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Table of Contents

ABSTRACT

Background: Abundant epidemiological and experimental evidence establishes alterations in cholesterol metabolism as a key driver of prostate cancer (PCa) aggressiveness. Our preliminary data shows cholesterol sulfotransferase (SULT) 2B1b, a global regulator of cholesterol metabolism, is overexpressed in human prostate neoplasia and PCa cell lines and that genetic knock down suppresses LNCaP growth and diminishes androgen receptor (AR) activity. It is hypothesized that SULT2B1b modulates PCa growth and phenotype via alterations in cholesterol metabolism. If validated, the studies will form the foundation for novel therapeutic intervention.

Objective: The primary objective of these studies is to define the role of SULT2B1b in PCa on growth, sensitivity to androgens, and apoptosis based on the central **hypothesis that SULT2B1b regulates malignant phenotypes via regulation of cholesterol metabolism**. The pivotal role of SULT2B1b in regulating cholesterol homeostasis in PCa is a novel observation that when better defined by the studies outlined in this application, will provide the foundation for new approaches for controlling cholesterol dysregulation in PCa.

Specific Aims:

Aim 1. To elucidate mechanism(s) by which SULT2B1b modulates cholesterol metabolism. Studies in this aim address the *hypothesis that SULT2B1b-mediated sulfonation of oxysterols and/or* SREPB-2 *is central to cholesterol dysregulation in PCa.*

Aim 2: To elucidate mechanism(s) of SULT2B1b-mediated alterations in apoptotic and AR responses. Studies in this aim address the *hypothesis that SULT2B1b modulates PCa growth via alterations in AR response that may or may not impact response to apoptotic stimuli.*

Aim 3: Validating the biological function of Sult2B1b via chemical probes. This aim describes a strategy to develop a small molecule inhibitor of SULT2B1b to probe biological function of SULT2B1b and to promote progression toward preclinical studies.

Study design: Impact of SULT2B1b on cholesterol metabolism and subsequent impact on cellular proliferation, apoptosis, and androgen responses will be evaluated in vitro human prostate cancer models after genetic (RNAi)-based modulation of endogenous SULT2B1b. The proposed studies include the development of a unique screening assay to identify lead compounds toward the development of a SULT2B1b inhibitor.

Innovation: Preliminary studies highlight an important and novel role for SULT2B1b as a pivotal regulator of cellular cholesterol metabolism in PCa. The conceptual hypothesis that SULT2B1b expression in PCa is central to dysregulation of cholesterol homeostasis is innovative. While a major focus of PCa research is on the AR and its impact on PCa growth, the proposed studies will probe the impact of SULT2B1b-mediated sulfation on pathways regulating cholesterol metabolism, including LXR and alternate non-LXR pathways. If validated, the studies will provide the foundation for developing novel approaches to controlling cholesterol dysregulation in PCa, which is known to contribute to AR activity as well as apoptosis and cellular proliferation. Thus, **establishing SULT2B1b as a key regulator of PCa growth and progression could potentially establish SULT2B1b a novel therapeutic target.**

Impact: Current treatments for organ-confined disease are associated with high morbidity. There are no effective treatments of hormone-independent disease. Understanding the factors that control the onset and progressing of indolent disease to lethal metastatic disease is key to reducing prostate cancer death. The impact of these studies, if successful, will be the identification of a novel regulator of prostate cancer growth and progression and allow for the potential development of better treatment options for organ-confined disease and novel options of treatment for hormone-independent, advanced metastatic disease.

Understanding the cellular mechanisms that control the transition of a normal cell to a cancer cell, and then from organ-confined disease to more lethal metastatic disease are key to developing more effective treatments for prostate cancer. The mechanisms that control these changes in tumor cells are often changes in how the cells respond to external signals such as hormones (androgens) and growth factors. Using a novel technology developed by a member of our research team, we discovered that choesterol-3-sulfate (CS), a cholesterol derivative produced in prostate cancer cells that is known to regulate cell growth and behavior in other cells, is uniquely expressed in human prostate cancer but not normal prostate tissue. Cholesterol and its derivatives is established as an important regulator of not only cell growth but also malignant behavior of cells including the ability to invade surrounding tissue and to escape the prostate and form tumors in different locations in the body (metastasis). Preliminary studies show that SULT2B1b, enzyme that produces CS in prostate cells, can control many malignant behaviors by controlling cholesterol and lipid metabolism via a pathway called LXR. We also show SULT2B1b can potentially regulate cholesterol and growth by as yet unknown mechanism as well. Based on previous studies in other cell types, we hypothesize that SULT2B1b can signal the tumor cell to alter prostate cancer cell behavior by several mechanisms. First, SULT2B1b can alter the function of LXR which in turn changes the way cholesterol is taken up and used by the cell to stabilize regions of cell membranes that control growth factor signaling. Second, because SULT2B1b alters cholesterol uptake and utilization, and cholesterol is the precursor of all androgens, the way the prostate cancer cells make its own androgens is altered. Third, SULT2B1b can directly regulate genes that directly control cell growth and death (apoptosis). Forth, SULT2B1b can directly change the behavior of the cell and allow the cells to grow in clusters and break away from the primary mass. Finally, SULT2B1b can alter gene regulation so that the tumor cells can better survive deprivation of oxygen and nutrients by increasing blood supply. All of these potential functions of SULT2B1b have been indentified in other cell types but never looked at in prostate cancer.

