Supporting Information

Revealing metabolic liabilities of ralaniten to enhance novel androgen receptor targeted therapies

AUTHORS: Jon K. Obst[1,2], Jun Wang[1], Kunzhong Jian[3], David E. Williams[3], Amy H. Tien[1], Nasrin R. Mawji[1], Teresa Tam[1], Yu Chi Yang[1], Raymond J. Andersen[3], Kim N. Chi[4], Bruce Montgomery[5] and Marianne D. Sadar*[1,2]

AFFILIATIONS:

[1]Department of Genome Sciences Centre, BC Cancer Research Centre, 675 W 10th Ave, Vancouver, BC, Canada V5Z 1L3.

[2]Department of Pathology and Laboratory Medicine, University of British Columbia, 2211 Westbrook Mall, Vancouver, BC, Canada V6T 2B5

[3]Departments of Chemistry and Earth, Ocean & Atmospheric Sciences, University of British Columbia, 2306 Main Mall, Vancouver, BC, Canada V6T 1Z1.

[4]BC Cancer Agency, 600 West 10th Ave, Vancouver, BC, Canada V5Z 4E6.

[5]University of Washington,1959 NE Pacific St, Seattle, WA, USA.

CORRESPONDING AUTHOR: Marianne D. Sadar. Department of Genome Sciences Centre, BC Cancer Research Centre 675 W 10th Ave, Vancouver, BC, Canada V5Z 1L3; Email: msadar@bcgsc.ca; PH: (604) 675-8157, FAX: (604) 675-8178

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Protein and gene expression assays

For western blot experiments, cells were seeded on 10 cm plates in complete media for 24 hrs before media was removed and replaced with RPMI 1640 to serum-starve cells for 24 hrs. Cells were pretreated with ralaniten (35 μM/16 hrs), EPI-045 (35 μM/16 hrs), EPI-002053 (35 μM/16 hrs) enzalutamide (5 μM/1 hr) or DMSO vehicle (16hrs) followed by stimulation with 1 nM R1881. Lysates were harvested 24 hrs after R1881 treatment in RIPA buffer and separated on a 10% SDS-PAGE gel and transferred to PVDF membrane (Millipore LTD,Cork, IRL). Cells used for qRT-PCR and microarray experiments were seeded onto 6-well and treated identically as described for the western blot studies. Total RNA was extracted using the RNeasy Micro Kit (Qiagen), cleaned using DNase I Kit, Amplification grade (MilliporeSigma) and reverse transcribed using the High-Capacity RNA-to-cDNA Kit (ThermoFisher Scientific). Diluted cDNA and Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) were combined with gene specific primers. Cells were harvested in 1 mL TRIzol reagent (Invitrogen) 24 hrs after R1881 treatment. Gene specific primer sequences given in Supplementary Table 1.

AR cloning and sequencing

Total RNA (1 μg) from LNCaP-RAL^{R} cells was treated with DNase I followed by reverse transcription using Superscript III First Strand Synthesis Kit (Invitrogen) with 2.5 μM oligo(dT)20. PCR amplifications were performed with Platinum DNA Taq Polymerase High Fidelity (Invitrogen). PCR amplifications were completed in 20 μL reactions with 1U Taq DNA High Fidelity Polymerase, 2 mM $MgSO_4$, 0.2 mM deoxynucleotide mix, and 2% DMSO with primers covering either the entirety of the AR (AR^{FL}) or the LBD (AR^{LBD}) with the following sequences:

AR^{FL} (**F**) 5'-AGGGGAGGCGGGGTAAGGGAAGTA-3; (**R**) 5'-CATGAGCTGGGGTGGGGAAATAGG-3'

AR^{LBD} (**F**) 5'GCGAAATGGGCCCCTGGATGGAT-3'; (**R**) 5'-CATGAGCTGGGGTGGGGAAATAGG-3'

The PCR cycle conditions consisted of 94ºC for 2 min, followed by 30 cycles of a denaturing step (94°C, 15 sec), an annealing step (60°C, 30 sec) and an elongation step (68°C, 1.5 min). The reaction was terminated with a final extension step at 68°C for 5 min. The PCR products were electrophoresed in a 0.8% agarose gel. Corresponding DNA fragments were isolated and cloned into PCR 2.1 TOPO cloning vector using the TOPO TA cloning kit (Invitrogen) and transformed into chemically competent TOPO10 cells according to manufacturer's protocol. Transformant plasmids containing DNA inserts were sequenced at the NAPS core unit at the University of British Columbia (Vancouver, BC, Canada; https://naps.msl.ubc.ca).

siRNA protein/mRNA expression and proliferation

For experiments examining protein or mRNA expression, cells were plated on 10 cm or 6-well plates respectively in complete media for 24 hrs prior to transfection. After 24 hrs, media was removed and replaced with Opti-MEM (Gibco) containing 10 nM (AR) or 15 nM (UGT2B15/17) siRNA/transfection reagent complexes (Lipofectamine RNAiMAX Transfection Reagent, Invitrogen). 24-96 hrs post transfection, protein or mRNA was harvested and analyzed as previously described. For the proliferation assay, cells were plated on 24-well plates and transfected as described above. After 24 hrs, cells were treated with 25 μM ralaniten and 0.1 nM R1881 (UGT2B15/17 knock down experiment), or 0.1 nM R1881 only (AR knock down experiment). After 72 hrs post treatment, cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 30 min at room temperature (RT) before being washed three times with distilled water. Cells were incubated with 0.1% crystal violet solution (Sigma) for 10 min at RT, followed by another three wash steps with distilled water. Dye was solubilised by adding a 1% SDS solution and incubating plates at RT for 30 min on an orbital shaker. 100 μL from each well was removed and added to a clear, polystyrene flat bottom 96-well microplate (Evergreen Scientific). Absorbance was read using a VersaMax Microplate Reader (Molecular Devices) at 595 nm.

