Drug Metabolism and Disposition DMD-AR-2022-001100

Supplemental Information

Application of Accelerator Mass Spectrometry to Characterize the Mass Balance Recovery and Disposition of AZD4831, a Novel Myeloperoxidase Inhibitor, Following Administration of an Oral Radiolabeled Microtracer Dose in Humans

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Supplemental Materials and Methods

Determination of total ^{14}C-concentrations in urine – extended sample processing

To maximize the extraction of ^{14}C from urine, the samples that were collected and stored in plastic tubes were transferred to glass tubes containing 1.5 mL of acetonitrile (MeCN). The original plastic storage sample tubes were sequentially extracted with 3 mL each of MeCN, methanol, 1 M aqueous hydrogen chloride, and 1 M aqueous sodium hydroxide. All extracts were added to the corresponding glass tube containing the urine sample in 1.5 mL of MeCN.

An aliquot of the combined extracts was analyzed using accelerator mass spectrometry (AMS) analysis. To generate a suitable current on the AMS, a fixed amount of ^{12}C was added to the cups prior to drying and AMS analysis. A quality control (QC) urine sample with a known amount of ^{14}C (derived from a ^{14}C-paracetamol solution [Hartmann Analytic, Braunschweig, Germany] diluted in urine to 195 mBq/mL) was analyzed with the study samples using the same procedure as previously described. The deviation from the nominal concentration in the extracted QC samples and the coefficient of variation were <5%; thus, the extended procedure was suitable to determine the ^{14}C levels in the extracted urine samples.

LC-MS/MS method for the investigation of racemization in the plasma samples from human subjects dosed with AZD4831

The objective of the configurational stability study was to quantitatively evaluate the potential for racemization of AZD4831 to its corresponding *S*-enantiomer and assess its impact on drug metabolism and pharmacokinetic analysis of AZD4831. Samples from humans following oral administration of AZD4831 during the multiple ascending dose (MAD) study (Nelander et al., 2021; Jurva et al., 2022) were assessed using a chiral liquid chromatography tandem mass spectrometry (LC-MS/MS) assay.

Synthesis of standards

The synthesis of the *S*-enantiomer of AZD4831 is described elsewhere (Inghardt et al., 2022). The synthesis of the stable labeled internal standard $^{13}C_3$, $^{15}N_2$-AZD4831 was performed at Pharmaron Beijing Co., Ltd (Beijing, China).

Calibration standards

Stock solutions of AZD4831 (*R*-enantiomer), the *S*-enantiomer, and internal standard $^{13}C_3$, $^{15}N_2$-AZD4831 were prepared in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA), and diluted appropriately to prepare calibration samples for AZD4831 and the *S*-enantiomer in human plasma on the day of analysis. The following calibration standards were prepared: 124.5, 62.0, 31.1, 15.6, 7.78, 3.89, 1.95, and 0.973 nM. QC samples were prepared by spiking a mixture of AZD4831 and the *S*-enantiomer in blank human plasma for a final concentration of 124.5 nM for

AZD4831 and 6.225 nM (5% of AZD4831) for the *S*-enantiomer. Calibration standards, QC samples, and plasma samples, including blanks, were processed by protein precipitation. This was achieved by mixing 50 µL of sample with 165 µL of MeCN containing 15 µL of 168 nM of $^{13}C_3$, $^{15}N_2$-AZD4831 as internal standard. Samples were vortex mixed and centrifuged to separate the organic layer, and then 150 µL was analyzed by LC-MS/MS.

Liquid chromatography

The analytes were separated with high-performance liquid chromatography (HPLC) on a Waters HPLC Aquity System (Waters Corp., Milford, MA, USA) using a CROWNPAK CR-1(+) column (150 × 3.0 mm, 5 µm, Chiral Technologies, Illkirch, France) kept at 25ºC. Chromatographic separation was achieved using a mobile phase consisting of MeCN/H_2O/trifluoracetic acid in a ratio of 96/4/0.5 at a constant flow rate kept at 0.15 mL/min. A C18 pre-column (Phenomenex, AJ0-9298, SecurityGuard™ ULTRA cartridges for EV0-C18 UHPLC, sub-2 µm and core-shell columns with 2.1 mm internal diameters) was applied before the chiral column. Autosampler temperature was kept at 12ºC.