We propose to investigate these putative functions of SULT2B1b in prostate cancer cells. We are also working towards developing a drug that interrupt SULT2B1b function and will test this compound in prostate cancer cells. Although it is too early for these studies to impact clinical treatment in the short term by discovering a novel regulator of prostate cancer growth, a new therapeutic target will have been identified so that better or novel treatments for both organ-confined and metastatic disease could be developed.

Rationale: Abundant epidemiological and experimental evidence establishes alterations in cholesterol metabolism as a key driver of prostate cancer (PCa) aggressiveness. Therapeutically targeting cholesterol metabolism in PCa through the use of cholesterol-lowering drugs (statins) decreases the occurrence of aggressive PCa. Our preliminary data show cholesterol sulfotransferase (SULT) 2B1b, a global regulator of cholesterol metabolism, is highly expressed in many clinical PCa specimens and PCa cell lines and that genetic knock down suppresses LNCaP growth and diminishes androgen receptor (AR) activity. It is hypothesized that SULT2B1b modulates PCa growth and phenotype via alterations in cholesterol metabolism. If validated, the studies will form the foundation for novel therapeutic intervention.

Hypothesis or objective: The primary objective of these studies is to define the role of SULT2B1b in PCa on growth, invasion, and sensitivity to androgens, and apoptosis in androgen responsive and non-responsive phenotypes based on the central **hypothesis that SULT2B1b regulates malignant phenotypes via regulation of cholesterol metabolism**. The pivotal role of SULT2B1b in regulating cholesterol homeostasis in PCa under standard growth conditions is a novel observation that when better defined by the studies outlined in this application, will provide the foundation for new approaches for controlling cholesterol dysregulation in PCa. To better define the role of SULT2B1b in PCa, the following goals are set forth herein: a) to validate the role of SULT2B1b in modulating cholesterol dysregulation, b) to elucidate the pathway(s) by which SULT2B1b modulates cholesterol levels in PCa, and c) to elucidate the mechanism(s) of SULT2B1b-mediated AR activity regulation. In addition, the potential role of SULT2B1b on regulating biosynthetic pathways associated with de novo androgen synthesis will be addressed based on the **hypothesis that SULT2B1b promotes PCa proliferation by impacting the biosynthesis and metabolism of androgen.** To accomplish these goals, the following specific aims are proposed:

Research Approach:

Aim 1. To elucidate mechanism(s) by which SULT2B1b modulates cholesterol metabolism. Stable and tetracycline (tet)-inducible PCa cell lines (LNCaP, VCaP, PC3, and DU145) expressing shRNA (short hairpin RNA) against SULT2B1b or full-length SULT2B1b cDNA have been developed and verified and will be used to assess impact of SULT2B1b modulation on PCa. Studies in this aim address the *hypothesis that SULT2B1b-mediated sulfonation of oxysterols and/or* SREPB-2 *is central to cholesterol dysregulation in PCa via limiting their agonistic effects on LXR* and will a) verify that modulation of SULT2B1b alters cholesterol levels in PCa cells; b) identify SULT2B1b-mediated signals that modulate LXR activity; c) evaluate SULT2B1b modulation of cholesterol homeostasis via the non-LXR-mediated pathway in which SULT2B1b controls cholesterol biosynthesis by direct regulation of SREBP-2 activity.

Aim 2: To elucidate mechanism(s) of SULT2B1b-mediated alterations in apoptotic and AR responses. Studies in this aim address the *hypothesis that SULT2B1b modulates PCa growth via alterations in AR response that may or may not impact response to apoptotic stimuli*. These studies will indentify SULT2B1b-mediated signals that alter AR activity via crosstalk between LXR and AR and regulation and function of AR co-factors.[11] Also, the impact on androgen concentration, AR expression, the binding of androgen to AR, the subcellular localization of AR, and the binding of AR to target gene promoters, and activation of the AR transcriptional activity will be assessed. These studies will also assess SULT2B1b impact on apoptosis by activation of death receptors via exposure to recombinant TRAIL, Fas Ligand and TNFα proteins.[18]

Aim 3: Validating the biological function of Sult2B1b via chemical probes. RNAi knockout of genes to probe biological function of a target protein is an important first step in probing biological function but it has its limitations due to disruption of all protein functions confounding

interpretation of the importance of the catalytic activity. An alternative approach to studying the importance of the catalytic activity of an enzyme to its biological function is to utilize small molecule inhibitors. Therefore, we will develop small-molecule inhibitors of SULT2B1b to probe its biological function. We developed a new fluorescence assay of Sult2B1b activity that can be used to determine the inhibitory potency of compounds and to perform high-throughput screening of compound libraries. We have also identified potential inhibitors via *in silico* screening using the available X-ray structure of SULT2B1b. We propose to use this new assay to determine the inhibitor potential of these compounds and to then test the best inhibitors for their ability to inhibit the formation of cholesterol sulfate in cell culture. We will then test the anti-proliferative activity of these inhibitors and will compare the results to those obtained via siRNA.

