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Role of OATP Transporters in Steroid Uptake by Prostate Cancer Cells *in Vivo*

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Abstract

Background—Epidemiologic and *in vitro* studies suggest *SLCO*-encoded OATP transporters influence the response of prostate cancer (PCa) to androgen deprivation by altering intra-tumor androgens. We have previously shown that castration resistant metastases express multiple *SLCO* transporters at significantly higher levels than primary PCa, suggesting 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 uptake of DHEAS, E1S and testosterone in LNCaP cells engineered to express OATP1B1, 1B3, 2B1 or 4A1. We measured 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 intra-tumor accumulation of administered steroids by mass spectrometry.

Results—OATP1B1 and 2B-expressing xenografts each showed a 3-fold 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 (~15ug/dL) 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

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CONFLICT OF INTEREST

The authors declare there are no competing financial interests in relation to the work described.

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 intra-tumor 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 AR signaling in PCa tumors, and support prospective evaluation of studies designed to test inhibition of OATP-mediated DHEAS uptake and utilization.

Keywords

SLCO; OATP; transporter; steroid uptake; prostate cancer; castration resistant; DHEA-S; xenograft

INTRODUCTION

Androgens play a critical role in prostate cancer (PCa) progression.[1] Androgen deprivation therapy (ADT) remains front-line therapy, but patients uniformly progress to castration resistance prostate cancer (CRPC). Residual intra-tumoral androgens play a critical role in maintaining ligand-dependent androgen receptor (AR) activation.[2] In particular, levels of prostatic androgens after castration are capable of activating AR and maintaining androgen-regulated gene expression,[3,4] and metastases from men with CRPC contain testosterone levels that significantly exceed prostate tissues of eugonadal men.[5]

The source of residual tissue androgens in castrate patients reflects uptake and intracellular conversion of adrenal androgens to testosterone or DHT, and/or de novo synthesis of androgens from cholesterol or progesterone precursors.[5,6] In particular, circulating 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 intra-tumoral conversion to downstream androgens in both castrate or abiraterone-treated men.[8]

The organic anion transporting polypeptides (OATP) are a superfamily of *SLCO*-encoded membrane transporters involved in transport of bile acids, steroid conjugates, xenobiotics and a variety of clinically important drugs.[9] Several family members mediate uptake of steroids and steroid precursors into steroidogenic tissues such as 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, green tea catechins (and even taxanes), all with known or postulated impacts on prostate carcinogenesis and/or progression.[13–17] Moreover, while 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 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 (ORF) for *SLCO1B*1, *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 ORF) 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). Virus was generated using the ViraPower System (Invitrogen/ Thermo Fisher, Waltham, MA, USA). LNCaP cells were obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA) and transduced at passage 25-30. Cell line authentication of stable lines was performed by STR profiling (DDC Medical, Fairfield, OH). OATP-overexpressing LNCaP cells were maintained in 10% FBS in RPMI-1640 and were not subjected to further subcloning prior to use.

De-glycosylation of OATP protein extracts and immunoblotting

Protein extracts were de-glycosylated using Protein Deglycosylation Mix (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. Briefly, whole cell lysates (30 ug) were made using RIPA buffer containing protease and phosphatase inhibitors (Roche Applied Sciences, Penzberg, Germany). De-glycosylation was performed for 5 hours at 37°C. Lysates were electrophoresed on 4-12% Bis-Tris gels (Invitrogen/ Thermo Fisher, Waltham, MA, USA) with MES buffer, transferred to nitrocellulose, blocked with 5% BSA in PBS/0.1% Tween-20 and probed with anti-V5 antibody (1:200; Invitrogen/ Thermo Fisher, Waltham, MA, USA) or anti-β-actin (1:500; Santa Cruz, Dallas, Texas, 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, Waltham, MA, USA) as described above, fixed for 15 min at RT in 1% paraformaldehyde and permeabilised with 0.2% Triton-X (BioRad, Hercules, CA, USA) for 5 min. Cells were blocked with 1%BSA in PBS for 30 min in RT and incubated with anti-V5 antibody (1:200, Invitrogen) for 1 hour followed by 30 min incubation with Alexa Fluor 594 conjugated anti-mouse secondary antibody (1:2000, Thermo Fisher, Waltham, MA, USA). Cells were mounted using Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA, USa), and visualized by IF microscopy.

