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

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ABSTRACT

The biotransformation and excretion of darolutamide were investigated in a phase I study. Six healthy male volunteers received a single dose of 300 mg 14C-darolutamide as an oral solution in the fasted state. Plasma, urine, and feces samples were analyzed for mass balance evaluation by liquid scintillation counting (LSC). Metabolite profiling and identification were determined using liquid chromatography mass-spectrometry with off-line radioactivity detection using LSC. Complete mass balance was achieved, with mean radioactivity recovery of 95.9% within 168 hours (63.4% in urine, 32.4% in feces). The administered 1:1 ratio of (S,R)- and (S,S)-darolutamide changed to approximately 1:5, respectively, in plasma. Darolutamide and the oxidation product, keto-darolutamide, were the only components quantifiable by LSC in plasma, accounting for 87.4% of total radioactivity, with a 2.1-fold higher plasma exposure for keto-darolutamide. Aside from darolutamide, the most prominent metabolites in urine were O-glucuronide (M-7a/b) and N-glucuronide (M-15a/b), as well as pyrazole sulfates (M-29, M-24) and glucuronides (M-21, M-22) resulting from oxidative cleavage of the parent. The darolutamide diastereomers were mainly detected in feces. In vitro assays showed that darolutamide metabolism involved a complex interplay between oxidation and reduction, as well as glucuronidation. Interconversion of the diastereomers involves oxidation to keto-darolutamide, primarily mediated by CYP3A4, followed by reduction predominantly catalyzed by cytosolic reductase(s), with aldo-keto reductase 1C3 playing the major role. The latter reaction showed stereoselectivity with preferential formation of (S,S)-darolutamide.

SIGNIFICANCE STATEMENT

The metabolism and excretion of darolutamide in humans revealed that oxidation (CYP3A4) and glucuronidation (UGT1A9, UGT1A1) were the main metabolic routes of elimination. Direct excretion also contributed to overall clearance. The two pharmacologically equipotent diastereomers of darolutamide interconvert primarily via oxidation to the active metabolite keto-darolutamide, followed by reduction predominantly by cytosolic reductase(s). The latter reaction showed stereoselectivity with preferential formation of (S,S)-darolutamide. Data indicate a low drug-drug interaction potential of darolutamide with inducers or inhibitors of metabolizing enzymes.

Introduction

Darolutamide (Nubeqa, formerly ODM-201) is a potent and selective nonsteroidal androgen receptor inhibitor developed as an immediate-release, oral, 300-mg tablet and is approved for the treatment of nonmetastatic castration-resistant prostate cancer (nmCRPC) (https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/212099Orig1s000lbl.pdf; https://www.ema.europa.eu/documents/product-information/nubeqa-epar-product-information_en.pdf; https://www.bayer.com/sites/default/files/2021-02/Bayer-Annual-Report-2020.pdf) based on significantly improved survival outcomes versus placebo in men with nmCRPC receiving androgen deprivation therapy (Fizazi et al., 2019, 2020). Darolutamide is a flexible pyrazole with polar substitutions and is structurally distinct from other androgen receptor inhibitors (Moilanen et al., 2015). Darolutamide is a 1:1 mixture of the two diastereomers, (S,R)-darolutamide and (S,S)-darolutamide, which interconvert via the major metabolite keto-darolutamide (Fig. 1) (Nykänen et al., 2020; Taavitsainen et al., 2020),...
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![Figure 1](image)

**Fig. 1.** Darolutamide diastereomers, (S,R)-darolutamide and (S,S)-darolutamide, interconvert via the major metabolite keto-darolutamide.

and all three compounds show similar pharmacological activity in vitro (Moilanen et al., 2015; Sugawara et al., 2019).

The present phase I study was conducted to fully characterize the pharmacokinetics, metabolic pathways, and excretion routes of darolutamide, determined after single administration of darolutamide to healthy male volunteers. The investigation of the pharmacokinetic characteristics of darolutamide after single intravenous and oral administration will be published separately. The evaluation of the metabolism and excretion of 14C-darolutamide in humans is reported herein. In addition, complementary in vitro studies conducted in human biomaterials to identify the relevant enzymes involved in the biotransformation of darolutamide and its major metabolite, keto-darolutamide, are presented.

**Methods**

**Phase I Study**

**Study Design.** The absorption, metabolism, routes of excretion, and mass balance of darolutamide and its metabolites were evaluated in a single-center, open-label phase I study performed by Quotient Clinical (Nottingham, UK) in six healthy male volunteers. The study was conducted in accordance with Good Clinical Practice Guidelines (https://database.ich.org/sites/default/files/E6_R2_Addendum.pdf). An independent ethics committee reviewed and approved the study protocol, and all subjects provided written, informed consent.

**Study Population.** Six healthy male subjects (five White, one Black) were enrolled in part 2 of the study and were included in the analyses. At baseline, mean age was 53.0 years (range 50.0–56.0 years), mean weight was 82.7 kg (range 63.7–91.0 kg), and mean body mass index was 27.1 kg/m² (range 24.0–30.4 kg/m²). None of the subjects were current smokers or consumed 14 units of alcohol per week. Subjects were discharged from the clinic on day 8, 168 hours postdose, having met the radioactivity release criteria, i.e., mass balance recovery of >90% had been achieved or two consecutive days showed <1% recovery.

**Study Medication.** On day 1, subjects received a single oral dose of approximately 300 mg 14C-darolutamide (actual range 301.3–302.2 mg) as an oral solution, containing on average 5.4-MBq 14C, after fasting for ≥10 hours overnight; the fasted state was maintained until approximately 4 hours postdose. The position of the 14C-label was at the phenyl moiety of darolutamide [the 14C-labeling of darolutamide and reference compounds are described in detail elsewhere (Taavitsainen et al., 2020)]. Dosimetry calculations were provided by Public Health England Centre for Radiation, Chemical and Environmental Hazards (Chilton, UK) according to local regulations. The associated radiation exposure fell within International Commission on Radiologic Protection (1992) guidelines for category IIA studies (0.1–1.0 mSv).

**Sample Collection.** Blood samples for evaluation of pharmacokinetic parameters, total radioactivity, and metabolite profiling and identification in plasma were collected predose and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 16, 24, 48, 72, 96, 120, 144, and 168 hours postdose. Whole blood was centrifuged to obtain the plasma fraction. In radioactive metabolite profiling, individual plasma samples were analyzed up to 48 hours postdose. For profiling and identification of metabolites that lacked the radioactive label because of metabolism, plasma samples (50 µL each) were pooled by individual time points across the subjects for analysis by liquid chromatography-mass spectrometry (LC-MS). Urine samples for determination of total radioactivity and metabolite profiling were collected over the following intervals: predose, 0–12 hours, and 12–24 hours after drug administration, and then for each 24-hour period up to 168 hours postdose. Each subject’s urine, collected in individual polypropylene containers during these intervals, was pooled prior to analysis to produce a single bulk sample for each interval per subject. Fecal samples for total radioactivity and metabolic profiling were collected predose and every 24 hours up to 168 hours postdose. Each subject’s samples were pooled per 24-hour

**ABBREVIATIONS:** ACN, acetonitrile; Aex, amount excreted; AKR, aldo-keto reductase; AUC, area under the concentration-time curve; eGFR, estimated glomerular filtration rate; HLC, human liver cytosol; HLM, human liver microsome; LC, liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; LSC, liquid scintillation counting; nmCRPC, nonmetastatic castration-resistant prostate cancer; P450, cytochrome P450; RAF, relative activity factor; SPE, solid phase extraction; t1/2, half-life; UGT, uridine-diphosphate glucuronosyltransferase.
time period prior to analysis of total radioactivity. All excreta samples were stored at least at -20°C until further analysis.