Innovation: Preliminary studies highlight an important and novel role for SULT2B1b as a pivotal regulator of cellular cholesterol metabolism in PCa. The studies outlined in this application employ standard analysis methods; however, the conceptual hypothesis that SULT2B1b expression in PCa is central to dysregulation of cholesterol homeostasis is innovative. While a major focus of PCa research is on the AR and its impact on PCa growth, the proposed studies will probe the impact of SULT2B1b-mediated sulfation on pathways regulating cholesterol metabolism, including LXR and alternate non-LXR pathways. If validated, the studies will provide the foundation for developing novel approaches to controlling cholesterol dysregulation in PCa, which is known to contribute to AR activity as well as apoptosis and cellular proliferation. Thus, **establishing SULT2B1b as a key regulator of PCa growth and progression could potentially establish SULT2B1b a novel therapeutic target.**

Impact/ Overarching Challenges and focus area. Cholesterol sulfate (CS) expression in prostate neoplasia appears to correlate to SULT2B1b activity suggesting a potential role of CS as a **biomarker** of prostate cells with altered growth rate and response to androgens. By better understanding how SULT2B1b 1) controls alterations in cellular responses and 2) regulates activity of immunosuppressive, tumor-derived factors such as oxysterols, implicate a role for SULT2B1b in prostate **tumor and microenvironment biology**. Validation of SULT2B1b as key controller of cholesterol dysregulation may also lead to development of better **therapeutics** for the treatment of both organ-confined disease and **advanced prostate cancer**.
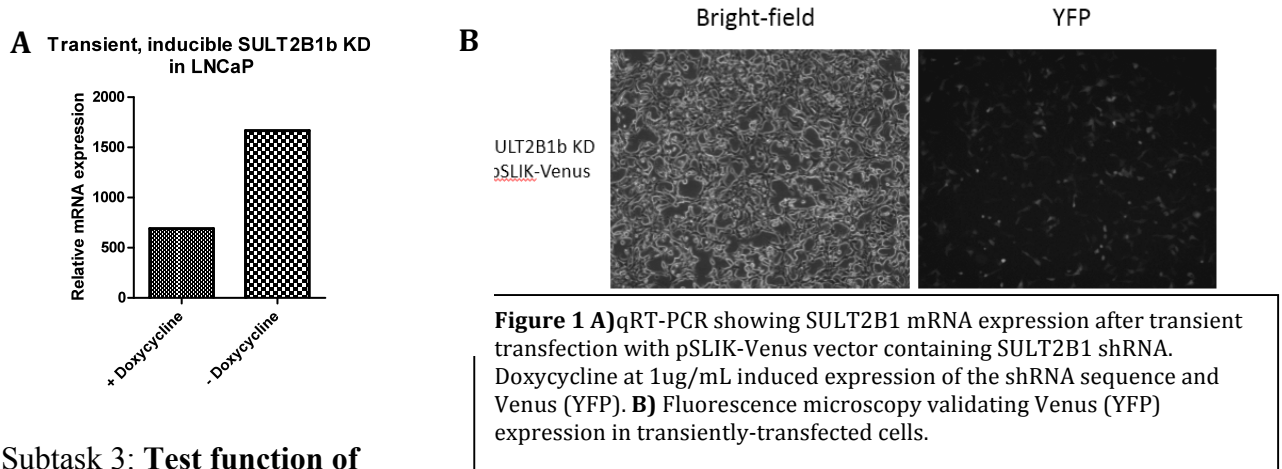
The studies proposed in these specific aims will be performed as accomplishing the following tasks as outlined in the approved statement of work (SOW). The following is a description of activities and accomplishments in the first year of the project:

Major Task 1: generation of SULT2B1b modulated PCa cell lines: Model development :

Subtask 1: Develop lentiviral plasmids containing inducible shRNA targeting SULT2B1b and SULT2B1b cDNA, under control of an inducible Tet- promoter. vectors used include pSlik and/or PLNKO (obtained from Addgene) , pLenti6 (purchased from Invitrogen),and pLenti-X (from Clontech) , Subtask completed? Yes, we successfully generated shRNA lentiviral plasmids based on the the pSLIK system (obtained from Addgene, the pSLIK system is a lentiviral plasmid with tet-inducible shRNA expression, with co-expression of YFP (venus) (http://www.pnas.org/content/103/37/13759.full%3Frelated-urls%3Dyes%26legid%3Dpnas%3B103/37/13759) that could modulate endogenous SULT2B1b in LNCaP via transient plasmid transfection , validating function of the shRNA sequence and plasmid construct. Figure 1 shows doxycycline exposure after plasmid transfection suppresses SULT2B1b expression in LNCaP

suffient tet-inducible expression. Validation of plasmid function allowed us to proceed to the next subtask.

Subtask 2: Production of infective Lentivirus using co-transfection of HEK293 (pCa cells LNCaP, PC3, DU145, and VCaP (purchased from ATCC) with lentivirus plasmids generated in Subtask1 and accessory lenitivirus plasmids (purchased from Clontech and Addgene) Subtask completed?: yes, we were able to generate infective lentivirus particles capable of infecting LNCaP with high efficiency, and observed greater that 60% knock down of endogenous SULT2B1b with exposure to doxycycline (Figure 1) following transient, (2 day) lentivirus transduction.

Figure 1 A)qRT-PCR showing SULT2B1 mRNA expression after transient transfection with pSLIK-Venus vector containing SULT2B1 shRNA. Doxycycline at 1ug/mL induced expression of the shRNA sequence and Venus (YFP). B) Fluorescence microscopy validating Venus (YFP) expression in transiently-transfected cells.