Lentiviral transduction of LNCaP-GFP, -2B15 and -2B17 cells

Stable expression of UGT2B15-HA, UGT2B17-HA, and GFP-HA was achieved by lentiviral infection. Recombinant lentiviral particles were produced by the cotransfection of 293T cells with 10 µg of expression vector UGT2B15-HA (LV352002), UGT2B17-HA (LV352008) or GFP-HA (LV032) in combination with 2nd Generation packaging mix (LV003) and transfected using LentiFectinTM transfection reagent (G074) according to the manufacturer's instructions. All components were purchased from Applied Biological Materials (ABM, Richmond, Canada), and the vector system was pLenti-GIII-CMV-C-term-HA. 48 hours post transfection, media was collected and concentrated by centrifugation at 25,000 rpm for 90 minutes at 4ºC.

LNCaP cells were transduced by plating 3x105 cells/well in 6-well plates and infecting with respective lentiviral particles (MOI: ~10^5-10^6; at a ratio of 1:1). After 24 hours of incubation at 37ºC at 5% CO2, media was removed and replaced with complete media. Following a further 24 hour incubation, media was changed once more to that containing 1.5 µg/mL puromycin to begin selection of transduced cells. After 7 days of selection, cells from 10 unique colonies for each expression vector were chosen for further expansion and aliquots were removed for initial confirmation of protein and mRNA expression. Clones were stored in liquid nitrogen.

Dose response assay

5,000 cells were plated per well in 96 well plates in 5% charcoal-stripped serum and incubated for 24 hrs to allow cells to attach. Treatments were prepared by serial dilution and cells were pretreated with DMSO, ralaniten (0.1, 0.5, 1, 5, 10, 25, 35 µM), enzalutamide (0.01, 0.05, 0.1, 0.5, 1, 5, 10 µM), bicalutamide (0.05, 0.01, 0.1, 0.5, 1, 5, 10, 25 µM) or EPI-045 (0.1, 0.5, 1, 5, 10, 25, 35 µM) for one hour prior to stimulation with 0.1 nM R1881 or EtOH vehicle. For experiments involving lentiviral transduced cells, 0.1, 1, 10, 25, 35 µM ralaniten was used. After 96 hrs cells were fixed and stained with 0.1% crystal violet and read on a VersaMax plate reader as described above. Data was background subtracted and normalized to DMSO/R1881.

For the initial characterization of EPI-045 (Supplementary Figure **S5**), 2,000 cells/well were seeded onto 96-well plates. Following a 24 hour incubation cells were pre-treated with DMSO vehicle, ralaniten or EPI-045 (0.1, 0.5, 5, 15, 35 μM) for one hour. Cells were stimulated with R1881 (0.1 nM) or EtOH vehicle. 72 hours post treatment, cell viability was measured using the Alamar Blue assay. 10 μL Alamar blue was added directly to wells and cells were incubated at 37°C for 2 hours. Plates were read using an Infinite M1000 microplate reader (TECAN) with Ex/Em=570/585 nm and bandwidth set at 5 nm. Data was background subtracted and normalized to DMSO/R1881 treatment to calculate androgen dependent growth.

Microsomal isolation and UGT2B enzymatic activity assay

Microsomes from cultured cells (LNCaP and LNCaP-RAL^R) and from mouse liver were isolated using sequential, high speed centrifugation. Buffers and a protease inhibitor cocktail were supplied with the Microsome Isolation Kit (Biovision), and the procedure was carried out according to manufacturers instruction. A bicinchoninic acid assay was used to determine protein concentration prior to beginning further experiments. Basal UGT2B enzymatic activity was measured in LNCaP and LNCaP-RAL^R cells using the UGT-Glo kit (Promega) according to manufacturers instruction. Luminescence was measured using a GloMax® 96 microplate luminometer (Promega, Madison, USA). For experiments measuring enzymatic activity of lentiviral transduced cells, the fluorometric UGT Activity Assay kit (Biovision, Milpitas, USA) was used according to manufacturer's instruction. Fluorescence was measured using a Safire2 Multi-Mode Plate Reader (Tecan, Männendorf, Switzerland). Measurements were taken every 2 minutes for 60 cycles with Ex/Em=415/502 nm and the bandwidth was set at 20 nm.