Accumulation of 3H-DHEA-S, 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, 2.5mM $CaCl_2$, 0.6 mM $MgSO_4$, 6 mM D-glucose, and 10 mM HEPES, pH 7.4) for 20 minutes, then incubated for 10 minutes in uptake buffer supplemented with 10 nM 3H-labeled DHEA-S, E1S, or testosterone (Perkin Elmer, Waltham, MA, USA). Cells were washed 3× in ice-cold PBS, solubilized in 0.2% SDS, mixed with scintillation fluid (National Diagnostics, Atlanta Georgia, USA), and radioactivity (cpm) measured via liquid scintillation counting (LS 6000IC scintillation counter, Beckman Coulter); cpm 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 matrigel) were subcutaneously injected into flanks of eugonadal male CB-17 8 week old SCID mice (Taconic, Hudson, NJ, USA). When tumors reached 300mm3 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.

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

After anesthetization mice were co-injected intravenously with 1,000,000 dpm C14-Sucrose (Perkin Elmer, Waltham, MA, USA) and 1,000,000 dpm of 3H-labeled DHEAS (specific activity (SA): 60-100 Ci/mmol), E1S (SA: 40-60Ci/mmol) or testosterone (SA: 85-105 Ci/mmol) in 0.2 ml of lactated Ringer's solution. After 5 minutes blood was collected, animals sacrificed and tumors resected. Triplicate tumor pieces (0.1gram each) and serum (50ul) were placed into vials with 0.75ml Solvable (Perkin Elmer, Waltham, MA, USA) at room temperature for 3-5 days with daily swirling. After tissues were dissolved, 7.5 mls of Opti-Fluor (Perkin Elmer, Waltham, MA, USA) was added, equilibrated overnight, and radioactivity (dpm) measured via liquid scintillation counting. Results were expressed as the ratio of radioactivity in the tumor/serum (DPM/gram)/(DPM/ul) of the 3H-labeled steroid corrected for the tumor/serum ratio (DPM/gram)/(DPM/ul) of the C14-Sucrose vascular marker.

Steroid accumulation in OATP expressing LnCaP xenografts *in vivo*

Tumor bearing mice were randomized to vehicle (corn oil, 100ul daily via intraperitoneal (i.p.) injection), DHEAS (10mg/kg/day daily i.p.) or testosterone (2.5mg/kg/day daily i.p.). After 14 days, mice were sacrificed and serum and tumor tissue snap frozen for measurement of steroids by mass spectrometry as we have previously published.[7,21]

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. Due to the greater variance between groups that characterize *in vivo* studies, p values were calculated from non-parametric Mann Whitney Rank Test s vs control. P values < 0.05 were considered significant, with p values ≤ 0.15 trending toward significance. Outlier observations (pre-defined as +/− 3 standard deviations) were excluded from 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 3H-steroid uptake give coefficients of variation in control tissues of ~30%. With n=10/group, this would permit detecting a ~50% increase in uptake with a power of 90-95%. We expect steroid uptake by tumor tissue to be several fold greater than in vector controls (consistent with in vitro findings) and therefore within power of our study.

