Absorption, distribution, metabolism and excretion of the oral prostaglandin D2 receptor 2 (DP_2) antagonist fevipiprant (QAW039) in healthy volunteers and in vitro

David Pearson, H. Markus Weiss, Yi Jin, Jan Jaap van Lier, Veit J. Erpenbeck, Ulrike Glaenzel, Peter End, Ralph Woessner, Fabian Eggimann, Gian Camenisch

Drug Metabolism and Disposition

SUPPLEMENTAL DATA

METHODS

Sample Collection and Aliquoting

Samples of whole blood, urine, and feces were collected over an 11-day period. For each scheduled PK sample, 30 mL (*) or 10 mL of venous blood was collected at each time point into ethylenediamine tetraacetic acid-containing tubes: pre-dose (0*), 0.25*, 0.5, 1*, 1.5, 2*, 3, 6*, 12, 24*, 36, 48*, 72*, 96, 120*, 144, 168*, 192, 216 and 240* post-dose (20 time points in total). Part of the blood was used for the analyses in whole blood (total radioactivity) and a major part was processed to plasma (total radioactivity, PK, metabolism). Each plasma sample was divided into two parts. One part of each plasma sample was acidified by addition of 1% volume of a 70% lactic acid solution. Both the acidified and non-acidified samples were then stored at -70°C.
After administration of the radiolabeled dose, and during the entire post-dose in-house observation period (Days 1–11, 0–240 hours), all urine and feces were collected completely. Urine portions were pooled per subject, within any planned collection interval (e.g. Days 2–11: 24-hour collection periods), accurately weighed and divided into two parts. One part of each urine sample was acidified by addition of 1% volume of a 70% lactic acid solution. Both the acidified and non-acidified samples were then stored at 0–4ºC. The individual feces sample containers were stored in a refrigerator (0–4°C) until transfer to the analysis laboratory, then were stored at -70°C pending processing. The complete set of fecal samples was processed and analyzed for radioactivity, as follows: pooling of feces per 24 hour collection period, dilution of the pools with 1–2 volumes of water, determination of the total weight of each diluted sample, thorough homogenizing of each diluted sample, determination of radioactivity (Liquid Scintillation Counting [LSC] section, see below), and aliquoting for radiometry and metabolism analyses. Processed sample aliquots were stored at -70°C.

Liquid Scintillation Counting

Radioactivity in blood, non-acidified plasma, non-acidified urine, and feces was measured under Good Laboratory Practice. Measurements were conducted by LSC, with a typical counting time of 10 minutes; low levels in blood were counted for 20

minutes. Before measurement, each blood sample (300 µL, measured in triplicate) was diluted with 200 µL blank whole blood, then was solubilized by addition of Solvable (1 mL, Perkin Elmer) and incubated at 60°C for one hour. Subsequently, Titriplex (100 µL, 0.1 M) was added, the sample was left for five minutes, hydrogen peroxide (100 µL) was added and the sample was incubated for 30 minutes at room temperature, 20 minutes at 45°C, then 60 minutes at 60°C. After cooling to room temperature, the sample was mixed with scintillation cocktail (Ultima Gold™; Perkin Elmer). Plasma samples (250 µL, measured in triplicate) were mixed directly with liquid scintillation cocktail (5 mL, Ultima Gold). Feces samples (approximately 0.5 g, weighed, measured in quadruplicate) were dried in a stove at 50°C for at least three hours, then Combusto aid (100 µL Perkin Elmer) was added and the sample was combusted in a sample oxidizer model 307 (Perkin Elmer). CarboSorb-E (7 mL, Perkin Elmer) was used as absorber agent for carbon dioxide. After combustion, the absorber was mixed with of the scintillant (13 mL, PermaFluor E). Urine samples (1 mL, measured in duplicate) were mixed with scintillation cocktail (5 mL, Ultima Gold).

The LLOQ of radioactivity was 84.1 ng-eq/g in whole blood, 31.1 ng-eq/g in plasma, 10.7 eg-eq/g in urine, and 42.8 ng-eq/g in feces.

In order to determine recoveries from sample preparations, radioactivity was measured offline using a Tri-Carb 2200CA or 2500TR liquid counter model Tri-Carb 3570TR/SL (Packard) operated by QuantaSmart™ software. Feces samples were measured after solubilization with Soluene 350 (Packard)/isopropanol (1:1, v/v), neutralization with hydrochloric acid, and addition of 15–20 mL of liquid scintillation cocktail IrgaSafe Plus (Zinsser Analytic Maidenhead, Berkshire, UK). Urine samples, plasma samples, and feces extracts were measured directly after addition of 5.5 mL LSC cocktail. Standard counting procedures were used and quench correction was performed by the external standard method. Fractions collected from ultra-high pressure liquid chromatography (UHPLC) (metabolite profiles) were monitored offline for radioactivity using TopCount™.

For UHPLC radiochromatograms, 3-second fractions were collected into 384 well LumaPlates™ (PerkinElmer). After evaporation of solvent, the plates were counted in Topcount NXT™ microplate scintillation and luminescence counter (Packard).

The specific radioactivity of the drug substance, the amount of drug substance in the capsule, the filling weight of the bottles, as well as the radioactivity of capsules, were measured before dosing.

Quantification of Fevipiprant and AG-metabolite in Plasma and Urine

Concentrations of fevipiprant and AG-metabolite in all acidified plasma samples up to 240 hours post-dose and in acidified urine were assessed by a validated specific liquid chromatography (LC)-mass spectrometry (MS)/MS assay. AG-metabolite was quantified as the sum of all isomers formed by acyl-glucuronide rearrangement. Acidified samples were analyzed to avoid AG-metabolite instability at neutral pH.

Plasma and urine samples (100 µL) were loaded onto a 96 well plate (Eppendorf), and 200 µL acetic acid (2%) containing internal standard (20 ng/mL [$^{13}CD_5$]fevipiprant) was added and the plate was centrifuged at 2250 × *g* for 10 minutes at 4°C. Extraction was performed, as follows: 96 well solid phase extraction (SPE) plates (Oasis HLB, Waters) were conditioned with methanol (200 µL), then ammonium acetate (0.5 M, 300 µL), then sample (250 µL) was loaded, wells were washed with phosphoric acid (0.85%, 200 µL), then methanol (2% in water, 200 µL), and finally analytes were eluted with methanol (2 × 150 µL). The

eluates were evaporated to dryness under a stream of nitrogen at 50°C, and then reconstituted in formic acid/methanol (150 µL, 50/50, 0.2% formic acid in water). The resulting extracts (20 µL) were injected onto an Symmetry C18 column (3.5 µm particles, 2.1 × 30 mm; Waters) on an Agilent 1200 HPLC system with mobile phases of formic acid (0.2 % in water, phase A) and methanol (phase B). Samples were eluted with the following gradient: 0 to 3 minutes: 5% B; 3 to 3.2 minutes: 5 to 90% B; 3.2 to 3.9 minutes: 90% B; 3.9 to 4 minutes: 90 to 5% B; 4 to 5 minutes: 5% B. The flow rate was 0.5 mL/minute. Eluate was infused into a triple quadrupole mass spectrometer (API 4000, Applied Biosystems) and ionized by ESI in positive ion mode; nitrogen gas was used for collisional activation. Fevipiprant and AG-metabolite were detected by selected reaction monitoring: transitions from m/z 427.1 to 145.0, from m/z 603.03 to 427.0 and from 433.2 to 145.0 were monitored for fevipiprant, the AG-metabolite, and the internal standard, respectively. Fevipiprant and the AG-metabolite were quantified by comparison with the internal standard peak area.

The lower limit of quantification was 2 ng/mL for fevipiprant and for the AG-metabolite using 0.100 mL of plasma, and it was 5 ng/mL for both analytes using 0.100 mL of urine.

Determination of Metabolite Profiles

Fevipiprant and metabolites were analyzed in acidified plasma, acidified urine pools and feces extract pools of individual subjects by LC-MS/MS, with offline radioactivity detection. Acidified samples were analyzed to avoid AG-metabolite instability at neutral pH.

Plasma samples of individual subjects were extracted using a SPE method, as follows: plasma aliquots were diluted 1.5 times with a 0.7% lactic acid solution. SPE cartridges HLB 6cc 200 mg (Waters, Milford, US) were washed with acetonitrile (3 mL) followed by lactic acid (3 mL, 0.7% in water). Diluted plasma was loaded onto the cartridge. Subsequently, the cartridges were washed with acetonitrile/lactic acid (4 mL, 80:20 acetonitrile/0.7% lactic acid in water), then eluted with acetonitrile/lactic acid (2 × 2 mL, 90:10 acetonitrile/0.7% lactic acid in water). The eluted fractions were concentrated under a flow of nitrogen to a volume of 150–300 µL. Extraction recoveries decreased over time (see Results section). Method development experiments (data not shown) showed that the AG-metabolite was stable (recovery >90%) during the extraction procedure.

Urine pools of individual subjects were prepared and extracted, as follows: for each subject, a 12–72 hours urine pool was prepared by combining identical percentages of the amounts of the different fractions; weighed aliquots of the 0–12 hours and 12–72 hours urine pools (10 mL) were diluted 1:1 (v/v) with lactic acid (10 mL, 0.7% in water). SPE cartridges HLB 20cc 1 g (Waters, Milford, US, Part 186000117) washed with acetonitrile (10 mL) followed by lactic acid (10 mL, 0.7% in water). Diluted urine was loaded onto the cartridge. Subsequently, the cartridges were washed with acetonitrile/lactic acid (10 mL, 80:20 acetonitrile/0.7% lactic acid in water), then eluted with acetonitrile/lactic acid (2 × 5 mL, 90:10 acetonitrile/0.7% lactic acid in water). The elute fractions were concentrated under a flow of nitrogen to a volume of 1.2 to 2.2 mL. The recovery of radioactivity from the sample was complete (>95%).