Synthesis of EPI-045, the mono glucuronides of ralaniten, and the monoglucuronides of EPI-045

The mono glucuronides of ralaniten (ralaniten G-1, ralaniten G-2, ralaniten G-3/**M15**) and EPI-045 (EPI-045-G-1, EPI-045-G-2) were synthesized as outlined in Supplementary Figures

S6 and **S7** respectively. Pure samples of the mono glucuronides of ralaniten (G-1, G-2, G-3/**M15**) were obtained by HPLC separation on a CSC-Insertsil 150A/ODS 2, 25 x 0.94 cm column using isocratic elution at 2 mL/min with either MeCN: H_2O (0.05% TFA) = 35%: 65% (ralaniten G-1 and ralaniten G-2) or MeCN: H_2O (0.05% TFA) = 32.5%: 67.5% (ralaniten G-3/**M15**) monitored at 230 nM. EPI-045 consisted of a mixture of four stereoisomers. Consequently, the monoglucuronides EPI-045 G-1 and EPI-045 G-2 were obtained as mixtures of diastereomers after C_{18} reversed-phase HPLC using a InertSustain 5 µm, 25 x 1.0 cm column with 13:7 (0.05%TFA/H_2O)/MeCN as eluent and UV detection at 201 nm. The diastereomeric mixtures of EPI-045-G-1i and EPI-045-G-1ii, and EPI-045-G-2i and EPI-045-G-2ii were isolated. The constitutions of EPI-045-G-1i, EPI-045-G-1ii, EPI-045-G-2i and EPI-045-G-2ii have been unambiguously assigned, but their absolute configurations have not been assigned. The constitutions of each of the pure monoglucuronides was confirmed by detailed analysis of their 1D and 2D nuclear magnetic resonance (NMR) and high resolution mass spectra (HRMS). Supplementary Figure **S4** shows the proton NMR spectrum of synthetic ralaniten G-3 (**M15**) compared with ralaniten G-3 isolated from a biological incubation experiment (RAL + UDPGA) shown in Figure **6**.

Tumor Immunohistochemistry

Sections (5 µm thick) were cut from formalin fixed paraffin-embedded tissues and deparaffinized in xylene and rehydrated in alcohols and distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water for 5 min, followed by washing in PBS three times. Incubation with the following primary antibodies: anti-AR N-20 (1:300; Santa Cruz), anti-PSA C19 (1:500; Santa Cruz), anti-UGT2B15 (1:100; Abcam) and anti-Ki67 (Dako envision system HRP/DAB kit) were incubated at 4°C overnight. Antigen was detected with 3,3-diaminobenzidine and counterstained with hematoxylin. The ApopTag® Fluorescein In Situ Apoptosis Detection Kit (MILLIPORE) was used for the TUNEL assay. Cells positive for Ki67 or

TUNEL staining were counted from three different sections of three different xenografts for each treatment. For Ki67 and TUNEL staining, data represents three sections from three different tumors per treatment group. Ki67: 2732 (vehicle); 2514 (ralaniten) and 2619 (EPI-045) cells in total were counted. For TUNEL staining, 3123 (vehicle); 3538 (ralaniten) and 3400 (EPI-045) cells were counted.

Supplementary Tables and Figures

Table S1. *Gene-specific primer sequences for qRT-PCR experiments.*

Target	Direction	Sequence
AR	FWD	AGGAACTCGATCCTATCATTGC
	REV	CTGCCATCATTTCCGGAA
AR-V7	FWD	CCATCTTGTCGTCTTCGGAAATGTTAT
	REV	TTTGAATGAGGCAAGTCAGCCTTTCT
FKBP5	FWD	CGCAGGATATACGCCAACAT
	REV	GAAGTCTTCTTGCCCATTGC
KLK2	FWD	TGTGTGCTAGAGCTTACTCTGA
	REV	CCACTTCCGGTAATGCACCA
NKX3.1	FWD	CCGAGACGCTGGCAGAGACC
	REV	GCTTAGGGGTTTGGGGAAG
PSA	FWD	TCATCCTGTCTCGGATTGTG
	REV	ATATCGTAGAGGGGGTGTGG
RHOU	FWD	CCCGTGAGACTCCAACTCTG
	REV	TGAAGCAGAGCAGGAAGATG
UGT2B7	FWD	ACGTATGGCTTATTCGAAAC
	REV	CATGTTACTGACCATTGACC
UGT2B11	FWD	CTAAGGAAATGGAGGAGTTTG
	REV	ATGTTACTTATCACTGACCCC
UGT2B15	FWD	CTTCTTGGTCATCCCAAAAC
	REV	GCAAATCTCTACTTGACATGG
UGT2B17	FWD	CTGAGCTTCCTTATGTTTCAC
	REV	ATTCTGCTCAAAATGAAGCC
UGT2B28	FWD	CAGTGAAGTTTTAGGAAGACC
	REV	AGGAAATTGAAAACTCCAGG
SDHA	FWD	CAGCATGTGTTACCAAGCTGT
	REV	CGTGTCGTAGAAATGCCACCT