Sample Preparation for Metabolite Profiling and Identification. Full details of equipment and materials for metabolite profiling and quantitation are provided in Supplemental Table 1. Plasma, urine, and fecal samples were prepared for liquid chromatography (LC) analysis with off-line radioactivity detection (method B, Supplemental Table 2). Plasma samples for radioactive metabolite profiling (0.25–0.5 mL) were treated with equal volumes of ammonium formate (25 mM) containing 5% acetonitrile at pH 4 (solvent A) and 1 mL of acetonitrile and chilled for 10 minutes. The plasma samples were homogenized, and the proteins were separated from the supernatant by centrifugation (10 minutes, 3000g, 4°C). Subsequently, the supernatants were evaporated to dryness and dissolved in solvent A (method B, Supplemental Table 2) prior to analysis. Individual urine samples (per subject and collection period) were mixed thoroughly and diluted with solvent A (1:1) prior to analysis (method B, Supplemental Table 2). Fecal samples were wet homogenized before analysis of total 14C radioactivity and metabolite profiling. For metabolite profiling, homogenized aliquots (0.35–0.50 g) were subsequently extracted with acetonitrile (5 mL) for 1 hour and then centrifuged, and the supernatants were concentrated by evaporation to 1–4 mL and then diluted 1:1 with solvent A before analysis (method B, Supplemental Table 2). The mean recovery of total radioactivity extraction for the different sampling time points amounted to 98.2%. For profiling and identification of metabolites that lacked the radioactive label because of cleavage of the central amide bond of darolutamide, samples were prepared for LC-MS analysis (method C, Supplemental Table 2). Plasma samples were diluted with ammonium acetate buffer, pH 4, and proteins were precipitated using acetonitrile (100 μL of plasma, 100 μL of solvent A, and 400 μL of acetonitrile). Samples were then centrifuged, the supernatant was filtered into protein precipitation plates and centrifuged, and then the filtrate was concentrated. Samples were diluted with ammonium acetate buffer, pH 4, centrifuged, and the supernatant was transferred to vials for LC-MS analysis. Urine and fecal samples were prepared using the same procedure as described for radiolabeled metabolites.

Fecal samples were also prepared to investigate the stability of keto-darolutamide ex vivo by mimicking the anaerobic microbial environment of the gut. Under anaerobic conditions, 14C-keto-darolutamide (1 mM in acetonitrile) was added to a slurry of fresh human feces in degassed water and incubated for 24 hours at 37°C. The incubation mixture was stopped by adding acetonitrile and centrifuged, and the supernatant was subjected to LC with off-line radioactivity detection (method B, Supplemental Table 2).

Determination of Total Radioactivity. Total radioactivity concentrations in body fluids and excreta, and mass balance calculations 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), with automatic quench correction by the external standard channel ratio method at 13°C using Atomlight as scintillation cocktail. Lower limits of quantitation for total 14C radioactivity were 30.57 ng eq/mL in plasma, 7.17 ng eq/mL in urine, and 64.32 ng eq/mL in feces.

Metabolite Profiling in Human Plasma, Urine, and Feces. Metabolic profiles were investigated by analytical LC on reversed phase columns, followed by off-line radioactivity detection (method B, Supplemental Table 2). Radioactivity was detected using a TopCount NXT Microplate Scintillation and Luminescence Counter (Perkin Elmer, Boston). In the radiochrograms obtained, the drug diastereomers were not separated and were analyzed as the sum of both. Semiquantitative LC-MS analysis was performed to measure metabolites lacking the radioactive label (method C, Supplemental Table 2); their concentrations were calculated based on a calibration curve prepared from respective reference standards. The synthesis of the respective metabolite reference standards is described in the Supplemental Methods 1.

Determination of Darolutamide and Keto-Darolutamide. Concentrations of (S,R)-darolutamide, (S,S)-darolutamide, and keto-darolutamide in plasma and concentrations of the diastereomers in urine were determined using a validated (plasma) or qualified (urine) high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) analytical method at PRA Health Sciences (Assen, The Netherlands) (method A, Supplemental Table 2). The lower limit of quantification for keto-darolutamide and both diastereomers in plasma and urine was 5 ng/mL. Concentrations of darolutamide were calculated as the sum of the two diastereomers, (S,R)- and (S,S)-darolutamide. Quality criteria of these bioanalytical methods are described in detail in the Supplemental Methods 2 and Supplemental Table 3. Measurement of (S,R)-darolutamide and (S,S)-darolutamide in samples from human feces was done by LC-MS analysis (method D, Supplemental Table 2).

Pharmacokinetic Evaluation. Pharmacokinetic analysis of the plasma concentration versus time data as well as from metabolic profiles was performed using appropriate noncompartmental techniques to obtain estimates of parameters including exposure [area under the concentration-time curve (AUC0–inf, AUC0–tlast, Cmax, time when maximum plasma concentration is observed, and apparent terminal half-life (t1/2)]. For urine, the amount excreted (A), the renal clearance, and the excretion t1/2 were calculated.

In addition, renal clearance of darolutamide was further investigated. The estimated glomerular filtration rate (eGFR) in the individual subjects was estimated by applying the Chronic Kidney Disease Epidemiology Collaboration formula (Levey et al., 2009) using individual physical information of the subjects (i.e., age, sex, and ethnic origin) and a normalized value for the body surface area of 1.73 m². This individual eGFR was used as a marker for passive glomerular filtration and tubular secretion. The renal clearance of (S,S)- and (S,R)-darolutamide was calculated based on drug amounts excreted and the unbound plasma exposure [AUC0–inf] (Brater, 2002).

In Vitro Studies

In Vitro Metabolic Profiling and Substrate Characteristics. Several studies were conducted to investigate the biotransformation of darolutamide and its diastereomers in vitro in human cryopreserved suspension hepatocytes (from different donors), subcellular liver preparations (pool), and recombinant enzymes. Full details of materials for the in vitro studies are provided in Supplemental Table 1. Depending on the purpose of the experiment, the starting material was darolutamide (14C-labeled or nonlabeled), its diastereomers, or keto-darolutamide (14C-labeled or nonlabeled), and the initial concentration range was 1–50 μM, with 1 μM used in most experiments. The total incubation time varied between different experiments. Several samples were typically withdrawn during incubation, which were quenched with acetonitrile and stored cold until analysis. After sample preparation using appropriate techniques, the supernatants were subjected to LC with LSC for radioactivity detection and LC-MS or LC-MS/MS analysis. For darolutamide, both nonchiral and chiral analyses were used. When both diastereomers were analyzed separately, total darolutamide was calculated as the sum of these two measured concentrations. 14C-Darolutamide was incubated in suspension hepatocytes from several donors for up to 4 hours to obtain quantitative in vitro metabolic profiles. Hepatocyte incubations in the presence of the CYP3A4 inhibitor itraconazole were performed to evaluate the proposed main metabolic pathway. To quantify substrate depletion and metabolic formation, 14C-darolutamide was incubated in human liver microsomes.
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(HLM) and a panel of 21 recombinant human P450 isoforms (Supplemental Table 1). CYP3A4-specific inhibitors (ketoconazole, cobicistat) were also applied in microsomal incubations. Additionally, incubations of human liver cytosol (HLC) and recombinant enzymes under different pH and cofactor conditions were used to evaluate the contribution of non-P450 enzymes in darolutamide oxidation.