Mass spectrometry

Analytes were detected in positive ionization mode using a Waters Xevo-TQS triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) with the following source parameters set: desolvation temperature at 500ºC; cone gas flow at 150 L/hr; nebulizer gas flow at 7 bar, and capillary voltage at 0.76 kv. The selected multiple

reaction monitoring transitions for AZD4831 and *S*-enantiomer of AZD4831 were: *m/z* 334.89 > 150.98; 335.34 > 317.92, and those for the internal standard $^{13}C_3$, $^{15}N_2$-AZD4831 were: *m/z* 340.02 > 150.99.

Data analysis

The enantiomeric separation and quantitation were performed in four batches on four different days. The range of standard curve for the *R*-enantiomer AZD4831 and *S*-enantiomer in human plasma were both 0.00389–0.1245 μmol/L. Data acquisition was performed with MassLynx V4.2 SCN986 software and data analysis was performed with TargetLynx XS V4.2 SCN986 software. The analytical run acceptance criteria for calibration standards was: 75% or better of back-calculated concentrations should be within ±20% (±25% at lower limit of quantification) of nominal values. The performance of the assay was assessed from the variability observed in the calibration curves as well as the accuracy of QCs. The acceptance criteria for QCs were: ≥67% of QCs should be ±20% of nominal value.

LC-MS method for in vitro phenotyping of UGT isoforms responsible for the formation of metabolite M7

The objective of the in vitro uridine diphosphate glucuronosyltransferase (UGT) phenotyping study was to identify whether AZD4831 is a substrate of any of the following human hepatic UGTs and, if so, estimate the relative contribution of each isoform: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7, and UGT2B15.

Chromatography

The chromatographic method was performed on a BEH C18 1.7 µm, 2.1 × 50 mm column heated to 40°C. The mobile phase was a mixture of solvent A (0.1% formic acid in water) and solvent B (100% MeCN). The gradient started with 5% of solvent B and was increased to 90% solvent B in 3 minutes. Afterwards, the gradient was instantaneously increased to 98% solvent B where it was kept until 4 minutes. The flow rate was set to 0.7 mL/min and the injection volume was 8 µL. The system was equilibrated for 0.5 minutes, with the mobile phase containing 5% solvent B before the next injection.

Mass spectrometry

Quadruple time-of-flight (QTOF) analysis was performed on an Acquity UPLC Xevo G2-S QTOF instrument (Waters Corp., Milford, MA, US) equipped with an Acquity UPLC I-Class (Waters Corp., Milford, MA, US) system and an electrospray ionization source. The system was run in sensitivity mode with a cone voltage of 20 V, capillary voltage of 0.5 kV, source temperature of 120°C, and desolvation temperature of 600°C. Mass accuracy was corrected using a lock mass solution, leucine-enkephalin (m/z 556.2771), 200 pg/mL in MeCN : water (50:50 v/v) containing 0.1% formic acid. Full scan (m/z range 80–1200) centroid mass spectrometric data were acquired using a fixed collision energy of 4 eV. The molecular ion of M7 at m/z 553.0801 $(M-H)^-$ and that of the internal standard 5,5-diethyl-1,3-diphenyl-2-iminobarbituric acid at m/z 334.1561 $(M-H)^-$ were monitored. The monitored molecular ions of the control substrates are shown in **Table 3**. Data acquisition was performed with MS software

MassLynx 4.1 (Waters Corp, MIlford, MA, US) and data analysis was performed using the QuanLynx module of the MassLynx 4.1 software.

Supplemental Tables

Table S1. Analytical equipment used in the hADME study (NCT04407091)