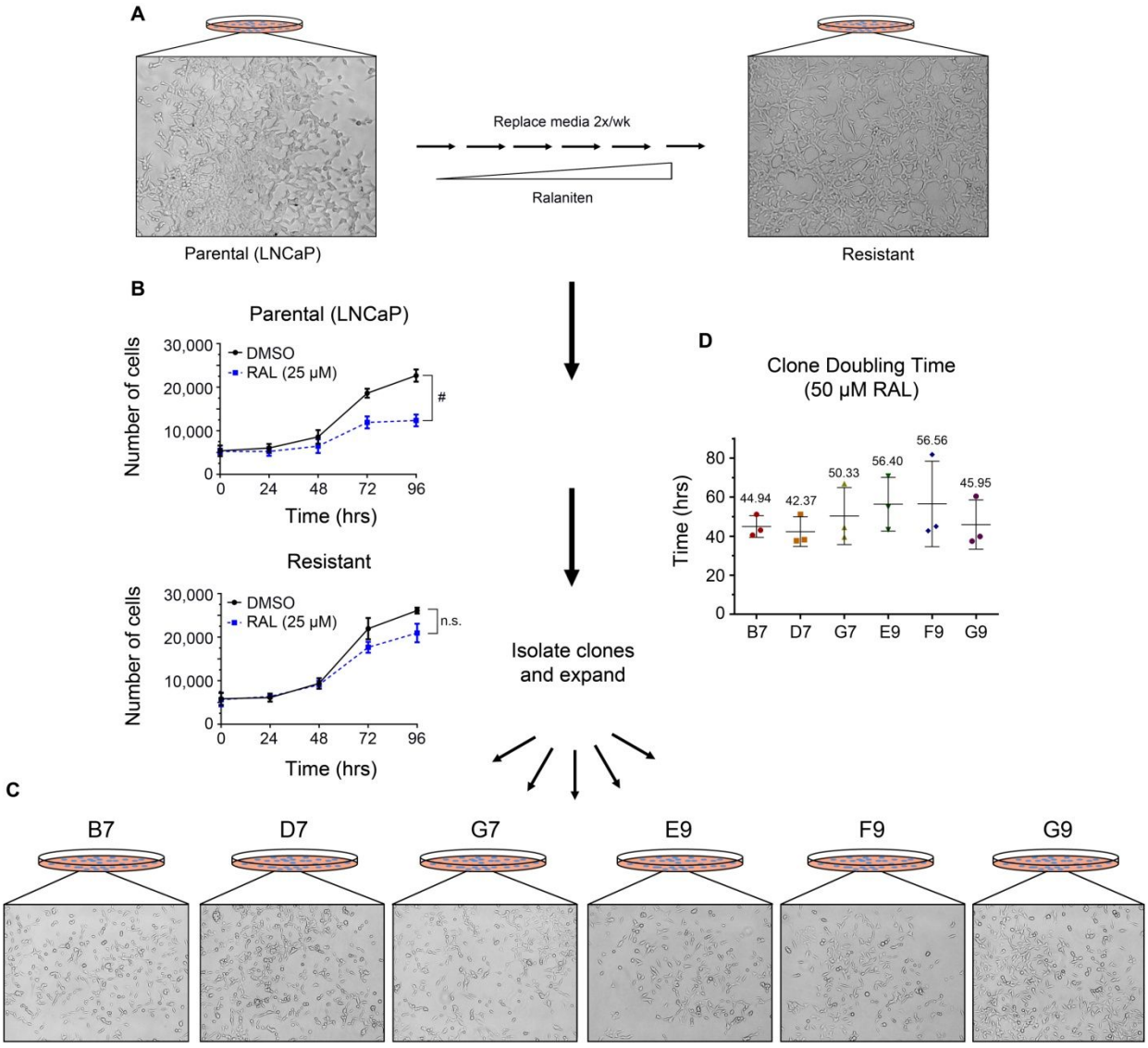


Figure S1. Generation of a model of acquired ralaniten resistance

(**A**) representative photographs at 20X magnification of the parental LNCaP and the initial Resistant cell line. The Resistant cell line was generated following continuous passage with increasing concentrations of ralaniten up to 25 μM. (**B**) Growth curves of each cell line treated with 25 μM ralaniten. Data are presented as mean ± SEM, n=3 independent experiments. $^{\#}p<0.001$; n.s. not significant. (**C**) Highly resistant clones were isolated from the Resistant line

and challenged with increasing concentrations of ralaniten, up to 50 μM. Representative photographs are shown at 20X magnification. (**D**) Doubling times of each of the clones were measured once stable growth was seen in the presence of 50 μM ralaniten. Clone D7 was chosen for further characterization (hereafter referred to as LNCaP-RAL^{R}) as it had the fastest doubling time. Data are presented as mean ± SD, n=3 independent experiments.

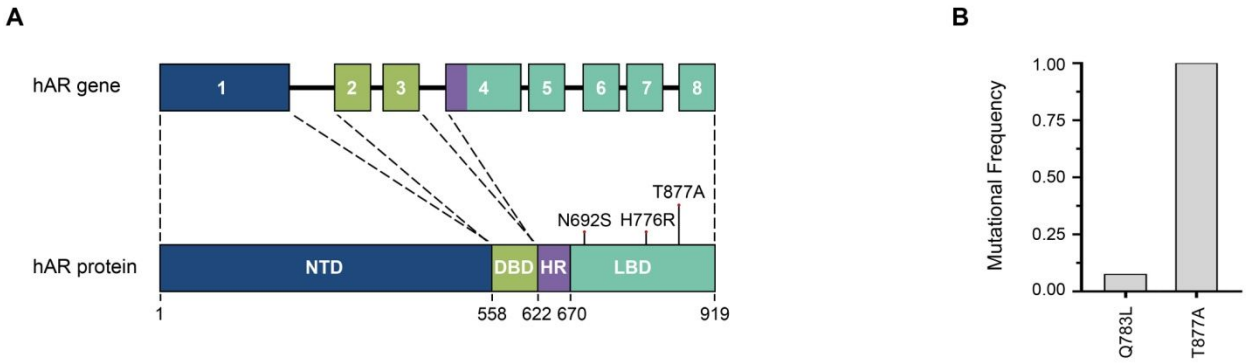


Figure S2. LNCaP-RAL^{R} cells do not harbor novel AR-NTD mutations

(**A**) Cartoon of the *AR* gene and protein showing functional domains. Sequencing the entire AR did not reveal any mutations in the NTD, although two were picked up in the LBD (N692S and H776R) in addition to the T877A mutation native to LNCaP cells. (**B**) When 26 additional bacterial clones were sequenced to confirm the authenticity of these mutations, only a new Q783L mutation was detected in more than one clone (2 of 26). The T877A mutation was seen in all 26, indicating that the others were likely the result of erroneous mistakes introduced during the PCR process.