Keto-darolutamide (14C-labeled or nonlabeled) metabolism was studied in incubations of hepatocytes, HLM, HLC, and several recombinant enzymes (P450, aldo-keto reductase (AKR)). The CYP3A4 inhibitor irtraconazole and AKR inhibitors flufenamic acid (AKR1C1-4) and EM1404 (AKR1C3; synthesized at Bayer AG, Germany) were included in selected incubations. Both darolutamide diastereomers and other metabolites were analyzed from these incubations.

The interconversion of darolutamide diastereomers via keto-darolutamide was investigated in suspension hepatocytes with (S,R)-darolutamide, (S,S)-darolutamide, or keto-darolutamide as starting material. In this context, the AKR1C3 inhibitor BAY 1128688 (synthesized at Bayer AG, Germany) and the CYP3A4 inhibitor cobicistat were used to evaluate the contribution of these pathways. Diastereomer-specific analytical methods were applied to these incubations.

To assess the role of glucuronidation in darolutamide biotransformation, darolutamide and both diastereomers were incubated with human liver, kidney, and intestinal microsomes and a panel of 13 recombinant uridine-diphosphate glucuronosyltransferase (UGT) isoenzymes (Supplemental Table 1). Incubations were fortified with uridine 5′-diphospho-glucuronic acid, 50 μg amalicetic acid per milligram microsomal protein, and MgCl2 (5 mM). Experiments were performed in the presence and in the absence of enzyme-selective inhibitors to evaluate the relevance of specific pathways. To confirm the relative contribution of UGT1A1 and UGT1A9 in the overall glucuronidation process of darolutamide, the relative activity factor (RAF) method was used (Gibson et al., 2013). In short, RAFs were determined based on propofol (UGT1A9) and estradiol (UGT1A1) glucuronidation activities in HLM relative to the activities in recombinantly expressed UGT1A9 and UGT1A1, respectively. Relative contribution was calculated by applying RAF values to the glucuronidation activity of (S,R)- and (S,S)-darolutamide determined in microsomes from baculovirus-transfected insect cells expressing human UGT isoenzymes.

For correlation analysis, HLM from 22 individual donors were characterized by incubation with the UGT isoform-selective substrates β-estradiol (UGT1A1), propofol (UGT1A9), R-flurbiprofen (UGT2B7), levomeodetomidine (UGT2B10), and S-oxazepam (UGT2B15). The glucuronidation activities were correlated with the formation rates of M-7a/b and M-15a/b (substrate BAY 1841788) in the respective panel of microsomes.

Suppliers of the materials used in in vitro assays are listed in Supplemental Table 1.

Quantification of Darolutamide and Metabolites In Vitro.

Metabolite profiles (radiochromatograms) were obtained using LC with radioactivity detection, with generation of metabolite pattern and integration of individual peaks (method F, Supplemental Table 4). LC-MS methods were applied for quantification of darolutamide and keto-darolutamide, chromatographic separation of the diastereomers, and quantitation of (S,R)- and (S,S)-darolutamide. Individual conditions of the employed methods (e.g., stereoselective LC) were dependent on the analytical question. For evaluation of the role of glucuronidation in darolutamide biotransformation, metabolites were quantified using LC-MS/MS methodology, and radioactivity was measured using LSC (method H, Supplemental Table 4). Bioanalytical methods are summarized in Supplemental Table 4.

Metabolite Identification. Chemical structures of the metabolites were identified or elucidated by LC-MS/MS based on retention times, exact mass, product ion spectra, and molecular formula and by comparison with authentic metabolite standards (M-1, M-26, M-30, M-32, M-33, and M-36). Structural proposals based on mass spectra interpretation were generated with a QExactive Plus high-resolution mass spectrometer (method B, Supplemental Table 2) using an electrospray ionization source and applying high collisional dissociation experiments to render product ion spectra. Electrospray ion voltage analysis was performed in both positive and negative ionization modes. In addition, in vitro samples of 14C-darolutamide in hepatocytes and recombinant P450 enzymes for metabolite profiling were analyzed by methods F and G, respectively (Supplemental Table 4). Furthermore, metabolites M-7a, M-15a, M-21, and M-22 were isolated from urine and characterized by NMR.

Isolation and NMR Identification of Metabolites M-7a, M-15a, M-21, and M-22. Selected metabolites were isolated from pooled human urine (day 1, 0–12 hours). Urine was diluted with an equal volume of 0.1% formic acid and applied onto a C18 solid phase extraction (SPE) cartridge. Washing and elution was performed with acetonitrile (ACN)/0.2% formic acid mixture by gradually increasing the solvent component up to 100% ACN. The fractions with the highest radioactivity were concentrated and subsequently applied onto a C18-E SPE cartridge for further purification. Again, the SPE fractions with the highest radioactivity were retained for metabolite isolation by high-performance LC with a diode-array detector using a preparative column (Nucleodur C18 Gravity, 250 × 21 mm, 5 μm). Using an isocratic elution with ACN/50 mM ammonium acetate (78:22, v/v) as mobile phase (flow: 20 mL/min), two fractions were collected containing respective diastereomeric mixtures of M-7a and M-7b (S,S, R = 92:8) and M-15a and M-15b (S,S, R = 87:13). Further purification of M-7a and M-15a was achieved by additional preparative high-performance LC with a diode-array detector using isocratic elution with ACN/0.2% formic acid (M-7a: 73:27 [v/v] and M15a: 75:25 [v/v]). M-21 and M-22 were isolated from different SPE fractions using a gradient elution with ACN/0.2% formic acid (20:80 at 0 minutes, 80:20 at 28 minutes). Purified fractions were then concentrated until dry under reduced pressure. Approximately 1 mg of each isolated metabolite was dissolved in CD3CN/D2O (70:30 v/v) for NMR on a 600-MHz Bruker Ultrashield Magnet operating with an Avance III Bruker console using a 5-mm QNP Cryo Probe. 1H spectra were recorded at 600.1 MHz, and 13C spectra were recorded at 150.9 MHz. The residual CD3CN signal was used for spectral reference. For structural elucidation, 1H, 13C, distortionless enhancement by polarization transfer 90 and 135, correlated spectroscopy, heteronuclear multiple-bond correlation, and heteronuclear single-quantum correlation spectra were recorded and analyzed.

