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Supporting Information

Hedgehog Proteins Consume Steroidal CYP17A1 Antagonists: Potential Therapeutic Significance in Advanced Prostate Cancer

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Supporting Information

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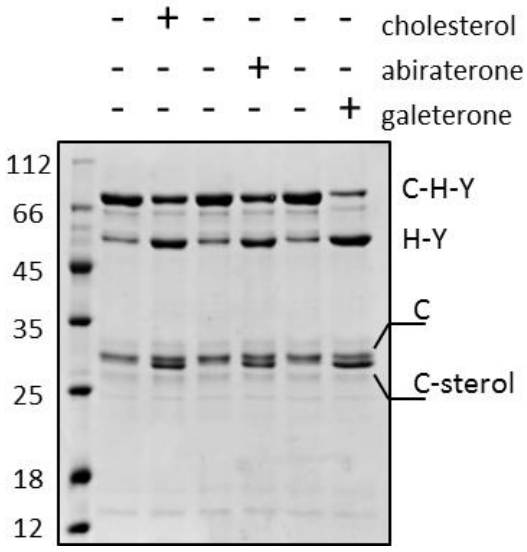
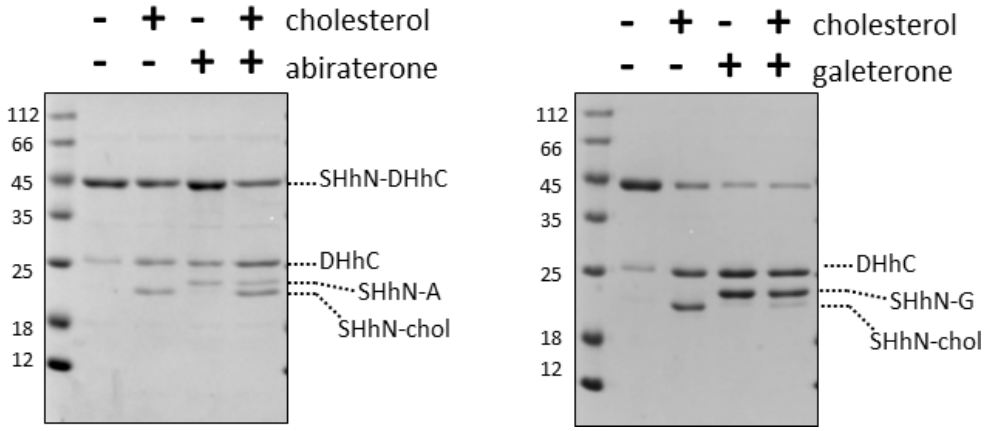
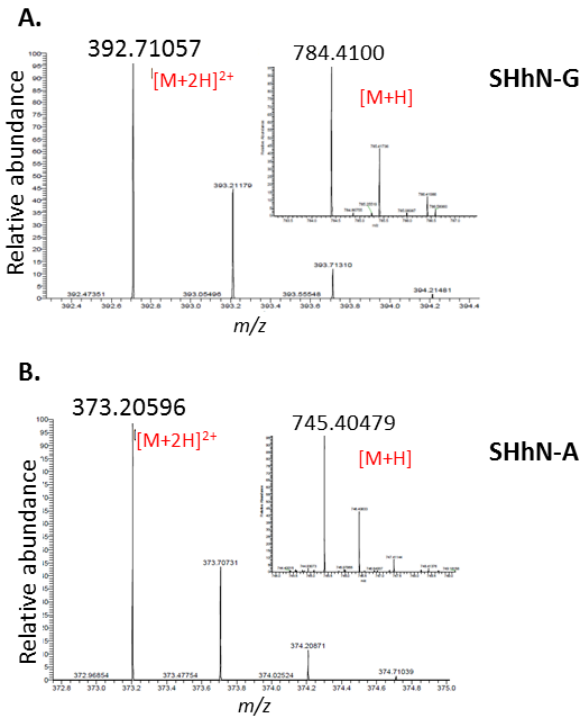


Supporting Figure 1: Cross validation of abiraterone and galeterone substrate activity. SDS-PAGE assay showing the activity of C-H-Y +/- sterols at 30 °C. Processing results