Subtask 3: **Test function of lentivirus-mediated, tet-inducible SULT2B1 overexpresssion or endogenous knock down by transient transduction using RT-PCR and western blotting in PCa cell lines LNCaP, PC3, and DU145 (all from ATCC).** Subtask completed, see description above. **Stable transduction, antibiotic selection and validation of tet-inducible modulation of sult2b1b.** Although the constructs and lentiviral particles produced in the first subtasks were effective in modulating endogenous SULT2B1b transiently. We were unable to produce stable clones of any of the human PCa lines with sufficient tet-inducible knockdown of SULT2B1b expression for further characterization. Alternatively, we were able to perform pathway analysis by transient transfection of siRNA. Using this method, we achieved high-level suppression of endogenous SULT2B1b (determined by qRT-PCR and western blotting, figure 2, in all PCa lines. We determined that the level of shRNA expression from a stably intergrated transgene gene delivered by lentivirus was too low (insufficient copy number?) to significantly modulate endogenous SULT2B1b expression in a high-level expressing cell line such as LNCaP. We are currently designing a tet-inducible CRISPR/CAS9-based method to directly edit the genome by inserting LoxP sites flanking critical exon(s) in the SULT2b1b gene and generating stable cell lines that over express an inducible CRE recombinase (ie tamoxifen-incucible CRE/ER-fusion protein)
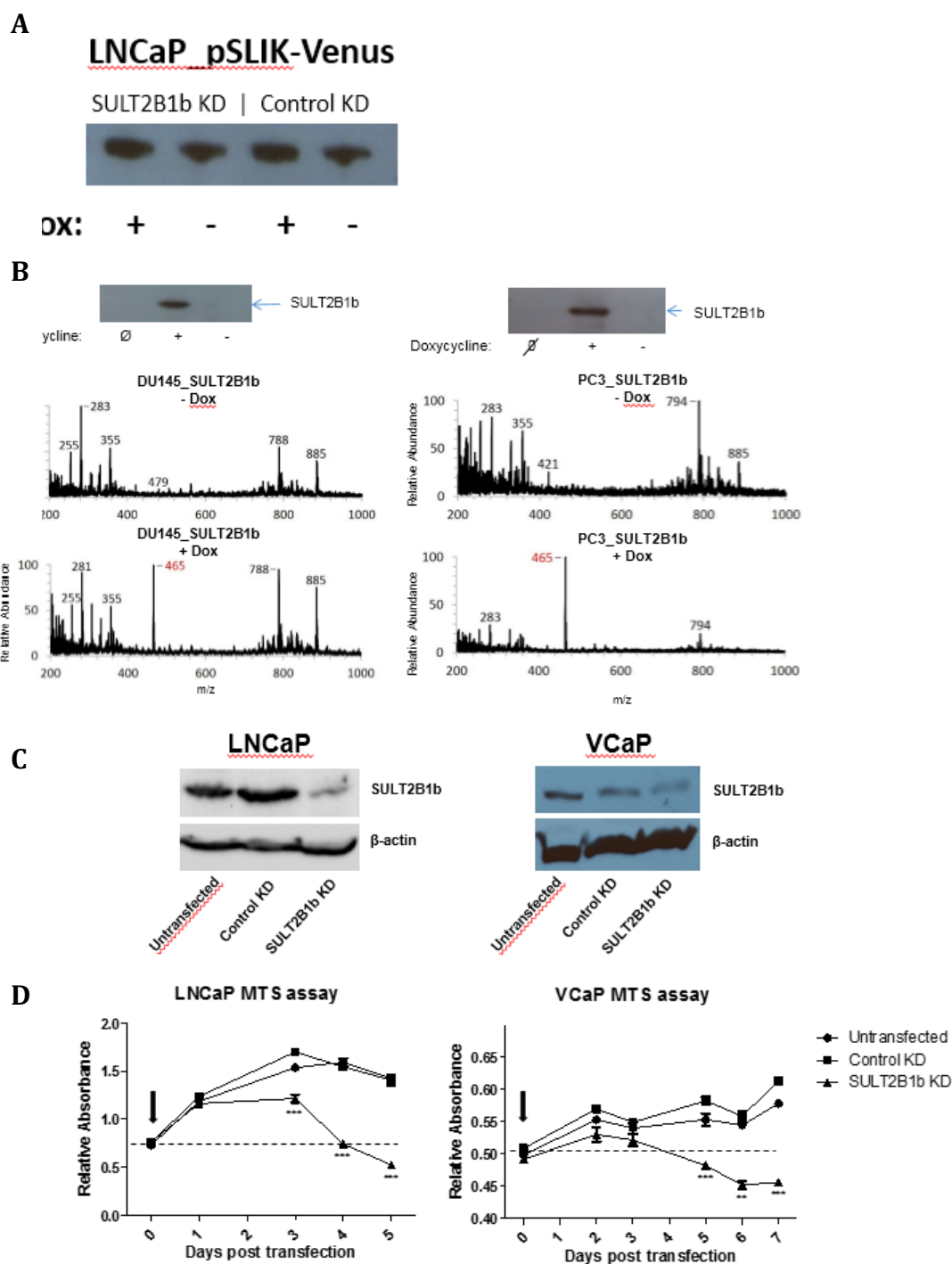
Figure 2. A) SULT2B1b protein expression by Western blot with or without induction of tet-inducible SULT2B1b shRNA in pSLIK-Venus vector. **B)** SULT2B1b expression by Western blot after tet-inducible SULT2B1b overexpression via CMV promoter in DU145 (left) and PC-3 (right) cells. Also included is functional analysis of SULT2B1b activity by accumulation of cholesterol sulfate (m/z=465) via DESI-MS. **C)** SULT2B1b expression by Western blot after 72 hours siRNA transfection in LNCaP and VCaP cells. **D)** MTS

