (mean 1.4, range 0.7–3.1; P = NS) (Figure 2b and Table 1). These are similar to E1S uptake reported in other cell models expressing 1B1 (4-40-fold), 1B3 (which showed 2–3-fold uptake in two studies, 32,33 but no uptake in three others, 4,34,35 and 4A1 (1.5-fold), 11,27,30,31

Testosterone, a neutral steroid, is thought to diffuse into cells, and in vitro studies have not identified testosterone transport by OATP1B1 or 2B1 in Xenopus and MDCKII cell lines. 36 However, consistent with the data of Hamada et al. 29 showing a 2-testosterone uptake by OATP1B3-expressing LNCaP cells, with no increase in other OATP cell lines.

Uptake of 3H-labeled steroids in OATP-expressing LNCaP xenografts in vivo

We next determined the impact of OATP expression on tumor androgens in OATP-expressing LNCaP xenografts in vivo using two approaches. Given the more extensive and consistent literature showing uptake of DHEAS by OATP1B1 and 2B1 vs 1B3, 28,30 and that the magnitude of testosterone uptake by OATP1B3 in our study was relatively low, we focused our in vivo analyses on evaluation of the OATP1B1- and 2B1-expressing lines. (Steroid uptake by OATP4A1 cells was attempted but assessments were limited owing to poor engraftment of this tumor line.)

To evaluate specifically the steroid uptake, we harvested serum and tumor pieces 5 min after mice were coinjected intravenously with C14-sucrose and 3H-labeled DHEAS, E1S or testosterone. Sucrose has a molecular weight similar to steroids, thus the extrasucrose space represents the specificity of tumor uptake for any given steroid. Figure 3 shows the tumor to serum ratio of radioactivity for each tritiated steroid (corrected for the C14-sucrose vascular marker) in the control tumors vs OATP1B1-, OATP2B1- or OATP4A1-expressing xenografts.

Consistent with our in vitro observations, the mean uptake of tritiated-DHEAS in OATP1B1, 2B1 and 4A1 xenografts was 3-fold (P = 0.002), 3.1-fold (P = 0.037) and 2.3-fold (P = 0.036) vs control (Figure 3a and Table 1). The mean uptake of E1S was also significantly increased in OATP1B1- and 2B1-expressing tumors, at 4.5-fold (P = 0.001) and 3-fold (P = 0.049) (data for 4A1 not available; Figure 3b and Table 1). Moderately increased uptake of testosterone was observed in the OATP1B1- and OATP4A1-expressing xenografts (2.6-fold, P = 0.011 and 2.6-fold, P = 0.009, respectively; Figure 3c and Table 1).

Tumor steroid levels in OATP-expressing LNCaP tumors treated with DHEAS or testosterone in vivo

We next evaluated the impact of OATP expression on intratumor steroid accumulation at serum steroid levels similar to those in men with PCa in vivo. Castrate mice implanted with OATP-expressing or control xenografts were treated with vehicle (corn oil, intraperitoneally), DHEAS (10 mg kg$^{-1}$ per day) or testosterone (2.5 mg kg$^{-1}$ per day) for 14 days. Mice were killed and serum and tumor tissues were snap frozen for the measurement of androgen levels by mass spectrometry. Transcript levels of SLCO1B1 and SLCO2B1 in overexpressing lines compared with vector controls (and to levels of these genes in our previously published CRPC metastases) are shown in Supplementary Figure 3. 23

The mean serum DHEAS level in DHEAS-treated mice was 2.7 μg dl$^{-1}$, which is lower but within the range of levels reported in abiraterone-treated men (mean15 μg dl$^{-1}$ or 0.4 μm). 7 The mean serum testosterone level in testosterone-treated mice was 479 ng dl$^{-1}$, which was similar to the levels in eugonadal men (~150–700 ng dl$^{-1}$ or 5–25 nm). Steroid levels in control Lncap tumors from intact, castrate, DHEAS and testosterone-treated mice are shown in Supplementary Figure 4. Mean steroid levels in