Feces pools of individual subjects were prepared and extracted, as follows: for each subject, a 0–96 hours feces homogenate pool was prepared by combining identical percentages of the amounts of the different homogenate fractions. Weighed aliquots of each pool (10 mL) were then mixed with acetonitrile (4 volumes) then agitated at 4°C for 12 hours; and centrifuged for 30 minutes at 3500 × *g* in a centrifuge model Minifuge RF (Heraeus). Subsequently, the supernatant was withdrawn and

concentrated under nitrogen flow to 10 mL. Finally, the sample was further purified by SPE, as described for urine extraction. Recoveries were calculated after extraction and concentration by measuring radioactivity in weighed aliquots performed in triplicate. The recovery of radioactivity from the sample was almost complete (>88%).

Extracted samples were subsequently analyzed by UHPLC with an Agilent 1290 system. Samples were injected onto a HSS T3 column (150 x 2.1 mm, 1.8 µm particles, Waters, Milford, MA, USA) with a corresponding guard column (3 x 2.1 mm), heated to 40°C. Separation was performed with a gradient of mobile phases ammonium acetate (10 mM, pH 5.0; phase A) and acetonitrile (phase B). The gradient was as follows: 0 to 1.5 minutes: 5% B; 1.5 to 2 minutes: 5 to 30% B; 2 to 5.5 minutes: 30 to 40% B; 5.5 to 5.7 minutes: 40 to 100% B; 5.7 to 7.5 minutes: 100% B; 7.5 to 7.6 minutes: 100 to 5% B; 7.6 to 12 minutes: 5% B. The eluate was split in a ratio of ~1:6. The smaller part was directed to an LTQ-Orbitrap XL mass spectrometer (ThermoFisher) for MS/MS analysis and the larger part was collected in 384 well LumaPlates® (PerkinElmer) for radioactivity detection. The fractions were evaporated to dryness and the radioactivity was counted in a Microplate scintillation counter model TopCount NXT (Packard).

Size Exclusion Chromatography of plasma samples

Selected plasma samples were analyzed by size exclusion chromatography (SEC) to investigate covalent binding to plasma proteins. Plasma samples (25 to 35 µL) were injected directly onto a BioSuite 125 UHR SEC column (4.0 µm particle size, 4.6 x 300 mm, Waters), with an Agilent 1200 HPLC system. The column was heated to 30°C. Analytes were separated with an isocratic gradient of 90:10 ammonium formate (50 mM)/acetonitrile at a flow rate of 0.33 mL/minute. The eluate was collected in 12-second fractions on a 96-well LumaPlate (PerkinElmer) for radioactivity detection. Multiple (5–10) injections were performed for each sample, with collection on the same plate. The fractions were evaporated to dryness and the radioactivity was counted in a microplate scintillation counter model TopCount NXT (Perkin Elmer).

In vitro Investigations

Blank urine acidification with lactic acid

Blank urine samples were collected from 11 healthy male volunteers. Urine samples were split into two groups, and one group was acidified as described for the clinical urine and plasma samples, whereas the second group remained untreated. pH was measured for each sample.

Assessment of AG-metabolite urine stability

AG-metabolite was added to human urine in triplicate (final concentration 130 ng/mL), then was acidified after 15 minutes, as described for clinical plasma samples. Samples were stored at room temperature, and aliquots were analyzed after zero and four weeks by LC-MS/MS, as described for quantification of fevipiprant and the AG-metabolite in clinical urine samples.

Assessment of AG-metabolite isomerization

Synthetic AG-metabolite (10 µM) as the pure 1-O-beta-acyl glucuronide isomer was incubated at 37°C in phosphate buffer (100 mM, pH 7.4), with shaking (1000 rpm) for up to 48 hours. Aliquots (50 µL) were taken at various time points and were quenched by the addition of 3 volumes of 0.7% lactic acid /acetonitrile (2:1). Samples were subsequently analyzed, as described for clinical plasma samples, to identify acyl-glucuronide isomers formed.

Incubations with uridine diphosphate glucuronosyltransferases (UGTs)

In vitro studies were performed to identify the human UGTs involved in the metabolism of fevipiprant. Recombinant UGTs (microsomes prepared from baculovirus-infected insect cells (BTI-TN-

5B1-4) expressing one single human UGT isoenzyme) were first pre-incubated with 200 µg alamethicin/mg protein, as follows per 200 µL of pre-incubation solution: UGT stock solution (100 µL, 5 mg protein/mL) was mixed with alamethicin stock solution (2 µL, 50 mg/mL in methanol) and incubated for five minutes at room temperature; afterwards, the mixture was diluted with ice-cold TRIS buffer (98 µL, 50 mM pH 7.5;) and pre-incubated on ice for 20–30 minutes until use for incubations. The methanol content in the pre-incubated enzyme stock solution was 1%.

Incubations were carried out for final [^{14}C]-fevipiprant concentrations of 25 and 1000 µM for screening with UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17; 10, 25, 50, 75, 100, 150, 200, 300, 500, 750, 1000, 2000, 4000, 7500 and 10000 µM for UGT1A3 kinetics; 10, 25, 50, 75, 100, 150, 200, 300, 500, 750 and 1000 µM for UGT2B7 kinetics; 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 300, 500, 750 and 1000 µM for UGT2B17 kinetics. The incubations were carried out in TRIS buffer (50 mM, pH 7.5). Incubations (typically 200 µL total volume) were prepared in the following manner: $MgCl_2$ solution (100 mM; final concentration in incubation: 5 mM), [^{14}C]-fevipiprant stock solution (final concentrations given above) and pre-incubated microsomes (described above; final concentration of 0.5 mg protein/mL for screening and 1.5 mg protein/mL for kinetics) were added to an appropriate volume of buffer; after three minutes pre-incubation of the admixture at 37°C (Eppendorf thermomixer comfort 5355, agitation set at 500 rpm), the reaction was started by addition of UDPGA in TRIS buffer (50 mM, pH 7.5) for a final UDPGA concentration of 5 mM for screening, 10 mM for kinetics. The samples were incubated at 37°C (Eppendorf thermomixer comfort 5355, agitation set at 500 rpm). After 60 minutes, the incubation reaction was stopped by addition of an equal volume of acetonitrile containing 0.4% acetic acid (v/v). After 30 minutes at -80°C (or overnight at -20°C), the samples were centrifuged at 30000 x g for 15 minutes. The supernatant was withdrawn and aliquots were analyzed by LSC (10 or 20 µL). For high performance liquid chromatography (HPLC) analysis, the supernatant was diluted with water to obtain a final solution containing <25% of the organic solvent. For samples with low substrate concentration, supernatants were evaporated to around 50% of the initial volume under nitrogen at 30°C with a Liebisch Evaporator (Fisher Scientific, Wohlen, Switzerland), then mixed with acetonitrile containing 0.4% acetic acid (v/v) and water, to achieve a final solution containing <25% of acetonitrile.

The residual pellet was rinsed twice with 0.5 mL water/acetonitrile (1:1, v/v) and dissolved (approximately one hour under shaking at 20°C) in 0.5 mL of a mixture containing 50% (v/v) Soluene-350 (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) and 50% isopropanol (v/v). Radiometry of aliquots of the supernatant and of the total amount of dissolved pellet was performed on a liquid scintillation counter (Tri-Carb 2500 TR, Packard Canberra Instr. Co. Meriden, CT, USA) after mixing with 10 mL LSC cocktail.

Supernatant samples were subsequently analyzed by HPLC with an Agilent 1100 system. Samples were injected onto an Atlantis T3 column (150 x 4.6 mm, 5 µm particles, Waters) with a corresponding guard column (20 x 4.6 mm), heated to 40°C. Separation was performed with a gradient of mobile phases ammonium acetate (20 mM, pH 5.0; phase A) and acetonitrile (phase B) at a flow rate of 1.0 mL/minute. The gradient was as follows: 0 to 5 minutes: 25% B; 5 to 34 minutes: 25 to 42% B; 34 to 36 minutes: 42 to 95% B; 36 to 43 minutes: 95% B; 43 to 44 minutes: 95 to 25% B; 44 to 50 minutes: 25% B. The eluate was directed to an on-line radioactivity detector (model Flow Star LB 513, Berthold Technologies GmbH, Regensdorf, Switzerland) with a 0.5 mL

liquid scintillator cell Z 500-4. The HPLC eluate was mixed with Rialuma®(Lumac, Groningen, Netherlands), pumped at a flow rate of 3 mL/minute (Berthold pump model LB 5036).

Radiochromatograms were analyzed using Radiostar (Berthold, Wildbad, Germany), Version 3.0. Rates of metabolism in each incubation were determined from fevipiprant concentrations after incubation. For kinetics incubations, rates at different fevipiprant concentrations were processed to determine Michaelis-Menten kinetic constants, using SigmaPlot Version 8.0 (S1), Enzyme Kinetics module Version 1.1 software (SPSS Science Inc., Chicago, IL, USA). The intrinsic clearance was calculated by the equation: $CL_{int} = V_{max}/K_m$.