Specific Aim 1: To characterize impact of sult2b1b on PCa growth and phenotype

Major Task2: Characterize impact of SULT2B1b modulation on lipid metabolism in human PCa cell lines

Subtask 2: Contact Roswell Park shared resource to arrange for sample analysis. Determine requirements for analysis: Completed, several samples of LNCaP with siRNA-based SULT2b1knocked down greater than 80% were submitted to The Roswell park shared resource for lipid analysis

Cell lines used: wt LNCaP, VCaP (from ATCC) and PCa lines with Tet-regulated SULT2b1b created in major task 1], because we were unable to produce the stable clones, we substituted transient siRNA knock-down PCa cells for the in vitro cellular analysis

Subtask 3: Characterize SULT2B1b as a regulator of PCa growth by modulating SULT2b1b in PCa cell lines and measuring tumor growth

Cell lines used: : Cell lines used: wt LNCaP, VCaP (from ATCC) with transient SULT2B1 modulation using siRNA- and PCa lines with Tet-regulated SULT2b1b created in major task 1] created in major task 1 and wt LNCaP and VCaP (ATCC) because we were unable to produce the stable clones, we were unable to complete this subtask substituted transient siRNA knock-down LNCaP cells.

Major Task 2: In Vitro testing/growth assays with PCa cell lines (Specific aim 1: To establish the critical role of SULT2B1b activity in PCa growth and phenotype *in vitro*. (Months 4-24)
- Subtask 1: Cell proliferation assays.
- Clonogenic/plating efficiency assays.
- Soft agar growth assays.
- LXR activity assays.
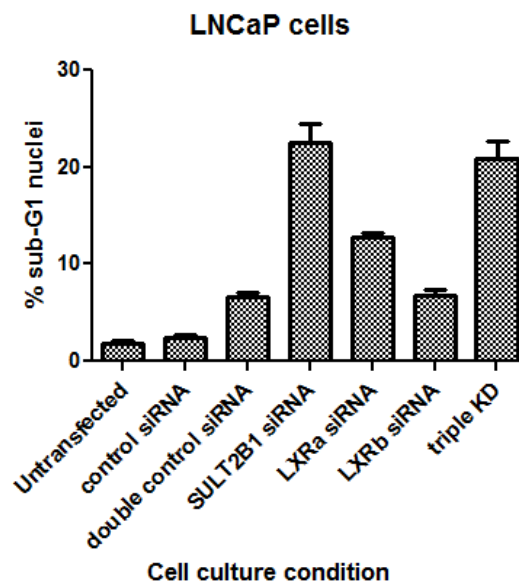- Akt phosphorylation assays

Figure 3. LNCaP cells were transfected with indicated siRNA combinations and cell cycle analysis was performed. Cells were harvested 72 hours after transfection, stained with propidium iodide, and analyzed by flow cytometry. Cells undergoing cell death are indicated by sub-G1 nuclei.

Cell lines used: wt LNCaP, VCaP (from ATCC) and PCa lines with Tet-regulated SULT2b1b created in major task 1]: because we were unable to produce the stable clones, we substituted transient siRNA knock-down PCa cell lines for the in vitro cellular analysis. Our data show knock down of endogenous SULT2B1b in all cell lines tested suppressed growth and greatly enhanced cell death, we performed further analysis to