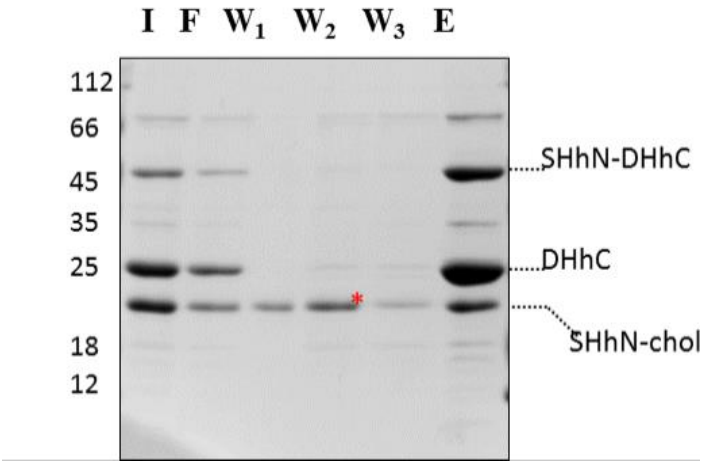


in the conversion of C-H-Y (86 kDa) into C-sterol (30 kDa) and H-Y (56 kDa).

Supporting Figure 2: Abiraterone and galeterone compete with cholesterol as substrates for hedgehog lipidation. Competition experiments used the chimeric precursor, SHhN-DHhC (1 µM) +/- sterols (25 x 10^{-6} M).



Supporting Figure 3: Characterization of galeterone and abiraterone conjugates by mass spectrometry. Expected/observed mass for the tryptic C-terminal fragment modified by abiraterone: 745.5/745.4; Expected/observed mass for the tryptic C-terminal fragment modified by galeterone: 784.3/784.4.



Supporting Figure 4: Analysis of Hh-sterol conjugates for cell-based signaling assays. Using the chimeric precursor, SHhN-DHhC, the protein was initially reacted with sterol (I); following dialysis and filtration, the sample was passed over a column with NiNTA resin (F); the resin was washed with imidazole containing buffer (W1-W-3); unreacted precursor and DHhC were eluted (E). Samples containing SHhN were collected and analyzed by SDS-PAGE and RP-HPLC before use in the cell reporter assays.

	K_m (μM)	k_{max} x10^{-3} (sec^{-1})	$t_{1/2}$ (min)
cholesterol	1 +/- 0.2	1.6 +/- 0.1	7
abiraterone	12 +/- 5	2.1 +/- 0.1	5
galeterone	3 +/- 0.6	12.9 +/- 2.2	1

Supporting Table 1: Kinetic parameters determined using C-H-Y (1x10^{-7} M) precursor protein in Bis-tris buffered solution, pH 7.1, containing NaCl (0.1 M), TCEP (1x10^{-3} M), EDTA (5x10^{-3} M), and Fos-choline-12 (1.5 x10^{-3} M), at 30 °C. Values represent the mean of at least three independent experiments +/- standard deviation.

Reagents: Antibiotics, Isopropyl β-D-1-thiogalactopyranoside (IPTG), granulated bacterial broth, and buffers used for protein purification and characterization were obtained from Thermo Fisher Scientific; p-nitrophenyl phosphate was purchased from Sigma; C3H10T1/2 cells and growth media were obtained from ATCC. Restriction enzymes and DNA ligase were purchased from New England Biolabs. Sterols were also obtained commercially: Abiraterone and galeterone (Adooq Bioscience), cholesterol (Sigma). Working stocks of the sterols were prepared fresh in ethanol at a final concentration of 10 mM. Fos-choline-12 was purchased from Anatrace Inc.

Hh signaling Assays: Activity of Hh protein +/- conjugated sterol was assessed using the Hh responsive mouse embryonic cell line, C3H10T1/2 (ATCC # CCL-226) [1-4]. Cells were maintained in Eagles Minimum Essential Media with 10% FBS, penicillin/streptomycin (ATCC). Signaling assays were conducted using low passage cells (4-6th passage) in 96 well dishes (Corning #3995), at an initial concentration of $5x10^3$ cells/well. Following overnight incubation, adherent cells in 100 µl of media were treated with serially diluted Hh protein from a 30 µm stock in EMEM media, or an equivalent volume of EMEM. Selected wells were treated with purmorphamine (10 µm, R&D Systems) as a positive control [5]. After 5 day incubation, media was removed, the cells were washed twice with Buffer A (200 µl/well), once with Buffer B (100 µl/well), followed by addition of Buffer C (100 µl/well). To enhance lysis, cells were subjected to two freeze/thaw cycles (15 min -80 °C, 15 min 37 °C). Resulting cell extract was pipetted up and down 10x, before transferring to a 96-well clear bottom plate (Corning #3598) for assaying alkaline phosphatase (AP). Activity of AP was determined after adding 100 µl of the chromogenic substrate p-nitrophenylphosphate (2 mg/ml in buffer C, made fresh). Product formation was monitored at 405 nm using a Biotek plate reader (28.5 °C). Final absorbance values at 405 nm were collected after 8 hrs, and normalized to total protein concentration for each well, determined separately by Bradford Assay. Buffers were prepared as follows: Buffer A: 20 mM $Na_2HPO_4^{2-}$, 150 mM NaCl pH 7.2; Buffer B: 50 mM Tris-HCl, 150 mM NaCl, pH 7.5; Buffer C: 100 mM Glycine 1 mM $MgCl_2$ pH 9.0.