Transporter Incubations using HEK293 cells (OAT1, OAT3, OCT, MATE1, MATE2-K, MRP2)

The potential of fevipiprant to be a substrate for organic anion transporter 1 (OAT1), organic anion transporter 3 (OAT3), organic cation transporter 2 (OCT2), multidrug and toxin extrusion protein 1 (MATE1) and multidrug and toxin extrusion protein 2-K (MATE2-K) was assessed using stably transfected HEK293 cells. Cells (0.22×10^6 cells per well) were seeded into pre-coated (poly-L-lysine/poly-L-ornithine, each 0.1 mg/mL in 10 mM phosphate buffer saline [PBS]) Costar 96 well flat bottom plates (Corning) in 200 µL of culture medium (DMEM with GlutaMax supplemented with 10% FBS, 1% penicillin-streptomycin (100 IU/mL penicillin and 100 µg/mL streptomycin), and 100 ng/µL Hygromycin B). Twenty-four hours later, the culture medium was aspirated and replaced with the final incubation solution, HBSS plus HEPES (12.5 mM, pH 7.4) containing:

For OAT1, [^{14}C]-fevipiprant (1, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100, 125, 150, 175, 200, 300 and 400 µM). 1 µM incubations were carried out in the presence and absence of inhibitor probenecid (100 µM). For OAT3, [^{14}C]-fevipiprant (0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100, 125, 150, 175, 200, 300 and 400 µM). 0.4 µM incubations were carried out in the presence and absence of inhibitor probenecid (100 µM). For OCT2, [^{14}C]-fevipiprant (0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100, 125, 150, 175, 200, 300 and 400 µM.) 1–8 µM incubations were carried out in the presence and absence of inhibitor phenoxybenzamine (50 µM). For MATE1 and MATE2K, [^{14}C]-fevipiprant (1, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100, 125, 150, 175, 200, 300 or 400 µM). 1–8 µM incubations (MATE1) and 1 µM incubations (MATE2K) were carried out in the presence and absence of inhibitor pyrimethamine (10 µM).

Each incubation was performed in triplicate, at both 37°C and at 4°C. Each experiment was terminated at designated time points (5 minutes for OAT1 and OAT3, 5 minutes for OCT2, 40 minutes for MATE1 and MATE2K) by removing the incubation solution. Subsequently, the wells were washed three times with ice-cold PBS buffer. The cell layer integrity was assessed optically and then NaOH (0.2 mL, 0.2 M) was added to each well. After incubation for 20 minutes at 37°C, aliquots (0.195 mL) were transferred into scintillation vials containing scintillation cocktail, then HCl (40 µL, 1 M) was added.

Radioactivity in samples was analyzed by LSC. Drug uptake clearance (PSapp, nL/min/mg protein) by the transfected cells was determined from the specific amount of radiolabeled fevipiprant inside the cells divided by the concentration in the incubation medium and normalized to the incubation time and the mean protein concentration measured in test wells. To take into account the initial concentration loss that likely results from nonspecific cellular binding events (Hassen et al., 1996), the initial (measured, apparent) uptake permeabilities PSapp for each fevipiprant concentration at 37°C were corrected by the 4°C data, as follows:

$$PS = PSapp_{37°C} - (PSapp_{4°C} - PSm_{4°C})$$

Equation 1

where PS is the corrected uptake clearance (µL/min/ mg protein), and PSm is the measured nonspecific (nonsaturable, passive) uptake clearance (µL/min/mg protein).

Kinetic uptake parameters were calculated by fitting PSapp and fevipiprant concentration data to the modified Michaelis-Menten equation (Sasaki et al., 2004):

$$\mathrm{PSapp} = \mathrm{PSm} \pm \mathrm{PSc} = \mathrm{PSm} \pm \frac{\mathrm{Vc,max}}{\mathrm{Km,app} + \mathrm{S}} = \mathrm{PSm} \pm \frac{\mathrm{PSc,max} \times \mathrm{Km,app}}{\mathrm{Km,app} + \mathrm{S}}$$

Equation 2

where PSc is the carrier-mediated (saturable) uptake permeability (nL/min/mg protein), PSc,max is the maximum carrier mediated uptake permeability (nL/min/mg protein), Vc,max is the maximum transporter uptake velocity/rate (fmol/min/mg protein), S is the applied substrate concentration (µM), and Km,app is the apparent Michaelis-Menten constant (µM).

Efflux transporter incubations

The affinity of fevipiprant for efflux transporters multi-drug resistance gene 1 (MDR1), multi-xenobiotic resistance (MXR) and multi-drug resistance protein 2 (MRP2) was assessed by measuring concentration-dependent fevipiprant uptake using recombinant LLCPK1 cells expressing MDR1, and MDCKII cells expressing MXR or MRP2. Cells (~0.6 x 10^5 cells per well) were seeded into Falcon clear bottom 96 well plates (Becton Dickinson) with 200 µL of culture medium (for LLCPK: medium 199 supplemented with 10% FBS, 50 µg/µl gentamycin and 100 µg/µl hygromycin B; for MDCKII: DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin, and 0.8 mg/ml G418) and incubated at 37°C with 5% CO_2. The assay was performed 24 hours (for LLCPK-MDR1 and MDCKII-MXR transfectants) or 72 hours (for MDCKII-MRP2 cells) later. On the day of the study, the culture medium was aspirated and replaced by pre-incubation solution (buffer system [for MDR1: Krebs-Henseleit, pH7.4; for MXR: cell culture medium; for MRP2: HBSS with HEPES 12.5 mM, pH 5] in the presence or absence of inhibitors of interest (for MDR1: cyclosporin A 10 µM, for MXR: fumitremorgin C 50 µM, for MRP2: MK571 10 µM]). Plates were subsequently incubated at 37°C for 10 minutes. The pre-incubation solution was then replaced with the final incubation solution (as pre-incubation solutions, but containing fevipiprant [for MDR1 and MXR: 2, 4, 6, 8, 10, 25, 50, 75, 100, 125, 150, 175 or 200 µM; for MRP2: 0.5, 1, 2, 4, 6, 8, 10, 25, 50, 75, 100, 125, 150, 175 or 200 µM]). Incubations with the lowest fevipiprant concentration were carried out in the presence and absence of the inhibitors described for the pre-incubation solutions. All incubations were performed in triplicate. Incubations were terminated after 40 minutes by removing the incubation solution. Subsequently, the cells were washed twice with ice-cold PBS buffer and 0.2 mL of NaOH solution (0.2N) was added to each well. After incubation for 20 minutes at 37°C, aliquots (0.195 mL) were transferred into scintillation vials containing scintillation cocktail, and radioactivity was measured by LSC. Fevipiprant uptake clearance parameters were calculated as described above for other transporter assays, except without correction for non-specific binding.

Hepatic uptake and organic anion-transporting polypeptide (OATP) uptake transporter incubations

The potential active uptake of fevipiprant by hepatocytes was studied in cryopreserved hepatocyte suspensions, by testing the concentration and temperature dependency of in vitro cellular uptake. The involvement of several candidate transporter families (OCT, OAT, OATP, Na^+-taurocholate cotransporting polypeptide [NTCP]) was

investigated using representative uptake transporter inhibitors.

Cryopreserved suspensions of isolated human hepatocytes were purchased from Biopredic International (35000 Rennes, France). Prior to starting the uptake studies, the cell suspensions were pre-warmed in an incubator at 37°C or 4°C. Subsequently, the hepatocyte concentration was adjusted to $2x10^6$ cells/mL with pre-warmed Krebs-Henseleit buffer (KHB). The uptake experiments were initiated by adding cell suspension (100 µL) to KHB buffer (50 µL) containing fevipiprant (1 and 200 µM) in the presence and absence of inhibitors of interest (rifamycin SV 20 µM and atorvastatin 10 µM, *p*-aminohippuric acid 3 mM, or tetraethylammonium 3 mM) at 37°C and 4°C. All incubations were performed in triplicate. After three minutes, the reaction was terminated by separating the cells from the substrate solution. For this purpose, 150 µL of incubation mixture was collected and carefully placed on top of a 100 µL filtration oil layer (a mixture of silicone oil and mineral oil with a density of 1.015) in 0.4 mL Easy-Cut microcentrifuge tubes (Becton Dickinson AG, Basel, Switzerland) containing NaOH (100 µL, 2 M) underneath the oil layer. The sample tubes were then quickly centrifuged at 14000 rpm for one minute. After two hours at room temperature, the centrifuged tubes were stored overnight at -80°C. Thereafter, each compartment of the tubes was cut, transferred into scintillation vials and mixed with scintillation cocktail. The compartment containing the dissolved cells was neutralized with HCl (100 µL, 1 M). Radioactivity was determined by LSC.

Fevipiprant uptake clearance (in µL/min/10^6 cells) was calculated, as described above for other transporter assays.

The affinity of fevipiprant for human OATP1B1, OATP1B3 and OATP2B1 transporters was investigated by measuring the concentration-dependent fevipiprant uptake using recombinant HEK293 cells expressing human OATP1B1, OATP1B3 and OATP2B1.

Cells (~2 x 10^6 cells per well for OATP1B1,OATP1B3 and OATP2B1 overexpressing cells) were seeded into precoated (poly-L-lysine/poly-Lornithine, 0.1 mg/ml of each in 10 mM PBS) Falcon clear bottom 96 well plates (Becton Dickinson) with 200 µL of culture medium (DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin, and 0.8 mg/µL G418) and were incubated at 37°C with 5% CO_2. In order to induce the expression of the OATP transporter systems, 48 hours after seeding, the cell culture medium was replaced with culture medium supplemented with 5 mM sodium butyrate and incubated for 24 hours. Seventy-two hours after seeding, the culture medium was aspirated and replaced with the final incubation solution (HBSS with 10 mM HEPES, 2% FBS, containing fevipiprant [2, 4, 6, 8, 10, 25, 50, 75, 100, 125, 150, 175, or 200 µM]). Incubations (2 µM) were carried out in the presence and absence of inhibitors rifamycin SV (20 µM) and atorvastatin (10 µM). Incubations were performed in triplicate. The experiment was terminated after three minutes by removing the incubation solution. The wells were washed twice with ice-cold PBS buffer and NaOH solution (0.2 mL, 0.2 M) was added to each well. After incubation for 20 minutes at 37°C, aliquots (0.195 mL) were transferred into scintillation vials containing scintillation cocktail. Radioactivity was measured by LSC.