### Table 1. Summary of DHEAS, E1S or T uptake by OATP-overexpressing LNCaP cell lines in vitro and in vivo

| Steroid   | Transporter | In vitro  | In vivo  | In vivo—mass spec |
|-----------|-------------|-----------|----------|-------------------|
|           |             | $^{3}$H-steroid | $^{3}$H-steroid/C14-sucrose$^{b}$ | Primary steroid | Downstream metabolite |
|           |             | Fold DHEAS | P-value  | Fold DHEAS | P-value  | Fold DHEAS | P-value  |
| DHEAS     | OATP1B1     | 4.0       | 0.0202   | 3.0       | 0.0017   | 3.9        | 0.0571   | 1.6        | 0.0571   |
| DHEAS     | OATP1B3     | 2.0       | 0.0849   | NA        | NA       | NA        | NA       |
| DHEAS     | OATP2B1     | 2.1       | 0.0394   | 3.1       | 0.0368   | 1.9        | 0.0476   | 2.7        | 0.0952   |
| DHEAS     | OATP4A1     | 1.7       | 0.0320   | 2.3       | 0.0364   | 1.2        | ns        | 1.1        | ns        |
| E1S       | OATP1B1     | 30.7      | 0.0006   | 4.5       | 0.0007   |            |          |
| E1S       | OATP1B3     | 1.4       | NS       | NA        | NA       |            |          |
| E1S       | OATP2B1     | 7.1       | 0.0323   | 3.0       | 0.0492   |            |          |
| E1S       | OATP4A1     | 1.4       | NS       | NA        | NA       |            |          |
| T         | OATP1B1     | 1.0       | NS       | 2.6       | 0.0112   | 1.2        | NS        | 0.5        | NS        |
| T         | OATP1B3     | 1.6       | 0.0113   | NA        | NA       | NA        | NA       |
| T         | OATP2B1     | 0.9       | NS       | 1.6       | NS       | 2.1        | 0.0635   | 1.8        | NS        |
| T         | OATP4A1     | 1.2       | NS       | 2.6       | 0.0091   | 2.3        | NS        | 2.7        | NS        |

Abbreviations: DHEAS, dehydroepiandrosterone; NA, not applicable; NS, nonsignificant; OATP, organic anion transporting polypeptide. $^{a}$Showing fold change vs vector controls (all P-values $\leq$ 0.25 are shown). $^{b}$Tumor: serum uptake ratio (g l$^{-1}$).
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Figure 3. Uptake of 3H-labeled steroids in OATP-expressing LnCaP xenografts in vivo. Castrate mice bearing OATP-expressing or vector control LnCaP cells were co-injected intravenously with C14-sucrose and 3H-labeled DHEAS, E1S or testosterone, and serum and tumor pieces were harvested at 5 min for liquid scintillation counting. The tumor to serum ratios of the 3H-labeled steroid (corrected for the C14-sucrose vascular marker) are shown for mice with tumors expressing the indicated constructs, treated with either 3H-labeled DHEAS (a), E1S (b) or testosterone (c). Individual values for replicate tumors of each OATP-expressing cell line are shown. P-values were calculated using the Mann–Whitney rank test between each set of OATP-expressing tumors vs the vector controls (P-values ≤ 0.05 were considered significant; all P-values ≤ 0.15 are shown). DHEAS, dehydroepiandrosterone; OATP, organic anion transporting polypeptide.