Data Analysis: Apparent EC50 values from dose-dependent cellular responses to added Hhs were calculated by non-linear regression to a three parameter equation (Graphpad Prism 6): Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X))).

Protein Expression: Recombinant, His-tagged C-H-Y protein, used in the fluorescence activity assays was expressed and purified from *E. coli* MC1061[6]; the chimeric His-tagged SHhN-DHhC construct, used for generating steroylated human ShhN was expressed and purified from *E. coli* BL21DE3 [7]. The plasmid used to generate steroylated Desert Hh, pet22b-Des_HhN-DHhC, was prepared from pet22b-SHhN-DHhC using NdeI and NcoI site that flank the SHhN segment.

Preparation of Hh-sterol conjugates: We used in vitro autoprocessing activity of the chimeric precursor, SHhN-DHhC to prepare cholesterol-modified SHhN (SHhN-chol), abiraterone-modified SHhN (SHhN-A), galeterone-modified SHhN (SHhN-G), and sterol-free SHhN. A typical reaction (5 mls) contained ShhN-DHhC (10 uM), sterol (250 µM), Triton-X 100 detergent (0.4%), Bis-Tris buffer with added NaCl and TCEP. After 4 h at 25 °C, the reaction mixture was dialyzed at 4 °C for 18 h into PBS buffer (pH 7.2), filtered using 0.22 um (PVDF, Celltreat), then treated with NiNTA resin to remove unreacted SHhN-DHhC and product HhC. The SHhN polypeptide eluted in column wash buffer. Cholesterol-modified Desert Hh was prepared in a

similar fashion. Sterol-free SHhN was prepared by cleaving SHhN from DHhC using hydroxylamine.

In addition to SDS-PAGE, purity of the Hh-sterol conjugates was assessed by reverse phase HPLC. Samples were separated with a Harmony C4 column (300 Å, 25 cm x 4 mm, ES Industries) by gradient elution (A: water, 0.1% TFA; B: acetonitrile, 0.1% TFA). Effluent was monitored at 220 nm. Elution times for ShhN and SHhN-chol are in good agreement with the data of Baker *et al.* [8].

Mass Spectroscopy: Molecular masses of the SHhN-A and SHhN-G conjugates were determined by ESI-MS/MS at the Cornell Proteomics Facility. Following separation by SDS-PAGE, gel slices containing Hh were washed, reduced with dithiothreitol, alkylated by iodoacetamide, and then digested with trypsin [9]. Digested peptides were extracted twice with 70 µl of 50% acetonitrile, 5% Formic Acid and once with 70 µl of 90% acetonitrile, 5% Formic acid. Lyophilized extracts were reconstituted in 20 µL of 0.5% FA for nanoLC-ESI-MS/MS analysis, using a LTQ-Orbitrap Elite mass spectrometer (Thermo-Fisher Scientific, San Jose, CA) equipped with a Dionex UltiMate3000RSLCnano system (Thermo, Sunnyvale, CA) and "CorConneX" nano ion source device (CorSolutions LLC, Ithaca, NY). The Orbitrap calibration and nanoLC-MS/MS operation have been described [10]. The instrument was operated in positive ion mode with nano spray voltage set at 1.5 kV and source temperature at 250 °C. All data are acquired with Xcalibur 2.2 software (Thermo-Fisher Scientific, San Jose, CA).

Kinetics of Hh autoprocessing:

FRET assay: Activity of Hh in the presence of cholesterol and the alternative substrates, galeterone and abiraterone, was determined optically using C-H-Y, a FRET-active Hh precursor protein. Details of the assay followed Owen *et al.* [7] with the exception that Fos-Choline-12, instead of Triton-X 100, was used for solubilizing the sterols. This substitution results in a 10-20 fold enhancement in Hh self-lipidation activity (manuscript in preparation).

Gel assay: To complement the optical assay, Hh activity was assessed by SDS-PAGE, which allows monitoring the appearance of DHhC and SHhN-sterol along with the loss of precursor SHhN-DHhC [7, 11]. Gels images were captured using BioRad Gel Doc EZ system.

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