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Drug Metabolism and Disposition

Supplement to:

Metabolism and Mass Balance of the Novel Nonsteroidal Androgen Receptor Inhibitor Darolutamide in Humans

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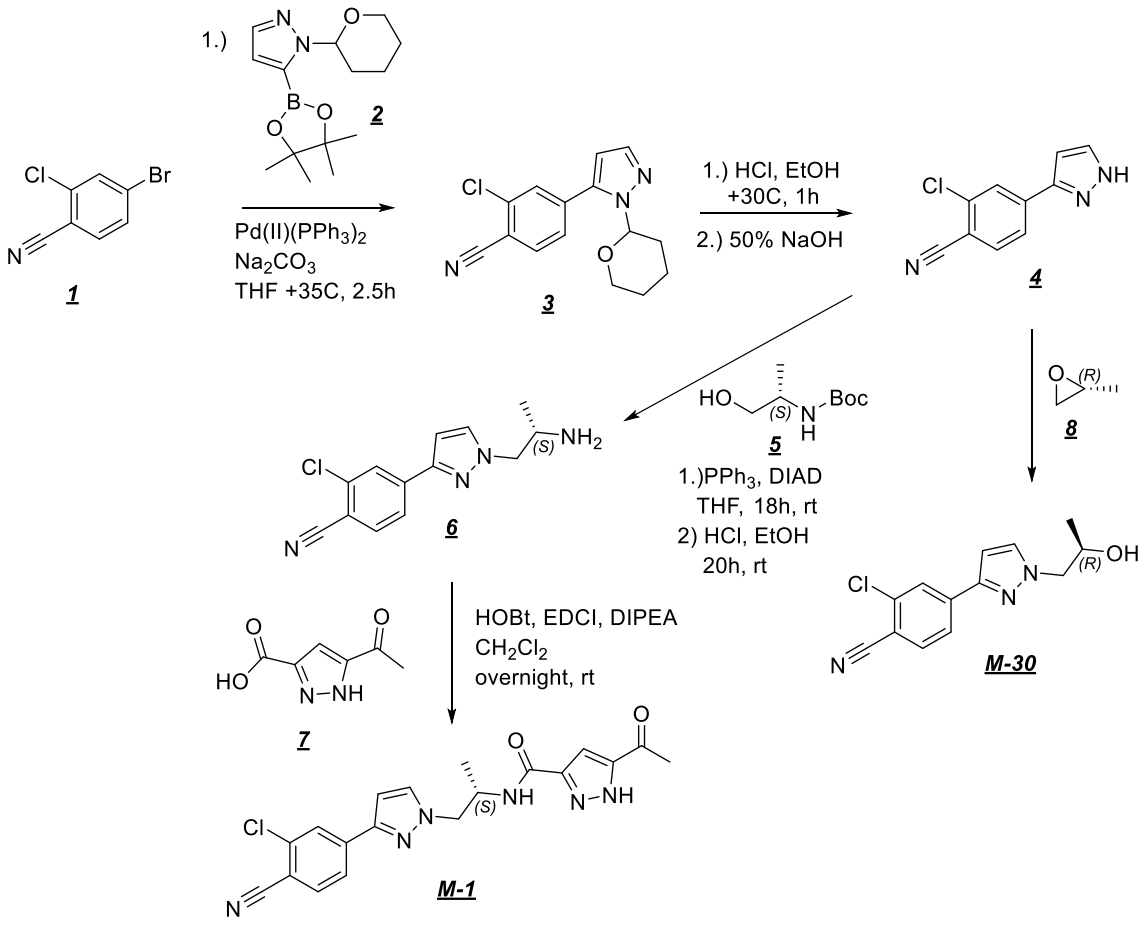
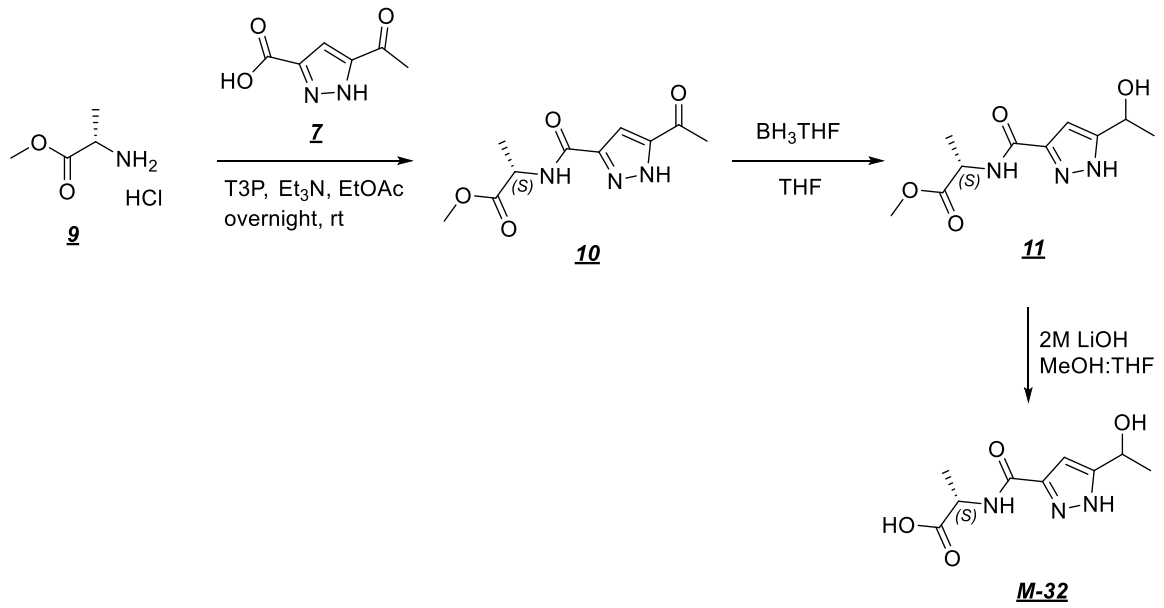
Supplementary Methods S1. Metabolite synthesis

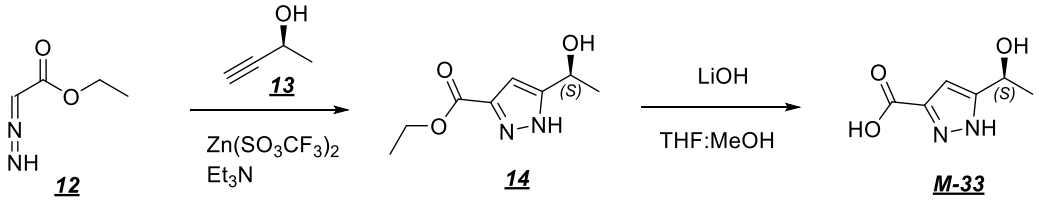
The following metabolites were synthesized at Orion Corporation: M-1, M-26, M-30, M-32, M-33; M-36 was synthesized at Bayer AG. Synthesis pathways are described in schemes 1-5 below, followed by experimental description where available.