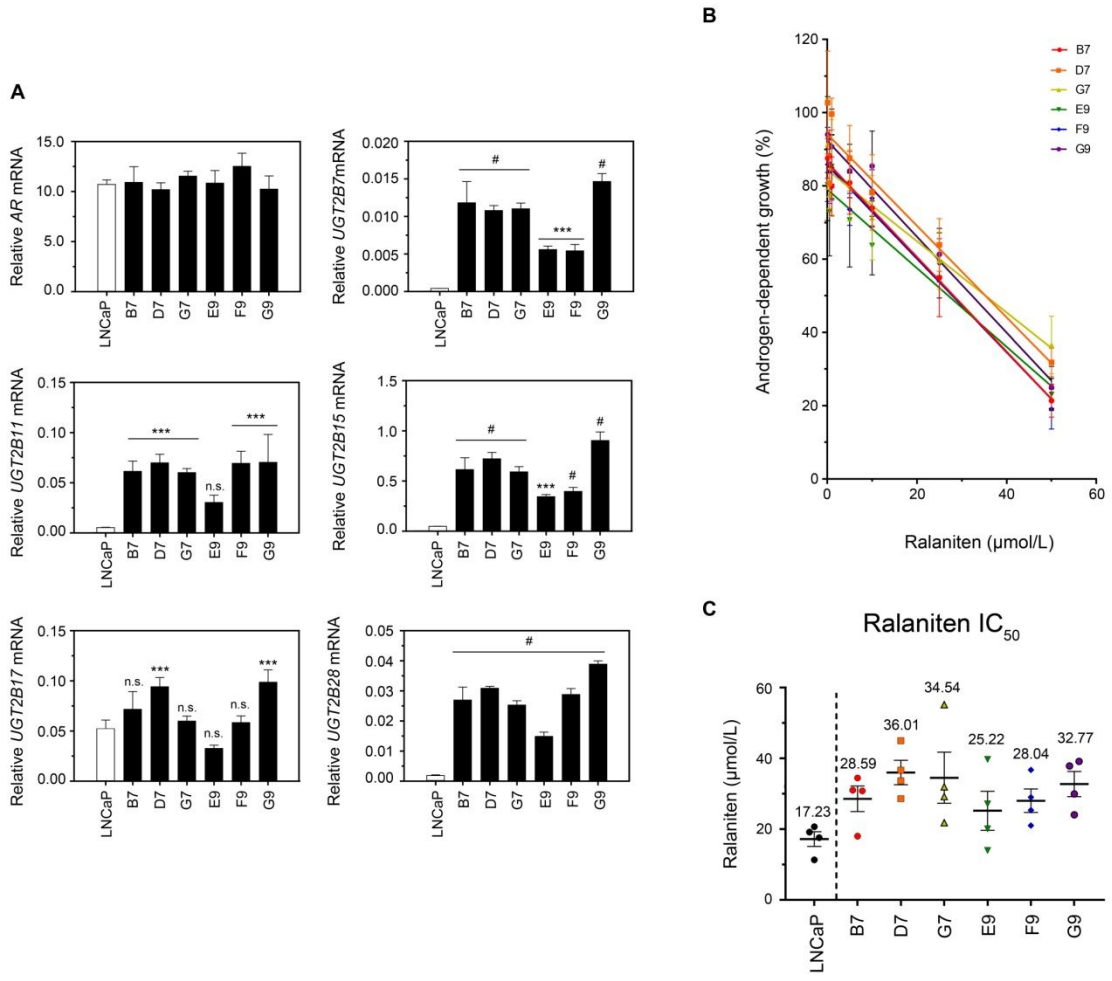


Figure S3. Enhanced UGT2B expression occurs in clones resistant to ralaniten

(**A**) Real-time PCR of *AR, UGT2B7, UGT2B11, UGT2B15, UGT2B17*, and *UGT2B28* transcript normalized to *SDHA* harvested from untreated LNCaP and individual clones isolated from the resistant cell line. While some variability exists with regards to UGT2B expression levels between the clones, all have higher expression than parental LNCaP cells. Conversely, *AR* levels continue to remain stable across clones. *n*=3 independent experiments, and data are presented as mean ± SD. ***p<0.001; #p<0.0001; n.s., not significant. (**B**) Dose response curves of LNCaP and resistant clones treated with ralaniten and stimulated with 0.1 nM R1881.

The IC_{50} values were calculated using a linear regression and interpolating where the line crossed 50% growth. (**C**) Graph of ralaniten IC_{50} values for each resistant clone compared to LNCaP. Clone D7 is LNCaP-RAL^R. *n*=4 independent experiments, and data are presented as mean ± SEM.