Equipment	Model and Vendor
AMS	1 MV multi-element AMS, model 4110 Bo, software versions for AMS01 B7679-2.0.0.79, for AMS02 B9188-2.0.0.614 (High Voltage Engineering, Amersfoort, Netherlands)
Mass spectrometer	Q Exactive orbitrap HRMS, Software: Xcalibur v2.2 sp1.48 (Thermo Fisher Scientific, Waltham, MA, USA) Waters Xevo-TQS triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA)
UPLC/HPLC	Aquity UPLC H Class Plus System (Waters Corp., Milford, MA, USA) Waters HPLC Aquity System (Waters Corp., Milford, MA, USA)
HPLC/Guard column	Aquity HSS T3 1.8 μM, 100 × 2.1 mm/VanGuard HSS Pre-Column T3 1.8 μM, 5 × 2.1 mm (Waters Corp., Milford, MA, USA) CROWNPAK CR-1(+) column, 150 × 3.0 mm, 5 μm (Chiral Technologies, Illkirch, France)
Fraction collector	Waters Fraction manager (WFM-A; Waters Corp., Milford, MA, USA) Split ratio was approximately 58% to the fraction collector and approximately 42% to HRMS

AMS, accelerator mass spectrometry; hADME, human absorption, distribution, metabolism, and excretion; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; UPLC, ultra-performance liquid chromatography.

Table S2. Key inclusion and exclusion criteria for participants in the hADME study (NCT04407091)

Inclusion criteria
Signed informed consent
Healthy male participants aged 18–65 years, with suitable veins for cannulation or repeated venepuncture
Body mass index of 18.0–30.0 kg/m^2 at screening
Regular bowel movements (i.e., average stool production of ≥1 and ≤3 stools per day)
Exclusion criteria
History of presence of gastrointestinal, hepatic, or renal disease, or any other condition known to interfere with absorption, distribution, metabolism, or excretion of drugs; uncontrolled or clinically significant thyroid disease; clinically significant illness, medical/surgery procedure, or trauma within 4 weeks
Clinically significant abnormalities in laboratory parameters, vital signs, electrocardiogram, or positive result on screening for serum hepatitis B surface antigen, hepatitis C virus antibody, and human immunodeficiency virus antibody
Current smoker or ex-smoker within 3 months, or current user or ex-user of e-cigarettes within 3 months; known or suspected history of alcohol or drug abuse or excessive intake of alcohol
Use of drugs or supplements with enzyme-inducing properties, such as St John's Wort, within 3 weeks
Use of any prescribed or non-prescribed medication, including antacids, analgesics (other than paracetamol/acetaminophen), herbal remedies, megadose vitamins and minerals, within 2 weeks or longer if the medication has a long half-life
Participants with pregnant or lactating partners
Radiation exposure, including from the present study, excluding background radiation but including diagnostic x-rays and other medical exposures, exceeding 5 mSv in the previous 12 months or 10 mSv in the last 5 years
Evidence of renal impairment at screening (estimated creatinine clearance of <80 mL/min)

hADME, human absorption, distribution, metabolism, and excretion.

Table S3. Analytical conditions for metabolite profiling in the hADME study (NCT04407091)

<table>
<tr><th>Instrument</th><th>Equipment Details</th><th colspan="3">Analytical Conditions</th></tr>
<tr><td rowspan="9">UPLC for metabolite profiling/identification</td><td rowspan="9">Column temperature: 45ºC

Flow rate: 0.5 mL/min

MPA: 0.1% formic acid in MilliQ water

MPB: MeCN ULC/MS-CC/SFC grade

Run time: 33 minutes

Split ratio between HRMS and fraction collector: approximately 58% to the fraction collector and approximately 42% to HRMS</td><td>Time (minutes)</td><td>MPA (%)</td><td>MPB (%)</td></tr>
<tr><td>0</td><td>95.0</td><td>5.0</td></tr>
<tr><td>1.00</td><td>95.0</td><td>5.0</td></tr>
<tr><td>13.00</td><td>83.0</td><td>17.0</td></tr>
<tr><td>22.00</td><td>63.0</td><td>37.0</td></tr>
<tr><td>23.00</td><td>10.0</td><td>90.0</td></tr>
<tr><td>28.00</td><td>10.0</td><td>90.0</td></tr>
<tr><td>28.10</td><td>95.0</td><td>5.0</td></tr>
<tr><td>33.00</td><td>95.0</td><td>5.0</td></tr>
<tr><td>Mass spectrometer</td><td>Thermo Q Exactive, software Xcalibur v2.2 sp1.48</td><td colspan="3">ESI +ve & -ve mode; calibrated within 3 ppm using calibration mixtures recommended by Thermo Fisher Scientific;

Full MS PRM mode and Full MS ddMS^{2}

[MS analysis based on quantification and characterization of metabolites of velsecorat (Holmberg et al., 2022)]</td></tr>
</table>

CC, convergence chromatography; ddMS^{2}, data-dependent MS^{2}; ESI, electrospray ionization; hADME, human absorption, distribution, metabolism, and excretion; HRMS, high-resolution mass spectrometry; MeCN, acetonitrile; MPA, mobile phase A; MPB, mobile phase B; MS, mass spectrometry; PRM, parallel reaction monitoring; SFC, supercritical fluid chromatography; ULC, ultra-liquid chromatography; UPLC, ultra-performance liquid chromatography.