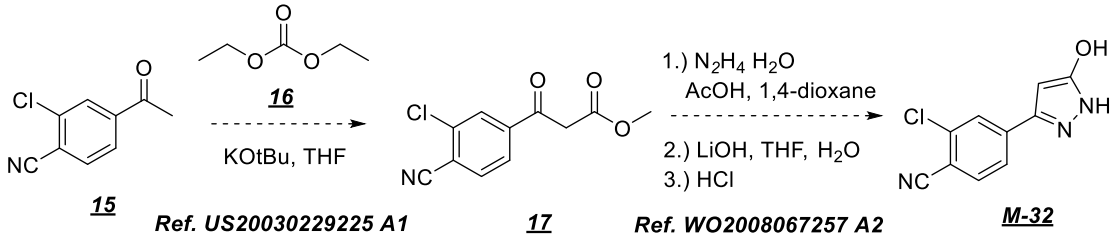
Scheme 1. Synthesis pathways to ***M-1*** and ***M-30***



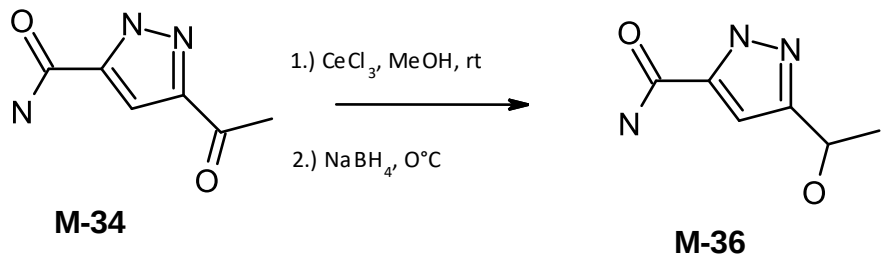
Scheme 2. Synthesis pathway to ***M-32***



Scheme 3. Synthesis pathway to ***M-33***



Scheme 4. Alternative synthesis pathway to ***M-32***



M-34 **M-36**

Scheme 5. Synthesis pathway to ***M-36***

Synthesis of metabolite ***M-1*** has been originally described in patent WO2011/051540 A1 as an example 34.

***(S)-3-Acetyl-N-(1-(3-(3-chloro-4-cyanophenyl)-1H-pyrazol-1-yl)propan-2-yl)-1H-pyrazole-5-carboxamide* M-1**

(a) 2- Chloro-4-(1-(tetrahydro-2H-pyran-2-yl)-1H-pyrazol-5-yl)benzonitrile ***3***

1-(tetrahydro-2H-pyran-2-yl)-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole ***2*** (6.5 g; 23.28 mmol) and 4-bromo-2-chlorobenzonitrile ***1*** (4 g; 18.48 mmol) were dissolved in THF (65 ml). To this mixture bis(triphenylphosphine)palladium(II) chloride (0.65 g; 0.92 mmol), sodium carbonate (4.7 g; 44.3 mmol) and 18 ml of water were added, and the reaction mixture was stirred at 35ºC for 2.5 h. The solvents were distilled to almost dryness, and water (48 ml) was added. After 30 min of stirring, the precipitated product was filtered and 32 ml of ethanol was added to the precipitation. The suspension was stirred for 15 min at room temperature (RT) and for 30 min at –10ºC before filtering to give 3.7 g of the product. ^{1}H-nuclear magnetic resonance (NMR; 400 MHz; d6-DMSO): δ 1.63-1.54 (m, 3H), 1.84-1.80 (m, 1H), 1.97-1.94 (m, 1H), 2.39-2.35

(m, 1H), 3.63-3.57 (m, 1H), 3.99 (m, 1H), 5.32-5.27 (m, 1H), 6.72 (d, 1H), 7.65 (d, 1H), 7.72 (m, 1H), 7.92 (d, 1H), 8.14 (d, 1H).

(b) 2- Chloro- 4-(1H-pyrazol-5-yl)benzonitrile **4**

2- Chloro-4-(1-(tetrahydro-2H-pyran-2-yl)-1H-pyrazol-5-yl)benzonitrile ***3*** (3.67 g; 12.75 mmol) was added to 8 ml of ethanol under nitrogen atmosphere. 15.5 ml of ~10 % HCl (g) in EtOH was slowly added, and the temperature was raised to 30ºC at which point the mixture was stirred for 1 h. The temperature was then lowered to –10ºC and the mixture was again stirred for 30 min, after which the product was precipitated as its HCl salt and was filtered and washed twice with 2 ml of ethanol. The product was dried in vacuo at +40ºC. Yield 2.8 g. 2- Chloro- 4-(1H-pyrazol-5-yl)benzonitrile hydrochloride (2.8 g; 11.47 mmol) was added to a mixture of 8 ml of water and 14 ml of MeOH under nitrogen atmosphere. To this, 50% sodium hydroxide (1.5 ml; 28.7 mmol) was added, with the temperature maintained under 25ºC during the addition. The mixture was stirred for 2 h, before the precipitate was filtered and washed twice with 3 ml of lukewarm water. The product was dried in vacuo at +40 ºC. Yield 1.97 g. ^{1}H-NMR (400MHz; d6-DMSO): δ 6.99 (t, 1H), 7.89 (m, 1H), 7.99 (d, 2H), 8.15 (s, 1H), 13.27 (s, 1H).

(c) (S)-4-(1-(2-aminopropyl)-1H-pyrazol-3-yl)-2-chlorobenzonitrile **6**

2-Chloro-4-(1H-pyrazol-3-yl)benzonitrile ***4*** (4.00g; 19.64 mmol), *(S)-tert*-butyl-1-hydroxypropan-2-yl carbamate ***5*** (3,79 g; 21.61 mmol) and triphenylphosphine were dissolved in dry THF under nitrogen atmosphere and stirred. Diisopropylazo-

dicarboxylate (7.74 ml; 39.3 mmol) was added dropwise and the reaction flask was cooled by ice bath. The reaction was stirred at RT overnight (18 h) and evaporated to dryness. For Boc deprotection, 200 ml of 10 % HCl/EtOH solution was added to the evaporation residue, stirred for 20 h at RT and evaporated to dryness. 100 ml of water was added to the evaporation residue and washed with 3 × 120 ml of DCM to remove reactant residues. The water phase pH was adjusted to ~12 by addition of 2 M NaOH. The product was washed with 3 × 80 ml of DCM and organic phase dried over Na_2SO_4. Organic phase was filtered and evaporated to give 2.605 g of the title compound.

(d) (S)-3-acetyl-N-(1-(3-(3-chloro-4-cyanophenyl)-1H-pyrazol-1-yl)propan-2-yl)-1H-pyrazole-5-carboxamide **M-1**

3-Acetyl-1H-pyrazole-5-carboxylic acid ***7*** (0.59 g; 3.84 mmol) and DIPEA (1.0 ml; 5.75 mmol) were dissolved in 4 ml of dry DCM. Anhydrous HOBt (0.78 g; 5.75 mmol) and EDCI (1.10 g; 5.75 mmol) were added at RT. (S)-4-(1-(2-aminopropyl)-1H-pyrazol-3-yl)-2-chlorobenzonitrile ***6*** (1.00 g; 3.84 mmol) was dissolved in 4 ml of DCM and the reaction was stirred for overnight at RT. 40 ml of DCM was added and organic layer washed with 3 × 15 ml of water. Combined water phases were washed with 2 × 20 ml of DCM. Both organic phases were dried over Na_2SO_4, filtered and evaporated to dryness. Both crude product fractions were combined and purified by CombiFlash (2% MeOH in DCM). Product fractions were combined and evaporated to give 497 mg of product. ^{1}H-NMR (400MHz; d6-DMSO): δ 1.16 (d, 3H, J=6.7 Hz), 2.49 (s, 3H), 4.31 (m, 2H), 4.46 (sept, 1H, J=6.7 Hz), 6.93 (d, 1H, J=2.4 Hz), 7.31 (s, 1H), 7.81 (d, 1H, J=2.4 Hz), 7.92

(d, 1H, J=7.9 Hz), 7.97 (d, 1H, J=8.1 Hz), 8.03 (d, 1H, J=1.3 Hz), 8.48 (d, 1H, J=8.5 Hz), 14.16 (s, 1H).