Results

Phase I Study

Darolutamide Pharmacokinetics. After administration of a single dose of 300 mg darolutamide as an oral solution, plasma concentrations were quantifiable (by method A in Supplemental Table 2) at the first time point (0.25 hours postdose), and Cmax was reached after 1.5 hours (range 1.0–3.0 hours) (Fig. 2; Table 1). Thereafter, plasma concentrations declined in a biphasic manner with a terminal t1/2 of 10.6 hours. Darolutamide was administered as a 1:1 mixture of the two diastereomers, (S,R)-darolutamide and (S,S)-darolutamide. In vivo, the ratio of drug diastereomers changed to 0.185 (approximately 1:5), based on the plasma AUC0–inf data, which were determined separately for both diastereomers in the LC-MS/MS bioanalytical determination (Table 1; method A in Supplemental Table 2). Mean terminal t1/2 for (S,R)-darolutamide was shorter than for (S,S)-darolutamide.

Urinary and Fecal Excretion of Darolutamide and Its Metabolites. After administration of 300 mg 14C-darolutamide, excretion of
radioactivity was rapid and relatively consistent across subjects. Mean recovery of 95.9% (range 92.6%–98.1%) was reached by the end of the sampling period (168 hours), indicating complete mass balance (Fig. 3A); 63.4% (range 59.5%–68.9%) of total radioactivity was recovered in the urine, and 32.4% (range 28.8%–35.9%) was recovered in the feces. Within 24 hours of dosing, 50.0% of the dose was recovered in the urine, with over 70% recovered in both urine and feces within 48 hours and <1% recovered in the urine after 3 days postdose.

Based on LC-MS/MS analysis (method A, Supplemental Table 2), the cumulative excreted amount of (S,R)-darolutamide was smaller than that of (S,S)-darolutamide in urine (Fig. 3B), which reflects the observed ratio of the two diastereomers in plasma. In this analysis, 7.2% of the administered dose of darolutamide was recovered as darolutamide diastereomers in the urine, consisting of 1.5% (S,R)-darolutamide and 5.7% (S,S)-darolutamide (Fig. 3B). Using LC separation with subsequent off-line radioactivity detection for the investigation of the metabolite profile resulted in a very similar value of 6.7% excreted as (S,R)-darolutamide in plasma (method B, Supplemental Table 2). The renal clearance of (S,R)-darolutamide and (S,S)-darolutamide in plasma was 1.58-fold (range 0.93–2.07) and 1.07-fold (range 0.65–1.32) greater, respectively, than the eGFR determined for the individual subjects. The mean ratio of (S,R)-darolutamide to (S,S)-darolutamide in feces was 1:7, determined by an exploratory LC-MS method (method D, Supplemental Table 2), also showing a preference for (S,S)-darolutamide.

**Metabolite Pattern of Darolutamide in Plasma and Excreta.** The profile of darolutamide and its metabolites in plasma obtained by LC with radioactivity detection (method B, Supplemental Table 2) is shown in Fig. 4A. Darolutamide and its major metabolite, the oxidation product keto-darolutamide, were the only components quantifiable by LC separation with off-line radioactivity detection in human plasma. Based on AUC_{0-inf} (Table 1), the sum of both compounds in plasma accounted for 87.4% (28.6% darolutamide and 58.8% keto-darolutamide) of total 14C-radioactivity [AUC_{0-inf}; 159 μg eq. h/mL]; plasma exposure was 2.1-fold higher for keto-darolutamide versus darolutamide. Other metabolites circulating in plasma, i.e., drug glucuronides of darolutamide as well as oxidation products, have only been identified by radioactivity detection at trace levels and did not contribute to the overall exposure to a relevant extent. Detection of nonradiolabeled pyrazole metabolites in the urine (see below), which are likely to be present in plasma prior to urinary excretion, prompted evaluation of plasma for the presence of these metabolites. LC-MS analysis (method C, Supplemental Table 2) detected low levels of the respective nonradiolabeled metabolites M-32, M-33, M-34, and M-36 in plasma.

Radioanalysis for metabolite profiling and identification showed that the urine excretion pattern of darolutamide was more complex than that observed in plasma. The radioactive dose was mostly excreted in urine as O-glucuronides (M-7a, M-7b; 25.6% of dose), N-glucuronides (M-15a, M-15b; 5.8% of dose), and the pyrazole sulfamate (M-29; 6.2% of dose), as well as darolutamide diastereomers (6.7% of dose, as noted above) (Fig. 4B; Table 2). No relevant amount of keto-darolutamide could be identified in the samples investigated. The absolute structure and stereochemistry of metabolite M-7a and M-15a was confirmed by NMR.

**TABLE 1**

Selected pharmacokinetic parameters of darolutamide, its diastereomers, and keto-darolutamide after a single dose of 14C-darolutamide (300 mg) as oral solution

| Plasma PK Parameter       | Total 14C Radioactivity | Parent Darolutamide | (S,R)-Darolutamide | (S,S)-Darolutamide | Keto-Darolutamide |
|---------------------------|-------------------------|---------------------|-------------------|-------------------|-------------------|
| AUC_{0-inf} (h.μg/mL)     | 159 (0.02)              | 45.6 (0.02)         | 7.11 (0.03)       | 38.3 (0.02)       | 93.7 (0.02)       |
| C_{max} (μg/mL)           | 16.5 (0.02)             | 3.82 (0.02)         | 1.81 (0.02)       | 2.49 (0.02)       | 11.7 (0.02)       |
| T_{max} (h)               | 1.5 (1.0–2.0)           | 1.5 (1.0–3.0)       | 1.0 (0.5–1.5)     | 3.0 (1.5–4.0)     | 1.5 (1.0–1.5)     |
| t_{1/2} (h)               | 11.5 (24.5)             | 10.6 (22.6)         | 6.58 (20.7)       | 10.7 (20.5)       | 10.7 (31.4)       |
| Analyte-to-parent ratio   | NC                      | 0.185 (14.7)        | 0.156 (12.4)      | 0.841 (2.2)       | 2.06 (11.1)       |

Further details are in the text.
with material isolated from urine (Supplemental Table 5). This material was further used to chromatographically assign and differentiate between the O- and N-glucuronide diastereomers M-7a/M-7b and M-15a/M-15b. Using LC high-resolution mass spectrometry (method C, Supplemental Table 2), metabolites that lost the 14C-label because of cleavage of darolutamide and/or keto-darolutamide were identified and evaluated in urine: these structural counterparts to metabolites as M-21, M-22, M-24, and M-29 accounted for 3.2%, 3.8%, 4.2%, and 6.2% of dose, respectively. Overall, 94.0% of the radioactivity present in urine samples (59.6% of the dose) could be assigned to known structures.

The ratios of O-glucuronides M-7a to M-7b and N-glucuronides M-15a to M-15b, identified using an LC-MS method enabling chiral separation (method E, Supplemental Table 2), increased over time in all subjects. Noting that “a”-suffixed glucuronides originate from (S,S)-darolutamide and “b”-suffixed glucuronides originate from (S,R)-darolutamide, there was an overall preference for formation of the (S,S)- over the (S,R)-diastereomer, with 14-fold (M-7a/M-7b) and 8-fold (M-15a/M-15b) greater amounts formed, respectively (Table 2).

The radioactivity found in fecal extracts was mainly assigned as darolutamide diastereomers, keto-darolutamide (M-1), and carboxylic acid (M-28, formed by oxidation of keto-darolutamide), with 30.3%, 0.6% and 1.0% of dose, respectively (Fig. 4C; Table 2). Overall, 98.4% of the radioactivity present in fecal samples (31.9% of dose) could be attributed to known structures. When the stability of keto-darolutamide was investigated in human feces samples under anaerobic conditions, results indicated that keto-darolutamide was prone to reduction to darolutamide in the environment of the gastrointestinal tract (Supplemental Fig. 1).