RESULTS

Overexpression of OATPs in LNCaP cells

We have previously shown increased expression of *SLCO* genes in CRPC metastases compared to 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).[20] To determine impact of these transporters on tumor steroid s *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 approximately 80 kDa 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 uptake of Fluo-3 in the OATP1B3 overexpressing cells vs. vector control, suggesting OATP1B3 produced in our over-expressing 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 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 approximately 4-fold (range 1.3-5.8; p=0.020), 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, Table 1). These fold changes are similar to DHEAS uptake observed in HEK293, xenopus, CHO or MDCKII cells expressing 1B1 (3-7 fold), 1B3 (2-5 fold), and 2B1 (1.5-3 fold), whereas DHEAS uptake by OATP4A1 has not been previously reported.[27,30,31] The increase in DHEAS by OATP2B1 is also consistent with the 2-fold increase previously reported for OATP2B1-expressing LnCaP cells.[18]

The mean increase in E1S in cells overexpressing OATP1B1 and 2B1 was 30-fold (range 22-39; p=0.001) and 7.1-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, 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[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 data of Hamada et al showing a 2-fold increase in OATP1B3-expressing COS-7 cells,[29] we observed a 1.6-fold (range 1.1-2.3; p=0.011) increase in 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 due to poor engraftment of this tumor line.)

To specifically evaluate steroid uptake, we harvested serum and tumor pieces 5 minutes after mice were co-injected intravenously with C14-Sucrose and 3H-labeled DHEAS, E1S or testosterone. Sucrose has a molecular weight similar to steroids so the extra-sucrose 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, 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, Table 1). 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, 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, 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 intra-tumor 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, i.p.), DHEAS (10mg/kg/day) or testosterone (2.5mg/kg/day) for 14 days. Mice were sacrificed and serum and tumor tissue snap frozen for measurement of androgen levels by mass spectrometry. Transcript levels of *SLCO1B1* and *SLCO2B1* in over-expressing lines compared to vector

controls (and to levels of these genes in our previously published CRPC metastases) are shown in Supplementary Figure 3.[20]

The mean serum DHEAS level in DHEAS-treated mice was 2.7ug/dL, which is lower but within range of levels reported in abiraterone-treated men (mean15ug/dL or 0.4uM).[7] The mean serum testosterone level in testosterone-treated mice was 479 ng/dL, similar to levels in eugonadal men (~150-700ng/dL or 5-25nM). Steroid levels in control LnCaP tumors from intact, castrate, DHEAS and testosterone-treated mice are shown in Supplementary Figure 4. Mean steroid levels in vehicle-treated tumors were 15 pg/mg (DHEAS), 0.3 pg/mg (DHEA), 0.06 pg/mg (AED), 0.08 pg/mg (testosterone), and 0.04 pg/mg (DHT). These are consistent with prior reports in LNCaP cells and similar prostate tissue levels from abiraterone-treated men (except for DHEAS levels in prostate tissue which were lower at ~0.008 pg/mg).[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, 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.[38,39] 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

Our data provide the first *in vivo* proof of principle demonstrating OATP-mediated effects on tumor androgen levels *in vivo*. We utilized 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 minutes 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 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 (15ug/dL or 0.4uM).[7] In contrast, even at the substantial circulating testosterone levels in eugonadal men (~500ng/dL), an increase in testosterone or downstream metabolites was not observed in testosterone-treated OATP1B1 or 2B1 expressing tumors.

Given the relative levels at which DHEAS (~200ug/dL or 5uM) and testosterone (20ng/dL or 0.7nM) circulate in castrate men,[7] the ability to facilitate uptake of DHEAS is quantitatively more likely to be clinically relevant in PCa progression than 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,[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 ADT, and has been attributed to genetic differences in OATP1B3-mediated testosterone uptake.[29,42] We did not evaluate steroid uptake by this transporter *in vivo*, but OATP1B3 has been shown to transport DHEAS *in vitro*,[22,23,32] 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 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,[27,33,43,44] and estrogen signaling has been implicated in prostate carcinogenesis and progression.[45] 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.[46,47]

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 DHEAS 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 than those in CRPC metastases for *SLCO2B1*, although similar to those detected for *SLCO1B1*.[20] 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.[20]