***(R)-2-chloro-4-(1-(2-hydroxypropyl)-1H-pyrazol-3-yl)benzonitrile* M-30**

2-Chloro-4-(1H-pyrazol-3-yl)benzonitrile ***4*** (100 mg; 0.49 mmol) was dissolved in MeOH (5 ml) under nitrogen atmosphere and cooled to 0˚C. K_2CO_3 (136 mg; 0.98 mmol) was added and then (R)-(+)-propylene oxide (0.17 ml; 2.46 mmol) dropwise. The reaction was stirred overnight at RT and evaporated to dryness. CH_2Cl_2 (15 ml) was added and organic phase was washed with 2 × 10 ml of water. Organic phase was filtered through phase separator and evaporated to dryness to obtain 100 mg of crude material. Crude material was dissolved in EtOAc (1 ml), and heptane (3 ml) was added dropwise to obtain precipitation. The mixture was stirred for 2 h at room temperature, cooled to 0˚C, filtered, and washed with cold heptane. The precipitation was dried under vacuum at +40 ˚C to obtain 38 mg of title compound as white solid. Right regioisomer was confirmed by NOESY-NMR experiment. ^{1}H-NMR (400 MHz; d6-DMSO): δ 1.07 (d, 3H, J=5.9 Hz), 3.95-4.14 (m, 3H), 4.95 (d, 1H, J=4.7 Hz), 6.95 (d, 1H, J=2.4 Hz), 7.81 (d, 1H, J=2.3 Hz), 7.94 (dd, 1H, J=8.2 Hz, J=1.5 Hz), 7.97 (d, 1H, J=8.1 Hz), 8.1 (d, 1H, J=1.2 Hz).

***(5-(1-hydroxyethyl)-1H-pyrazole-3-carbonyl)-L-alanine* M-32**

(a) Methyl-(5-acetyl-1H-pyrazole-3-carbonyl)-L-alaninate **10**

L-Alanine methylester hydrochloride ***9*** (452 mg; 3.24 mmol), EtOAc (10 ml), Et_3N (1.4 ml; 9.73 mmol) and finally 3-acetyl-1H-pyrazole-5-carboxylic acid ***7*** (0.500 g; 3.24 mmol)

were charged in the reaction flask under nitrogen atmosphere. The mixture was cooled to 0˚C, T3P (50% EtOAc solution; 2.3 ml; 3.89 mmol) was added, and the reaction was stirred at RT overnight. The reaction mixture was evaporated to dryness, dissolved in CH_2Cl_2 (25 ml), and washed with water (2 × 15 ml). Organic phase was dried with phase separator cartridge and evaporated to dryness. Evaporation residue was dried under vacuum at +50˚C to obtain 449 mg of crude material. CombiFlash purification (RediSep Column: Silica 40g Gold; CH_2Cl_2 - CH_2Cl_2:MeOH 9:1) gave 372 mg of title compound. ^{1}H-NMR (400 MHz; d-$CDCl_3$): δ 1.54 (d, 3H, J=7.2 Hz), 2.57 (s, 3H), 3.80 (s, 3H), 4.78-4.90 (m, 1H), 7.35 (s, 1H), 7.65 (br s, 1H), 12.26 (br s, 1H).

(b) Methyl-(5-(1-hydroxyethyl)-1H-pyrazole-3-carbonyl)-L-alaninate **11**

Methyl-(5-acetyl-1H-pyrazole-3-carbonyl)-L-alaninate ***10*** (200 mg; 0.84 mmol) was dissolved in dry THF (2 ml) and cooled to 0˚C. BH_3THF (1M; 1 ml; 1.00 mmol) was added in small portions, and the reaction was stirred overnight at RT. Additional BH_3THF (1M; 1 ml; 1.00 mmol) was added, and the reaction was stirred overnight and heated for 2 hours at +50˚C and again overnight at RT. A third amount of BH_3THF (1M; 1 ml; 1.00 mmol) was added slowly, and the reaction was heated at +50˚C for 4 hours and then at RT for 3 days. The reaction was evaporated to dryness, CH_2Cl_2 (25 ml) was added, and organic phase was washed carefully with water (2 × 15 ml). The product was in water phase (pH ~4.5), which was evaporated to dryness. CombiFlash chromatographic purification (C18; ACN-water) provided 30 mg of title product as a diastereomeric mixture. ^{1}H-NMR (400 MHz; d-$CDCl_3$): δ 1.45-1.58 (m, 6H), 3.76-3.81 (m, 3H), 4.69-4.84 (m, 1H), 4.91-5.04 (m, 1H), 6.52 (br s, 1H), 7.46-7.55 (m, 1H).

(c) (5-(1-hydroxyethyl)-1H-pyrazole-3-carbonyl)-L-alanine **M-32**

Methyl-(5-(1-hydroxyethyl)-1H-pyrazole-3-carbonyl)-L-alaninate ***11*** (25 mg; 0.10 mmol) was dissolved in 1 ml of THF:MeOH (1:1), and 2M LiOH (aq. 0.10ml; 0.21 mmol) was added. The reaction was stirred overnight at RT to complete. pH was adjusted carefully below 7 with 2M HCl, and the product was evaporated to dryness and dried in vacuum at +40˚C. Dry MeOH was added, and the product was filtered and evaporated to dryness to obtain 12.6 mg of title compound. ^{1}H-NMR (400 MHz; d-$CDCl_3$): δ 1.46 (d, 3H; J=7.0 Hz), 1.51 (d, 3H, J=6.6 Hz), 4.43 (q, 1H, J=7.0 Hz), 4.90-4.97 (m, overlapping with MeOH signal and confirmed by ^{1}H-^{13}C-HMBC NMR experiment), 6.66 (br s, 1H).

(S)-5-(1-hydroxyethyl)-1H-pyrazole-3-carboxylic acid **M-33**

(a) Ethyl (S)-5-(1-hydroxyethyl)-1H-pyrazole-3-carboxylate **14**

Zinc trifluoromethanesulfonate (0.259 g; 0.71 mmol), (*S*)-(-)-3-butyn-2-ol ***13*** (0.250 g; 3.57 mmol) and Et_3N (0.75 ml; 5.35 mmol) were charged in reaction flask under nitrogen atmosphere. Ethyldiazoacetate ***12*** (0.45 ml; 4.28 mmol) was added slowly and the reaction was heated to +100˚C for 2 hours. The reaction was cooled down to RT, and 5 ml of water added slowly. CH_2Cl_2 (15 ml) and an additional 5 ml of water were added and phases were separated. Water phase was washed twice with CH_2Cl_2. Organic phases were added and dried by filtration through phase separator cartridge and evaporated to dryness to obtain 523 mg of crude material. CombiFlash purification (RediSep Column: Silica 12g Gold; CH_2Cl_2 - CH_2Cl_2:MeOH 9:1) gave 165 mg of title compound. ^{1}H-NMR (400 MHz; d6-DMSO): δ 1.18 (t, 3H, J=7.3 Hz), 1.25-1.42 (m, 3H),

3.11 (q, 2H, J=7.3 Hz), 4.20-4.33 (m, 2H), 5.42 (br d, 1H, 4.9 Hz), 6.54 (s, 1H), 13.28 (br s, 1H).

^{1}H-NMR indicated the presence of 2 pyrazole tautomers, and the signals reported above were identified for the major one. Tautomers can be obtained in pyrazole ring signals; in addition to 6.54 ppm, minor tautomer can also be detected as a broad singlet at 6.72 ppm (in 4:1 ratio), and a ring NH minor tautomer can be obtained as a broad singlet at 13.60 ppm (also in 4:1 ratio).

(b) (S)-5-(1-hydroxyethyl)-1H-pyrazole-3-carboxylic acid **M-33**

(*S*)-ethyl-5-(1-hydroxyethyl)-1H-pyrazole-3-carboxylate ***14*** (430 mg; 2.34 mmol) was dissolved in EtOH (1ml) and THF (4ml) mixture. 2M NaOH (aq. 8.17 ml; 16.34 mmol) was added and stirred overnight at RT. The reaction was carefully adjusted to slightly acidic with HCl and evaporated to dryness. 1.36 g of crude material was obtained. Identification with MS was positive.

2-chloro-4-(5-hydroxy-1H-pyrazol-3-yl)benzonitrile **M-26**

A detailed synthesis description was not available by the time of publication.

3-(1-Hydroxyethyl)-1H-pyrazole-5-carboxamide **M-36**

3-Acetyl-1H-pyrazole-5-carboxamide ***M-34*** (209 mg; 1.36 mmol) was suspended in MeOH (11 ml) under nitrogen atmosphere. $CeCl_3$ (353 mg; 1.43 mmol) was added

and the resulting solution cooled to 0˚C. $NaBH_4$ (54 mg; 1.43 mmol) was slowly added, and the reaction was stirred for 1 h at 0˚C. 100 ml of water was added, the pH of water phase was adjusted to ~9 by addition of 2 M NaOH and then extracted 3 × 80 ml n-butanol.