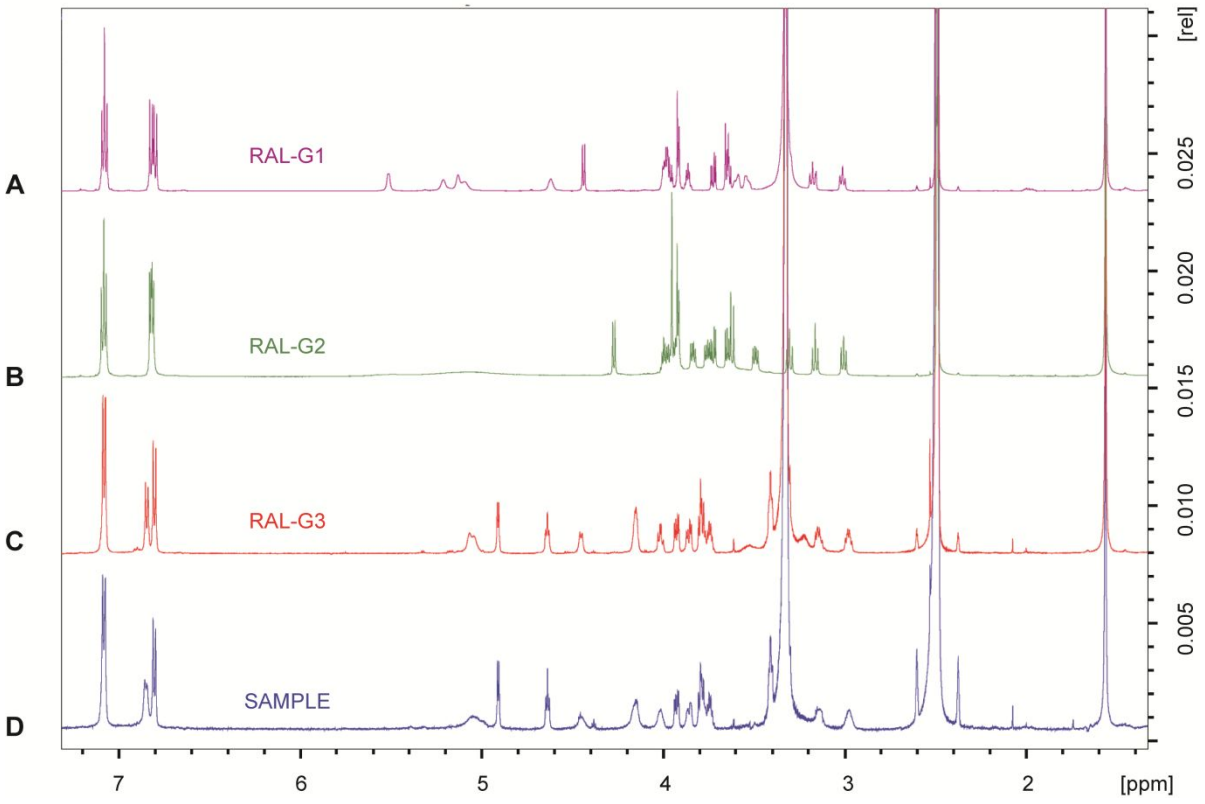


Figure S4. Glucuronidation of ralaniten occurs on the secondary alcohol on C20

^{1}H NMR spectra of synthetic ralaniten RAL-G1 (**A**), RAL-G2 (**B**), and RAL-G3 (**C**) (100 µg/600µL) and that of ralaniten G-3 isolated after biological incubation (RAL + UDPGA) of 250 µg ralaniten (**D**) at 600 MHz in DMSO-d_6. Ralaniten G-3 represents the metabolite formed following glucuronidation of ralaniten on the secondary alcohol on C20.