Overall, the metabolite profiles of darolutamide in urine and feces revealed darolutamide diastereomers, O- and N-glucuronides of the

![Graph A](image_url)  
**Fig. 3.** Cumulative (A) excretion of total radioactivity in urine and feces and (B) excretion and recovery of darolutamide and its diastereomers in urine after a single dose of 14C-darolutamide (300 mg) as oral solution. Data are presented as arithmetic means for cumulative percentage of amounts excreted (n = 6).
Fig. 4. 14C-Darolutamide metabolic profiles in (A) plasma collected at 3 hours postdose, (B) urine collected from 12 to 24 hours postdose, and (C) feces collected from 48 to 72 hours postdose from an individual participant after oral administration of 300 mg darolutamide containing 5.4 MBq 14C-darolutamide (obtained using nonchiral LC with off-line radioactivity detection).
diastereomers (M-7 and M-15), and several phase I biotransformation products resulting from cleavage of the drug molecule into separate moieties, with the labeled metabolites M-21, M-22, M-24, and M-29 on one side and the corresponding unlabeled metabolites M-32, M-33, M-34, and M-36 on the other side. Molecular and key fragment ions of darolutamide and metabolites are presented in Supplemental Table 6. A schematic representation of the proposed metabolic pathway is depicted in Fig. 5.

**In Vitro Studies**

**Metabolite Profile in Human Hepatocytes.** After an incubation of \(^{14}\)C-darolutamide with human hepatocytes, the most abundant metabolites were keto-darolutamide and \(O\)-glucuronides (M-7 isomers; analysis using method G, Supplemental Table 4). Only minor formation of other metabolites was found (Table 3). Applying itraconazole as a CYP3A4 inhibitor in hepatocytes, the metabolic clearance of darolutamide was reduced by approximately 30% (Supplemental Table 7), confirming involvement of CYP3A4.

**Oxidative and Reductive Metabolism and Interconversion of** Diastereomers of Darolutamide. Oxidative and reductive metabolism of darolutamide and the mechanisms of the interconversion of the two diastereomers were investigated in various in vitro assays (analyzed with LC-MS method I and J, Supplemental Table 4).

The oxidation of darolutamide was studied by incubation of \(^{14}\)C-darolutamide in HLM as well as a panel of 21 recombinant human P450 isoforms. CYP3A4 and, to a lesser extent, CYP1A1 were the main enzymes catalyzing the depletion of \(^{14}\)C-darolutamide, with predominant formation of keto-darolutamide (Fig. 6). In addition, formation of the metabolites M-26, M-30, and M-31 by further oxidation and cleavage of the molecule was observed (Fig. 5). Oxidative formation of these metabolites was also mediated via CYP3A4 and to a very minor extent via other recombinant P450 isoforms. The role of CYP3A4 in the formation of keto-darolutamide was confirmed using CYP3A4-specific inhibitors (itraconazole, cobicistat) in microsomal incubations.

Minor amounts of keto-darolutamide were also observed in HLM and HLC incubations of darolutamide in the presence of the cofactors NAD and NADP. Despite the very slow formation rate compared with P450-mediated metabolism, these results suggest that other cytosolic and membrane-associated enzymes can also form keto-darolutamide (Fig. 7). After applying different assay conditions, the results suggest that alcohol dehydrogenase (pH- and NAD-dependent metabolism) and AKRs (NADP-dependent metabolism) are able to form minor amounts of keto-darolutamide from darolutamide. Notably, when incubating the diastereomers individually, the (S,S)-diastereomer appears to be the preferred substrate for these non-P450 oxidation pathways (Supplemental Fig. 2).

The oxidation and reduction of keto-darolutamide was studied in HLM, HLC, and several recombinant enzymes. The reduction back to darolutamide was efficient in HLC in the presence of reduced NADP (NADPH), and it was sensitive to the AKR inhibitors flufenamic acid (AKR1C1–4) and EM1404 (AKR1C3) (Fig. 8). Diastereomer-specific analysis suggested that (S,S)-darolutamide is specifically produced by cytosolic enzymes, whereas both diastereomers were detected in incubations of HLM and hepatocytes (Fig. 9). Recombinant AKR1C1–1C4 incubations confirmed the dominant role of AKR1C3 in reductive metabolism (Fig. 8). Furthermore, AKR1D1 was observed to have some activity (data not shown). HLM incubation using keto-darolutamide as substrate revealed both reduction and oxidation (M-30, M-31, M-26, M-25; Fig. 5). Several P450 isoforms are able to metabolize keto-darolutamide, as shown in incubations applying recombinant enzymes (Supplemental Fig. 3).

The interconversion of diastereomers was studied in human hepatocytes using diastereomer-specific LC-MS analytics (method I, Supplemental Table 4) after incubation of the substrates (S,R)-darolutamide, (S,S)-darolutamide, and keto-darolutamide in the presence and absence of AKR1C3 inhibitor BAY 1128688 or CYP3A4 inhibitor cobicistat. In the presence of cobicistat, depletion of (S,R)-darolutamide and (S,S)-darolutamide was less pronounced compared with the presence of no inhibitor (Fig. 10A), indicating the involvement of CYP3A4 in the metabolism of both diastereomers. The depletion of keto-darolutamide was not significantly impacted by cobicistat, whereas the addition of BAY 1128688 resulted in a distinctly slower depletion of keto-darolutamide (Fig. 10A), indicating that reduction via AKR1C3 plays the major role in the metabolism of keto-darolutamide. In the presence of BAY 1128688, there was minor inhibition of (S,R)-darolutamide depletion, and further depletion of (S,S)-darolutamide

### TABLE 2

Metabolite profile of darolutamide in excreta, expressed as percentage of the administered dose, as found in urine and feces 0–168 h after administration of \(^{14}\)C-darolutamide (300 mg) as oral solution

| Component | Type | Dose<sup>a</sup> | %   | %   |
|-----------|------|-----------------|-----|-----|
| M-1       | Oxidation product (keto-darolutamide) | -<sup>b</sup> | 0.6 |     |
| M-2       | Hydroxylated and glucuronidated metabolite | 1.3 | -<sup>c</sup> |     |
| M-7a      | O-glucuronide | 23.8 | -<sup>c</sup> |     |
| M-7b      | O-glucuronide | 1.8 | -<sup>c</sup> |     |
| M-10      | Glucuronidation product of M-1 | 0.6 | -<sup>c</sup> |     |
| M-15a     | N-glucuronide | 5.0 | -<sup>c</sup> |     |
| M-15b     | N-glucuronide | 0.7 | -<sup>c</sup> |     |
| M-21      | Dealkylated product of oxidative cleavage | 3.2 | -<sup>c</sup> |     |
| M-22      | Dealkylated product of oxidative cleavage | 3.8 | -<sup>c</sup> |     |
| M-24      | Dealkylated product of oxidative cleavage | 4.2 | -<sup>c</sup> |     |
| M-28      | Oxidation product of M-1 | 2.4 | 1.0 |     |
| M-29      | Dealkylated product of oxidative cleavage | 6.2 | -<sup>c</sup> |     |
| Darolutamide | -<sup>c</sup> | 6.7 | 30.3 |     |
| Other     | -<sup>c</sup> | 3.8 | 0.5 |     |
| % A<sub>U</sub> (% of dose) | 63.4 | 32.4 |     |     |

<sup>a</sup> Percentage of oral dose.
<sup>b</sup> Not found in urine.
<sup>c</sup> Not found in feces.
occurred. Formation of the respective metabolites in this interconversion process is summarized in Fig. 10B. Applying (S,R)-darolutamide as starting material, clear time-dependent formation of keto-darolutamide as well as (S,S)-darolutamide was observed, whereas the formation of (S,R)-darolutamide from starting material (S,S)-darolutamide was much lower. This was confirmed when keto-darolutamide was used as a substrate. A rapid increase in (S,S)-darolutamide was observed, reaching maximum concentration after 30 minutes of incubation, with very low simultaneous formation of (S,R)-darolutamide. This observation indicates preferred formation of (S,S)-darolutamide in hepatocytes.