Fevipiprant uptake clearance and kinetic parameters were calculated, as described above for other transporter assays, except without correction for non-specific binding.

Synthesis of AG-metabolite

In a shaker 750 mL reaction mixture [50 mM UDPGA * 3 Na (SIGMA U6751), 25 mM $MgCl_2$ (Sigma 208337), 250 mM HEPES (SIGMA H3375), 40 mM NaCl aq. pH 7.5] were mixed with 500 mL of pig liver S9

fraction ((Kittelmann et al., 2003)) and 25 mL fevipiprant solution (100 mM in DMSO). The reaction was divided in 6 aliquots of approximately 212 mL and incubated with closed cap in six 500 mL Polyethylene tubes (Beckman Coulter JLA-10.500 369681) at 37°C and 150 rpm for 14 hours. After the end of the incubation time each reaction aliquot was mixed with 4 mL acetic acid, 250 mL acetonitrile and stirred at room temperature for 30 minutes. The broth was centrifuged at 10000 g for 5 min and the supernatant was filtrated through a paper filter. The filtrate was then mixed with 7.5 Liters of aqueous TFA solution 0.05 % and pumped directly on a RP18 chromatography column. The conditions for preparative HPLC were: self-packed steel column 200 x 50 mm; stationary phase LiChroprep RP18 (MERCK KGaA 1.13900); solvent A: aqueous TFA 0.05 %; solvent B: acetonitrile; gradient: 0 - 5 min 25 % B, 35 min 95 % B; flow rate of 150 mL/min; room temperature; detection at 220 nm; fraction size 60 mL. The product eluted at 65 % B. The product containing fractions were combined and the solvents were evaporated under reduced pressure to a final volume of 250 mL. The remaining raw product solution was divided in four equal aliquots. Each aliquot was used for an identical chromatography. The second preparative HPLC was performed with a 250 x 21 mm Nucloeodur 100-10 C18 ec column (Macherey-Nagel, Düren, Germany) under the same conditions as described before, but with a flow rate of 40 mL/min. The product containing fractions were again combined, concentrated to about 150 mL and dried by lyophilization overnight. The product, 462 mg (31%), was obtained with > 99 % purity (HPLC/full DAD). ^{1}H NMR (600 MHz, DMSO-d6) δ ppm 2.28 (s, 3 H) 3.17 (t, 1 H) 3.27 (s, 3 H) 3.30 (m, 2 H) 3.68 (d, 1 H) 3.93 (q, 2 H) 5.40 (d, 1 H) 5.76 (s, 2 H) 6.47 (d, 1H) 7.10 (dd, 1 H) 7.96 (d, 1 H) 8.03 (d, 1 H) 8.13 (d, 1 H) 8.24 (s, 1 H).
13C NMR (101 MHz, DMSO-d6) δ ppm 9.7, 29.2, 40.9, 42.9, 71.1, 72.1, 75.5, 75.8, 94.3, 102.9, 116.1, 120.1, 123.4, 124.8, 126.4, 127.4, 131.7, 135.3, 140.0, 141.8, 142.5, 146.9, 169.9, 170.1.

Radiolabeled AG-metabolite was prepared similarly but with [^{14}C]-fevipiprant as starting material.

Synthesis of lactone metabolite

Fevipiprant (200 mg, 0.469 mmol) was taken into 1,2-dimethoxyethane (5 ml) and m-chloroperoxybenzoic acid (168 mg, 0.750 mmol) was added. The reaction was stirred at room temperature for 16 hours then added to water, extracted with dichloromethane three times and evaporated. The resultant solid was initially purified on a C-18 reverse phase column eluting with a gradient of 0-40% acetonitrile-water. Product containing fractions were extracted with dichloromethane three times and the combined organic phases were washed with saturated aqueous $NaHCO_3$ and evaporated to afford rac-(3a*R*,8a*S*)-3a-hydroxy-8a-methyl-8-(4-(methylsulfonyl)-2-(trifluoromethyl)benzyl)-3,3a,8,8a-tetrahydro-2H-furo[3',2':4,5]pyrrolo[2,3-b]pyridin-2-one as a white solid (85 mg, 41%). LCMS: MH^+ 443. ^{1}H NMR (DMSO-d6, 400 MHz) 1.62 (3H s), 3.30 (2H d J=6.2 Hz), 3.34 (3H s), 4.84 (1H d J=18.1 Hz), 4.95 (1H d J=18.1 Hz), 6.57 (1H s), 6.89 (1H dd J=7.2, 5.2), 7.85 (2H overlapping d, apparent J=8.6 Hz), 8.04 (1H d J=8.1 Hz), 8.17 (1H d J=8.1 Hz), 8.23 (1H s)

Synthesis of fevipiprant

Fevipiprant was prepared as described in (Bala et al., 2005). [^{14}C]-fevipiprant was prepared as shown in Figure S10.

RESULTS

Table S1. Mass spectral data of [^{14}C]-fevipiprant and metabolites

Component	Matrix [a)]	Elementary composition of $[M+H]^+$	Observed LC/MS(MS) data			
			m/z $[M+H]^+$	Mass accuracy (ppm)	Rt (min)	Major MS/MS signals (m/z)
AG metabolite	p, u	$C_{25}H_{25}N_2O_{10}F_3S$	603.1281	1.1	4.1	427
Lactone metabolite	u, f	$C_{19}H_{17}N_2O_4F_3S$	443.0888	1.2	6.5	423, 405, 403, 397, 383, 377,361, 341
Fevipiprant	p, u, f	$C_{19}H_{17}N_2O_4F_3S$	427.0936	0.4	5.5	407,381, 347, 190, 145

[a)] p, plasma; u, urine; f, feces

Table S2. Excretion of radioactivity in excreta (0–240 h)

Subject	Excretion of radioactivity (% of dose)		
	Urine	Feces	Total
1	39.9	53.8	93.6
2	37.3	57.3	94.6
3	44.7	46.0	90.7
4	46.5	50.3	96.9
Mean ± SD	42.1 ± 4.26	51.9 ± 4.84	93.9 ± 2.57

Table S3. Biotransformation of fevipiprant by recombinant uridine diphosphate glucuronosyltransferase (UGTs)

Enzyme	Supplier (Gentest) Catalogue Number	Supplier (Gentest) Lot Number	Metabolism Rate (pmol/min/mg protein)	
			25 µM fevipiprant	1000 µM fevipiprant
Human liver microsomes	457081	82087	21.5	393
UGT insect cell control	456400	3	n.d.	n.d.
UGT1A1	456411	61013	n.d.	n.d.
UGT1A3	456413	70200	8.50	487
UGT1A4	456414	21161	n.d.	n.d.
UGT1A6	456416	9	0.17	n.d.
UGT1A7	456407	30991	0.92	n.d.
UGT1A8	456418	05599	2.83	13.3
UGT1A9	456419	59841	1.58	n.d.
UGT1A10	456410	96097	n.d.	n.d.
UGT2B4	456424	56983	1.92	16.7
UGT2B7	456427	03362	2.96	28.3
UGT2B15	456435	5	1.54	6.67
UGT2B17	456437	4	18.0	61.7

n.d., not detectable.

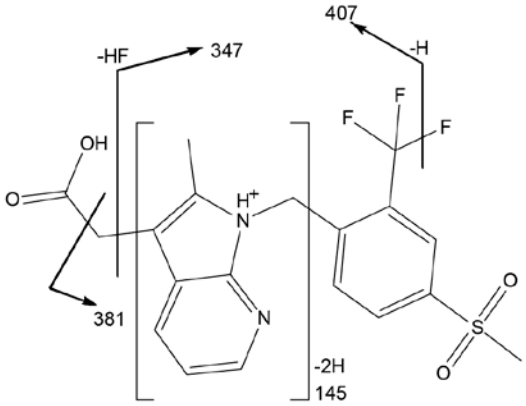
Table S4. Hepatic fevipiprant uptake by human hepatocytes

Fevipiprant (µM)[a)]	Temperature (°C)	Inhibitor	CLapp [µl/10^6 cells][b,c,d)]	CL [µl/10^6 cells][e)]	CL [µl/min/10^6 cells][e)]
1	37	-	33 ± 1	30 ± 2	10 ± 1
200	37	-	15 ± 5	15 ± 6	5 ± 2
1	37	RSV/ATO	17 ± 1	14 ± 2	5 ± 1
1	37	TEA	25 ± 1	22 ± 2	7 ± 1
1	37	PAH	29 ± 2	26 ± 3	9 ± 1
1	4	-	11 ± 2	-	-
200	4	-	8 ± 2	-	-
1	4	RSV/ATO	9 ± 1	-	-
1	4	TEA	11 ± 0	-	-
1	4	PAH	13 ± 1	-	-