determine mechanism of growth control and determined cell death was independent of LXR modulation (figure 3)suggesting that the growth inhibitory function of SULT2B1b knockdown was not significantly impacting cell growth via regulation of LXR agonists (oxysterols). Our additional analysis shows direct regulation of androgen receptor mRNA and protein levels, supporting a hypothesis of SULT2B1b impacting gene regulatory pathways associated with AR activity (figure 4).
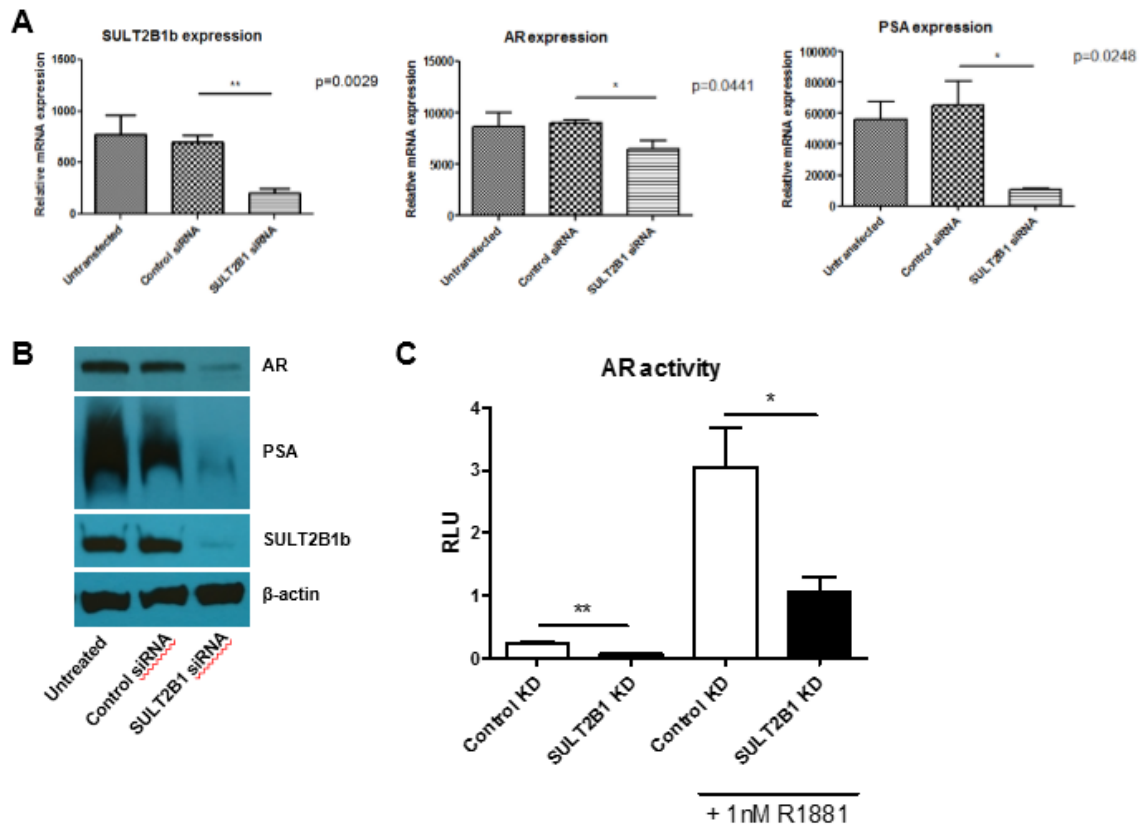

Figure 4. A) qRT-PCR shows mRNA expression of cells with indicated treatments at 72 hours. **B)** Western blot at 72 hours after indicated treatments. **C)** A luciferase reporter construct controlled by the AR-responsive portion of the PSA promoter was transfected into stably selected control or SULT2B1b shRNA LNCaP cells. Firefly luciferase activity was normalized to Renilla. Relative luciferase units (RLU) is shown for cell conditions with or without addition of 1nM R1881 to stimulate AR activity.

Based on these novel data, we wanted to identify global gene pathways associated with SULT2B1b modulation and performed single cell RNA sequencing. We are currently working with our bioinformatics consultant and are just now beginning to analyze the data. The accomplishment of these tasks have allowed us to produce a manuscript describing our novel observations of SULT2B1b modulation impact on AR expression and activity. We currently estimate the submission of the manuscript within the month.

Major Task 3: In vivo xenograft model (Months 4-36,)

Subtask 1: Establish xenograft model, preliminary tumorgenic studies to establish basic parameters needed for statistical validation of the tumor studies including implantation efficiency

> **Subtask 2: Implantation of stable cell lines produced in task 1, and monitoring of survival in mice** because we were unable to produce the stable clones, we were able to substitute transient siRNA knock-down in all PCa cell lines for the in vitro cellular analysis but the in vivo analysis outlined in major task 3 will be delayed until we can generate the stable inducible cell lines using the CRISPR/CAS9 method described above in subtask 2.

Major Task 4: **Development of small molecule SULT2B1b inhibitor: Testing of identified lead compounds**

- *Subtask 1:* Assessment of compound effects on SULT2B1b enzyme activity of cholesterol sulfate production in vitro.

