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Title page

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

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Running Title Page

Fevipiprant ADME in healthy volunteers and in vitro

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Abbreviations: ADME, absorption, distribution, metabolism, and excretion; AE, adverse event; $Ae_{0-240\ h}$, amount of drug excreted into the urine from time zero to 240 hours post-dose; AG, acyl-glucuronide; AUC, area under the concentration-time curve; AUC_{0-t}, the area under the concentration-time curve from time zero to t; AUC_{inf}, the area under the concentration-time curve from time zero to infinity; BMI, body mass index; CL/F, the systemic clearance CL of the drug from the plasma divided by the bioavailability F; CLr, renal clearance; C_{max}, the maximum (peak) plasma or blood drug concentration after single administration; CRTh2, chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells; DDI, drug-drug interaction; DMPK, Drug Metabolism and Pharmacokinetics; DP_2, prostaglandin D_2 receptor 2; EU,

European Union; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; IL, Isotope Laboratory; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LSC, liquid scintillation counting; MATE, multidrug and toxin extrusion protein; MDR, multi-drug resistance gene; NOAEL, no observed adverse effect level; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; PK, pharmacokinetic(s); SEC, size exclusion chromatography; TRD, Technical Research and Development; T_{max}, the time to reach peak or maximum concentration following drug administration; $T_{1/2}$, terminal half-life of elimination; UGT, uridine 5'-diphospho (UDP)-glucuronosyltransferase; Vz/F, the apparent volume of distribution during the terminal phase divided by the bioavailability F.

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Abstract

Fevipiprant is a novel oral prostaglandin D_2 receptor 2 (DP_2; also known as CRTh2) antagonist, which is currently in development for the treatment of severe asthma and atopic dermatitis. We investigated the absorption, distribution, metabolism, and excretion properties of fevipiprant in healthy subjects after a single 200 mg oral dose of [^{14}C]-radiolabeled fevipiprant. Fevipiprant and metabolites were analyzed by liquid chromatography coupled to tandem mass spectrometry and radioactivity measurements, and mechanistic in vitro studies were performed to investigate clearance pathways and covalent plasma protein binding. Biotransformation of fevipiprant involved predominantly an inactive acyl glucuronide (AG) metabolite, which was detected in plasma and excreta, representing 28% of excreted drug-related material. The AG metabolite was found to covalently bind to human plasma proteins, likely albumin; however, in vitro covalent binding to liver protein was negligible. Excretion was predominantly as unchanged fevipiprant in urine and feces, indicating clearance by renal and possibly biliary excretion. Fevipiprant was found to be a substrate of transporters organic anion transporter 3 (OAT3; renal uptake), multi-drug resistance gene 1 (MDR1; possible biliary excretion), and organic anion-transporting polypeptide 1B3 (OATP1B3; hepatic uptake). Elimination of fevipiprant occurs via glucuronidation by several uridine 5'-diphospho glucuronosyltransferase (UGT) enzymes, as well as direct excretion. These parallel elimination pathways result in a low risk of major drug-drug interactions or pharmacogenetic/ethnic variability for this compound.

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Introduction

The prevalence of allergic diseases is increasing worldwide, with the World Health Organization estimating that 400 million people globally will be asthma sufferers by 2025 (Pawankar, 2014). In the European Union (EU), 44–76 million individuals of the 217 million EU employees suffer from allergic diseases of the airways or the skin and up to 90% of these individuals are untreated or insufficiently treated (Zuberbier et al., 2014).

Fevipiprant (QAW039; [(2-[2-methyl-1-(4-[methylsulfonyl]-2-[trifluoromethyl]benzyl)-1H-pyrrolo(2,3-b)pyridin-3-yl] acetic acid)]) is a potent and highly selective novel antagonist of the human prostaglandin D_2 receptor 2 (DP_2, also known as CRTh2), which is a class A G protein–coupled receptor involved in the modulation of inflammatory responses (Sykes et al., 2016). DP_2 is also expressed on innate immune cells, such as eosinophils and ILC-2 cells, and plays a role in the pathophysiology of respiratory disease (Townley and Agrawal, 2012; Xue et al., 2014). Recently, human functional studies across diverse cellular systems have shown that fevipiprant shows high potency for competitive inhibition of disease-relevant DP_2-mediated responses in human cells, such as Th2 cell cytokine production, and eosinophil activation (Sykes et al., 2016). Fevipiprant is currently in development as a once-daily oral therapy for respiratory and dermatologic disorders, such as severe asthma and atopic dermatitis (Sykes et al., 2016).

Two Phase 1 studies investigated the pharmacokinetics (PK), safety, and tolerability of fevipiprant after single and multiple ascending doses in healthy subjects (Erpenbeck et al., 2016). On administration of single and multiple oral doses, fevipiprant peak plasma concentrations were observed 1–3 hours post-dose, and the apparent terminal half-life was approximately 20 hours. Steady state was achieved within four days, with less than two-fold accumulation. An acyl glucuronide (AG) metabolite without DP_2 antagonist activity was detected in plasma. Fevipiprant was well-tolerated at single and multiple oral doses up to 500 mg/day (Erpenbeck et al., 2016).

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With any new chemical entities, it is important to identify and quantify all relevant metabolites and elimination pathways, for assessment of possible metabolite pharmacology, safety (FDA, 2008; ICH, 2009; Gao et al., 2013), clearance mechanisms and drug-drug interactions (DDIs) (EMA, 2012; FDA, 2012), including planning of suitable clinical DDI and hepatic/renal impairment studies.

We report the assessment of human absorption, distribution, metabolism, and excretion (ADME) of a single oral 200 mg dose of [^{14}C]-fevipiprant in healthy male subjects, as well as identification of enzymes and transporters involved in the human PK of fevipiprant, and characterization of covalent binding to plasma proteins in humans.

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Materials and Methods

The primary objectives of the clinical ADME study were to identify and quantify fevipiprant and metabolites in plasma and excreta for analysis of absorption, PK, and elimination pathways.

Study Drug

The parent batch of [^{14}C]-radiolabeled fevipiprant was prepared by the Isotope Laboratory (IL), Drug Metabolism and Pharmacokinetics (DMPK), Novartis, Switzerland. This batch was adjusted to a final specific radioactivity of 15.7 kBq/mg by dilution with non-radiolabeled fevipiprant, produced under Good Manufacturing Practice and released for human use by Technical Research and Development (TRD), Novartis. The chemical structure of the compound and the position of the radiolabel are shown in **Figure 1**.

Chemical and radiochemical purity 99.0% (with no single impurity ≥ 1%) was verified by high performance liquid chromatography (HPLC) and stability was ascertained from the period from manufacturing to dose administration. The radiolabeled study drug was provided as individually manufactured doses of four 50 mg (monohydrate; 787.5 kBq) [^{14}C]-fevipiprant capsules per bottle providing a total radioactive dose of 3.15 MBq (IL and TRD departments, Novartis).

Chemicals and Standards

Radiolabeled and non-radiolabeled fevipiprant, radiolabeled and non-radiolabeled AG metabolite and non-radiolabeled lactone metabolite were synthesized as described in (Bala et al., 2005) and (Supplemental Data). All other reagents were of analytical grade and obtained from commercial sources.

Subjects and Study Design

This was an open-label, single-center, single-arm study to investigate the ADME of a single oral dose of 200 mg [^{14}C]-fevipiprant in four healthy male subjects. Informed consent was obtained from each subject in writing before any assessment was performed. The study (registered with

EudraCT as 2011-002842-10) was conducted at PRA International, Early Development Services, Zuidlaren, The Netherlands, from February 17 through March 26, 2012, according to the ethical principles of the Declaration of Helsinki. The study protocol was reviewed by the Independent Ethics Committee of Stichting Beordeling Ethiek Biomedisch Onderzoek, Assen, The Netherlands.