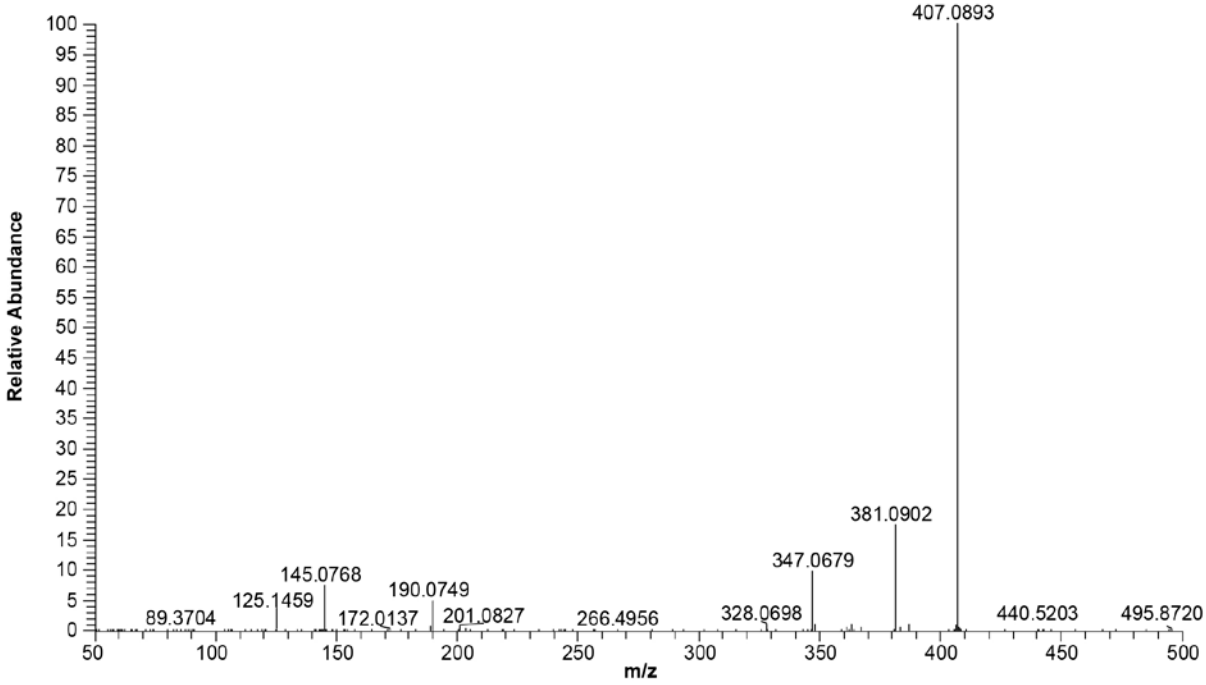
RSV, rifamycin SV; ATO, atorvastatin; PAH, *p*-aminohippuric acid; TEA, tetraethylammonium.

[a)]Nominal values; [b)]Mean ± SD of n=3 determined after 3 minutes of incubation; [c)]Average protein concentration was 1.49 mg for 0.2x10^6 cells; [d)] V_{app}/S, where V_{app} was calculated from the slope of the time-dependent fevipiprant uptake [pmol/10^6 cells] between 0 and 3 minutes; [e)]Corrected uptake data calculated according to Equation 1 assuming CLapp,4°C at 200 µM to be equal to CLm,4°C

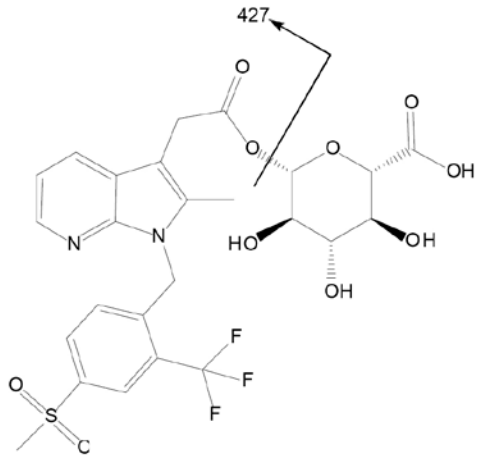
A)



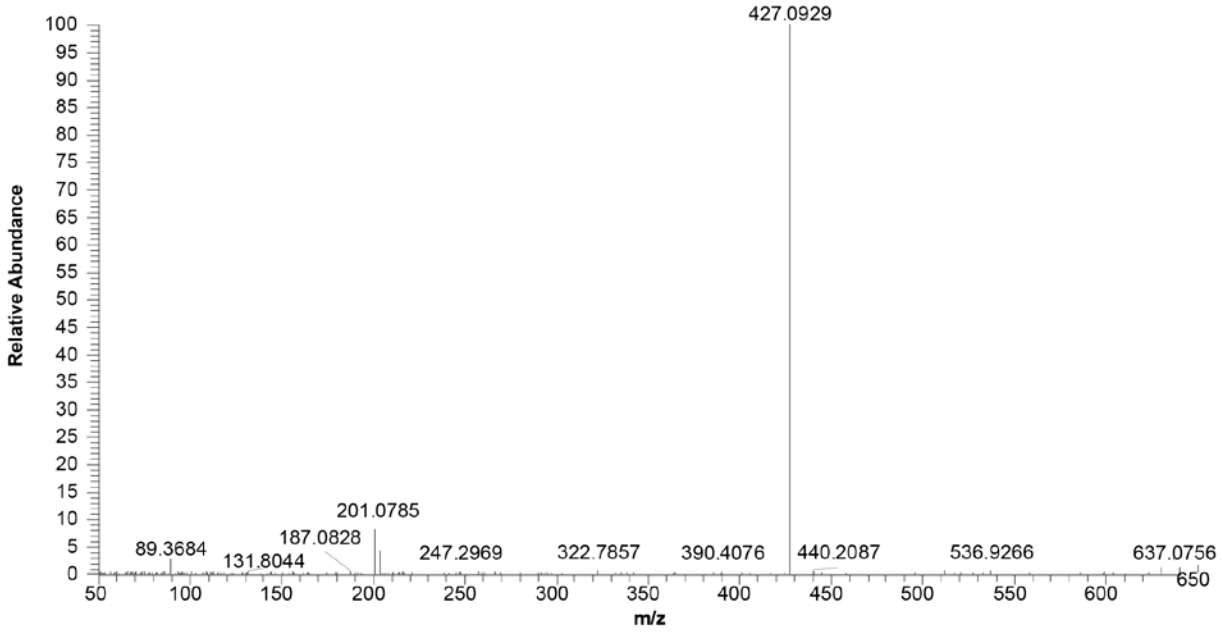
A2104_009 #671-678 RT: 5.51-5.59 AV: 4 NL: 2.27E8
F: FTMS + cESI Full ms2 427.09@cid35.00 [50.00-500.00]



B)



A2104_009 #568-580 RT: 3.90-4.04 AV: 6 NL: 1.05E6
F: FTMS + c ESI Full ms2 603.13@cid35.00 [50.00-650.00]



C)

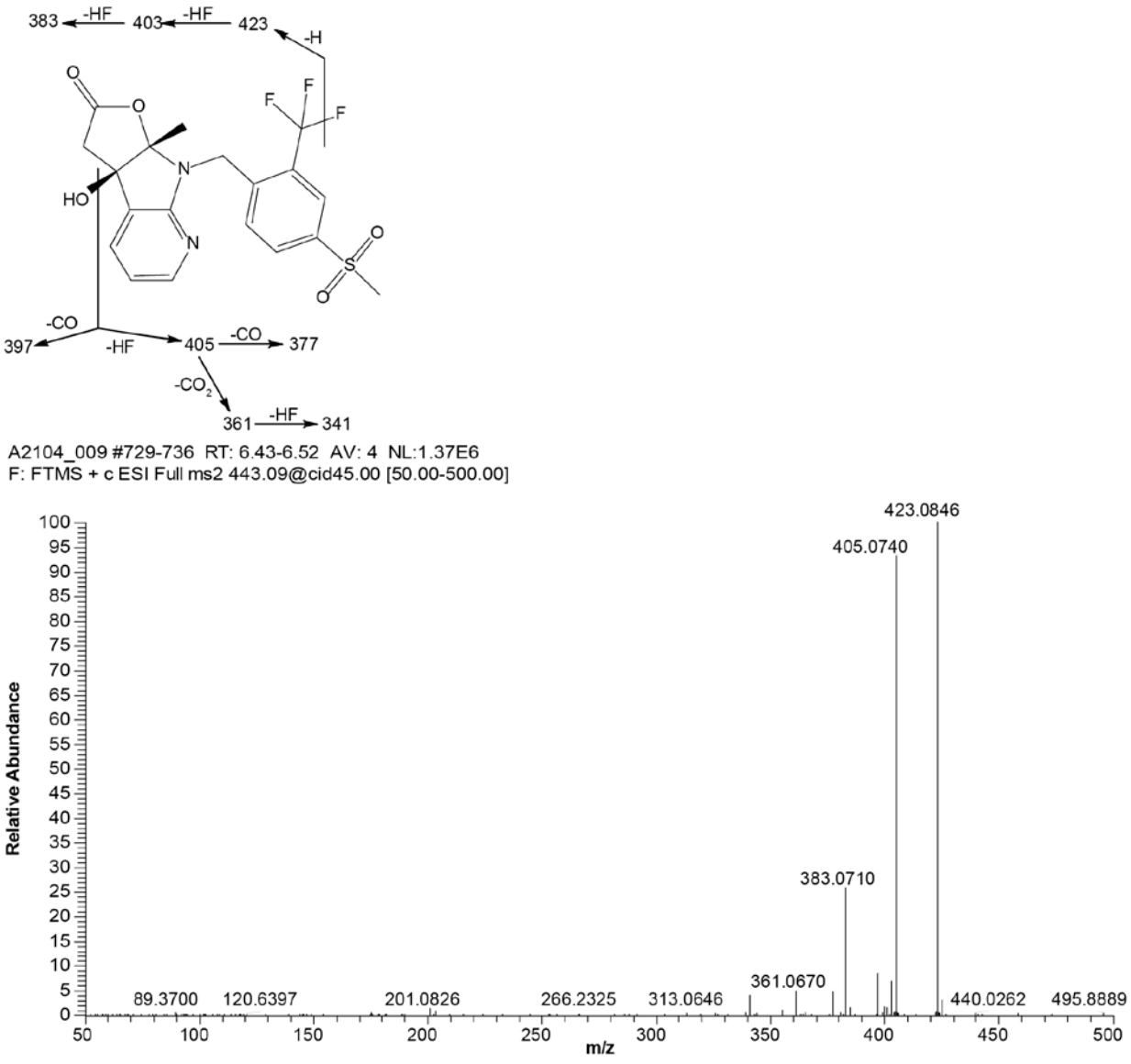


Figure S1. MS/MS spectra and proposed fragmentation of A) fevipiprant B) AG metabolite and C) lactone metabolite