Table S4. Summary of control substrates and metabolites used for the in vitro determination of UGT isoform involved in glucuronide metabolite formation

Isoform	Substrate	Substrate Concentration (μM)	Metabolite Monitored	Monitored Metabolite Ion (m/z) Analyzed by QTOF
UGT1A1	β-estradiol	5	β-estradiol 17-β-D-glucuronide	447.2019 $(M-H)^-$
UGT1A3	4-methyl umbelliferone	50	4-methylumbelliferone-β-d-glucuronide	351.0716 $(M-H)^-$
UGT1A4	Lamotrigine	200	Lamotrigine N-2-glucuronide	432.0477 $(M+H)^+$
UGT1A6	1-naphthol	2.5	1-naphthol O-glucuronide	319.0818 $(M-H)^-$
UGT1A9	Propofol	25	Propofol β-D-glucuronide	353.1601 $(M-H)^-$
UGT2B7	Zidovudine	150	3'-azido-3'-deoxythymidine β-D-glucuronide	442.1210 $(M-H)^-$
UGT2B15	Oxazepam	50	Oxazepam glucuronide	461.0752 $(M-H)^-$

QTOF, quadrupole time of flight; UGT, UDP-glucuronosyltransferase.

Table S5. Summary of substrates and metabolites used for the RAFs previously determined by Busse et al. (Busse et al., 2020)

Isoform	Substrate	Metabolite	RAF
UGT1A1	β-Estradiol	β-Estradiol 17-β-D-glucuronide	1.1
UGT1A3	CDCA	CDCA 24-Acyl-β-D-glucuronide	0.060
UGT1A4	Midazolam	Midazolam N-β-D-glucuronide	0.48
UGT1A6	Serotonin	Serotonin β-D-glucuronide	4.8
UGT1A9	Propofol	Propofol β-D-glucuronide	0.24
UGT2B7	Zidovudine	3'-Azido-3'-deoxythymidine β-D-glucuronide	1.1
UGT2B15	S-Oxazepam	Oxazepam glucuronide	6.5

CDCA, chenodeoxycholic acid; RAF, relative activity factor.

Table S6. Demographics and baseline characteristics of healthy participants in hADME study (NCT04407091)

Characteristic	Statistic	Participants (*N* = 6)
Age, years	Mean	31.0
	SD	7.2
	Median	32.0
	Min	22
	Max	39
Height, cm	Mean	174.7
	SD	3.8
	Median	174.5
	Min	170
	Max	179
Weight, kg	Mean	83.9
	SD	4.1
	Median	84.2
	Min	78.8
	Max	89.6
Body mass index, kg/m^2	Mean	27.5
	SD	1.3
	Median	27.8

	Min	25.2
	Max	29.1
Sex, n (%)	Male	6 (100)
Race, n (%)	Black or African American	2 (33.3)
	White	4 (66.7)
Ethnic group, n (%)	Not Hispanic or Latino	6 (100)

hADME, human absorption, distribution, metabolism, and excretion; SD, standard deviation.