Subjects were male, aged 18 to 55 years, weighed ≥50 kg, with a body mass index (BMI) of 18 to 29 kg/m^2, and could communicate well with the Investigator, and understand and comply with the requirements of the study. The subjects were nonsmokers, had no history of alcoholism or drug abuse, and did not use any prescription drugs or herbal medication within four weeks prior to dosing and/or over-the-counter medication or dietary supplements (vitamins included) within two weeks prior to dosing.

The study consisted of a screening visit (between Day -14 and Day -2), a baseline visit (on Day -1), a single-dose treatment (on Day 1), 240 hours in-house observation period (Days 1–11), optional collections and assessments (at Day 14 and Day 21), and a study Completion Visit (Day 25). Each subject received a single oral [^{14}C]-radiolabeled dose of 200 mg fevipiprant (3.15 MBq, 85 µCi), as four 50 mg capsules, in the morning, after an overnight fast of at least 10 hours. Subjects continued to fast for four hours post-dose (water was allowed after two hours).

The radiation exposure was not deemed to pose any relevant health risk to the subjects. Based on human PK data (with non-radiolabeled fevipiprant) and animal mass balance data (with [^{14}C]-fevipiprant), the expected effective radiation dose was estimated to be up to 0.87 mSv, lower than the recommended limit of 1 mSv/year defined by the International Commission on Radiological Protection (ICRP, 2007). It was not expected that the clinical and laboratory staff handling the radioactive materials and samples would be exposed to any radiation and associated health risk.

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Safety assessments included the monitoring and recording of all adverse events (AEs), regular checks of routine blood chemistry, hematology and urine values, electrocardiogram recordings, measurements of vital signs, and physical examinations.

Sample Collection and Aliquoting

Samples of whole blood, urine, and feces were collected over an 11-day period. Part of each whole blood sample was processed to plasma. Each urine and plasma sample was divided into two parts, and one part of each sample was acidified by addition of 1% volume of a 70% lactic acid solution, in order to stabilize the known AG metabolite. Details of sample collection and processing are given in the (Supplemental Data).

Quantification of total radioactivity, fevipiprant and metabolites

Total radioactivity in blood, non-acidified plasma, non-acidified urine, and feces was measured by liquid scintillation counting (LSC). 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. The AG metabolite was quantified as the sum of all isomers formed by acyl glucuronide rearrangement. Fevipiprant and metabolites were analyzed in acidified plasma, acidified urine and feces extract pools of individual subjects by LC-MS/MS, with offline radioactivity detection. Selected plasma samples were analyzed by size exclusion chromatography (SEC), with off-line radioactivity detection. Details of these analytical methods are provided in the (Supplemental Data).

Structural Characterization of Metabolites

The structural characterization of metabolites in plasma and excreta was carried out by MS/MS analysis after LC separation of analytes (described above).

Single stage and product ion spectra with exact mass measurements were obtained in positive ion mode. The structures of the metabolites were derived from their product ion mass spectra,

the elemental composition determined by exact mass measurement and comparison with synthetic standards.

Pharmacokinetic Evaluations

The present study evaluated the following PK parameters, which were determined from blood (total radioactivity), plasma, and urine: C_{max} (the maximum [peak] plasma or blood drug concentration after single administration [amount × volume^{-1}]), T_{max} (the time to reach peak or maximum concentration following drug administration [time]), $T_{½}$ (the elimination half-life associated with the terminal slope [lambda z], respectively, of a semi-logarithmic concentration-time curve [time]), AUC_{0-t} (the area under the concentration-time curve from time zero to t [amount × time × volume-1]), AUC_{inf} (the area under the concentration-time curve from time zero to infinity [amount × time × volume^{-1}]), CL/F (the systemic clearance CL of the drug from the plasma [volume × time^{-1}] divided by the bioavailability F), Vz/F (the apparent volume of distribution during the terminal [lambda z] phase divided by the bioavailability F [volume]), $Ae_{0-240\ h}$ (amount of drug excreted into the urine from time zero to 240 hours post-dose [% of dose]), CLr (renal clearance; calculated as $Ae_{0-240\ h}/AUC_{0-240\ h}$). PK parameters were calculated using Phoenix WinNonlin 6.3 with noncompartmental analysis.

In vitro Investigations

Fevipiprant covalent binding assay in microsomes and hepatocytes

[^{14}C]-fevipiprant (5 µM, 4.9 MBq/mg) was incubated with human liver microsomes (0.5 mg/mL) at 37°C in sodium phosphate buffer (0.1 M, pH 7.4) in the presence of several sets of cofactors (a) glutathione [GSH, 5 mM]; b) *β*-nicotinamide adenine dinucleotide phosphate [NADPH, 1 mM]; c) NADPH and uridine 5'- diphosphoglucuronic acid [UDPGA, 4 mM]; d) NADPH, UDPGA and GSH). Aliquots were taken after 1 hour and precipitated with 3 volumes of acetonitrile.

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[^{14}C]-fevipiprant (10 µM, 4.9 MBq/mg) was incubated with cryopreserved human hepatocytes (0.5x10^6 cells/mL) 37°C, 95% humidity and 5% CO_2, in Williams E medium. Aliquots were taken after 0, 1.5 and 3 hours, and precipitated with 4 volumes of acetonitrile.

The precipitate obtained from each experiment was filtered and washed extensively with 90% methanol, then the filters were removed and protein precipitate was solubilized in Solvable, then analyzed by LSC. Covalent protein binding was calculated as pmol/mg protein and pmol/10^6 cells for microsomes and hepatocytes, respectively.

Incubations of radiolabeled fevipiprant and AG-metabolite with human plasma

[^{14}C]-fevipiprant (4.9 MBq/mg) and [^{14}C]-AG metabolite (3.3 MBq/mg) were each incubated with 1:1 human plasma/PBS at a final concentration of 20 µM at 37°C for 24 hours. Incubation of [^{14}C]-AG metabolite at 100 µM, and control incubations without plasma were also performed. During the 20 µM incubations, aliquots were removed, precipitated by addition of 5 volumes of acetonitrile and incubation at 4°C for 60 minutes, and then filtered. Filters were washed with methanol/water (90:10) until eluate contained less than 5 pmol/mL radioactivity, as measured by LSC. Retained radioactivity in the filters was then measured by dissolution of filters in Solvable (Perkin Elmer) at room temperature for 16 hours, followed by addition of HCl (1M, 0.25 mL), then scintillant (Irgasafe plus, Zinsser Analytic, 5 mL) was added and radioactivity was measured by LSC. Retained radioactivity was assumed to be bound to proteins.

Aliquots from 21-hour incubations were further analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): 5, 10 and 20 µg aliquots were prepared in XT sample buffer (BioRad) supplemented with 10 mM DTT and heated at 95°C for 5 minutes. Subsequently, prepared samples were loaded to a CriterionTM XT precast gradient Midi gel 4-12% Bis Tris (BioRad). The gel was run for 35 minutes at 200 Volt in Mes buffer (BioRad). The gel was blotted to a PVDF membrane (Transblot Turbo, BioRad) and the resulting membrane was exposed for 4 days to a radio imaging plate (Fujifilm, BAS-IP TR 2040) to detect the radio-

labeled proteins. Molecular weight and intensities of detected bands was measured by labeling the size marker (all blue standard, BioRad) on the membrane with 3H supplemented ink and the analysis tool of the AIDA software (v.4.25One®).

Additionally, aliquots from 21-hour incubations were depleted of albumin and analyzed by SDS-PAGE: 10 µL aliquots of 20 µM incubations were purified using ProteoExtract Albumin Removal Kit (Calbiochem #122640). Sample aliquots were diluted with albumin Binding Buffer (350 µL) and purified according to the kit manual. The volumes of each eluate were reduced to 110 µL by centrifuged at 14000 × *g* in 30 kDa Amicon spin filters (Millipore). Protein content of the samples was determined using the Protein DC Assay (BioRad) and a BSA standard in PBS. Samples were then analyzed by SDS-PAGE, as described above.