We developed a coupled-enzyme assay to measure the activity of Sult2B1b in order to replace the cumbersome end-point assay that uses radioactivity (^{35}S-labeled PAPS). The assay system is diagramed below in Figure 5.
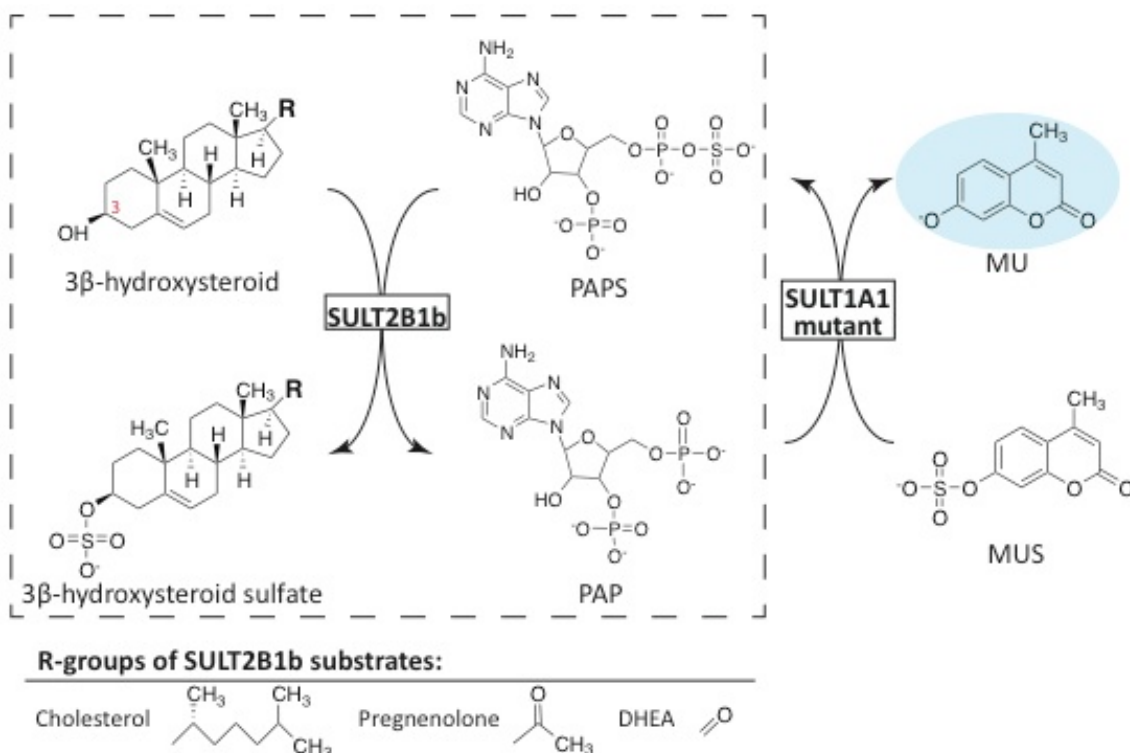

Figure 5. Scheme of the coupled-enzyme assay for measuring the activity of SULT2B1b. The reaction in dashed box is catalyzed by SULT2B1b. The coupled-reaction is catalyzed by SULT1A1-mut, resulting in the accumulation of fluorophore MU.

We adapted and optimized the assay shown in Figure 5 for high-throughput screening (HTS) and we screened 7,040 compounds that were selected from our LOPAC, Life Chemicals and Asinex small molecule libraries. We used a two pronged approach for evaluating 'hit' compounds, i.e. inhibitors. First, we did a prescreen by monitoring the background activity of Sult1A1, the coupling enzyme, for any sign of inhibition by the library compounds. We only looked for compounds that produced no inhibition, +/- 5% inhibition. We next added the Sult2B1b enzyme and looked for compounds that inhibited the activity by 60% or higher. We found that 15 compounds out of the 7,040 satisfied this criteria. We then rechecked these 15 compounds using compound from the original compound library plates and we found that 3 of the 15 compounds showed reproducible inhibition in the assays. We then purchased these 3 compounds in powder form from various vendors and we rechecked the compounds for inhibition in the primary screening assay.

- **Subtask 2:** Confirm specificity by evaluation of sulfonation activity on other sterols using in vitro assays.

The 3 confirmed hit compounds were then tested for their ability to inhibit the coupling-enzyme, Sult1A1, using different detergent conditions and concentrations of MUS and PAP. We found that two of the compounds under conditions of higher detergent showed observable inhibition of Sult1A1 so these compounds were dropped from further study. However, one compound (Figure 6), YC-1, showed little to no inhibition of Sult1A1 under the same conditions tested (Figure 7a) and it also showed significant inhibition of Sult2B1b suggesting that YC-1 selectively targets Sult2B1b (Figure 7b).
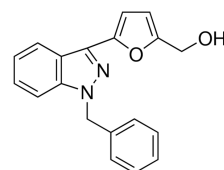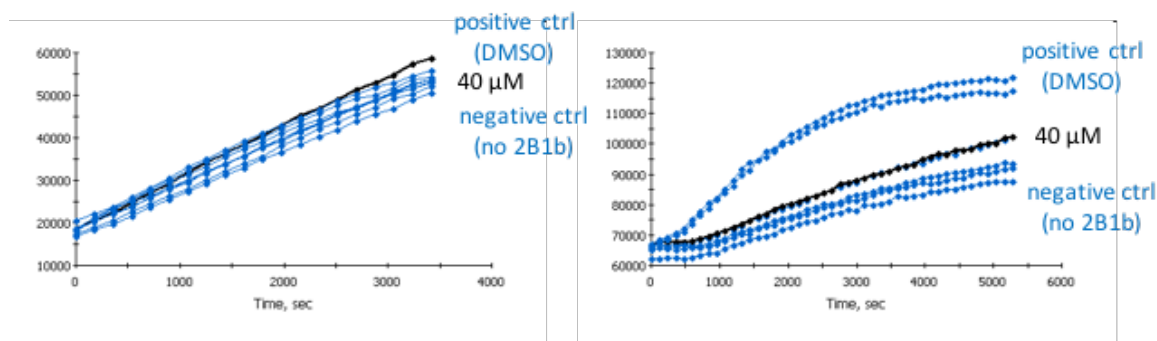

Figure 6.
Structure of YC-1

Figure 7. Inhibition of Sult1A1 and Sult2B1b by compound YC-1. (A) Control reaction to show that the YC-1 does not inhibit Sult1A1. (B) Inhibition of Sult2B1b by YC-1 with the substrate pregnenolone.

Since the substrate cholesterol is insoluble at higher concentrations and it, as well as the substrate DHEA, are just as active as pregnenolone with Sult2B1b, we no routinely use

prenenolone in our assays.