Given the number of endogenous and therapeutic substrates transported via OATPs, attempting to target OATP transport in general is unlikely to be feasible. However, Harshmann et al have recently demonstrated that statins inhibit DHEAS uptake by OATP2B1,[48] suggesting statins could play a role in decreasing tumor androgen uptake in CRPC patients. A treatment strategy utilizing 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 intra-tumoral uptake and conversion of the residual DHEAS levels present in abiraterone-treated patients.[8,49] Our data demonstrating that OATP-mediated DHEAS uptake is capable of modifying intra-tumor androgen levels *in vivo* strongly supports the prospective evaluation of clinical studies designed to test these hypotheses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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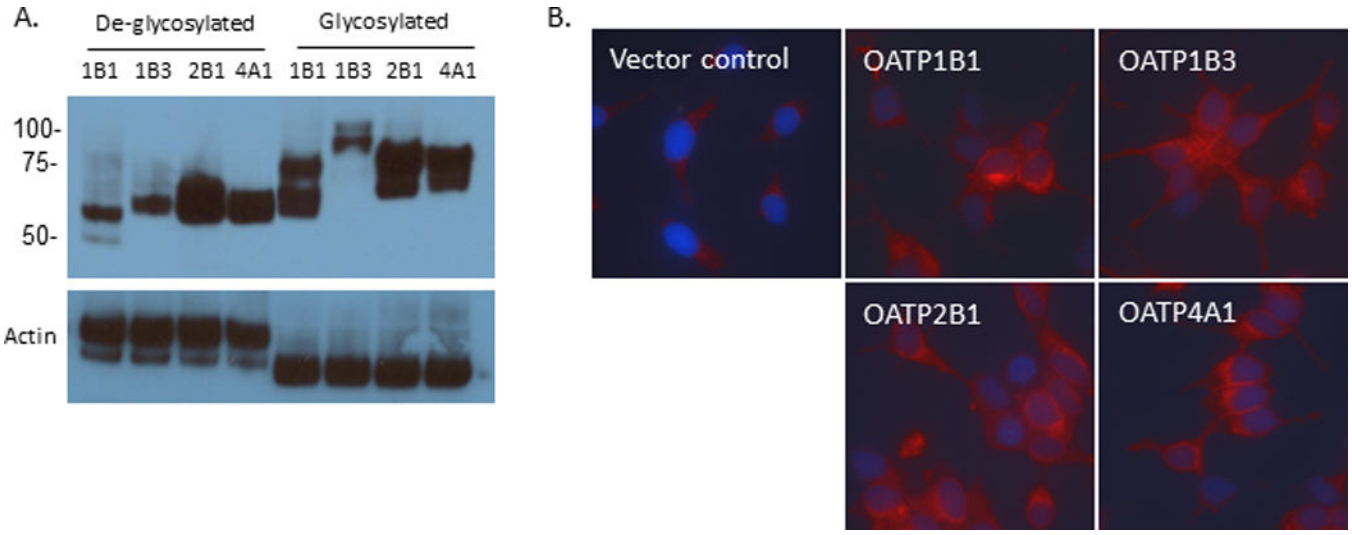

Figure 1. Stable overexpression of V5-tagged OATP1B1, 1B3, 2B1, 4A1 in LNCaP cells

(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 de-glycosylated 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 nuclei. Figures shown at 40× magnification.