SDS-PAGE gels were additionally analyzed by gel excision and protein characterization, as follows: the radiolabeled band was excised and digested with trypsin, as described in the In-gel tryptic digestion kit (Thermo Scientific) procedure. The bands were first destained, reduced, alkylated, and washed, as recommended in the procedure before digestion. Digestions with trypsin and chymotrypsin were performed in ammonium bicarbonate (500 mM, pH 8) for 24 hours at 37°C. After incubation, incubates were acidified by adding 1% volume of lactic acid (70% in water). Acidified incubates were analyzed by LC-MS/MS with an Acuity I class system (Waters). Samples were injected onto an Acuity BEH130 C18 column (150 x 2.1 mm, 1.7 µm particles, Waters) with a corresponding guard column (10 x 2.1 mm), heated to 30°C. Separation was performed with a gradient of mobile phases formic acid (0.1%; phase A) and acetonitrile (phase B) at a flow rate of 0.2 mL/minute. The gradient was as follows: 0 to 2 minutes: 5% B; 2 to 70 minutes: 5 to 50% B; 70 to 75 minutes: 50 to 75% B; 75 to 76 minutes: 75 to 5% B; 76 to 86 minutes: 5% B. The eluate was directed to an LTQ-Orbitrap XL mass spectrometer (ThermoFisher) for MS/MS analysis. Protein fingerprint analysis was then performed using ProteinProspector MS-Fit software version 2.

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Aliquots from the 24-hour 100 µM incubation of the AG-metabolite were analyzed by SEC, as described for plasma samples (Supplemental Data).

Other in vitro methods

Descriptions of all other in vitro methods are provided in the (Supplemental Data).

Demographics

A total of four male Caucasian subjects were enrolled and all completed the study. Subjects had a mean age of 43.0 years (range: 27–54), weight of 85.6 kg (range: 77.9–93.5), height of 183.3 cm (range: 176–192), and BMI of 25.5 kg/m^2 (range: 23.8–28.3). Only male subjects were selected in order to perform a detailed analysis in a well-defined uniform group, and as the study size is insufficient to make statistical comparisons of sub-populations such as genders. No significant differences in PK or metabolism are known or anticipated between males and females.

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Results

Safety and Tolerability Data

Two (50%) of the subjects reported a total of four AEs during the study, which were considered to be mild in severity. There were no serious AEs or discontinuations due to AEs. Two AEs (headache and somnolence) were considered to be treatment-related. No clinically significant abnormalities or changes were observed in clinical laboratory assessments, vital signs, or electrocardiogram parameters.

Pharmacokinetics of Total Radioactivity, Fevipiprant, and AG metabolite

Key PK variables calculated for total radioactivity, fevipiprant and AG metabolite are summarized in **Table 1** and **Figure 2**. Values for AG metabolite represent the sum of AG metabolite and all isomers formed by acyl glucuronide rearrangement (assigned by comparison with rearrangement products formed from synthetic AG metabolite).

Following oral administration of [^{14}C]-fevipiprant, levels of radioactivity in blood and plasma, and levels of fevipiprant and the AG metabolite in plasma, were initially aligned and reached a maximum at approximately 3 hours post-dose. Radioactivity was detectable in blood and plasma for up to 240 hours post-dose, with a mean terminal half-life in plasma of 254 hours (230 hours in blood). Contrastingly, fevipiprant and its major metabolite, the AG metabolite, were only detected in plasma up to 96 and 120 hours post-dose, respectively, with average half-lives of approximately 12 hours. The total exposure ($AUC_{0-240\ h}$) and C_{max} of radioactivity in plasma were approximately two-fold higher than observed in blood, indicating that blood radioactivity was almost entirely located in plasma. Inter-subject variability was low to moderate.

Metabolite Identification and Profiles, and Metabolite Pharmacokinetic Analysis

Plasma, urine and feces extracts were analyzed by LC-MS/MS with radioactivity detection for metabolite identification and profiles. The structures of all identified metabolites could be

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confirmed by comparison of retention time and mass spectral data with synthesized standards, and are given in **Figure 1**. Mass spectral data and representative product ion mass spectra of fevipiprant and metabolites are shown in **Table S1** and **Figure S1** (Supplemental Data). The only abundant metabolite detected was the AG metabolite. A minor lactone metabolite was also identified, likely resulting from oxidative ring-closure of fevipiprant (see discussion section for more detail). The structures of minor components P5.3 and P8.5 could not be determined.

Metabolite profiles were determined after extraction of samples by solid phase extraction. The extraction recoveries of total radioactivity were found to decrease with time post-dose for all subjects. The average percentage of total radioactivity recovery at 2 hours was 92.6%, but at 48 hours was only 11.0%. Due to the low extractable radioactivity in plasma samples at later time points, radiochromatograms were only analyzed up to 12 hours post-dose. Exposure (as $AUC_{0-12\,h}$) of fevipiprant and its metabolites, based on the radiochromatogram profiles, is shown in **Table 2**. A representative plasma radiochromatogram for one subject is shown in **Figure 3**. The AG metabolite and fevipiprant represented the main proportion of radioactivity in plasma (total 72% of [^{14}C]-$AUC_{0-12\,h}$) based on radiochromatograms up to 12 hours. However, fevipiprant and the AG metabolite accounted for a much lower proportion of the total plasma [^{14}C]-$AUC_{0-240\,h}$ (around 12%) based on LC-MS/MS quantification up to 240 hours (**Table 1**). Correspondingly, the loss of radioactivity observed during sample processing represented a major proportion of radioactivity, amounting to 26.6 ± 2.7% of the plasma [^{14}C]-$AUC_{0-12\,h}$ and estimated at 87.7% of the plasma [^{14}C]-$AUC_{0-240\,h}$ based on LC-MS/MS quantification of fevipiprant and the AG metabolite. To further investigate the loss of radioactivity during sample processing, additional plasma analysis and in vitro experiments were performed (Covalent Binding and Acyl glucuronide Stability Investigations section).

Excretion of radiolabeled components

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After oral administration of [^{14}C]-fevipiprant, 42.1 ± 4.3% of the dose was recovered in urine and 51.9 ± 4.8% was recovered in feces over 240 hours post-dose (**Table S2** (Supplemental Data); **Figure 4**), indicating that radioactive drug-related material was primarily excreted via renal and biliary/fecal excretion. The overall recovery of drug-related material was near complete (93.9 ± 2.6%). The majority of the radioactivity was recovered within 144 hours post-dose, with subsequent samples up to 240 hours containing less than 1% of the dose.

Metabolite profiles were also determined in urine and feces pools (**Figure 3**). Extraction recovery was high for all excreta samples (>88%). Urinary and feces excretion data for fevipiprant and all metabolites based on metabolite profiles, and urinary excretion data for fevipiprant and the AG metabolite based on LC-MS/MS quantification over 240 hours, are presented in **Table 3**. In urine, fevipiprant and AG metabolite were the major components (total approximately 40% of dose). Urinary excretion data for fevipiprant and the AG metabolite over 240 hours were similar to the 0–72 hours values (**Table 3**). In feces, fevipiprant was the major component excreted. Overall, fevipiprant and AG metabolites represented approximately 85% of the dose excreted in urine and feces, and other metabolites were only present in traces. Based on urinary excretion data for the period 0–240 hours (**Table 3**), the renal clearance of fevipiprant and AG metabolite were calculated to be 8.92 L/h and 7.99 L/h, respectively.

Absorption was estimated to be at least 43.5%, based on the total radioactive dose recovered from urine (42.1%), and the metabolites excreted in feces (approximately 1.4%).