Figure 4. Fold increase in intratumor steroid levels in subcutaneous OATP-expressing LnCaP tumors treated with DHEAS or testosterone in vivo. Castrate mice bearing OATP-expressing or vector control LnCaP xenografts were treated with vehicle alone (corn oil, intraperitoneally), DHEAS (10 mg kg\(^{-1}\) per day) or testosterone (2.5 mg kg\(^{-1}\) per day) for 14 days. (a) The fold increase in intratumor steroid levels in DHEAS-treated over vehicle-treated mice of each xenograft, showing levels of DHEAS (in box) and the metabolites to which it can be converted (DHEA, AED and testosterone). Notably, OATP-mediated DHEAS uptake may be particularly relevant in the high progesterone setting associated with abiraterone, as progesterone has been shown to enhance OATP-mediated DHEAS uptake in non-PC models.35,36 These data provide a mechanistic rationale for the observed association between higher circulating adrenal androgen levels and improved vehicle-treated tumors were 15 pg mg\(^{-1}\) (DHEAS), 0.3 pg mg\(^{-1}\) (DHEA), 0.06 pg mg\(^{-1}\) (AED), 0.08 pg mg\(^{-1}\) (testosterone) and 0.04 pg mg\(^{-1}\) (DHT). These are consistent with prior reports in LNCaP cells and similar to prostate tissue levels from abiraterone-treated men (except for DHEAS levels in prostate tissue, which were lower at ~0.008 pg mg\(^{-1}\)).21,37

DHEAS levels were increased in DHEAS-treated xenografts expressing OATP1B1 and 2B1 by 3.9-fold (P = 0.057) and 1.9-fold (P = 0.048) (Figure 4a and Table 1). This was accompanied by a trend toward increased levels of steroids downstream of DHEAS, particularly DHEA for 1B1 (1.6-fold, P = 0.057) and 2B1 (2.7-fold, P = 0.095), with weaker trends for AED and testosterone in 1B1 (P = 0.130 and P = 0.114), consistent with known expression of the requisite transforming enzymes in LNCaP cells.36,37 We observed limited accumulation of testosterone and downstream androgens in testosterone-treated tumors (Figure 4b), even at the substantial circulating testosterone levels in eugonadal men. Table 1 summarizes findings from each of the preceding in vitro and in vivo studies.

DISCUSSION
We believe our data provide the first in vivo proof of principle demonstrating OATP-mediated effects on tumor androgen levels in vivo. We used two distinct methods to evaluate steroid uptake in our OATP-expressing xenografts. To isolate an effect of OATP expression on steroid uptake from time-dependent effects of steroid accumulation and/or potential metabolism, we administered 3H-labeled steroids to tumor-bearing mice and evaluated radioactivity in tumors resected at 5 min after injection. In separate experiments, we determined whether expression of OATPs at physiologically relevant levels of administered DHEAS or testosterone did, in fact, result in the accumulation of the administered steroid or its downstream metabolites.

Both sets of in vivo studies showed a 2–3-fold increase in DHEAS levels in LNCaP xenografts expressing OATP1B1 or 2B1. Notably, this occurred at circulating DHEAS levels in the range observed in abiraterone-treated men (15 μg dl\(^{-1}\) or 4 μM).3 In contrast, even at the substantial circulating testosterone levels in eugonadal men (~500 ng dl\(^{-1}\) or 0.7 nM), DHEAS levels in prostate tissue, which were lower at ~0.008 pg mg\(^{-1}\)).

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DISCUSSION
We believe our data provide the first in vivo proof of principle demonstrating OATP-mediated effects on tumor androgen levels in vivo. We used two distinct methods to evaluate steroid uptake in our OATP-expressing xenografts. To isolate an effect of OATP expression on steroid uptake from time-dependent effects of steroid accumulation and/or potential metabolism, we administered 3H-labeled steroids to tumor-bearing mice and evaluated radioactivity in tumors resected at 5 min after injection. In separate experiments, we determined whether expression of OATPs at physiologically relevant levels of administered DHEAS or testosterone did, in fact, result in the accumulation of the administered steroid or its downstream metabolites.

Both sets of in vivo studies showed a 2–3-fold increase in DHEAS levels in LNCaP xenografts expressing OATP1B1 or 2B1. Notably, this occurred at circulating DHEAS levels in the range observed in abiraterone-treated men (15 μg dl\(^{-1}\) or 4 μM).3 In contrast, even at the substantial circulating testosterone levels in eugonadal men (~500 ng dl\(^{-1}\) or 0.7 nM), the ability to facilitate uptake of DHEAS is quantitatively more likely to be clinically relevant in PCa progression than the uptake of testosterone. Notably, OATP-mediated DHEAS uptake may be particularly relevant in the high progesterone setting associated with abiraterone, as progesterone has been shown to enhance OATP-mediated DHEAS uptake in non-PC models.35,36 These data provide a mechanistic rationale for the observed association between higher circulating adrenal androgen levels and improved
response to CYP17A inhibition,\textsuperscript{40,41} and emphasize the ongoing need to inhibit ligand-mediated AR signaling in PCa tumors, even at the circulating androgen levels achieved in abiraterone-treated patients.