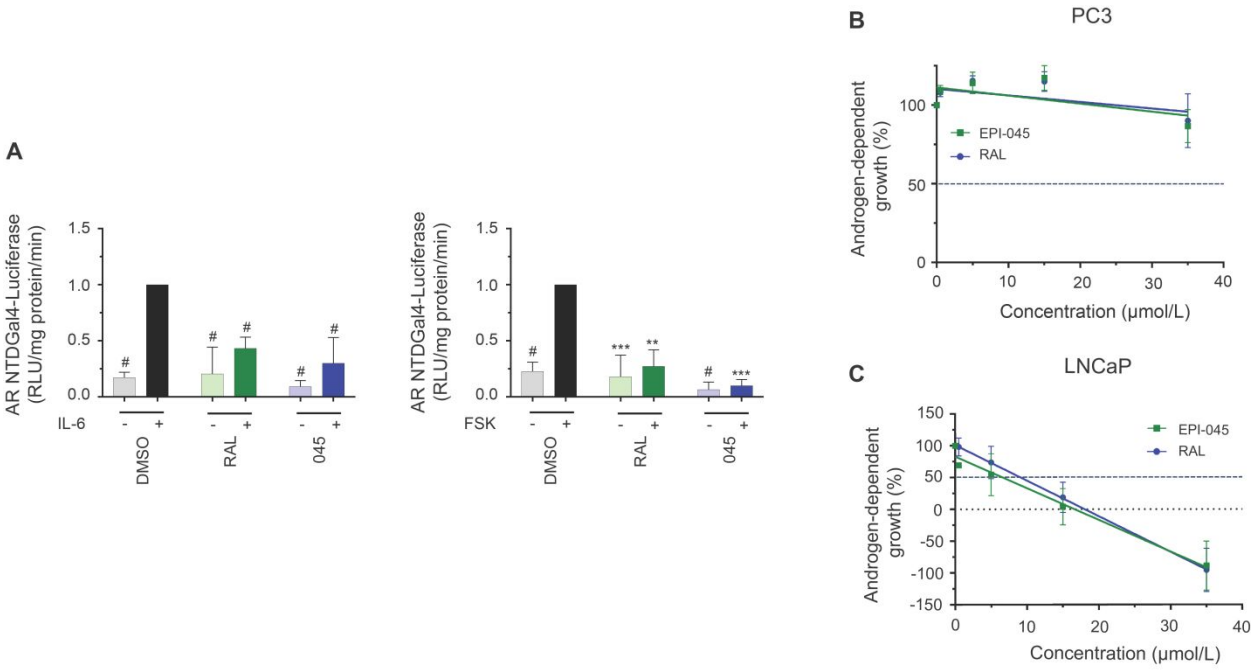
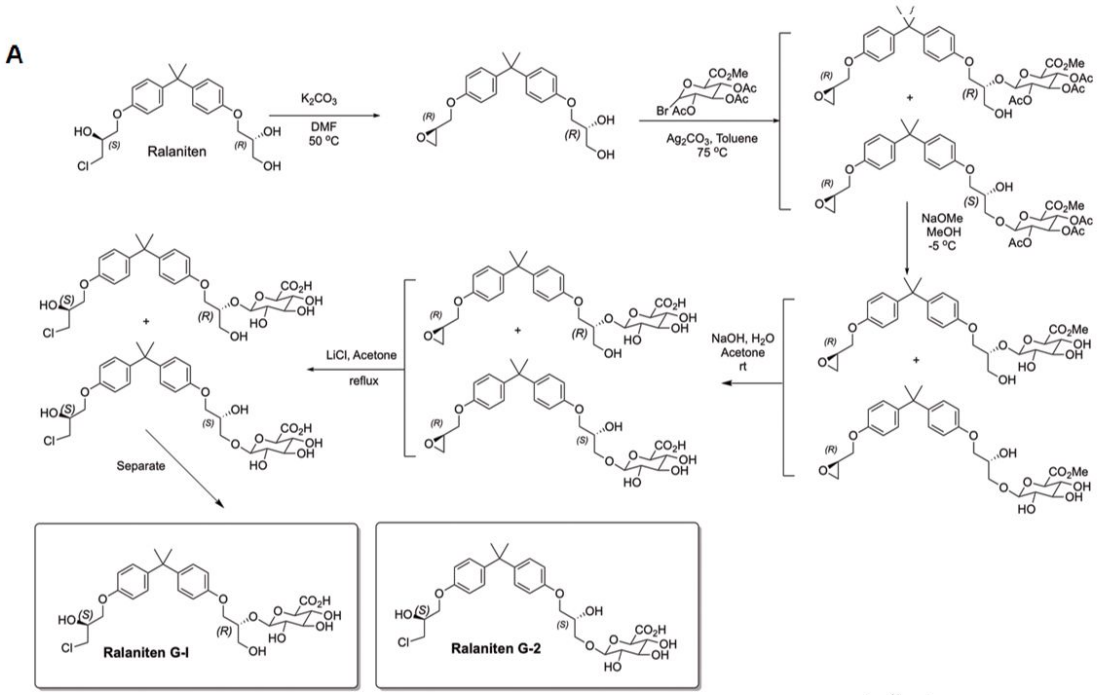
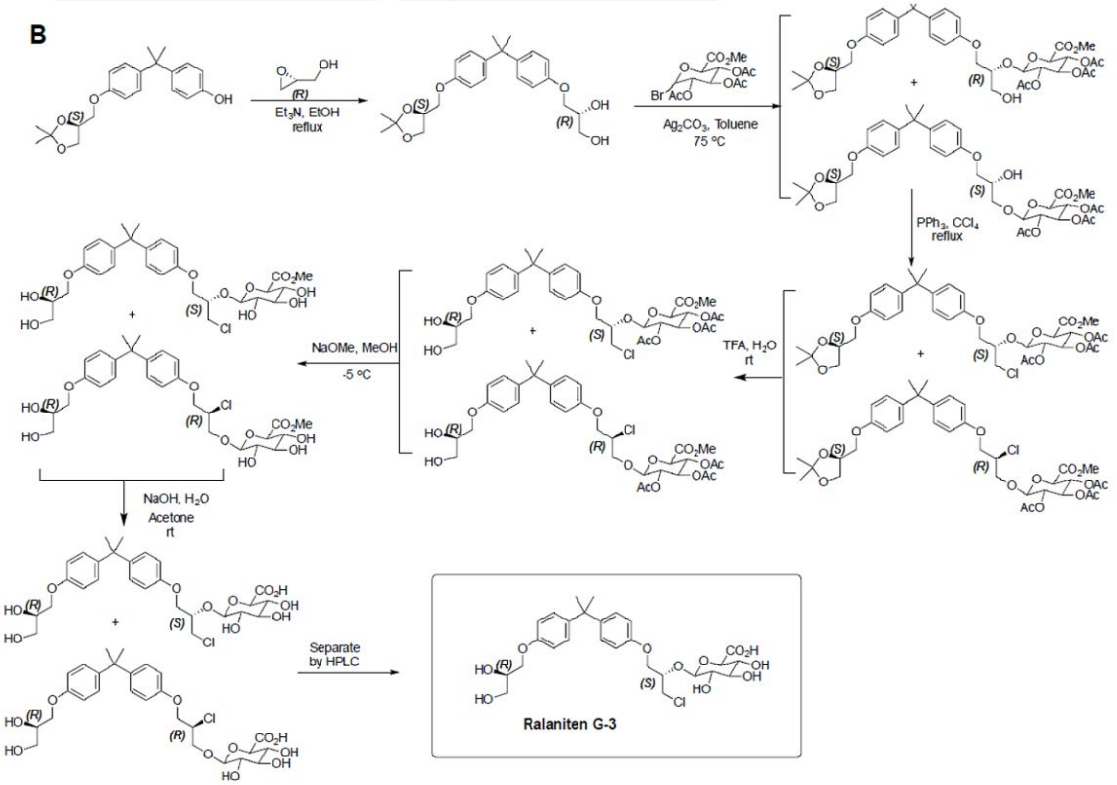