Covalent Binding and Acyl glucuronide Stability Investigations

Due to the observed long retention of radioactivity in plasma and low extractability of plasma samples, we hypothesized that the AG metabolite could covalently bind to plasma proteins (Regan et al., 2010), leading to long-lived radioactive metabolite-protein conjugates, as

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observed for other compounds, such as ibuprofen (Castillo et al., 1995), tolmetin (Zia-Amirhosseini et al., 1994), and bilirubin (Weiss et al., 1983). Additional in vitro experiments were performed to investigate covalent binding. Initially, [^{14}C]-fevipiprant was incubated with human liver microsomes (HLM) and human hepatocytes to investigate possible covalent binding of fevipiprant or metabolites to liver proteins mainly via oxidative processes. Additionally, incubations of [^{14}C]-fevipiprant and [^{14}C]-AG metabolite in human plasma were performed, followed by precipitation and LSC of proteins to identify whether radiolabeled protein conjugates were formed.

When probing for covalent binding of fevipiprant or metabolites to human liver microsomes and hepatocytes in the presence of various cofactors (**Table 4**) only trace levels of covalent binding were observed in comparison to positive control compounds. Additionally, no increase in covalent binding was observed with the addition of glucuronidation cofactor UDPGA, suggesting that covalent binding to liver microsomes associated with the AG metabolite is insignificant.

In incubations with plasma, fevipiprant showed very low covalent binding to human plasma proteins, while the AG metabolite showed 11-fold higher covalent binding after 24 hours incubation (**Figure S2** (Supplemental Data)), indicating that covalent binding in plasma occurs via the AG metabolite rather than the parent compound.

Subsequently, to identify proteins involved in plasma covalent binding gel electrophoresis of the AG metabolite plasma incubate was carried out. Radio-imaging of the resulting gel (**Figure 5A**, **lanes A1**–**3**) showed that only one protein of around 68 kDa size was conjugated to the AG metabolite. Peptide fingerprint analysis of the labeled protein band by LC-MS after gel excision and trypsin/chymotrypsin digest identified that the abundant plasma protein human serum albumin was present. Based on this result, gel electrophoresis of AG metabolite plasma incubates after depletion of albumin was also performed. The intensity of the radio-signal

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detected was significantly reduced by albumin depletion of the respective samples (**Figure 5A**, **lanes B1–3**). These data show that the AG metabolite forms a conjugate with albumin in vitro.

To evaluate whether this in vitro finding corresponds to the in vivo observations, size exclusion chromatography (SEC) of the AG metabolite plasma incubates and of the clinical plasma samples was performed (gel electrophoresis could not be performed on the in vivo samples, due to insufficient sensitivity). SEC of the AG metabolite in vitro incubation showed the presence of fevipiprant, AG metabolite, and an earlier eluting peak assigned to a protein conjugate (**Figure 5B**). Analysis of in vivo human plasma samples showed similar profiles (representative samples are shown in **Figure 5C**), with an increase of the protein conjugate peak over time post-dose. These data provide evidence that the in vivo plasma covalent binding also involves albumin.

The recoveries of radioactivity for all plasma samples analyzed by SEC were between 90 and 110%, confirming that the low extraction recoveries for clinical plasma samples were explained by loss of drug related material covalently bound to plasma protein. Correspondingly, the amount of protein-conjugate detected by SEC at each time point was similar to the amount of radioactivity lost on extraction.

Acyl glucuronides are often unstable at neutral pH (Bailey and Dickinson, 2003) but can be stabilized by acidification of clinical samples (Ebner et al., 2010; Wang et al., 2011). As this instability was observed for the AG metabolite in pre-clinical experiments, an aliquot of all plasma and urine samples for analysis of fevipiprant and metabolites was acidified by addition of 1% volume of a 70% lactic acid solution. Experiments in blank urine samples showed that a pH in the range of 3.1–5.2 was obtained after acidification (compared with 5.3–7.3 before acidification), and the AG metabolite was confirmed to be stable to hydrolysis for four weeks at

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room temperature in acidified urine (less than 1% degradation to the parent compound measured). However, isomerization of the acyl-glucuronide was not measured here, and may have occurred during the incubation. All clinical data reported here were obtained using the acidified aliquots, except for total radioactivity measurements.

To assess potential acyl-glucuronide isomerization, incubations were performed with a synthesized standard of the 1-O-beta isomer of the AG metabolite to identify other acyl glucuronide isomers formed. Several isomers of the AG metabolite were detected, and were assigned to acyl glucuronide rearrangement products (Bailey and Dickinson, 2003). As these isomers were considered to be degradation products of the AG metabolite that could not be accurately quantified if formed during sample collection or processing, they were quantified together with the AG metabolite for all analyses in this report.

Fevipiprant as a Substrate of Enzymes and Transporters

Based on the clinical excretion data, fevipiprant clearance pathways include glucuronidation and direct excretion of the parent drug. To determine which enzymes and transporters are involved in these processes, a number of in vitro investigations were carried out, as follows:

For the determination of the enzymes catalyzing the formation of the AG metabolite, in vitro biotransformation of [^{14}C]-fevipiprant, catalyzed by 12 recombinant uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase; UGT) enzymes, was investigated. Fevipiprant was metabolized by a number of the enzymes (**Table S3** (Supplemental Data)), with UDP-glucuronosyltransferase 1A3 (UGT1A3), UDP-glucuronosyltransferase 2B7 (UGT2B7), and UDP-glucuronosyltransferase 2B17 (UGT2B17) showing the highest activities. Michaelis-Menten enzyme kinetic parameters are shown in **Table 5** and the kinetic profiles are presented in **Figures S3–S5** (Supplemental Data).

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For evaluation of hepatic uptake, [^{14}C]-fevipiprant uptake into hepatocytes was measured in the presence and absence of transporter inhibitors. Hepatic uptake clearance was determined to be approximately 10 µL/min/10^6 cells (**Table S4** (Supplemental Data)). Uptake was slower at 4°C or in the presence of organic anion-transporting polypeptide (OATP) transporter inhibitors, indicating a mixture of passive and active (most likely OATP-mediated) hepatic uptake.

For the clarification of transporters involved in hepatic uptake as well as renal, biliary and intestinal secretion, uptake of [^{14}C]-fevipiprant into cells overexpressing the corresponding transporters was measured at a range of fevipiprant concentrations in the presence and absence of inhibitors of these transporters. Based on these data (**Figures S6**–**8** (Supplemental Data)), fevipiprant was determined to be a substrate of transporters OATP1B3 (hepatic uptake; K_m =16 µM), organic anion transporter 3 (OAT3) (renal secretion; K_m = 3.2 µM) and multi-drug resistance gene 1 (MDR1) (biliary/intestinal efflux; K_m > 200 µM, exact value not calculable due to lack of saturation), but not a substrate of transporters OATP1B1, OATP2B1, organic anion transporter 1 (OAT1), organic cation transporter 2 (OCT2), multidrug and toxin extrusion protein 1 (MATE1) or multidrug and toxin extrusion protein 2-K (MATE2-K). This data suggest that fevipiprant is actively taken up into hepatocytes by OATP1B3, and actively excreted into bile and into the GI tract via MDR1 and into urine via OAT3 transport.

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Discussion

In the present study, the absorption of fevipiprant was estimated to be at least 43.5%, as a minimum estimate based on metabolite excretion and renal fevipiprant excretion. It is likely that the actual absorption value is higher, as AG metabolites would likely hydrolyze to fevipiprant in the gastrointestinal tract after biliary excretion, and in addition direct biliary excretion of fevipiprant is possible. Preclinical data from rat support this hypothesis, with absorption of around 60%, and bile-duct cannulated rat studies after intravenous dosing showing excretion of both fevipiprant (25% of dose) and AG-metabolite (33% of dose) in bile.