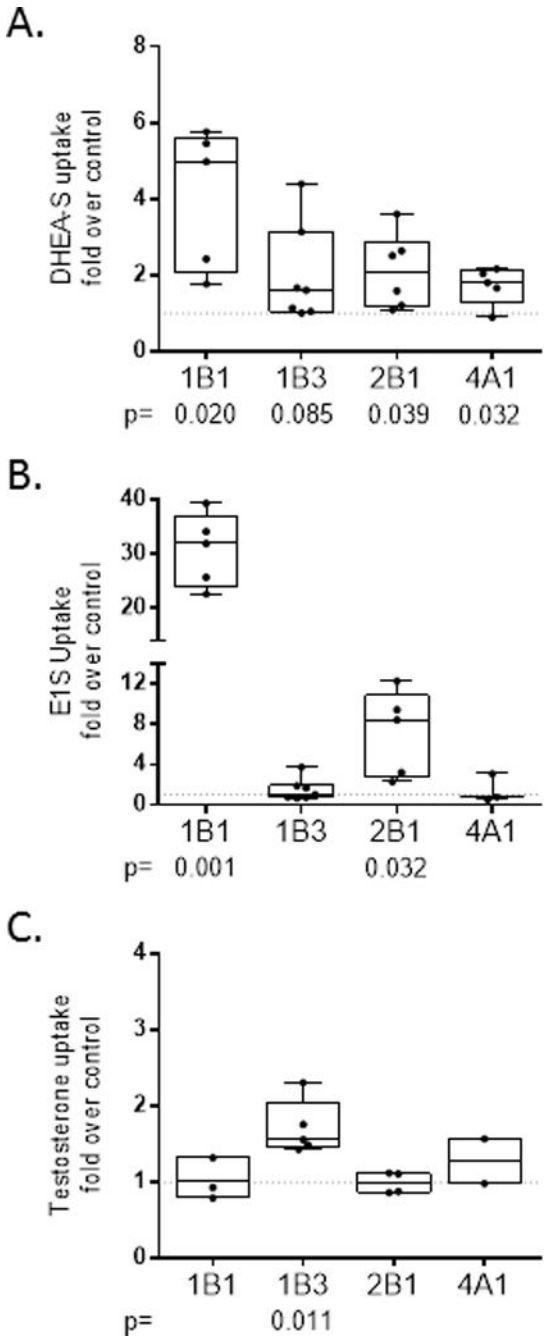

Figure 2. Mean steroid hormone accumulation in OATP overexpressing LNCaP cells *in vitro*

Cells were incubated at 37ºC for 10 minutes with 10nM 3H-labeled (A) DHEAS, (B) E1S, and (C) testosterone and counts 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 calculated using a one sample t test vs a hypothetical value

representing a mean fold change of one (P values ≤0.05 were considered significant; all p values ≤ 0.15 are shown).