Thus, the oxidative metabolism in hepatocytes of both darolutamide diastereomers leading to formation of keto-darolutamide is mediated by CYP3A4, AKR1C3 stereoselectively catalyzes the reduction of keto-darolutamide to (S,S)-darolutamide, and other reductases contribute to formation of (S,R)-darolutamide.

Glucuronidation. In experiments with recombinantly expressed UGTs, glucuronidation of darolutamide to yield the diastereomeric O-glucuronides M-7a and M-7b was predominantly catalyzed by UGT1A9 and, to a lesser extent, by UGT1A8, UGT2B17, UGT1A7, UGT1A3, and UGT1A1 (Supplemental Fig. 4). Formation of the diastereomeric N-glucuronides M-15a and M-15b was mediated solely by UGT2B10. Experiments in which the single diastereomers were investigated separately were in good agreement with those performed with darolutamide.

Greater amounts of the drug glucuronides were formed in liver microsomes than in either renal or intestinal microsomes, and the metabolite pattern differed among the tested tissues (Fig. 11; analyzed with method H, Supplemental Table 4). In liver microsomes, the O-glucuronide M-7a and N-glucuronides M-15a [glucuronides of (S,S)-darolutamide] were formed in an approximately 1:1 ratio, and their formation was more pronounced than that of M-7b and M-15b [glucuronides of (S,R)-darolutamide]. Renal and intestinal microsomes showed a high preference for the formation of M-7a over M-7b, and levels of M-15a and M-15b were very low because of the absence of UGT2B10 in these tissues.

In vitro experiments using HLM in the presence of UGT isoform-selective inhibitors further evaluated the relative contribution of single UGT isoforms to darolutamide glucuronidation. Partial inhibition of M-7a and M-7b formation by atazanavir (a UGT1A1 and UGT1A3 inhibitor; 10 μM) and niflumic acid (a UGT1A9 inhibitor; 10 μM) indicated that UGT1A9, UGT1A1, and UGT1A3 are predominantly responsible for O-glucuronidation of darolutamide. The combination of both inhibitors reduced M-7a and M-7b formation by approximately 80% and 75%, respectively. In the presence of the UGT2B10-selective inhibitor desloratadine, formation of M-15a and M-15b was reduced versus control by 79% and 62%, respectively, confirming that UGT2B10 is the major isoform mediating N-glucuronidation. A correlation analysis of experiments using HLM from individual donors (see Supplemental Table 8) confirmed the involvement of UGT2B10 in M-15a and M-15b formation ($r^2 = 0.83$ and $r^2 = 0.84$, respectively; substrate: levomedetomidine).

To further evaluate the relative contribution of UGT1A1 and UGT1A9 to the glucuronidation of darolutamide, RAFs in HLM were determined for both diastereomers of darolutamide (see Supplemental Table 9). The relative contributions of UGT1A1 and UGT1A9 to (S,R)-

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**TABLE 3**

Metabolite profiles in incubations of $^{14}$C-darolutamide (5 μM) with human hepatocytes from three donors (suspension incubation for 3 h, 1 million cells/mL, selected metabolites)

| Metabolite                  | Mean of Total Radioactivity (Yield) (%) |
|----------------------------|----------------------------------------|
| Darolutamide               | 70.7                                   |
| Keto-darolutamide          | 21.5                                   |
| M-7a                       | 3.7                                    |
| M-7b                       | 2.2                                    |
| Others*                    | 1.8                                    |

*Coelution with two additional hydroxylated metabolites (M-6, M-5).

*Comprises M-2, M-3, M-8, and other unidentified compounds.
and (S,S)-darolutamide glucuronidation were in good accordance with the values calculated based on chemical inhibition experiments.

**Discussion**

The presented phase I study in which healthy male subjects received 300 mg $^{14}$C-darolutamide as an oral solution, supported by in vitro data also presented here, together provide extensive information about the pharmacokinetics, biotransformation, and excretion of darolutamide and its metabolites.

After oral solution administration, darolutamide was rapidly absorbed. Parent drug and keto-darolutamide accounted for over 87% of total $^{14}$C-radioactivity in plasma, with keto-darolutamide contributing the majority. The equal ratio of the two diastereomers in the administered dose changed to a 1:5 ratio favoring (S,S)-darolutamide in plasma. In vitro studies in human hepatocytes showed that this change is due to an enzymatic interconversion process: the conversion of (S,R)-darolutamide to (S,S)-darolutamide was rapid and distinct, whereas the conversion from (S,S)-darolutamide to (S,R)-darolutamide was less pronounced. Furthermore, the (S,R)-diastereomer was found to have a shorter half-life in vivo than the (S,S)-diastereomer. We have previously shown in rats in vivo that, after dosing of one individual diastereomer, the other diastereomer also appears, with a slight preference for (S,S)-darolutamide formation after repeated dosing (Taavitsainen et al., 2020). The diastereomer interconversion takes place in each species studied (including mice, rats, dogs, and humans), with the prevailing diastereomer differing between species (Nykänen et al., 2020; Taavitsainen et al., 2020) (data on file).

Excretion of darolutamide and its biotransformation products is rapid and complete. Approximately two-thirds of the administered dose was excreted via the kidneys in urine. Only small amounts of approximately 7% of the administered dose were excreted as the two diastereomers in

![Fig. 6. Depletion of $^{14}$C-darolutamide (1 $\mu$M) after 1-hour incubation with a complete panel of all available recombinant P450 enzymes (100 pmol/mL) or human liver microsomes (0.5 mg/mL). CYP, cytochrome P450; w/o, without.](image)

![Fig. 7. Formation of keto-darolutamide (M1) from darolutamide (1 $\mu$M) in cell-free reaction with recombinant CYP3A4 (50 pmol/mL), human liver microsomes (0.5 mg/mL), and human liver cytosol (0.5 mg/mL) after 60 minutes of incubation, dependent on cofactor supplementation. MS, mass spectrometry.](image)
urine, demonstrating extensive metabolism of darolutamide. The renal clearance of the diastereomers was similar to the estimated eGFR with a tendency for a slightly higher renal clearance of (S,R)-darolutamide. The data indicate that darolutamide is mainly excreted by passive glomerular filtration, although a contribution of transporter-mediated secretion cannot be excluded in the case of (S,R)-darolutamide. In line with the 1:5 plasma ratio of (S,R)-darolutamide to (S,S)-darolutamide, the excreted amount of (S,R)-darolutamide in urine was smaller than that of (S,S)-darolutamide, with an observed ratio of 1:4.