Organic phase was dried over Na_2SO_4, filtered and evaporated to dryness. Crude product was purified by Biotage SNAP 10 g (50 % MeOH in DCM). Product fractions were combined and evaporated to give 77 mg of product. 1H-NMR (600 MHz, DMSO-d6): δ [ppm] = 1.34 (br d, 1H), 1.39 (d, 3H), 4.70 (quin, 1H), 4.79 (quin, 1H), 5.08 (br d, 1H), 5.41 (d, 1H), 6.43 (d, 1H), 6.77 (s, 1H), 7.11 (br s, 1H), 7.40 (br s, 1H), 7.84 (br s, 1H), 13.00 (br s, 1H), 13.11 (br s, 1H).

Supplementary Methods S2. Bioanalytical Methods for Human Samples

A quantitative liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was established for the determination of (*S,R*)-darolutamide, (*S,S*)-darolutamide, and keto-darolutamide in human plasma (all) and urine (darolutamide diastereomers only). Concentrations of darolutamide were calculated as the sum of the two diastereomers, (*S,R*)-darolutamide and (*S,S*)-darolutamide. The plasma method used solid-phase extraction followed by chiral high-performance LC-MS/MS detection (method A in Supplementary Table S2 below), quantitation being achieved by weighted linear regression using ^{13}C-labeled internal standards. Method validation and study sample analysis were performed in accordance with pertinent guidelines by PRA Health Sciences (European Medicines Agency, 2011; Food and Drug Administration, 2018). The determined analyte concentrations in study samples were verified by assaying quality control samples of blank matrix spiked with known concentrations of the respective analytes. Concentrations below the lower limit of quantification (LLOQ) were omitted. Concentrations above the LLOQ were determined with a precision better than 15% and an accuracy within 85–115%, with concentrations at the LLOQ being determined with a precision of 20% and accuracy within 80–120%, in accordance with standard operating procedures and pertinent method validation guidelines. Bioanalytical results are summarized in Supplementary Table S3 below. Total radioactivity concentrations in whole blood and plasma after oral solution dosing were determined by Quotient Bioresearch Ltd (Rushden, UK) using liquid scintillation counting (LSC) in a liquid scintillation spectrometer Tri-Carb 2900 TR Liquid Scintillation Analyzer (Perkin Elmer, Shelton, USA) with automatic quench correction by the external standard channel ratio method at 13°C using Atomlight™, high-performance LSC-cocktail, as scintillation cocktail. LLOQs for total ^{14}C-radioactivity were 30.57 ng eq/mL in plasma, 100.44 ng eq/mL in blood.

Supplementary Table S1. Suppliers of materials for in vitro studies

Material	Supplier
Human male hepatocytes, cryopreserved	
Donor TWT	Celsis In Vitro Technologies, Baltimore, MD, USA
Donor TZU	Bioreclamation IVT, Baltimore, MD, USA
Donor HUM4070B	Triangle Research Labs, Triangle Research Park, NC, USA
Donors: GMK, NIQ and VCM	Celsis, Brussels, Belgium
Pooled human liver microsomes (200 mixed gender donors): Xtreme 200 lot 1010420 and lot 1210223 **Pooled human liver cytosol (50 mixed gender donors): H0610.C, lot 1310087, and lot 1410012** **Pooled human intestinal microsomes (15 mixed gender donors): lot 510408** **Pooled human renal microsomes (15 mixed gender donors): lot 510251**	XenoTech LLC, Lenexa, KS, USA
Human liver microsomes (single donor): HH13, H023, HK25, H030, H032, H066, H088, H089, H093	BD Gentest Corp., Woburn, MA, USA
Human liver microsomes (single donor): M003, M027, M028, M029, M030, M031, M032, M055, M056, M057, M058, M059, M060, M061	Cytonet, Weinheim, Germany

Human liver cytosol	Bioreclamation IVT (formerly Celsis In VitroTechnologies, Baltimore, MD, USA)
Recombinant CYP isoforms (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, 3A7, 4A11, 4F2, 4F3A, 4F3B, 4A12, 19A1); Supersomes™ **Recombinant UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, 2B17); Supersomes™**	Corning, Woburn, MA, USA
Recombinant AKR isoforms AKR1C1, 1C2, 1C3, 1C4	Bayer AG, Berlin, Germany
UDPGA, NADP, NAD	Sigma-Aldrich GmbH, Steinheim, Germany
NADPH	Sigma, Zwijndrecht, Netherlands

Supplementary Table S2. LC methods for measurement of darolutamide and metabolites in plasma, urine and feces

	A. LC-MS/MS	B. Analytical LC	C. LC-MS	D. LC-MS	E. LC-MS
Compounds analyzed	(*S,S*)-darolutamide, (*S,R*)-darolutamide, and keto-darolutamide	Total ^{14}C-radioactivity, metabolite profiling, structure elucidation	Darolutamide and metabolites M-32, M-33, M-34, and M-36	(*S,S*)-darolutamide and (*S,R*)-darolutamide	Drug glucuronide diastereomers M-7a/b and M-15a/b
Sample	Plasma (all), urine (not keto-darolutamide)	Plasma, urine, feces	Urine	Feces	Urine
HPLC system	Shimadzu LC-10AD VP Series (SHIMADZU SCIENTIFIC INSTRUMENTS, INC., Columbia, MD, USA)	Agilent 1200 (Agilent Technologies, Waldbronn, Germany) and Waters Acquity (Eschborn, Germany)	Waters Acquity (Eschborn, Germany)	Waters Acquity (Eschborn, Germany)	Waters Acquity (Eschborn, Germany)
HPLC column	Plasma: Chiral AGP, 150 × 4 mm, 5 µm (Chromtech, Apple Valley, MN, USA) Urine: CHIRALPAK®AGP, 150 × 4 mm, 5 µm (Sigma	Pursuit 3 C8, 150 × 3 mm, 3 µm (Agilent, Santa Clara, CA, USA)	Pursuit 3 C8, 150 × 3 mm, 3 µm (Agilent, Santa Clara, CA, USA)	Accucore C4, 150 × 3 mm, 2.6 µm (ThermoFisher Scientific Inc., Waltham, USA)	Betasil Phenyl-Hexyl, 100 × 2.1 mm, 3 µm (ThermoFisher Scientific Inc., Waltham, USA)

	Aldrich, Saint Louis, MO, USA)				
Column temperature	40°C	30°C	30°C	10°C	55°C
Gradient elution flow rate	1.00 mL/min	0.35 mL/min	0.35 mL/min	0.35 mL/min	0.30 mL/min
Solvent A	Water	25 mM ammonium formate pH4 (5% acetonitrile)	10 mM ammonium formate pH4 (5% acetonitrile)	10 mM ammonium formate pH4	Water (0.1% Formic acid)
Solvent B	Ethanol	Acetonitrile + 1% formic acid	Acetonitrile	Methanol	Methanol
Injection volume	10 µL	100 µL (plasma), 50–100 µL (urine), 50 µL (feces)	50 µL	10 µL	10 µL
Mass spectrometer	API4000 (AB Sciex, Framingham, MA, USA)	QExactive Plus (ThermoFisher Scientific Inc., Waltham, MA, USA)	QExactive Plus (ThermoFisher Scientific Inc., Waltham, MA, USA)	Fusion™ Lumos™ Tribrid™ (ThermoFisher Scientific Inc., Waltham, MA, USA)	QExactive Plus (ThermoFisher Scientific Inc., Waltham, MA, USA)
Radiochemical detector	n/a	Topcount NXT™ Microplate Scintillation & Luminescence	n/a	n/a	n/a

	Counter (Perkin Elmer, Boston, USA)

HPLC, high-performance liquid chromatography; LC-HMRS, liquid chromatography–high resolution mass spectrometry; LC-MS, liquid chromatography–mass spectrometry; n/a, not applicable.