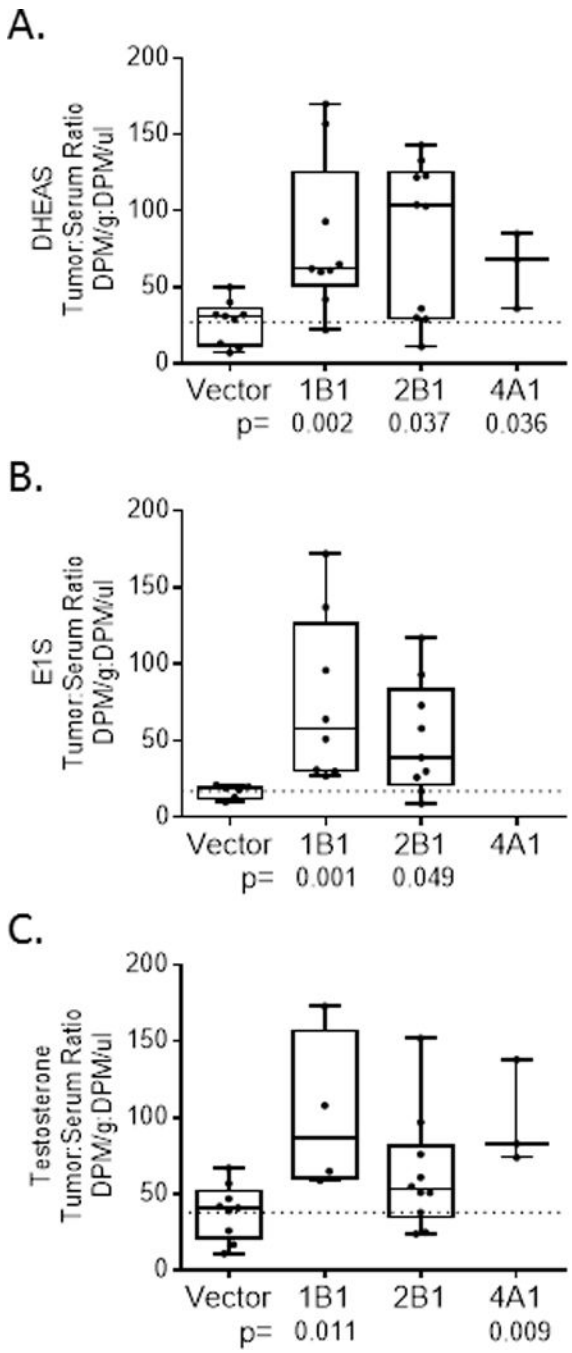

Figure 3. Uptake of 3H-labeled steroids in OATP expressing LnCaP xenografts *in vivo*

Castrate mice bearing OATP expressing or vector control LNCaP cells were co-injected intravenously with C14-Sucrose and 3H-labeled labeled DHEAS, E1S or testosterone, and serum and tumor pieces were harvested at 5 minutes 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 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).