The AG metabolite was the only major metabolite detected in plasma. In preclinical species, the AG metabolite was also a major metabolite in plasma and/or excreta, but comprised a lower proportion of total plasma AUC (<30%). However, in toxicity studies exposure to the AG metabolite at the NOAEL was similar or higher than human exposure at the highest phase III dose. The abundance of the AG metabolite was not well predicted from in vitro data. While the AG metabolite was formed in hepatocytes of all species investigated (mouse, rat, dog, monkey, human), the calculated intrinsic clearance was in all cases low (e.g. 0.35 µL/min/10^6 cells in human). It is possible that the in vitro underestimation of metabolic clearance is due to reduced activity of OATP1B3-mediated uptake in cryopreserved hepatocyte suspensions in vitro, in comparison to the in vivo situation where transporters are fully active (Lundquist et al., 2014).

Aside from the AG metabolite, we detected a minor lactone metabolite. Due to its low abundance, the formation of this metabolite was not investigated in more detail. However, several possible formation pathways can be envisaged (see **Figure S9** (Supplemental Data)) involving epoxidation, followed by either nucleophilic ring-closing or epoxide hydrolysis and lactonization. It is unlikely that this possible epoxide intermediate is involved in the formation of

the observed covalent adduct in plasma, as oxidative covalent binding in microsomes and hepatocytes was negligible (**Table 4**), and relevant reactive metabolites were not detected in plasma.

Total radioactivity was found to decline slowly in blood and plasma after the elimination of the majority of fevipiprant from plasma, likely due to the formation of protein conjugates by covalent binding of the AG metabolite to human serum albumin. The observed terminal half-life of radioactivity in plasma (254 h or 10.6 days) was in the range of, but shorter than, the half-life reported for human albumin (19 days (Peters T., 1995)). Consequently, the terminal half-life of total radioactivity, although calculated using data covering a relatively short time period, is likely to be accurate or somewhat underestimated. Preclinical data in rat and dog show much shorter terminal half-life values for total radioactivity, potentially due to a shorter half-life of albumin in these species (e.g. 2.5 days in rat (Car et al., 2006)). The concentration of radioactivity remaining in plasma after 240 h (500 pmol/mL) corresponds to around 1.5 µmol or 0.65 mg fevipiprant equivalent, assuming 3 L human plasma volume. Assuming that approximately 60% of albumin is distributed to extracellular space (Peters T., 1995), the amount of drug-albumin conjugate remaining in the body at 240 hours post-dose is therefore approximately 1.6 mg corresponding to 0.8% of dose (or 0.3% assuming no extra-vascular distribution of the protein conjugate). Given total body albumin of around 360 g (Peters T., 1995), the extent of covalent binding is then around 0.07% of total body albumin. Consequently, repeated dosing with fevipiprant is unlikely to lead to the covalent modification of a large proportion of albumin in the body.

The stability of acyl glucuronides has been previously investigated in detail (Regan et al., 2010). Key factors proposed to be associated with AG stability are steric accessibility and electronic properties of the carboxylic acid/ester functionality. Fevipiprant contains a sterically unhindered carboxylic acid as part of an aryl-acetic acid moiety, similar to diclofenac and tolmetin, which

also form unstable AG-metabolites (Zia-Amirhosseini et al., 1994; Castillo et al., 1995). It is therefore unsurprising that the AG-metabolite of fevipiprant is also unstable.

Acyl glucuronide-derived drug-protein conjugates have been observed for other drugs such as ibuprofen and tolmetin, as well as bilirubin (Weiss et al., 1983; Zia-Amirhosseini et al., 1994; Castillo et al., 1995), which has been reviewed extensively (Regan et al., 2010). A number of AG-forming drugs have been associated with clinical AEs, such as hepatotoxicity. However, no clear causal link of AEs to the reactivity or covalent protein binding of the AGs has been established. In comparison with other covalent binding compounds (Usui et al., 2009) including compounds that form AG metabolites (Darnell et al., 2015), the in vitro covalent binding of fevipiprant to liver microsomes and hepatocytes (**Table 4**) is negligible. Additionally, no covalent binding to liver microsomes associated with glucuronidation was observed, suggesting that the AG-metabolite does not rapidly bind to liver proteins after its formation. From this it can be inferred that covalent binding to albumin occurs in plasma rather than in the liver, likely due to the high concentrations of both AG metabolite and albumin in plasma.

As of January 2017, over 1700 subjects have been exposed to fevipiprant in the clinical program. Phase 3 trials in asthma are currently ongoing. There have been no AEs of idiopathic drug reactions or liver toxicity in these clinical studies. Overall, the in vivo nonclinical assessments and clinical safety data available to date indicate a low risk of idiosyncratic drug-induced liver injury or other idiosyncratic drug reactions potentially associated with covalent drug-protein binding, in line with the apparently negligible reactive metabolites formed by oxidative pathways.

Excretion and metabolism data from this study indicate that fevipiprant is eliminated by several pathways. Following oral administration, 57% of the dose was detected as unchanged fevipiprant in urine and feces, indicating direct renal and possibly biliary excretion. In addition, 29% of the dose was excreted as metabolites. The exact amounts of dose eliminated by

metabolism and direct excretion are not clear, as back-conversion of the AG metabolite to fevipiprant during/after excretion is possible. Data from preclinical species (mouse, rat and dog) showed similar excretion routes, except with relatively higher fecal excretion. In mouse, rat and dog, fecal/biliary excretion was around 80% of dose (after both intravenous and oral administration) and renal excretion was 1% in mouse, 6-7% in rat and 11-13% in dog. Urinary excretion in rat and dog consisted mainly of the parent drug, suggesting that the higher overall renal excretion in human is due to a fast renal (rather than biliary) excretion of the AG metabolite, or due to a more extensive formation of AG metabolite in human, in line with the high plasma concentrations of the AG metabolite.

We demonstrated in vitro that fevipiprant was a substrate of several human UDP-glucuronosyltransferases, as well as transporters involved in tubular secretion in the kidney (OAT3), active hepatic uptake (OATP1B3), and biliary excretion (MDR1). The OAT3 data show that the elimination of fevipiprant involves direct renal secretion. This is in line with the measured renal clearance value (~9 L/h), which is higher than the expected glomerular filtration clearance (GFR) (0.9 L/h, based on a GFR of 7.5 L/h (Davies and Morris, 1993) multiplied by the fraction unbound (fu) of fevipiprant (0.118)), as expected in the case of active renal secretion. As fevipiprant is a substrate of MDR1, biliary or intestinal secretion is also likely: this would contribute to the large amount of unchanged fevipiprant excreted in feces. The UGT phenotyping data show that at least three hepatic and extrahepatic (Tukey and Strassburg, 2000) UGT isoenzymes are involved in the metabolic clearance pathways of fevipiprant. Based on the range of elimination pathways and isoenzymes involved in the clearance of fevipiprant, a low risk of DDI or variability due to genetic differences is expected. However, in order to address possible effects of the inhibition of these pathways on the PK of fevipiprant, clinical DDI studies are planned or ongoing with inhibitors of OAT3, UGT enzymes, OATP1B3 and MDR1.

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Additionally, the potential of fevipiprant and its AG-metabolite to inhibit these or other enzymes and transporters involved in drug disposition has been assessed (Barve et al., 2016).

For the AG metabolite, the renal clearance was similar to fevipiprant (~8 L/h) and the expected clearance by glomerular filtration is 1.76 L/h (0.234 [fu] × 125 mL/min, as described above for fevipiprant). As the expected glomerular filtration clearance is five-fold lower than the observed renal clearance, an active tubular secretion of the AG metabolite is also likely.

In conclusion, we demonstrated that fevipiprant is eliminated via various metabolic enzymes and direct excretion and hence this novel compound is unlikely to be a victim of a strong drug interaction or to display major variability or ethnic sensitivity in PK due to genetic polymorphism. The major metabolite is an acyl glucuronide which forms covalent adducts to albumin in human plasma.