Supplementary Table S3. Range of accuracy and precision data of LC-MS/MS methods for (*S,R*)-darolutamide, (*S,S*)-darolutamide, and keto-darolutamide across all validation quality control replicates

	(*S,R*)-darolutamide			(*S,S*)-darolutamide			Keto-darolutamide		
	Range (ng/mL)	**Accuracy, %**	**Precision, %**	**Range (ng/mL)**	**Accuracy, %**	**Precision, %**	**Range (ng/mL)**	**Accuracy, %**	**Precision, %**
Human plasma (PRA)	5–5000	97.8–117.0	1.1–2.5	5–5000	98.9–117.2	1.1–2.0	5–5000	98.0–118.0	1.2–2.7
Human plasma (Celerion)	4.94–4940	86.5–109.1	1.4–6.0	5.06–5060	88.3–109.4	1.1–6.2	10.0–10000	89.1–107.9	1.1–6.6
Human urine (PRA)	5–5000	93.3–109.6	1.1–4.1	5–5000	93.2–108.4	0.6–3.5	N/A	N/A	N/A

N/A, not available.

Supplementary Table S4. LC-MS methods for quantitation of darolutamide and metabolites in vitro

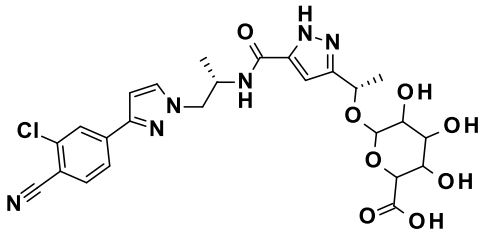
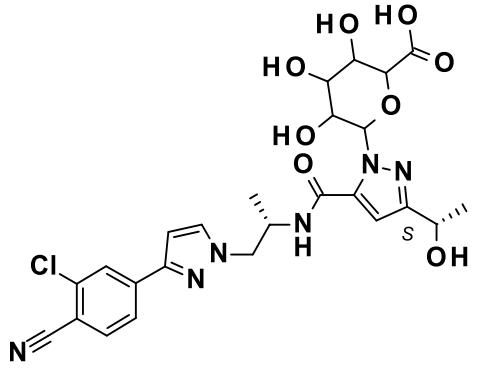
	Metabolite profiling		Role of glucuronidation	Metabolite profiling	
Compounds analyzed	**F.** Total ^{14}C-radioactivity, metabolite profiling, (*S,S*)-darolutamide, (*S,R*)-darolutamide, and keto-darolutamide	**G.** Total ^{14}C-radioactivity, metabolite profiling	**H.** Darolutamide (1:1 mixture of (*S,S*)-darolutamide, and (*S,R*)-darolutamide)	**I:** (*S,S*)-darolutamide, (*S,R*)-darolutamide, and keto-darolutamide	**J:** Darolutamide and keto-darolutamide
Sample	Human hepatocytes and subcellular liver preparations, recombinant CYPs	Human hepatocytes	Human liver, intestinal or renal microsomes, recombinant UGTs	Human liver microsomes and cytosol, human hepatocytes, and recombinant aldo keto reductase	Recombinant aldo keto reductase, human liver microsomes, and cytosol
HPLC	Agilent HP 1290 (Agilent Technologies, Waldbronn, Germany)	Agilent 1100 (Agilent Technologies, Waldbronn, Germany)	Agilent HP 1290 Infinity (Agilent Technologies, Waldbronn, Germany)	Agilent HP 1290 (Agilent Technologies, Waldbronn, Germany)	Agilent HP 1290 (Agilent Technologies, Waldbronn, Germany)
HPLC column	Pursuit 3 C8, 150 × 3.1 mm, 3 µm	XBridge C18, 4.6 × 100 mm, 3.5 µm, with pre-column Waters	Betasil Phenyl-Hexyl, 100A × 2.1 mm, 3 µm with pre-column of	Accucore C4, 150 × 3 mm, 2.6 µm (ThermoFisher Scientific	Aquity BEH C18,100 x 2.1 mm 1.7µm (Waters

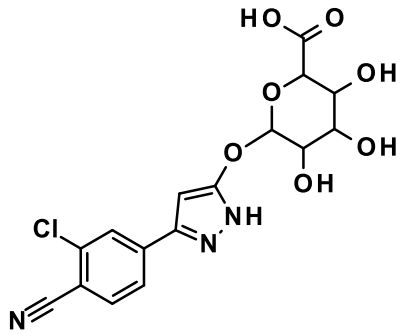
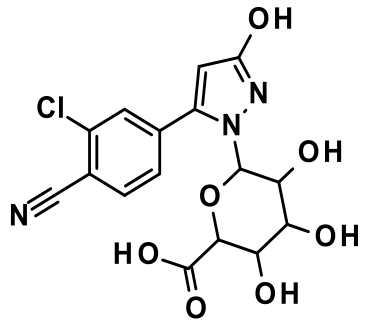
	(Agilent, Santa Clara, CA, USA)	XBridge 3.5 µM 4.6 × 20 mm (Waters Corporation, Milford, MA, USA)	betasil phenyl-hexyl, 10 × 2.1 mm, 3 µm (ThermoFisher Scientific Inc., Waltham, MA, USA)	Inc., Waltham, MA, USA)	Corporation, Milford, MA, USA)
Column temperature	30°C	30°C	55°C	10°C	40°C
Gradient elution flow rate	0.35 mL/min	1 mL/min	0.40 mL/min	0.35 mL/min	0.35 mL/min
Solvent A	10 mM ammonium formate + 5% acetonitrile at pH4	0.1% formic acid	Water (0.05% Formic acid)	10 mM ammonium formate + 5% acetonitrile at pH4	10 mM ammonium formate + 5% acetonitrile at pH4
Solvent B	Acetonitrile + 0.1% formic acid	Acetonitrile	Methanol	Methanol	Acetonitrile + 0.1% formic acid
Injection volume	40 µL	10–20 µL	5 or 20 µL	10 µL	40 µL
Mass spectrometer	Exactive, Q Exactive™ or Q Exactive™ Plus mass spectrometer (Thermo Scientific, Waltham, MA, USA)	Sciex QTRAP 4000 (Sciex, Concord, Ontario, Canada)	QTRAP 6500 mass spectrometer (Applied Biosystems MDS Sciex, Ontario, Canada)	Exactive, Q Exactive™, or Q Exactive™ Plus mass spectrometer (Thermo Scientific, Waltham, MA, USA)	Exactive, Q Exactive™, or Q Exactive™ Plus mass spectrometer (Thermo Scientific, Waltham, MA, USA)

Radiochemical detector	Topcount NXT™ (Perkin Elmer, Waltham, MA, USA)	βRAM/Sofie (LabLogic, Sheffield, UK)	Canberra Packard TriCarb® 2900TR or 3100TR (Perkin Elmer/Canberra Packard, Rodgau-Jüdesheim, Germany)	n/a	n/a

HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; n/a, not applicable.

Supplementary Table S5. NMR data of metabolites M-7a, M-15a, M-21 and M-22 isolated from pooled human urine

Metabolite	Structure	Chemical shifts
M-7a		^{1}**H NMR:** (600 MHz, D_3-ACN/D2O 70:30 v/v): δ/ppm = 7.93 (d, 1H), 7.81 (dd, 1H), 7.76 (d, 1H), 7.65 (d, 1H), 6.69 (d, 1H), 6.61 (s, 1H), 5.01 (q, 1H), 4.48-4.42 (m, 1H), 4.31-4.22 (m, 3H), 3.75 (d, 1H), 3.46 (t, 1H), 3.30 (t, 1H), 3.22 (dd, 1H), 1.45 (d, 3H), 1.15 (d, 3H). ^{13}**C NMR** (150 MHz, D3-ACN/D2O 70:30 v/v): δ/ppm = 172.2, 162.9, 149.3, 148.3, 145.8, 140.3, 137.6, 135.8, 134.4, 127.2, 125.1, 117.4, 111.7, 105.1, 104.5, 101.6, 76.3, 75.6, 73.8, 72.2, 70.0, 56.8, 46.6, 22.3, 17.9.
M-15a		^{1}**H NMR**: (600 MHz, D_3-ACN/D_2O 70:30 v/v): δ/ppm = 7.96 (m, 1H), 7.81 (m, 1H), 7.76 (m, 1H), 7.65 (d, 1H), 6.72 (m, 1H), 6.67 (m, 1H), 5.96 (m, 1H), 4.81 (m, 1H), 4.41 (m, 1H), 4.26 (m, 2H), 4.06 (m, 1H), 3.90 (m, 1H), 3.55 (m, 1H), 3.50 (m, 1H), 1.37 (m, 3H), 1.16 (m, 3H). ^{13}**C NMR**: (150 MHz, D_3-ACN/D_2O 70:30 v/v): δ/ppm = 172, 160.4, 157.9, 149.4, 140.3, 139.2, 137.6, 135.9, 134.4, 127.3, 125.2, 117.4, 111.8, 106.6, 105.4, 86.3, 78.0, 77.1, 72.2, 71.9, 64.6, 56.7, 47.1, 23.2, 17.6.