Table S7. Arithmetic mean (SD) cumulative fraction of ^{14}C dose (%) excreted as total ^{14}C in urine, feces, and total (urine + feces) following a single oral dose of 10 mg of ^{14}C-AZD4831 (as a solution) over 0–336 hours in healthy volunteers

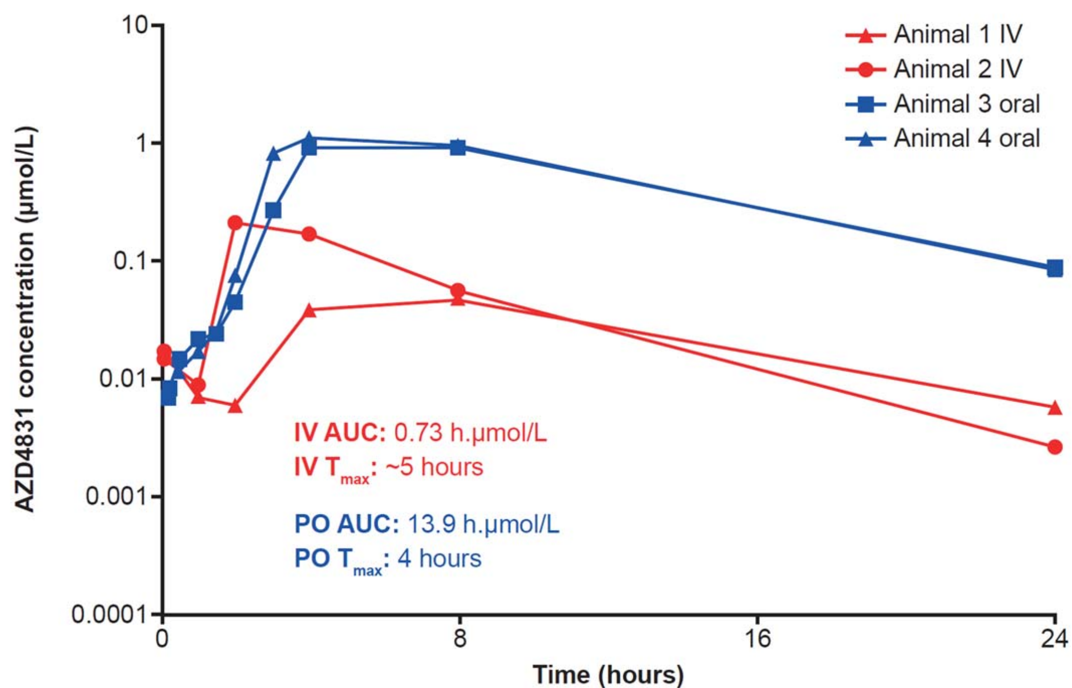
Timepoint (hours)	Mean (SD) cumulative fraction of ^{14}C dose (%) excreted		
	Urine	Feces	Total (urine + feces)
Pre-dose	NQ (NC)	NQ (NC)	NQ (NC)
0–6	7.6 (2.7)	–	–
0–12	13.3 (2.9)	–	–
0–24	17.9 (2.7)	5.0 (5.1)	22.9 (6.2)
0–36	26.0 (3.7)	–	–
0–48	29.1 (4.3)	9.7 (8.0)	38.8 (8.0)
0–72	34.5 (5.1)	15.7 (6.3)	50.1 (7.2)
0–96	38.7 (5.2)	19.0 (7.6)	57.7 (8.5)
0–120	41.5 (5.4)	21.5 (5.2)	63.1 (5.9)
0–144	43.9 (5.4)	24.4 (2.7)	68.3 (5.0)
0–168	45.4 (5.5)	26.4 (2.6)	71.8 (3.3)
0–192	46.9 (5.8)	28.4 (2.6)	75.3 (3.9)
0–216	48.1 (5.9)	29.5 (3.3)	77.5 (4.2)
0–240	49.0 (6.0)	30.1 (3.0)	79.0 (4.2)
0–264	49.7 (6.1)	30.9 (3.4)	80.6 (4.4)
0–288	50.3 (6.1)	31.5 (3.4)	81.8 (4.6)

0–312	50.8 (6.2)	32.0 (3.7)	82.8 (5.0)
0–366	51.2 (6.3)	32.4 (3.9)	83.6 (5.3)

NC, not calculated; NQ, not quantifiable; SD, standard deviation.

Supplemental Figures

Fig. S1. LC-MS/MS chromatograms monitoring the concentrations of AZD4831 (µmol/L) in rat plasma following a single intravenous or oral administration of M7 in Hans Wistar rats



AUC, area under the plasma concentration-time curve; IV, intravenous; LC-MS/MS, liquid chromatography tandem mass spectrometry; PO, oral; T_{max}, time at maximum plasma concentration.

Supplemental References

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