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Authorship Contributions

Participated in research design: Jin, van Lier, Erpenbeck, End, Glaenzel, Woessner

Conducted experiments: van Lier

Contributed new reagents or analytic tools: Eggimann

Performed data analysis: Pearson, Weiss, Jin, End, Glaenzel

Wrote or contributed to the writing of the manuscript: Pearson, Weiss, Jin, Erpenbeck, Glaenzel, End, Eggimann, Camenisch

References

Bailey MJ and Dickinson RG (2003) Acyl glucuronide reactivity in perspective: biological consequences. *Chem Biol Interact* **145:**117-137.

Bala K, Leblanc C, Sandham DA, Turner KL, Watson SJ, Brown LN, and Cox B (2005) Pyrrolopyridines as CRTh2 receptor antagonists, their preparation, pharmaceutical compositions, and use in therapy, WO2005123731A2.

Barve A, Tillmann H-C, Ilsley E, Vemula J, Nica A, Imbert G, Elbast W, Schiller H, Camenisch G, and Woessner R (2016) Impact of co-administration of fevipiprant (QAW039) and SLCO1B1 genotype on the PK of simvastatin and rosuvastatin. *European Respiratory Journal* **48:**1108.

Car BD, Eng VM, Everds NE, and Bounous DI (2006) Chapter 5 - Clinical Pathology of the Rat A2 - Suckow, Mark A, in: *The Laboratory Rat (Second Edition)* (Weisbroth SH and Franklin CL eds), pp 127-146, Academic Press, Burlington.

Castillo M, Lam YW, Dooley MA, Stahl E, and Smith PC (1995) Disposition and covalent binding of ibuprofen and its acyl glucuronide in the elderly. *Clin Pharmacol Ther* **57:**636-644.

Darnell M, Breitholtz K, Isin EM, Jurva U, and Weidolf L (2015) Significantly Different Covalent Binding of Oxidative Metabolites, Acyl Glucuronides, and S-Acyl CoA Conjugates Formed from Xenobiotic Carboxylic Acids in Human Liver Microsomes. *Chem Res Toxicol* **28:**886-896.

Davies B and Morris T (1993) Physiological parameters in laboratory animals and humans. *Pharm Res* **10:**1093-1095.

Ebner T, Wagner K, and Wienen W (2010) Dabigatran acylglucuronide, the major human metabolite of dabigatran: in vitro formation, stability, and pharmacological activity. *Drug Metab Dispos* **38:**1567-1575.

EMA (2012) Guideline on the investigation of drug interactions.

Erpenbeck VJ, Vets E, Gheyle L, Osuntokun W, Larbig M, Neelakantham S, Sandham D, Dubois G, Elbast W, Goldsmith P, and Weiss M (2016) Pharmacokinetics, Safety, and Tolerability of Fevipiprant (QAW039), a Novel CRTh2 Receptor Antagonist: Results From 2 Randomized, Phase 1, Placebo-Controlled Studies in Healthy Volunteers. *Clin Pharmacol Drug Dev* **5:**306-313.

Evans DC, Watt AP, Nicoll-Griffith DA, and Baillie TA (2004) Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chem Res Toxicol* **17:**3-16.

FDA (2008) Guidance for industry: safety testing of drug metabolites.

FDA (2012) Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations.

Gao H, Jacobs A, White RE, Booth BP, and Obach RS (2013) Meeting report: metabolites in safety testing (MIST) symposium-safety assessment of human metabolites: what's REALLY necessary to ascertain exposure coverage in safety tests? *AAPS J* **15:**970-973.

ICH (2009) Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals M3 (R2).

ICRP (2007) The 2007 Recommendations of the International Commission on Radiological Protection, in: *Ann ICRP*

Lundquist P, Loof J, Sohlenius-Sternbeck AK, Floby E, Johansson J, Bylund J, Hoogstraate J, Afzelius L, and Andersson TB (2014) The impact of solute carrier (SLC) drug uptake transporter loss in human and rat cryopreserved hepatocytes on clearance predictions. *Drug Metab Dispos* **42:**469-480.

Pawankar R (2014) Allergic diseases and asthma: a global public health concern and a call to action. *World Allergy Organ J* **7:**12.

Peters T. J (1995) Metabolism: Albumin in the Body, in: *All about albumin*, pp 188-250, Academic Press.

Regan SL, Maggs JL, Hammond TG, Lambert C, Williams DP, and Park BK (2010) Acyl glucuronides: the good, the bad and the ugly. *Biopharm Drug Dispos* **31:**367-395.

Sykes DA, Bradley ME, Riddy DM, Willard E, Reilly J, Miah A, Bauer C, Watson SJ, Sandham DA, Dubois G, and Charlton SJ (2016) Fevipiprant (QAW039), a Slowly Dissociating CRTh2 Antagonist with the Potential for Improved Clinical Efficacy. *Mol Pharmacol* **89:**593-605.

Townley RG and Agrawal S (2012) CRTH2 antagonists in the treatment of allergic responses involving TH2 cells, basophils, and eosinophils. *Ann Allergy Asthma Immunol* **109:**365-374.

Tukey RH and Strassburg CP (2000) Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol* **40:**581-616.

Usui T, Mise M, Hashizume T, Yabuki M, and Komuro S (2009) Evaluation of the potential for drug-induced liver injury based on in vitro covalent binding to human liver proteins. *Drug Metab Dispos* **37:**2383-2392.

Wang L, Munsick C, Chen S, Bonacorsi S, Cheng PT, Humphreys WG, and Zhang D (2011) Metabolism and disposition of 14C-labeled peliglitazar in humans. *Drug Metab Dispos* **39:**228-238.

Weiss JS, Gautam A, Lauff JJ, Sundberg MW, Jatlow P, Boyer JL, and Seligson D (1983) The clinical importance of a protein-bound fraction of serum bilirubin in patients with hyperbilirubinemia. *N Engl J Med* **309:**147-150.

Xue L, Salimi M, Panse I, Mjosberg JM, McKenzie AN, Spits H, Klenerman P, and Ogg G (2014) Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *J Allergy Clin Immunol* **133:**1184-1194.

Zia-Amirhosseini P, Ojingwa JC, Spahn-Langguth H, McDonagh AF, and Benet LZ (1994) Enhanced covalent binding of tolmetin to proteins in humans after multiple dosing. *Clin Pharmacol Ther* **55:**21-27.

Zuberbier T, Lotvall J, Simoens S, Subramanian SV, and Church MK (2014) Economic burden of inadequate management of allergic diseases in the European Union: a GA(2) LEN review. *Allergy* **69:**1275-1279.

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Footnotes

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DP, MW, YJ, VE, UG, PE, RW, FE and GC are employees of Novartis Pharma and may hold shares in Novartis.

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Legends for Figures

Figure 1. Structural formula of fevipiprant and proposed biotransformation pathways of fevipiprant in humans. *Position of [^{14}C]-radiolabel

Figure 2. Plasma concentrations of fevipiprant, AG-metabolite and total radioactivity after oral administration of a 200 mg dose of [^{14}C]-fevipiprant to four healthy subjects. Units for fevipiprant and AG-metabolite concentrations are ng/mL, for total radioactivity concentrations ng-eq/mL. Concentrations of fevipiprant an AG metabolite were determined by LC-MS/MS, concentrations of total radioactivity were determined by LSC.

Figure 3. Representative radiochromatograms in plasma (3 h post dose), urine (time pooled 0-72 h), and feces extracts (time pooled 0-96 h) for one subject after oral administration of a 200 mg dose of [^{14}C]-fevipiprant. dpm: disintegrations per minute

Figure 4. Cumulative excretion of radioactivity in urine and feces after oral administration of a 200 mg dose of [^{14}C]-fevipiprant to four healthy subjects. Radioactivity was determined by LSC.

Figure 5.