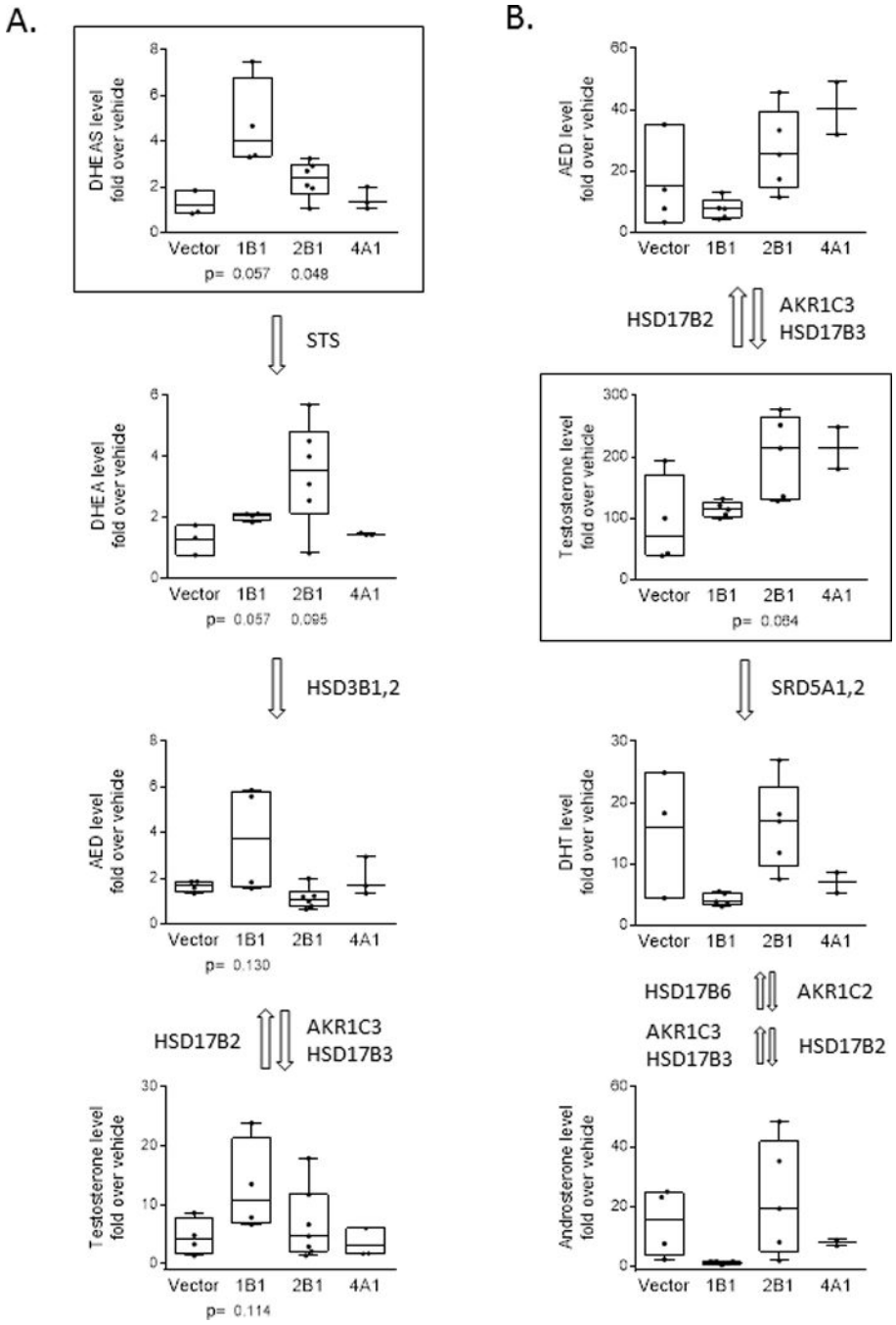

Figure 4. Fold increase in intra-tumor steroid levels in subcutaneous OATP-expressing LnCaP tumors treated with DHEAS or testosterone *in vivo*

Castrate mice bearing OATP expressing or vector control LNCaP xenografts were treated with vehicle alone (corn oil, i.p.), DHEAS (10mg/kg/day) or testosterone (2.5mg/kg/day) for 14 days. (A) The fold increase in intra-tumor steroid levels in DHEAS-treated over vehicle-treated mice of each xenograft type, showing levels of DHEAS (in box) and the metabolites to which it can be converted (DHEA, AED, and testosterone). (B) The fold increase in tumor steroid levels in testosterone-treated over vehicle-treated mice of each xenograft, showing levels of testosterone (in box) and the metabolites to which it can be converted (AED, DHT

and androsterone). Also shown are the steroidogenic enzymes mediating the metabolic conversion of DHEAS or testosterone. Individual values for replicate tumors of each OATP expressing cell line are shown. P values 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). Mean tumor steroid levels in castrated vehicle-treated tumors were 15 pg/mg (DHEAS), 0.3 pg/mg (DHEA), 0.06 pg/mg (AED), 0.08 pg/mg (testosterone), and 0.04 pg/mg (DHT).

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Table 1

Summary of DHEAS, E1S or T uptake by OATP overexpressing LNCaP cell lines *in vitro* and *in vivo* showing fold change vs vector controls (all p values ≤ 0.25 are shown)

		in vitro		***in vivo***		***in vivo* - Mass Spec**			
		3H-steroid		**3H-steroid/14C-sucrose[1]**		**primary steroid**		**downstream metabolite**	
Steroid	**Transporter**	**fold DHEAS**	**p value**	**fold DHEAS**	**p value**	**fold DHEAS**	**p value**	**fold DHEA**	**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
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
		fold E1S	p value	fold E1S	p value				
E1S	OATP1B1	30.7	0.0006	4.5	0.0007				
E1S	OATP1B3	1.4	ns		NA				
E1S	OATP2B1	7.1	0.0323	3.0	0.0492				
E1S	OATP4A1	1.4	ns		NA				
		fold T	p value	fold T	p value	fold T	p value	fold AED	p value
T	OATP1B1	1.0	ns	2.6	0.0112	1.2	ns	0.5	ns
T	OATP1B3	1.6	0.0113		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

[1]Tumor:Serum uptake ratio (g/uL) for the indicated 3H-labeled steroid corrected for the C14-Sucrose vascular marker