Figure S5. EPI-045 retains specificity against the AR

(**A**) Transactivation assays of the AR NTD were performed in LNCaP cells cotransfected with Gal4UAS-TATA-luciferase and AR-(1-558)-Gal4 DBD prior to incubation with FSK or IL-6 or vehicle for 24 hr. DMSO, ralaniten (25 μM) or EPI-045 (25 μM) were added 1h before the addition of FSK or IL-6. Dose response curves showing androgen stimulated growth of PC3 (**B**) and LNCaP (**C**) cells treated with ralaniten or EPI-045 and stimulated with 0.1 nM R1881. IC_{50} values were calculated using a linear regression and interpolating/extrapolating where the line crossed 50% growth. **LNCaP; RAL:** 9.15 μM, **EPI-045:** 6.65 μM, p=0.194. **PC3; RAL:** 145.3 μM, **EPI-045:** 119.9 μM, p=0.862. *n*=3 independent experiments for each cell line and data are presented as mean ± SEM.


A
Ralaniten
K_2CO_3
DMF
50 °C
Ag_2CO_3, Toluene
75 °C
NaOMe
MeOH
-5 °C
NaOH, H_2O
Acetone
rt
LiCl, Acetone
reflux
Separate
Ralaniten G-1
Ralaniten G-2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reflux
TFA, H2O
rt
NaOMe, MeOH
-5 °C
NaOH, H2O
Acetone
rt
Separate
by HPLC
Ralaniten G-3

Figure S6. Synthetic routes to ralaniten monoglucuronides

(**A**) Synthesis of ralaniten G-1 and ralaniten G-2 Synthetic samples of ralaniten G-1 and G-2 were separated by C_{18} reversed-phase HPLC using an InertSustain, 5 µm, 25 x 0.4 cm column, with 67:33 (0.05 % TFA/H_2O)/MeCN as eluent at a flow rate of 1 mL/min. Retention times: ralaniten G-1: 21.81 min, ralaniten G-2: 24.57 min. (**B**) Synthesis of ralaniten G-3 (**M15**). Ralaniten G-3 and incubated samples of ralaniten were separated and analyzed by C_{18} reversed-phase HPLC using an InertSustain, 5 µm, 25 x 1 cm column, with 67:33 (0.05 % TFA/H_2O)/MeCN as eluent at a flow rate of 2 mL/min for 40 mins followed by a linear gradient to 3:7 (0.05 % TFA/H_2O)/MeCN at 2 mL/min over 15 mins. Retention times: ralaniten-G-3 36.40 min, ralaniten: 52.92 mins.

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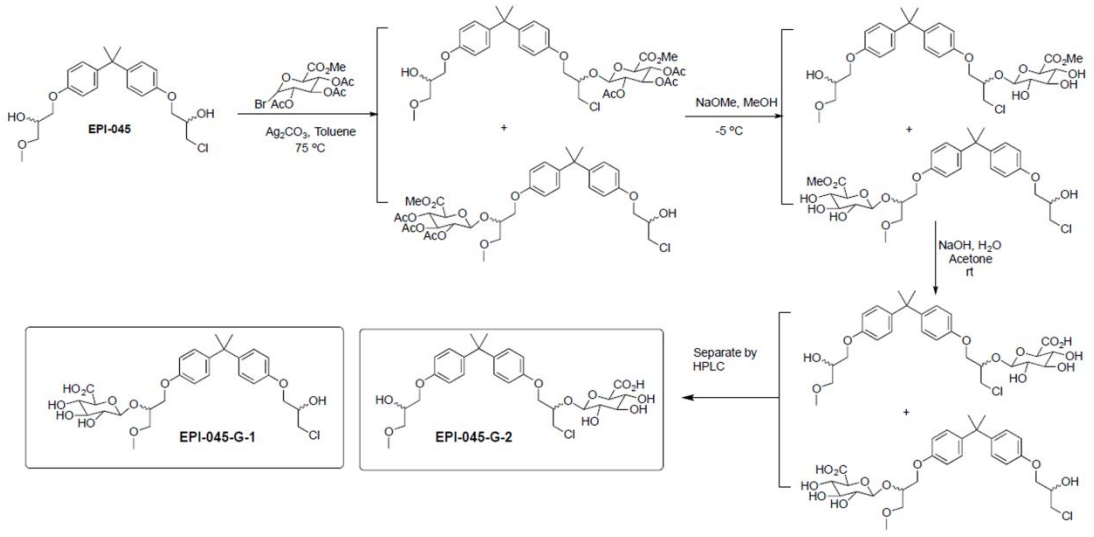

Figure S7. Synthetic route to EPI-045 monoglucuronides