A) SDS-PAGE analysis of covalent protein binding after 24 h incubation of [^{14}C]-AG-metabolite (20 µM) with 1:1 human plasma/PBS at 37 °C. Lanes A1-3: untreated incubation sample; Lanes B1-3: incubation sample after albumin depletion using a ProteoExtract Albumin Removal Kit (Calbiochem). 5, 10, and 20 µg of protein was analysed in lanes A1/B1, A2/B2 and A3/B3, respectively. Protein content was determined using a Protein DC Assay (BioRad). Relative intensities of bands calculated using AIDA software: A1, 5878; A2, 9787; A3, 17839; B1, 2791; B2, 6435; B3, 10635. The molecular weight markers on the left side of the gel were drawn with ^{3}H-supplemented ink to mark the location of visible marker proteins (all blue standard, BioRad).

B) SEC analysis after 24 h incubation of [^{14}C]-AG-metabolite (100 µM) with 1:1 human plasma/PBS at 37 C.

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C) Representative SEC analyses of plasma samples from one healthy subject 2, 6, and 120 h after oral administration of a 200 mg dose of [^{14}C]-fevipiprant

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Tables

Table 1. PK parameters of total radioactivity in whole blood and plasma and of fevipiprant and its main circulating metabolite (AG metabolite) in plasma (mean ± SD)

	Total radioactivity in whole blood	**Total radioactivity in plasma**	**Fevipiprant in plasma**	**AG metabolite in plasma**
T_{max} (h)[a)]	3.00 (3.00–6.00)	3.00 (3.00–6.00)	2.50 (2.00–6.00)	3.00 (3.00–6.00)
C_{max} (ng/mL or ng-eq/mL) [b)]	923 ± 216	1750 ± 396	348 ± 139	1450 ± 353
$AUC_{0–240\ h}$ (ng*h/mL or ng-eq*h/mL) [c)]	36400 ± 6960	76500 ± 9740	2490 ± 312	9840 ± 1770
AUC_{inf} (ng-eq*h/mL)	69700 ± 10600	156000 ± 20700	-	-
CL/F (L/h)	-	-	80.5 ± 9.42	-
Vz/F (L)	-	-	1370 ± 459	-
$T_{1/2}$ (h)	230 ± 54.6	254 ± 24.8	12.3 ± 5.95	11.8 ± 5.19

[a)] T_{max} is presented as median (range). [b)] ng/mL for fevipiprant and AG-metabolite, ng-eq/mL for total radioactivity. [c)] ng*h/mL for fevipiprant and AG-metabolite, ng-eq*h/mL for total radioactivity.

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Table 2. Fevipiprant and metabolites in plasma, based on metabolism profiles (mean ± SD)

Component	[^{14}C]-AUC$_{0–12\ h}$ [a)]	
	ng/mL or ng-*eq**h/mL [b)]	% of total
Fevipiprant	1690 ± 266	15.3 ± 2.33
AG metabolite	6240 ± 922	56.5 ± 5.65
P8.5	125 ± 145	1.18 ± 1.37
Sum of additional components	47.1 ± 52.9	0.405 ± 0.426
Total components detected	8100 ± 852	73.4 ± 2.73
Lost during sample processing	2920 ± 210	26.6 ± 2.73
Total radiolabeled components in sample	11000 ± 837	100

[a)]Calculated using the linear trapezoidal method; concentrations at time zero taken as zero. [b)] ng/mL for fevipiprant, ng-eq/mL for other components.

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Table 3. Fevipiprant and metabolites in excreta (mean ± SD)

Component	Excretion (% of dose)[a)]			Excretion (% of dose)[b)]
	Urine 0–72 h	Feces 0–96 h	Total	Urine 0–240 h
Fevipiprant	12.7 ± 2.21	44.5 ± 6.12	57.2 ± 6.29	11.0 ± 1.56
AG metabolite	26.9 ± 2.58	1.24 ± 0.244	28.1 ± 2.40	27.6 ± 3.53
Lactone metabolite	0.133 ± 0.0458	0.177 ± 0.355	0.310 ± 0.389	n/a
P5.3	0.0956 ± 0.0121	0.00	0.0956 ± 0.0121	n/a
Total components detected	39.8 ± 3.35	46.0 ± 6.36	85.7 ± 5.16	n/a
Total excretion in time period[c)]	41.2 ± 3.78	50.5 ± 6.26	91.7 ± 4.97	n/a

[a)]Quantified from radioactivity profiles; [b)]Quantified by LC-MS/MS; [c)]The difference between total excretion and total components detected corresponds to losses during sample preparation.

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Table 4. Covalent binding of fevipiprant to microsomes and hepatocytes in vitro

Incubation conditions	Amount of covalent drug protein adducts
HLM, GSH	1 ± 0.0 pmol/mg protein
HLM, NADPH	3 ± 0.4 pmol/mg protein
HLM, NADPH, UDPGA	3 ± 0.3 pmol/mg protein
HLM, NADPH, UDPGA, GSH	1 ± 0.5 pmol/mg protein
HH	8 ± 1.4 pmol/10^6 cells

HLM: human liver microsomes; HH: human hepatocytes; GSH: glutathione; NADPH: nicotine adenine dinucleotide phosphate; UDPGA: uridine diphosphoglucuronic acid.

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Table 5. Michaelis-Menten enzyme kinetic parameters for biotransformation of fevipiprant by UGT enzymes.

	UGT1A3	UGT2B7	UGT2B17
V_{max} (mean ± SD, pmol/min/mg)	5634 ± 1603	35.2 ± 6.8	43.3 ± 0.9
K_m (mean ± SD, µM)	13352 ± 5854	320 ± 143	53.3 ± 4.5
Derived intrinsic clearance (V_{max}/K_m, µL/mg/min)	0.422	0.11	0.812