Variation in SLCO1B3 has also been linked with time to progression in men on androgen deprivation therapy, and has been attributed to genetic differences in OATP1B3-mediated testosterone uptake.\textsuperscript{29,42} We did not evaluate steroid uptake by
this transporter in vivo, but OATP1B3 has been shown to transport DHEAS in vitro, raising the possibility that the association of OATP1B3 with outcomes could in some part be related to DHEAS uptake (although an impact of genotype on OATP1B3-mediated DHEAS uptake has not been reported).

Our findings also demonstrate the uptake of E1S in OATP1B1- and OATP2B1-expressing cells in vivo. OATP-mediated E1S uptake has been identified as an important mediator of estrogen-dependent growth in breast cancer, and estrogen signaling has been implicated in prostate carcinogenesis and progression. Whether OATP-mediated E1S uptake contributes to PCa development in eugonadal men or to disease progression in castrate men has not been explored, but represents an intriguing hypothesis.

An important limitation of this work is the inherent variability that characterizes in vivo vs in vitro studies, which may explain the varying results obtained in the testosterone uptake studies. As such, our data do not preclude a change in testosterone uptake within the standard deviation of the observed values. Notably, despite this variability, we observed a consistent impact of OATP1B1 and 2B1 on DHEA uptake across the in vitro and in vivo studies, supporting the robustness of this observation and the potential clinical impact of this phenomenon.

The contribution of a single OATP to steroid accumulation in our study was relatively small (on the order of 2–3-fold). Transcript levels in the overexpressing cell lines were lower compared with those in CRPC metastases for SLCO2B1, although similar to those detected for SLCO1B1. As such, the clinical impact of OATP transport on tumor steroid levels in PCa is most likely to be relevant at high substrate concentrations (such as for DHEAS, as discussed above) and/or when transporter expression is increased or multiple transporters are expressed. In this regard, we have previously found that CRPC metastases are characterized by the expression of multiple OATP transporters and at much higher levels than in primary PCa, consistent with the hypothesis that OATP-mediated steroid transport is most biologically relevant in castration-resistant disease.

Given the number of endogenous and therapeutic substrates transported via OATPs, attempting to target OATP transport in cancer is generally not likely to be feasible. However, Hashmann et al. have recently demonstrated that statins inhibit DHEAS uptake by OATP2B1, suggesting that statins could have a role in decreasing tumor androgen uptake in CRPC patients. A treatment strategy using statins to inhibit OATP-mediated DHEAS uptake, combined with a steroid sulfatase inhibitor (to prevent conversion of DHEAS to DHEA) or an AKR1C3 inhibitor (to prevent conversion of DHEA to downstream metabolites), might be particularly effective in preventing intratumoral uptake and conversion of the residual DHEAS levels present in abiraterone-treated patients. Our data demonstrating that OATP-mediated DHEAS uptake is capable of modifying intratumor androgen levels in vivo strongly supports the prospective evaluation of clinical studies designed to test these hypotheses.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We acknowledge the expert technical assistance provided by Steve Mongovin, Brandy Olin, Bea Binang and Dominic Tran. This work was supported by Pacific Northwest Prostate Cancer SPORE P50 CA97186 (to EAM); NIH/NCI Cancer Center Support Grant SP30CA15704-40 (to EAM, AK) and Department of Defense CDMRP W81XWH-11-2-0154 (to EAM).
Supplementary Information accompanies the paper on the Prostate Cancer and Prostatic Diseases website (http://www.nature.com/pcan)