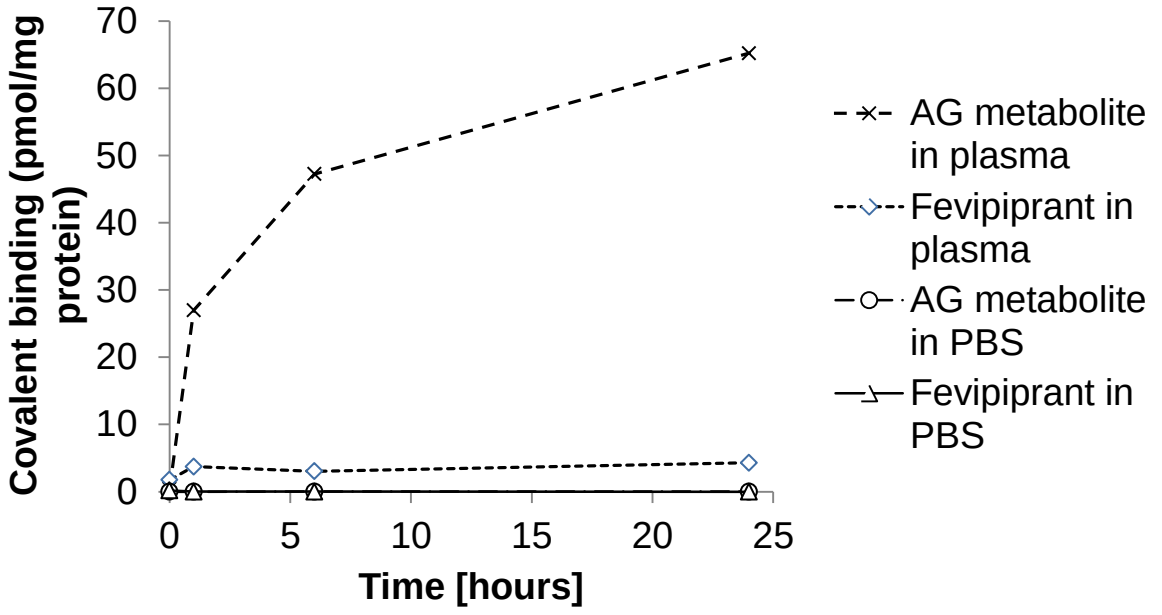


Figure S2. Covalent protein binding after incubation of [^{14}C]-fevipiprant (20 µM) or [^{14}C]-AG metabolite (20 µM) with 1:1 human plasma/PBS or PBS at 37 ºC, followed by protein precipitation, filtration and LSC quantification of protein-bound radioactivity. PBS, phosphate buffered saline

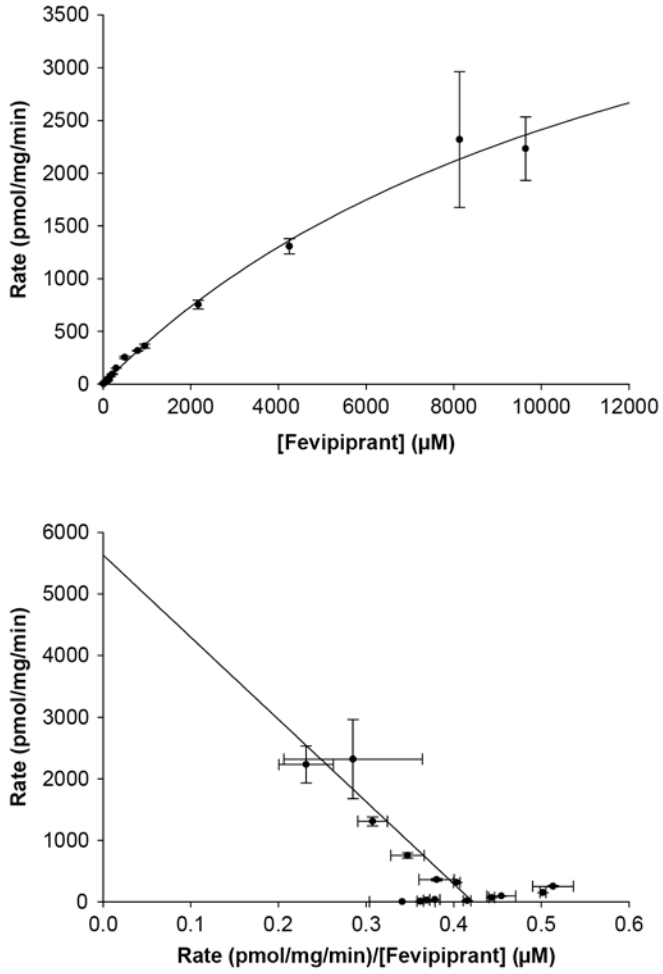


Figure S3. Enzyme kinetics of [^{14}C]-fevipiprant metabolism by recombinant UGT1A3, presented as reaction rate vs substrate concentration, and as reaction rate vs rate/substrate concentration. Reaction rates were measured by incubation of [^{14}C]-fevipiprant with recombinant UGT1A3 in TRIS buffer at 37 °C for 60 minutes, followed by extraction and HPLC with radioactivity quantification. The rate was calculated from the decrease in fevipiprant concentration based on the HPLC metabolite profile generated. Data fitting was performed using Sigmaplot Enzyme Kinetics module based on Michaelis-Menten kinetics.

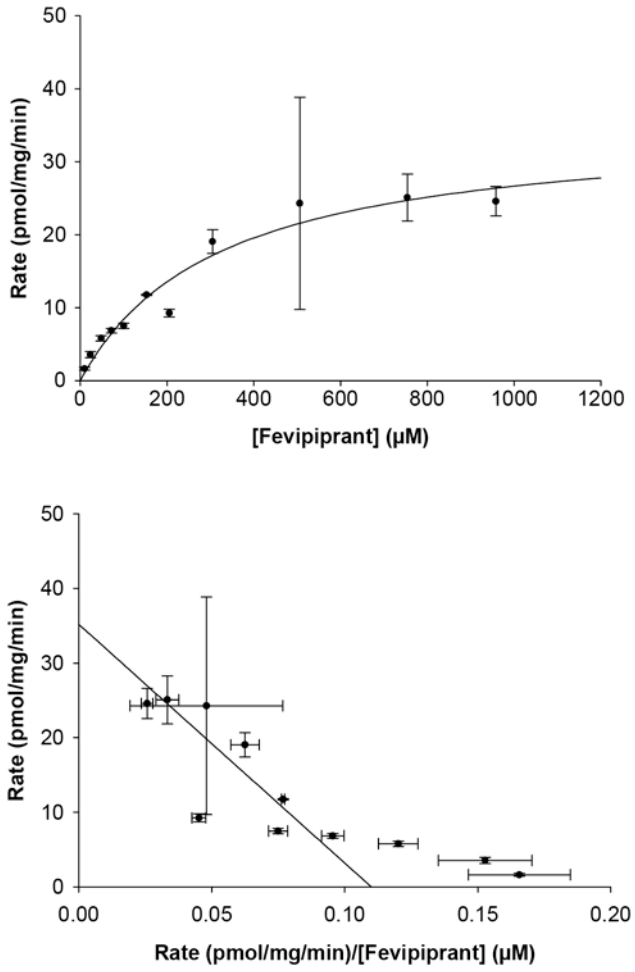


Figure S4. Enzyme kinetics of [^{14}C]-fevipiprant metabolism by recombinant UGT2B7 presented as reaction rate vs substrate concentration, and as reaction rate vs rate/substrate concentration.

Reaction rates were measured by incubation of [^{14}C]-fevipiprant with recombinant UGT2B7 in TRIS buffer at 37 °C for 60 minutes, followed by extraction and HPLC with radioactivity quantification. The rate was calculated from the decrease in fevipiprant concentration based on the HPLC metabolite profile generated. Data fitting was performed using Sigmaplot Enzyme Kinetics module based on Michaelis-Menten kinetics.