Excretion of the radioactive dose into urine occurred mainly as O-glucuronides of the darolutamide diastereomers, with smaller amounts as N-glucuronides, pyrazole sulfate, and darolutamide. In addition, nonlabeled pyrazole amide was measured at a relatively high proportion in urine. Metabolite profiling in urine indicated a preference for (S,S)- over (S,R)-darolutamide to form the respective O-glucuronides, as the difference in urine concentrations of M-7a and M-7b was almost 14-fold. In vitro investigations in HLM provided further support for more pronounced O-glucuronidation of (S,S)-darolutamide versus (S,R)-darolutamide. Although the majority of drug-related radioactivity was excreted renally, the high degree of metabolite formation suggests that hepatic metabolism contributes to darolutamide clearance more than renal elimination.

Approximately one-third of the administered dose was excreted with feces. Darolutamide was the predominant component in fecal extracts. Keto-darolutamide and, to a lesser extent, the carboxylic acid metabolite of darolutamide were identified as the most relevant metabolites but amounted to less than 2% of the dose. Excretion of drug diastereomers in the feces could result via three different clearance scenarios: both drug diastereomers are excreted directly into feces via biliary transporter-mediated pathways [darolutamide has been identified as a substrate for P-glycoprotein (Zurth et al., 2019)], the observed instability of keto-darolutamide in the reducing anaerobic microbiome in the human gastrointestinal tract could account for the high proportion of darolutamide, or secreted drug glucuronides are cleaved in the presence of the microbial gut flora back to the respective drug isomers. Biliary excretion has been studied in rats dosed with 14C-darolutamide (Taavitsainen et al., 2020). The rat metabolite profile in bile supports direct biliary excretion.
excretion of darolutamide diastereomers (approximately 8% of dose) as well as excretion of O-glucuronide conjugates (approximately 6% of dose). However, at least in the rat, the proportion of keto-
darolutamide in bile was very small, being only approximately 0.1% of the dose (Taavitsainen et al., 2020). Differences in the glucuronidation of (S,S)-darolutamide and (S,R)-darolutamide may contribute to the 1:7 ratio of (S,R)- versus (S,S)-darolutamide seen in feces, a more pronounced difference than that seen in plasma and urine. The slightly higher proportion of (S,S)-darolutamide in fecal extracts compared with plasma may be due to breakdown of the 
O-glucuronides by β-glucuronidases. Based on the studies presented, the actual in vivo situation for excretion of the diastereomers may result from a combination of all three of the proposed scenarios. However, in patients with nmCRPC receiving darolutamide in tablet formulation with food, for which absorption is 60%–70% (Massard et al., 2016) (in contrast to the complete absorption of the oral solution used in this study), unabsorbed drug can also contribute to the amount of darolutamide excreted in feces.

Further characterization of the metabolic profile of darolutamide in vitro indicated that the interconversion of the two diastereomers is

![Fig. 9. Formation of diastereomers from keto-
darolutamide (1 μM) in vitro with recombinant 
AKR1C3 (50 nM), human liver cytosol (0.5 mg/mL), 
human liver microsomes (0.5 mg/mL), and human 
hepatocytes (10^6 cells/mL). MS, mass spectrometry.]

![Fig. 10. Interconversion of darolutamide diastereomers and keto-darolutamide in the presence and absence of the CYP3A4 inhibitor (cobicistat) or the AKR1C3 inhibitor (BAY1128688, synthesized at Bayer AG, Germany): (A) time-dependent depletion of (S,R)-darolutamide, (S,S)-darolutamide, and keto-darolutamide (1 μM starting concentration for each, individual incubations) in presence of a CYP3A4 inhibitor (red), an AKR1C3 inhibitor (green), and no inhibitor (blue); (B) time-dependent metabolite and diastereomer formation in human hepatocytes by applying 1 μM starting material of (S,R)-darolutamide [analytes: (S,S)-darolutamide, keto-darolutamide], (S,R)-
darolutamide [analytes: (S,R)-darolutamide, keto-darolutamide], and keto-darolutamide [analytes: (S,R)- and (S,S)-darolutamide] in presence or absence of CYP3A4 or 
AKR1C3 inhibitor. Data points for (S,R)-darolutamide are indicated by open squares, for (S,S)-darolutamide by open diamonds, and for keto-darolutamide by filled triangles throughout. MS, mass spectrometry.]
a two-step process starting with oxidation to keto-darolutamide followed by reduction. This process is catalyzed by an interplay of membrane-associated enzymes (mainly CYP3A4, minor CYP1A1) as well as cytosolic enzymes. The role of CYP3A4 in the oxidative metabolism of darolutamide was supported by phase I drug-drug interaction studies (Zurth et al., 2019). In addition, as a P450-independent pathway, alcohol dehydrogenase also contributes to oxidation of darolutamide to keto-darolutamide to a minor extent. Cytosolic NADP-dependent AKR activity also made a minor contribution to the oxidative pathway, forming keto-darolutamide from (S,S)-darolutamide as the preferred substrate. In hepatocytes, keto-darolutamide is very rapidly reconverted to darolutamide with a preferred formation of (S,S)-darolutamide. This reduction is predominantly catalyzed by cytosolic reductase(s), with AKR1C3 playing the major role. Furthermore, other reductases present in cytosol along with microsomal reductases may also contribute to this biotransformation reaction to a minor degree. The stereoselectivity of cytosolic AKR1C3 results in the preferential formation of (S,S)-darolutamide from keto-darolutamide, and the increased clearance of (S,S)-darolutamide as compared with (S,R)-darolutamide in the presence of an AKR1C3 inhibitor in hepatocytes is attributed to the fact that this diastereomer is a more preferred substrate for oxidative metabolism as well as for glucuronidation.

Clearance of darolutamide by glucuronidation, leading to O-glucuronide M-7 isomers, is mainly mediated by UGT1A9, UGT1A1, and UGT1A3. N-glucuronidation to form M-15 isomers, however, appears to be mediated by UGT2B10 only, a primarily hepatic enzyme, which explains the lower amounts of glucuronides formed in other tissues (renal, intestinal) (Kaivosaari et al., 2011). Glucuronidation is a major elimination route of darolutamide, as the fraction metabolized via glucuronidation is at least one-third (assuming none of the darolutamide observed in feces was due to hydrolysis of glucuronides in the gut) or at most two-thirds (assuming darolutamide observed in feces was completely formed by hydrolysis of the glucuronides in the gut) (Fig. 12). Regarding the drug-drug interaction potential related to the glucuronidation pathway, the maximal effect of UGT inhibitors is theoretically predicted by a simplified static model to increase darolutamide exposure by a factor of 2.6-fold. In clinical studies, strong inhibition of the UGT1A9 substrate dapagliflozin, which has a similar metabolized fraction of 0.66, with mefenamic acid increased the exposure of dapagliflozin mildly, by 1.5-fold (Kasichayanula et al., 2013; https://
and possibly also P-glycoprotein, since the exposure of the sensitive et al., 2019), this is far more likely to be due to the induction of CYP3A4, and possibly also P-glycoprotein, since the exposure of the sensitive

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Authorship Contributions

Kuss I, et al.; ARAMIS Investigators (2019) Darolutamide in nonmetastatic, castration-resistant prostate cancer. N Engl J Med 380:1235–1246.