We next tested the ability of YC-1 to inhibit the wild type and S384D mutant Sult2B1b enzymes. The S384D mutant has been reported to mimic the phosphorylated form of Sult2B1b. We deterlmined the IC50 values for YC-1 from a dose-response study and the results are summarized the table below.   We find that YC-1 equally inhibits wild-type and the S384D mutant suggesting that this compound could inhibit both forms of the enzyme.

Enzyme	IC$_{50}$ (µM)	Max *I(%)*
Wild-type SULT2B1b	20.5 ± 9.6	141.8 ± 31.7
SULT2B1b S348D mutant	13.9 ± 4.9	106.3 ± 16.0

- ***Subtask 3:*** Testing of lead compounds on endogenous SULT2B1b in LNCaP cell culture.

   Since compound YC-1 inhibited Sult2b1b enzyme activity in vitro, we next tested whether it would inhibit the growth of LNCaP cells in cell culture. We tested for the ability of YC-1 to inhibit the growth of LNCaP cells over 4 days and at 7 different concentrations of YC-1. The results are shown in Figure 8.

We found that YC-1 does indeed inhibit the growth of LNCaP cells at all concentrations tested down to 3.1 uM.

Figure 8. Inhibition of growth of LNCaP cells by compound YC-1.

We next wanted to test whether YC-1 would prevent the formation of cholesterol-sulfate in cells by inhibiting Sult2B1b. We therefore utilized the DESI-MS approach, which originally detected the large increase in cholesterol-sulfate concentrations in LNCaP cells compared to normal cells. We treated LNCaP cells
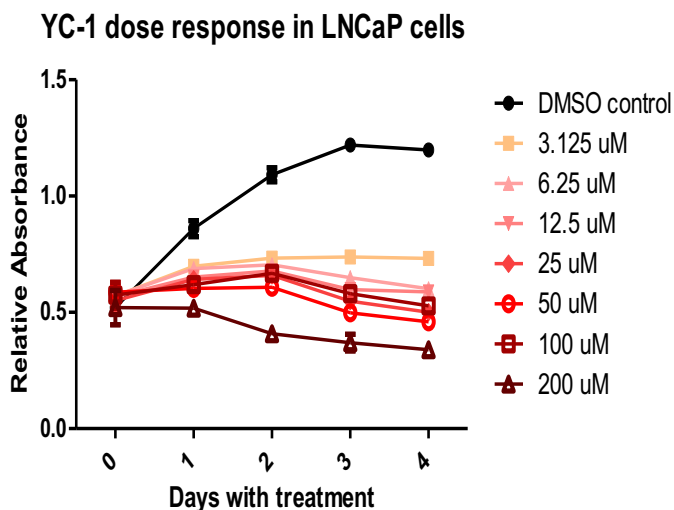
with 1 to 20 uM of YC-1 but failed to see a decrease in the MS peak of 465.42 which is indicative of cholesterol sulfate.
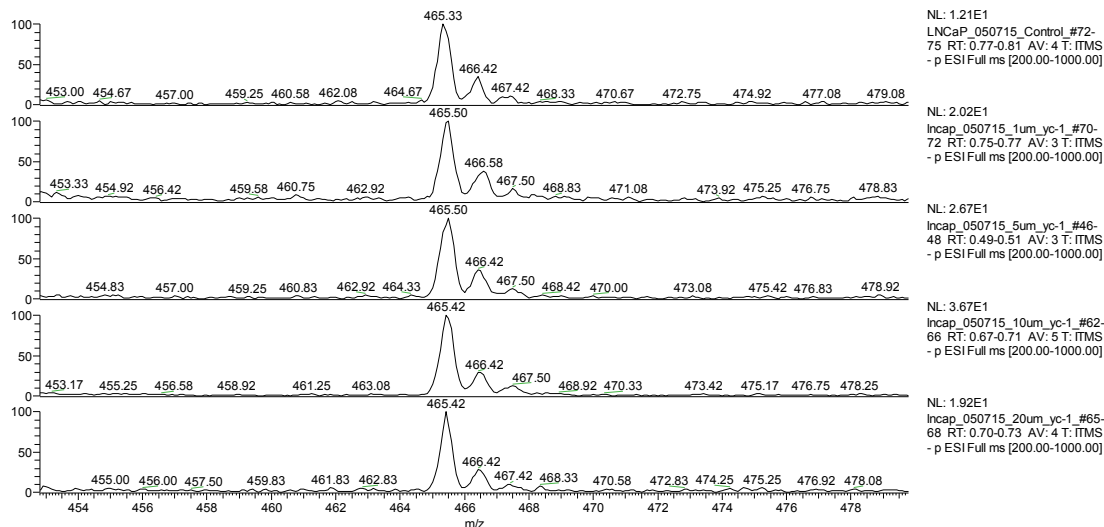

Figure 9. DES-MS Spectra of LNCaP cells treated with various concentrations of YC-1.

These results suggest that YC-1 may be acting on another target or that it may form a product-bound inhibitor complex with Sult2B1b whereby YC-1 and cholesterol sulfate bind to the enzyme. We will test the latter mechanism via enzyme kinetic studies. In case it is the former mechanism, we will continue to screen our compound libraries for new hit molecules that will show on-target effects in the DESI-MS based assay.