M-21		**1H NMR**: (600 MHz, D_3-ACN/D_2O 70:30 v/v): δ/ppm = 7.88 (br s, 1H), 7.80 (d, 1H), 7.70 (dd, 1H), 6.34 (s, 1H), 5.11 (d, 1H), 3.97 (d, 1H), 3.55 (t, 1H), 3.49 (t, 1H), 3.45 (t, 1H). **13C NMR** (150 MHz, D_3-ACN/D2O 70:30 v/v): δ/ppm = 171.6, 162.4, 142.8, 137.8, 136.4, 136.0, 127.3, 125.1, 117.0, 112.7, 101.8, 91.1, 76.2, 75.9, 73.5, 72.1.
M-22		^{1}**H NMR**: (600 MHz, D_3-ACN/D_2O 70:30 v/v): δ/ppm = 7.87 (d, 1H), 7.71 (s, 1H), 7.54 (d, 1H), 5.89 (s, 1H), 5.00 (d, 1H), 4.04 (t, 1H), 3.86 (d, 1H), 3.56 (t, 1H), 3.42 (t, 1H). ^{13}**C NMR** (150 MHz, D_3-ACN/D_2O 70:30 v/v): δ/ppm = 172.0, 162.5, 145.3, 137.6, 136.3, 135.8, 131.0, 129.0, 116.9, 113.8, 95.5, 85.9, 77.4, 76.9, 72.0, 71.8.

Supplementary Table S6. Molecular ions and characteristic fragment ions of darolutamide and metabolites detected in biological samples

Assignment	Matrix	Calc. mass [M+H]+ [m/z]	Meas mass [M+H]+ [m/z]	Calc. mass [M-H]- [m/z]	Meas. mass [M-H]- [m/z]	Mass shift to drug [Da]	Molecular formula	Key fragment ions (parent *m/z* → M+H]+)	Key fragment ions (parent *m/z*→ [M-H]-)
Darolutamide		399.1331	399.1328	397.1185	397.1187	-	C19H19N6O2Cl	381, 244, 196, 178	353, 202
Keto-darolutamide (M-1)		397.1180	397.1164	395.1023	395.1030	-2	C19H17N6O2Cl	244, 194, 136	202, 192, 152
M-2		591.1606	591.1601	589.1450	589.1465	+192	C25H27N6O9Cl	415, 397, 196, 178	413
M-7a		575.1657	575.1649	573.1501	573.1505	+176	C25H27N6O8Cl	399, 381, 244, 196, 178	379, 193
M-7b		575.1657	575.1656	573.1501	573.1513	+176	C25H27N6O8Cl	399, 381, 244, 196, 178	379, 193
M-10		573.1501	573.1496	571.1344	571.1357	+174	C25H25N6O8Cl	397, 194	395, 202
M-15a		575.1652	575.1650	573.1501	573.1505	+176	C25H27N6O8Cl	399, 381, 196, 178	397, 202, 175
M-15b		575.1652	575.1650	573.1501	573.1505	+176	C25H27N6O8Cl	399, 381, 196, 178	397, 202, 175
M-21		396.0599	396.0591	394.0442	394.0448	-3	C16H14N3O7Cl I	220	218, 175, 113
M-22		396.0599	396.0593	394.0442	394.0448	-3	C16H14N3O7Cl	220	260, 218, 175, 113
M-24		299.9846	299.9839	297.9689	n/a	-99	C10H6N3O4ClS	220, 190, 175, 162, 136	n/a
M-25		220.0278	220.0276	218.0121	n/a	-179	C10H6N3OCl	190, 175, 162, 136	n/a

Assignment	Matrix	Calc. mass [M+H]+ [m/z]	Meas mass [M+H]+ [m/z]	Calc. mass [M-H]- [m/z]	Meas. mass [M-H]- [m/z]	Mass shift to drug [Da]	Molecular formula	Key fragment ions (parent *m/z* → M+H]+)	Key fragment ions (parent *m/z*→ [M-H]-)
M-26		204.0329	204.0324	202.0172	202.0165	-195	C10H6N3C	n/a	184, 166, 89
M-28		399.0972	399.0964	397.0816	397.0824	0	C18H15N6O3Cl	244, 196	353, 202, 150, 110
M-29		299.9846	n/a	297.9689	297.9696	-99	C10H6N3O4ClS	n/a	218
M-30		262.0747	262.0739	n.d.	n/a	-137	C13H13N3OCl	244, 204	n/a
M-31		260.0591	258.0440	n.d.	n/a	-139	C13H11N3OCl	216, 215	n/a
M-32		n.d.	n/a	226.0828	226.0815	-171	C9H13N3O4	n/a	182, 138
M-33		n.d.	n/a	153.0306	153.0285	-244	C6H6N2O3	n/a	109, 82
M-34		n.d.	n/a	152.0465	152.0445	-245	C6H7N3O2	n/a	109, 67
M-36		n.d.	n/a	154.0622	154.0623	-243	C6H9N3O2	n/a	111, 110, 67

n/a, not applicable; n/d not detected, .

Supplementary Table S7. Depletion of ^{14}C-darolutamide in human hepatocytes of two donors (HH-TZU, HH-TWT) in the absence and presence of the CYP3A4 inhibitor itraconazole for up to 60 minutes incubation time

Donor	Inhibitor	$t_{½}$ (min)	Intrinsic clearance (µL/min/10^6 cells)	Ratio to control	Blood clearance ('well stirred' model) (L/h/kg)	Ratio to control
HH-TZU	None	286	2.42	–	0.27	–
	Itraconazole 2 µM	457	1.52	0.63	0.18	0.67
HH-TWT	None	119	5.84	–	0.50	–
	Itraconazole 2 µM	209	3.31	0.57	0.34	0.68

The intrinsic CL values were calculated and converted into CL involved by using the equations describing the well stirred model of hepatic CL involved by using the equations describing the well stirred model of hepatic CL (Pang and Rowland, 1977). Values of 21 g liver/kg of body weight, 110 Mio cell per g liver and 1.32 L/h/kg for hepatic blood flow were used for the calculations of all hepatocyte incubations.