Kuss I, et al.; ARAMIS Investigators (2020) Nonmetastatic, castration-resistant prostate cancer and survival with darolutamide. N Engl J Med 383:1040–1049.

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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 \textbf{M-1} and \textbf{M-30}
Scheme 2. Synthesis pathway to **M-32**

![Chemical structure of M-32 with reaction details]

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

![Chemical structure of M-33 with reaction details]

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

![Chemical structure of M-32 with alternative synthesis details]
Scheme 5. Synthesis pathway to \textbf{M-36}

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

\textbf{(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 \textbf{3}

1-(tetrahydro-2H-pyran-2-yl)-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole \textbf{2} (6.5 g; 23.28 mmol) and 4-bromo-2-chlorobenzonitrile \textbf{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 35°C 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 –10°C 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. 1H-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 (4.00 g; 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 Na₂SO₄. 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 Z (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 Na₂SO₄, 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. ¹H-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-(1-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$_2$CO$_3$ (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$_2$Cl$_2$ (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; d$6$-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$_3$N (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$_2$Cl$_2$ (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$_2$Cl$_2$ - CH$_2$Cl$_2$: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$_3$THF (1M; 1 ml; 1.00 mmol) was added in small portions, and the reaction was stirred overnight at RT. Additional BH$_3$THF (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$_3$THF (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$_2$Cl$_2$ (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. 1H-NMR (400 MHz; d6-CDCl3): 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 1H-13C-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-butyne-2-ol 13 (0.250 g; 3.57 mmol) and Et3N (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. CH2Cl2 (15 ml) and an additional 5 ml of water were added and phases were separated. Water phase was washed twice with CH2Cl2. 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; CH2Cl2 - CH2Cl2:MeOH 9:1) gave 165 mg of title compound. 1H-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. CeCl₃ (353 mg; 1.43 mmol) was added
and the resulting solution cooled to 0°C. NaBH₄ (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 Na₂SO₄, 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                                   | Celsis In Vitro Technologies, Baltimore, MD, USA                                                   |
| Donor TWT                                                              | Bioreclamation IVT, Baltimore, MD, USA                                                              |
| Donor TZU                                                               | Triangle Research Labs, Triangle Research Park, NC, USA                                             |
| Donor HUM4070B                                                         |                                                                                                     |
| Donors: GMK, NIQ and VCM                                               | Celsis, Brussels, Belgium                                                                          |
| Pooled human liver microsomes (200 mixed gender donors): Xtreme 200 lot 1010420 and lot 1210223 | XenoTech LLC, Lenexa, KS, USA                                                                      |
| 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      |                                                                                                     |
| 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 Vitro Technologies, 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™ | Corning, Woburn, MA, USA |
| Recombinant UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, 2B17); Supersomes™ | |
| 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

| Compounds analyzed | A. LC-MS/MS | B. Analytical LC | C. LC-MS | D. LC-MS | E. LC-MS |
|--------------------|-------------|------------------|----------|----------|----------|
| (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) |
| HPLC column | Plasma: Chiral AGP, 150 x 4 mm, 5 μm (Chromtech, Apple Valley, MN, USA) | Pursuit 3 C8, 150 x 3 mm, 3 μm (Agilent, Santa Clara, CA, USA) | Pursuit 3 C8, 150 x 3 mm, 3 μm (Agilent, Santa Clara, CA, USA) | Accucore C4, 150 x 3 mm, 2.6 μm (ThermoFisher Scientific Inc., Waltham, USA) | Betasil Phenyl-Hexyl, 100 x 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 |

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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

| Compounds analyzed                                                                 | Metabolite profiling                                                                 | Role of glucuronidation                                                                 | Metabolite profiling                                                                 |
|------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| 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)                           |
|                                                                                   |                                                                                      |                                                                                      |                                                                                      |
| 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                                                            |
|                                                                                   |                                                                                      |                                                                                      |                                                                                      |
|                                                                                   |                                                                                      |                                                                                      |                                                                                      |
|                                                                                   |                                                                                      |                                                                                      |                                                                                      |
| 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       | ![Structure of M-7a](image1.png) | **1H NMR:** (600 MHz, D$_3$-ACN/D$_2$O 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).  
**13C NMR** (150 MHz, D$_3$-ACN/D$_2$O 70:30 v/v): δ/ppm = 172, 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      | ![Structure of M-15a](image2.png) | **1H NMR:** (600 MHz, D$_3$-ACN/D$_2$O 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).  
**13C NMR** (150 MHz, D$_3$-ACN/D$_2$O 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 | \[
\begin{align*}
\text{1H NMR: } & (600 \text{ MHz, } \text{D}_3\text{-ACN/D}_2\text{O }70:30 \text{ v/v}): \delta/\text{ppm} = 7.88 \text{ (br s, 1H), 7.80 (d, 1H), 7.70 (dd, 1H), 6.34 (s, 1H), 5.11 (d, 1H), 3.97 (d, 1H), 3.55 (t, 1H), 3.49 (t, 1H), 3.45 (t, 1H).} \\
\text{13C NMR (150 MHz, } \text{D}_3\text{-ACN/D}_2\text{O }70:30 \text{ v/v): } & \delta/\text{ppm} = 171.6, 162.4, 142.8, 137.8, 136.4, 136.0, 127.3, 125.1, 117.0, 112.7, 101.8, 91.1, 76.2, 75.9, 73.5, 72.1.
\end{align*}
\] |
|---|---|
| M-22 | \[
\begin{align*}
\text{1H NMR: } & (600 \text{ MHz, } \text{D}_3\text{-ACN/D}_2\text{O }70:30 \text{ v/v}): \delta/\text{ppm} = 7.87 \text{ (d, 1H), 7.71 (s, 1H), 7.54 (d, 1H), 5.89 (s, 1H), 5.00 (d, 1H), 4.04 (t, 1H), 3.86 (d, 1H), 3.56 (t, 1H), 3.42 (t, 1H).} \\
\text{13C NMR (150 MHz, } \text{D}_3\text{-ACN/D}_2\text{O }70:30 \text{ v/v): } & \delta/\text{ppm} = 172.0, 162.5, 145.3, 137.6, 136.3, 135.8, 131.0, 129.0, 116.9, 113.8, 95.5, 85.9, 77.4, 76.9, 72.0, 71.8.
\end{align*}
\] |
### 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] | Molecular formula | Mass shift to drug [Da] | 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 | 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] | 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 | 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 | 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 | 299.9846 n/a 297.9689 297.9696 -99 | C10H6N3O4ClS | n/a | 218 |
| M-30       | 262.0747 262.0739 n.d. n/a -137 | 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 | 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 | 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 | 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 | 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 | 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 | $t_{1/2}$ (min) | Intrinsic clearance (μL/min/10⁶ 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 (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 (specific substrate) | Correlation coefficient ($r^2$) |
|---------------------------------|---------------------------------|
|                                 | 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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