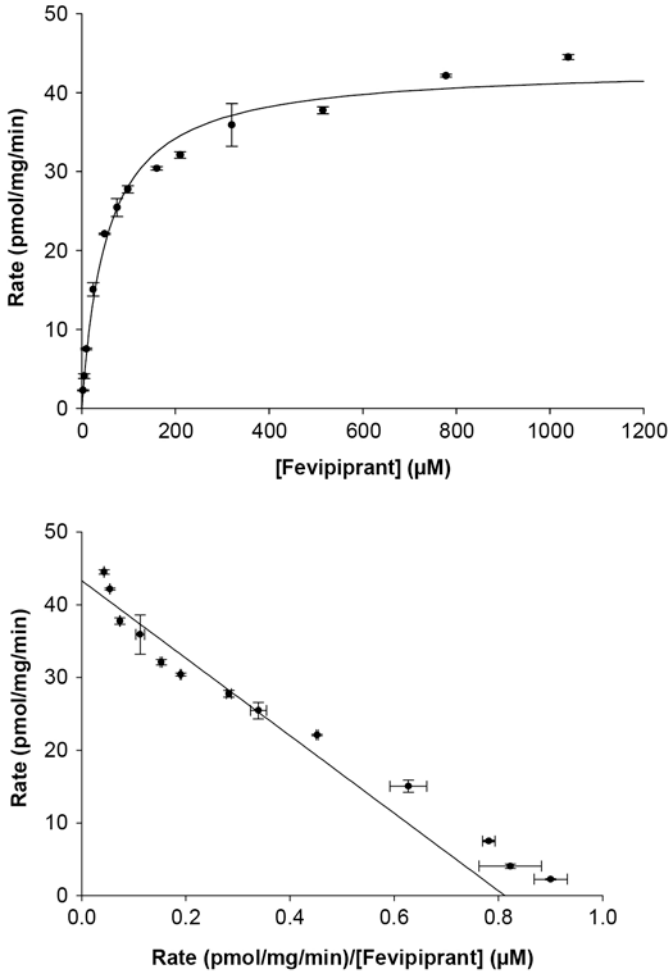


Figure S5. Enzyme kinetics of [14C]-fevipiprant metabolism by recombinant UGT2B17 presented as reaction rate vs substrate concentration, and as reaction rate vs rate/substrate concentration. Reaction rates were measured by incubation of [^{14}C]-fevipiprant with recombinant UGT2B17 in TRIS buffer at 37 °C for 60 minutes, followed by extraction and HPLC with radioactivity quantification. The rate was calculated from the decrease in fevipiprant concentration based on the HPLC metabolite profile generated. Data fitting was performed using Sigmaplot Enzyme Kinetics module based on Michaelis-Menten kinetics.

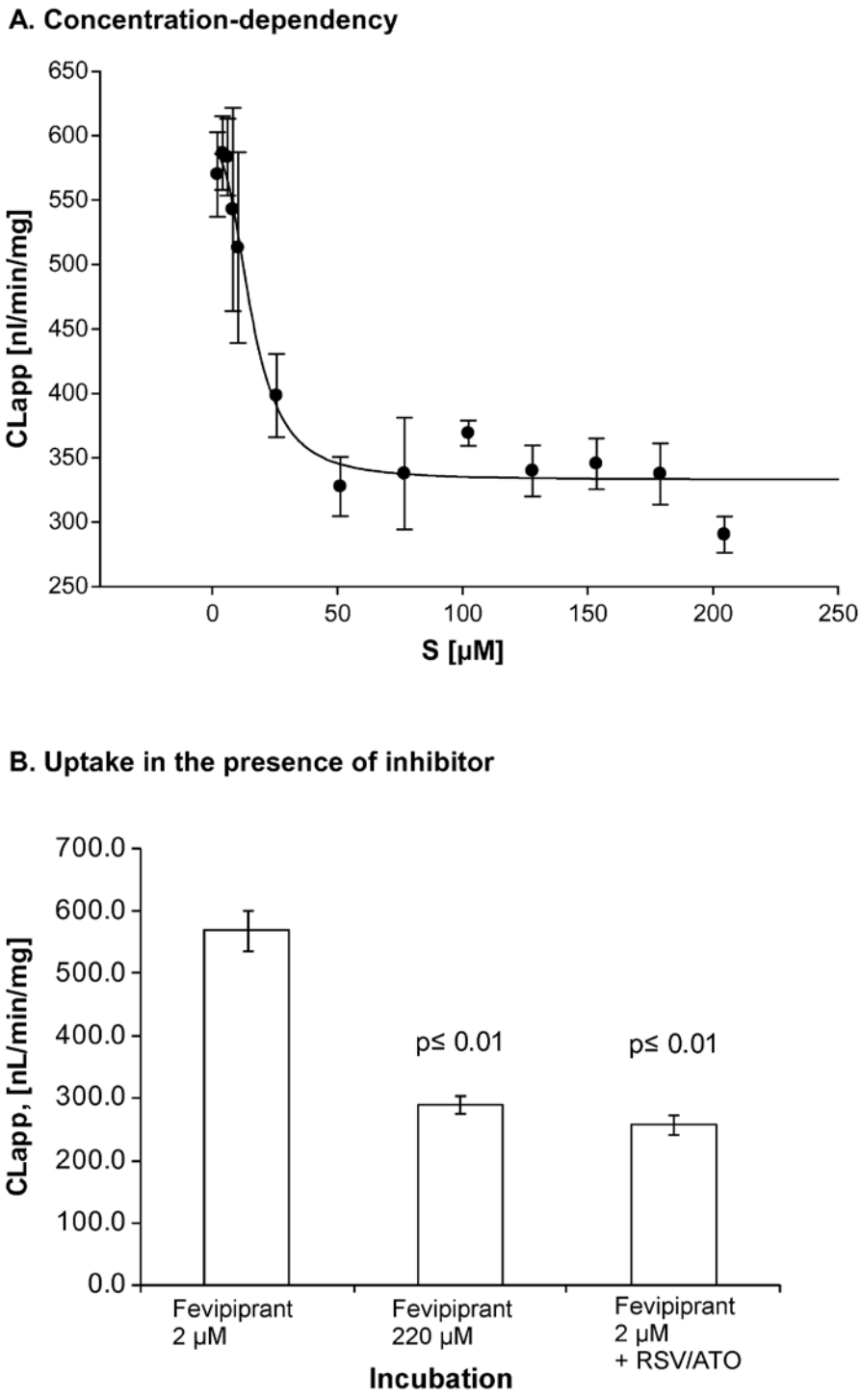


Figure S6 A, B. Uptake of fevipiprant by OATP1B3-transporter expressing HEK293 cells presented as CLapp for various substrate concentrations and in the presence and absence of inhibitors of OATP1B3. CLapp was calculated from the amount of radioactivity present in

OATP1B3-expressing HEK293 cells after incubation with [^{14}C]-fevipiprant at 37ºC for 3 minutes, then fitting data to Equation 2 (Supplementary methods section)RSV, rifamycin SV; ATO, atorvastatin.

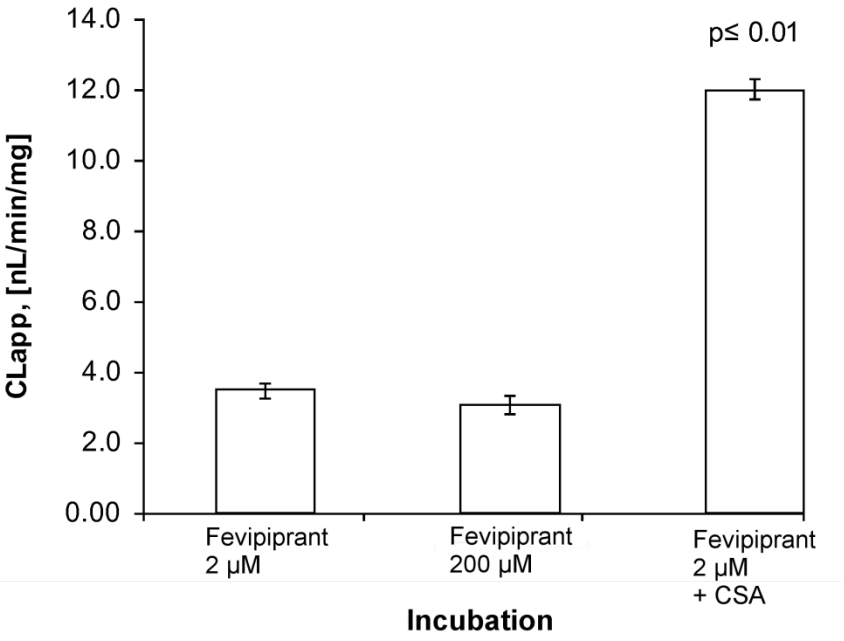


Figure S7. Uptake of fevipiprant by MDR1-transporter expressing LLCPK1 cells presented as CLapp for two substrate concentrations and in the presence and absence of an inhibitor of MDR1. CLapp was calculated from the amount of radioactivity present in MDR1-expressing LLCPK1 cells after incubation with [^{14}C]-fevipiprant at 37ºC for 40 minutes, then fitting data to Equation 2 (Supplementary methods section). CSA: Cyclosporine A

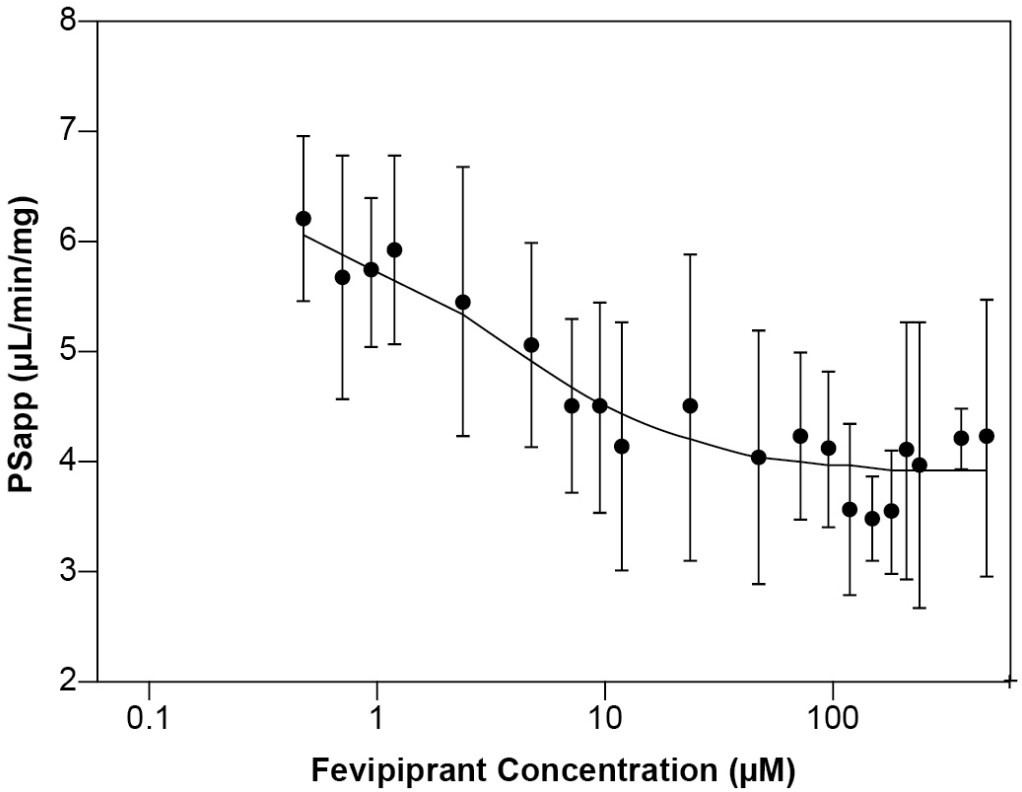


Figure S8. Uptake of fevipiprant by OAT3 transporter expressing HEK293 cells presented as PSapp for various substrate concentrations. PSapp was calculated from the amount of radioactivity present in OAT3-expressing HEK293 cells after incubation with [^{14}C]-fevipiprant at 37ºC for 3 minutes, by fitting data to Equation 2 (Supplementary methods section)