Supplementary Table S8. Correlations between UGT isoform-selective activity and formation of *O*- and *N*-glucuronides in human liver microsomes

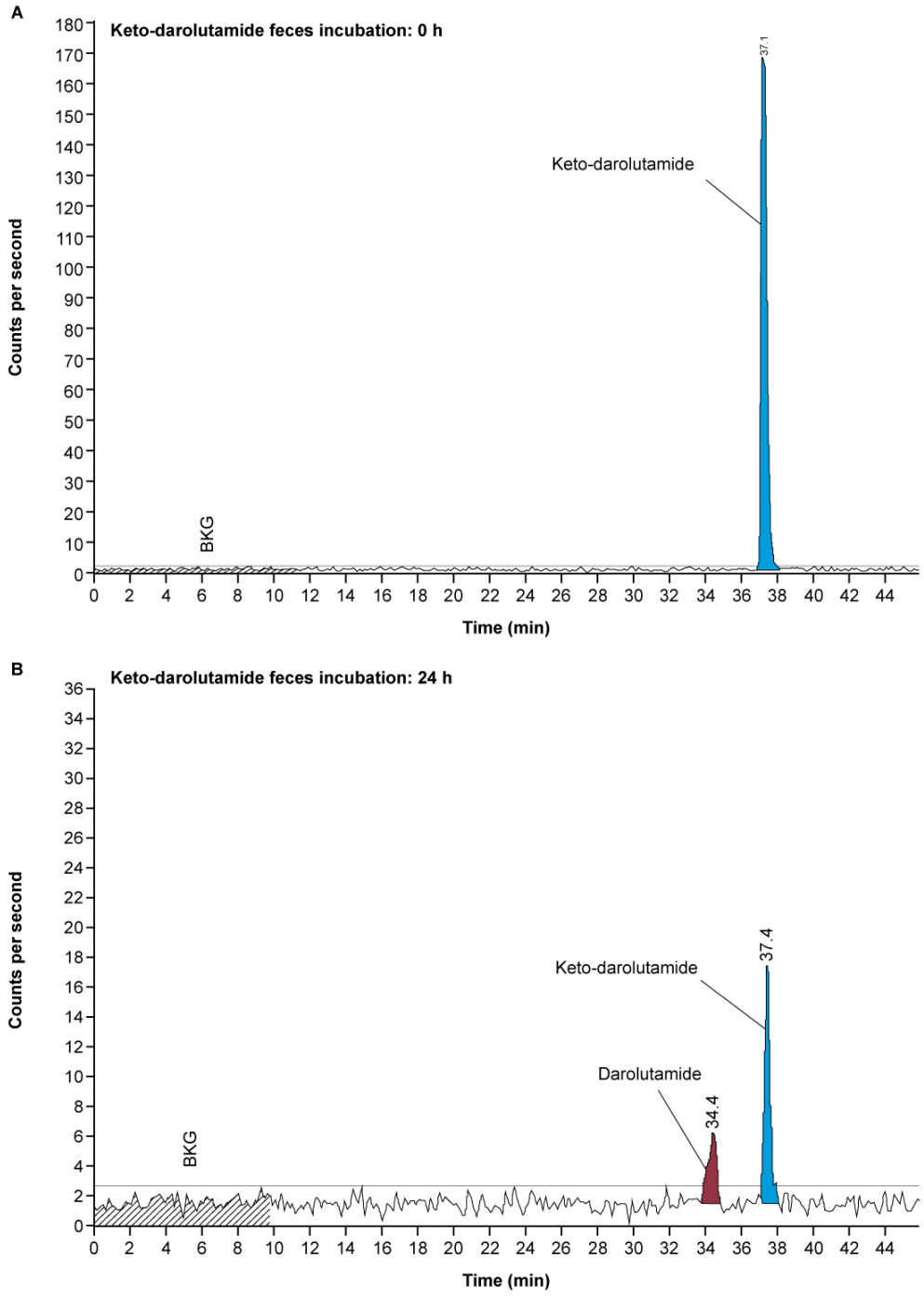
UGT isoform	**Correlation coefficient (r^2)**			
(specific substrate)	**M-7a**	**M-7b**	**M-15a**	**M-15b**
UGT1A1 (β-Estradiol)	0.38	0.64	0.11	0.13
UGT1A9 (Propofol)	0.54	0.65	0.10	0.12
UGT2B7 (*R*-Flurbiprofen)	0.44	0.28	0.53	0.51
UGT2B10 (Levomedetomedine)	0.57	0.59	0.83	0.84
UGT2B15 (*S*-Oxazepam)	0.34	0.19	0.48	0.46

UGT, Uridine-diphosphate-glucuronosyltransferase.

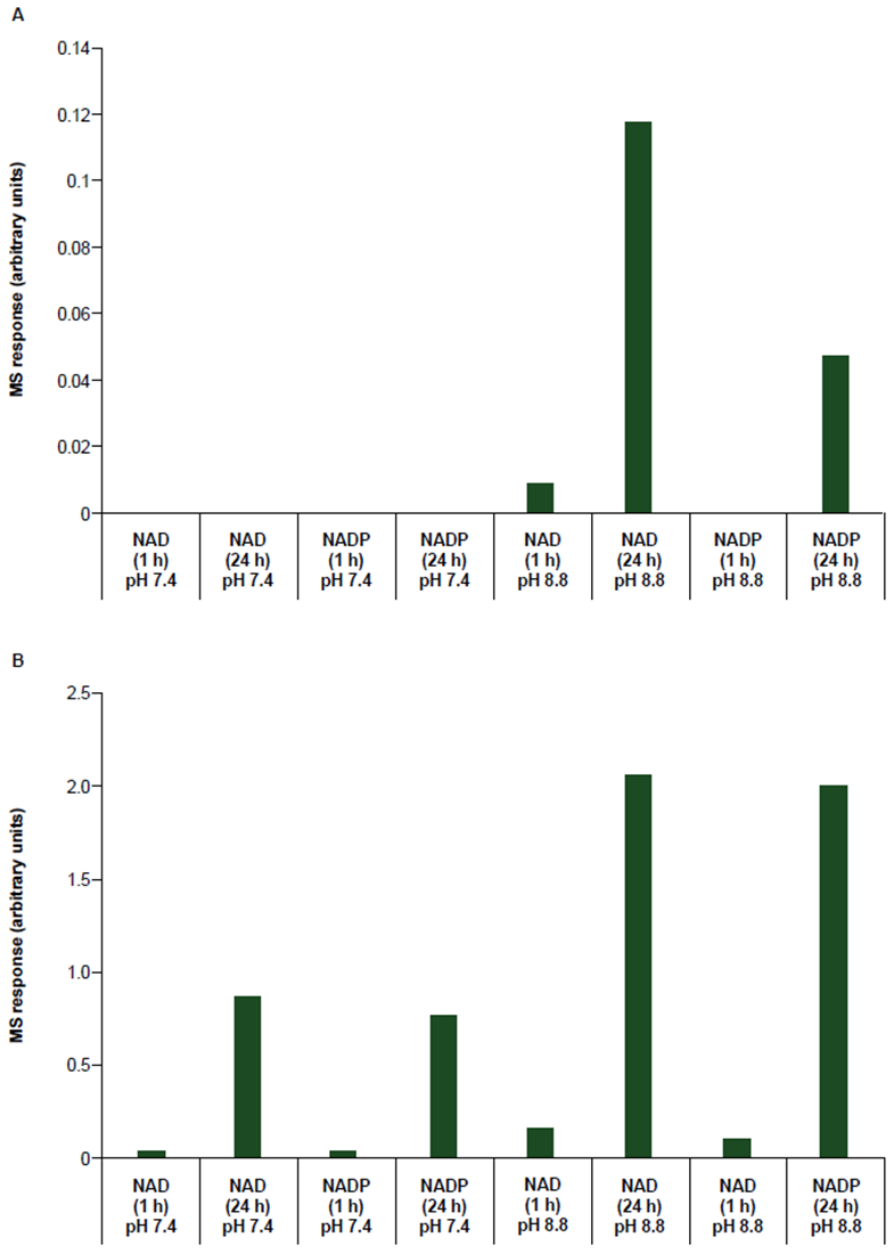
Supplementary Table S9. Contribution of UGT1A1 and UGT1A9 to M-7a and M-7b formation calculated based on experiments with chemical inhibitors and by relative activity factors (RAF)

	Chemical inhibition	**RAF**
	Relative contribution to M-7a formation (%)	
UGT1A1	22	33
UGT1A9	78	67
	Relative contribution to M-7b formation (%)	
UGT1A1	56	66
UGT1A9	44	34

Supplementary Fig. S1. HPLC pattern of ^{14}C-keto-darolutamide stock solution before (A) and after (B) incubation for 24 hours at 37°C with human feces at a concentration of 2 µM.

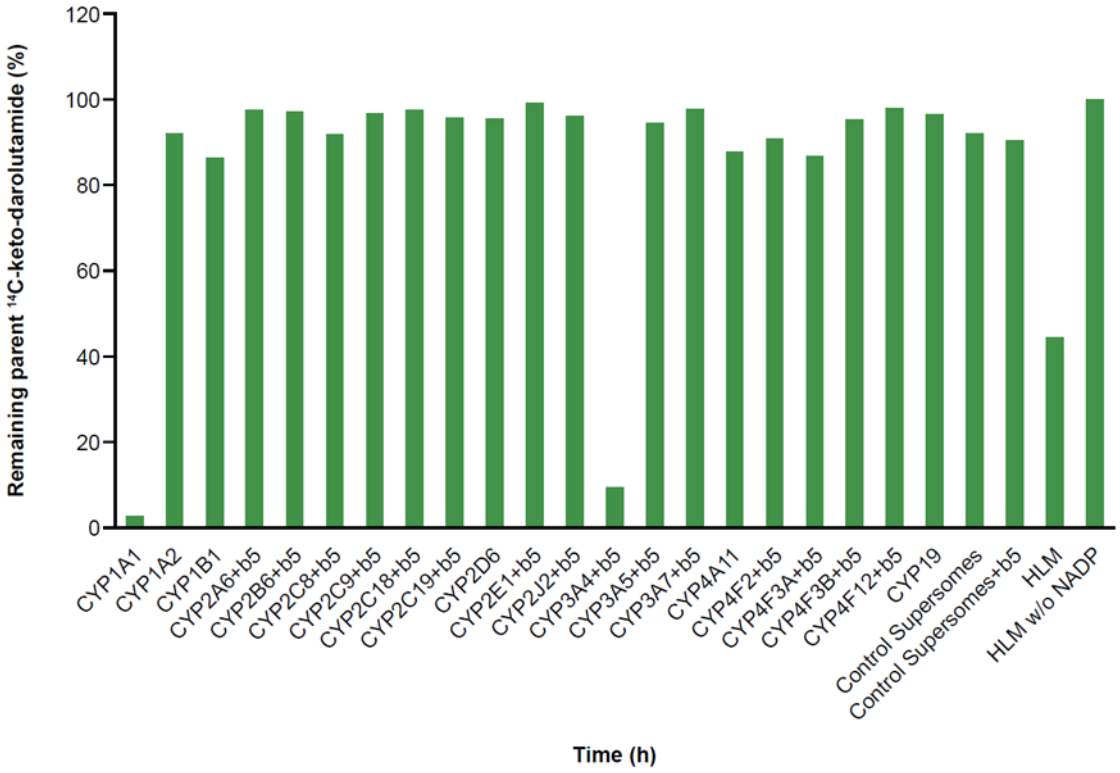


Supplementary Fig. S2. Oxidation of 1 µM (*S,R*)-darolutamide (A) and 1µM (*S,S*)-darolutamide (B) to keto-darolutamide in human liver cytosol in the presence of cofactors (NAD, NADP).



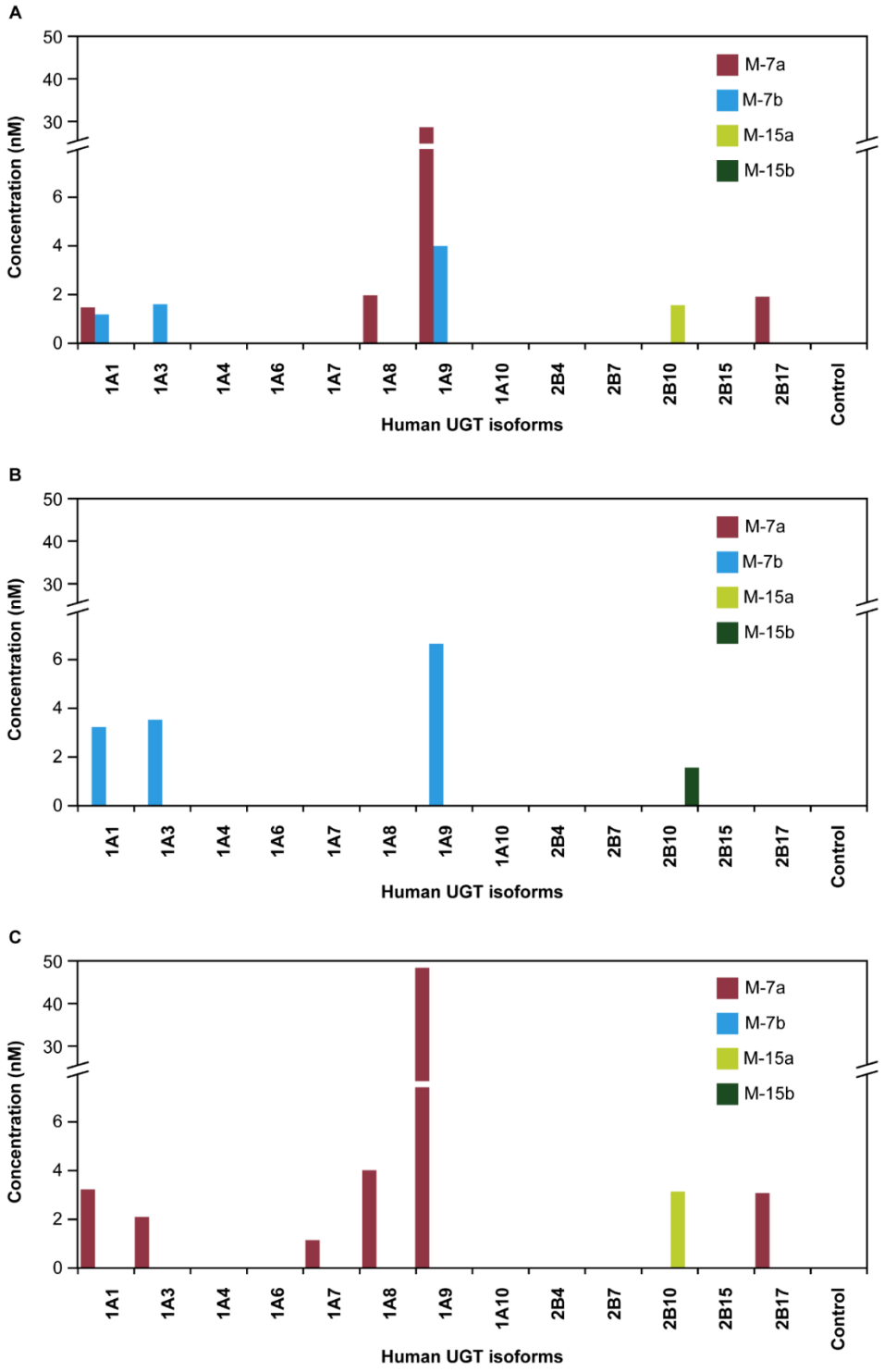
MS, mass spectrometry; NAD, nicotinamide adenine dinucleotide; NADP nicotinamide adenine dinucleotide phosphate.

Supplementary Fig. S3. Depletion of ^{14}C-keto-darolutamide (1 µM) after 1-hour incubation with a complete panel of all available recombinant CYP enzymes or human liver microsomes.



CYP, cytochrome P450; HLM, human liver microsomes; NADP, nicotinamide adenine dinucleotide phosphate.

Supplementary Fig. S4. Formation of glucuronides M-7a/b and M-15a/b catalyzed by various recombinant human UGT isoforms applying 1 µM darolutamide (A), (*S,R*)-darolutamide (B), and (*S,S*)-darolutamide (C). Only relevant activities above 1 nM glucuronide (0.1% substrate conversion) are shown.



Supplementary References

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Food and Drug Administration (2018) Bioanalytical method validation: guidance for industry.

Pang KS and Rowland M (1977) Hepatic clearance of drugs. III. Additional experimental evidence supporting the "well-stirred" model, using metabolite (MEGX) generated from lidocaine under varying hepatic blood flow rates and linear conditions in the perfused rat liver in situ preparation. *J Pharmacokinet Biopharm* **5**:681-699.