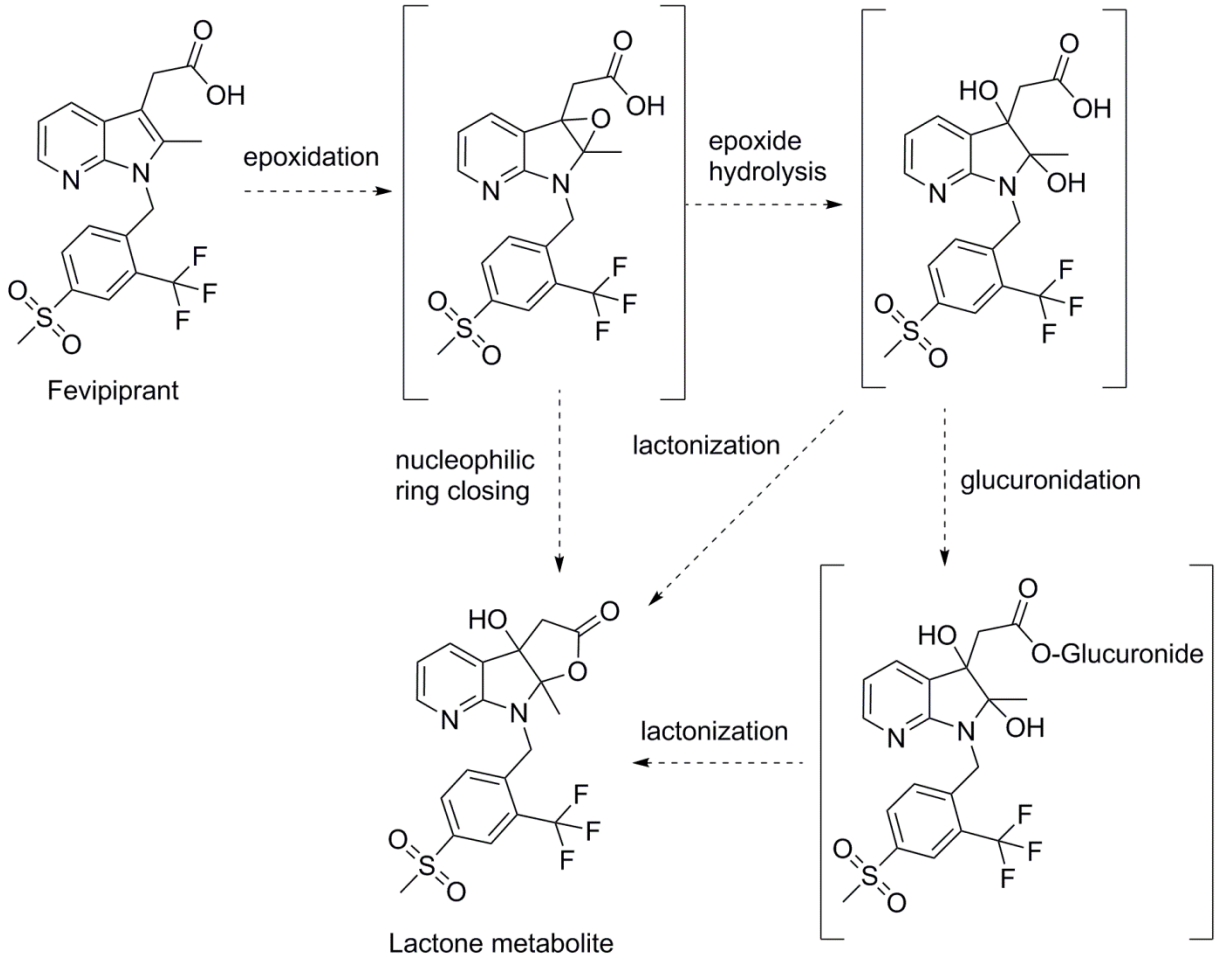


Figure S9. Proposed possible pathways of lactone metabolite formation

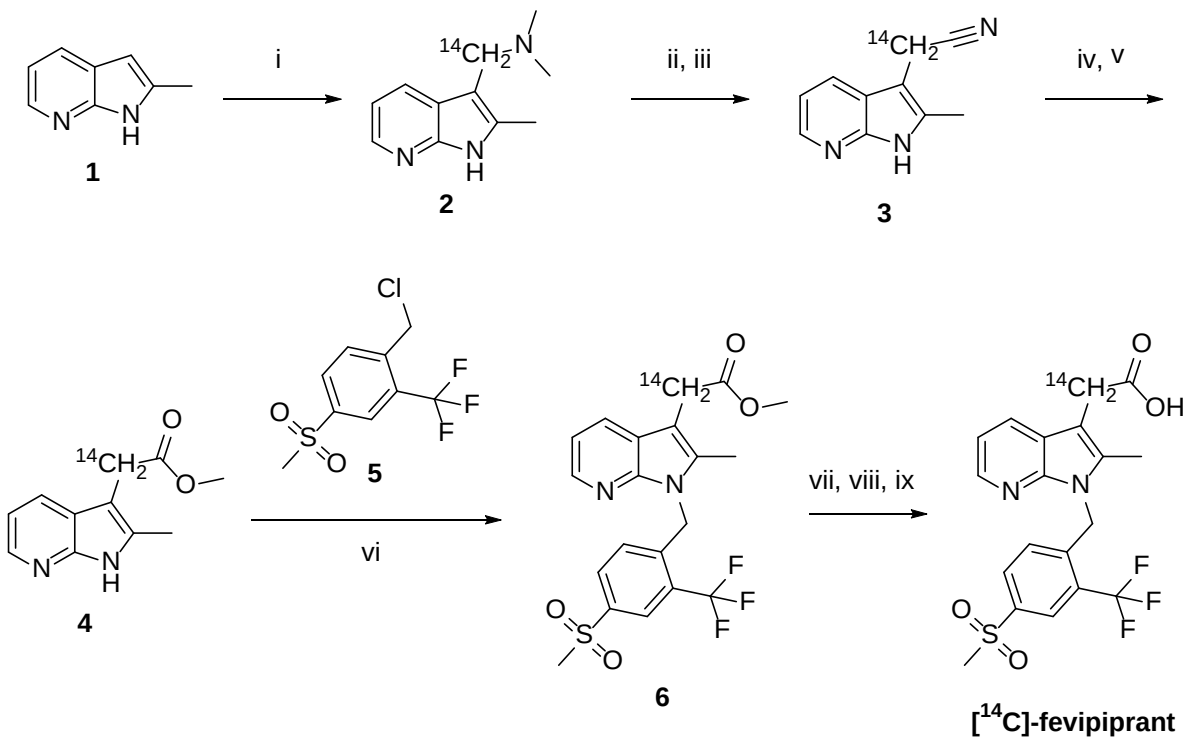


Figure S10. Synthetic scheme for [^{14}C]-fevipiprant. i) ($^{14}CH_2O$)n, H_2O, $Me_2NH.HCl$; ii) MeI, EtOH, 25°C; iii) NaCN, DMF, 100°C; iv) HCl, 100°C; v) MeOH, H_2SO_4, 100°C; vi) 5, $CsCO_3$, acetone, reflux; vii 12% NaOH, rt; viii) HCl; ix) crystallization EtOH/H_2O.

References

Bala, K., Leblanc, C., Sandham, D. A., Turner, K. L., Watson, S. J., Brown, L. N., & Cox, B. (2005). WO2005123731A2.

Hassen, A. M., Lam, D., Chiba, M., Tan, E., Geng, W., & Pang, K. S. (1996). Uptake of sulfate conjugates by isolated rat hepatocytes. *Drug Metab Dispos, 24*(7), 792-798.

Kittelmann, M., Reinegger, U., Espigat, A., Oberer, L., Aichholz, R., Francotte, E., & Ghisalba, O. (2003). Preparative enzymatic synthesis of the acylglucuronide of mycophenolic acid. *Adv. Synth. Catal., 345*, 825-829.

Sasaki, M., Suzuki, H., Aoki, J., Ito, K., Meier, P. J., & Sugiyama, Y. (2004). Prediction of in vivo biliary clearance from the in vitro transcellular transport of organic anions across a double-transfected Madin-Darby canine kidney II monolayer expressing both rat organic anion transporting polypeptide 4 and multidrug resistance associated protein 2. *Mol Pharmacol, 66*(3), 450-459. doi: 10.1124/mol.66.3.