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Figures

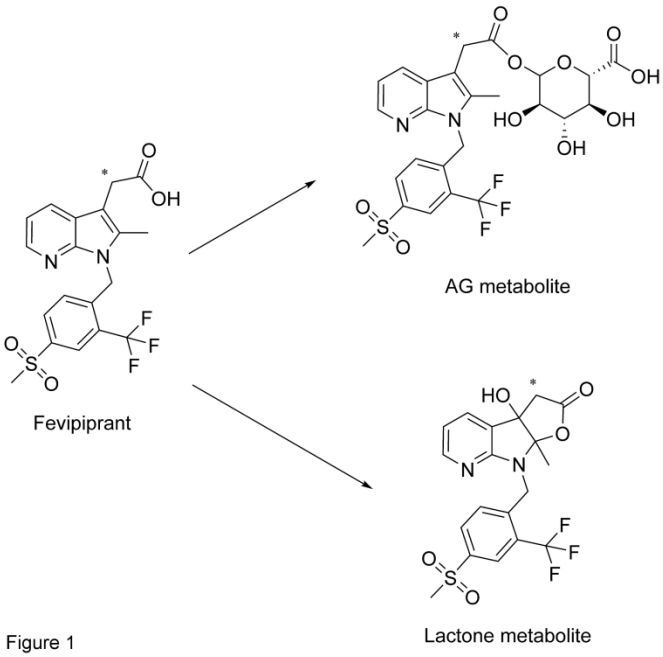

Figure 1

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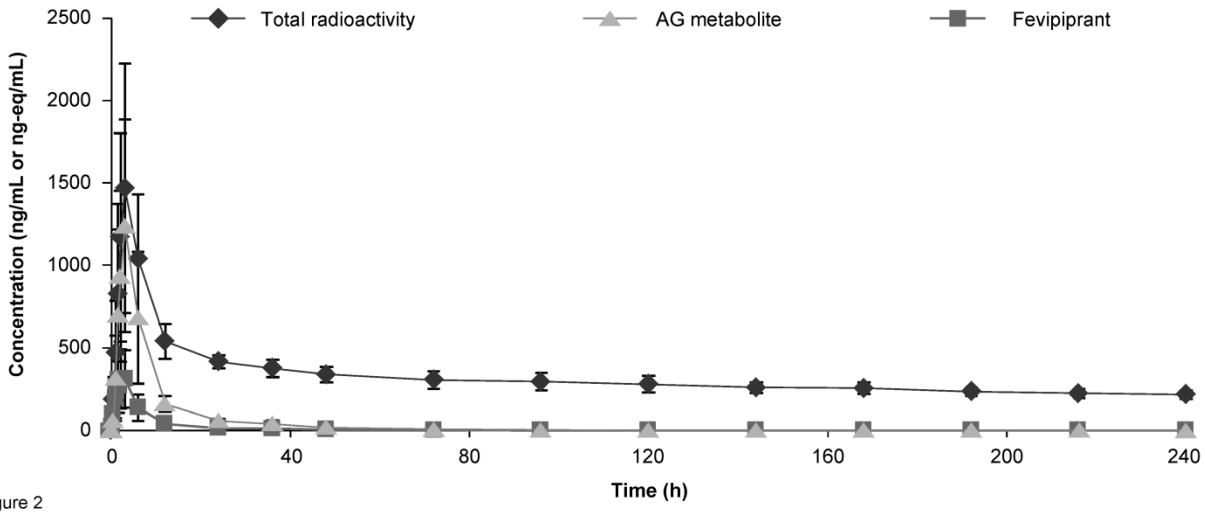

Figure 2

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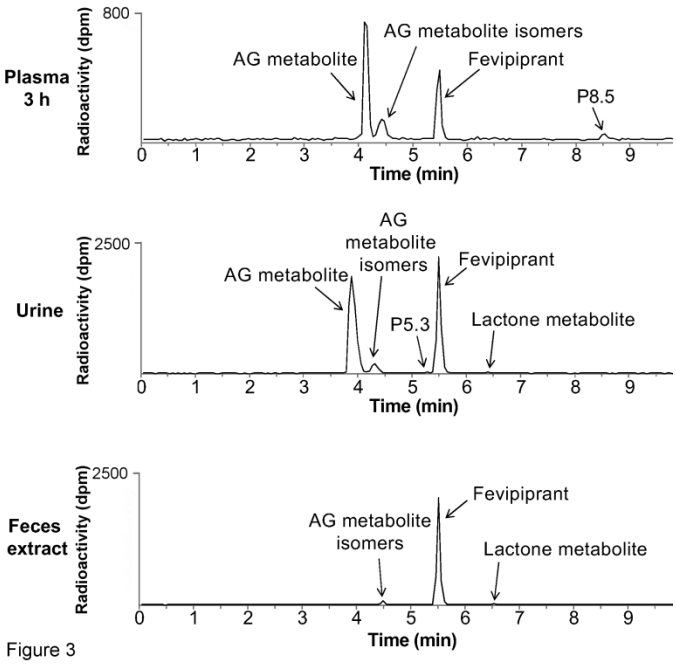

Figure 3

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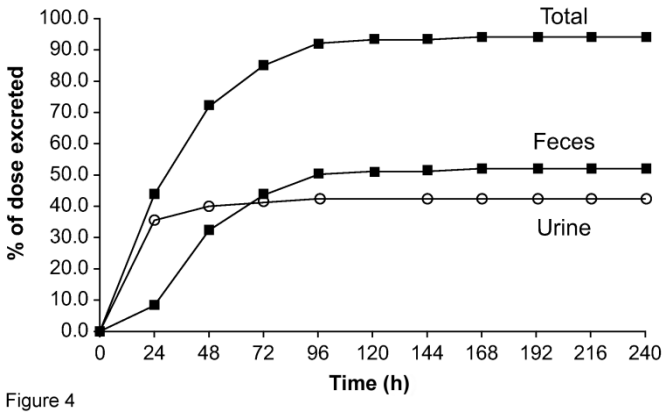

Figure 4

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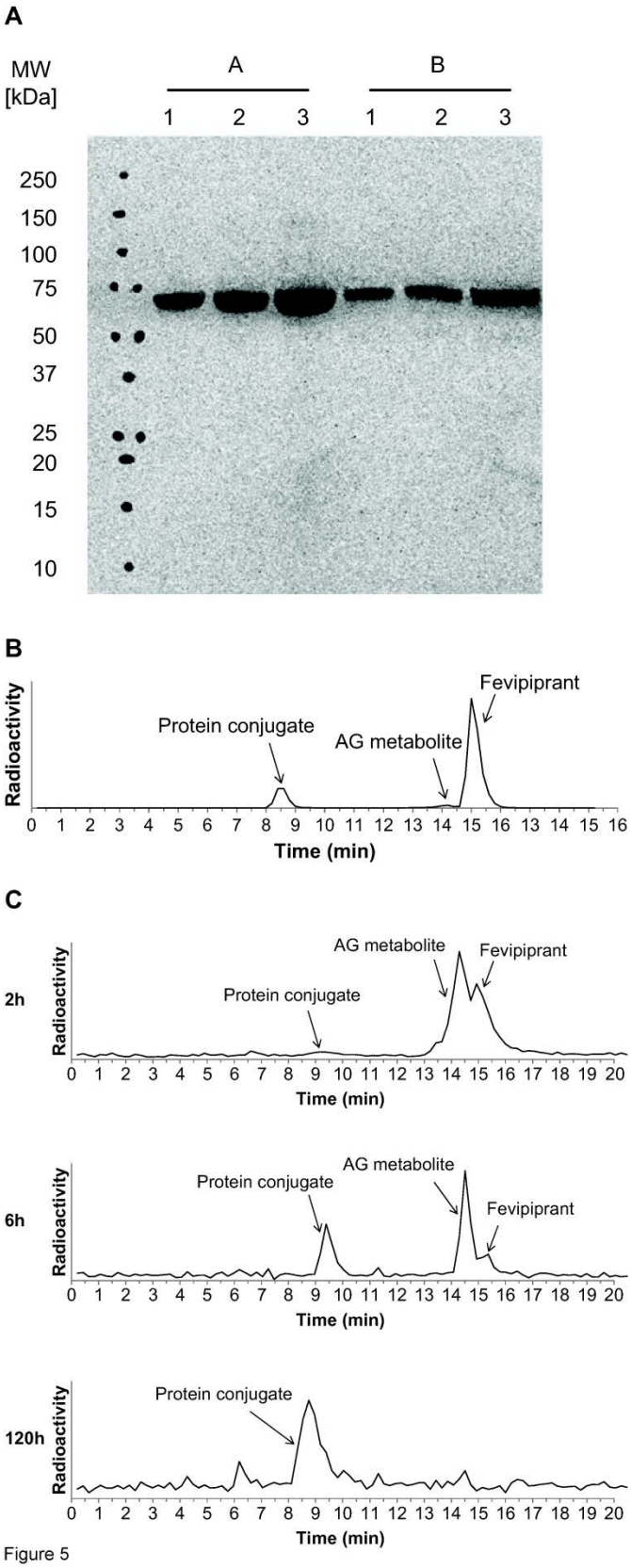

Figure 5

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

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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.

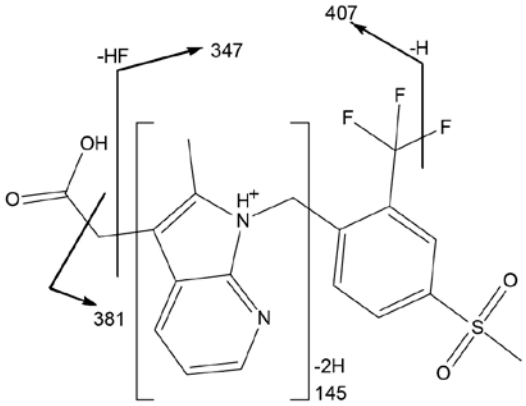
Table S4. Hepatic fevipiprant uptake by human hepatocytes

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)



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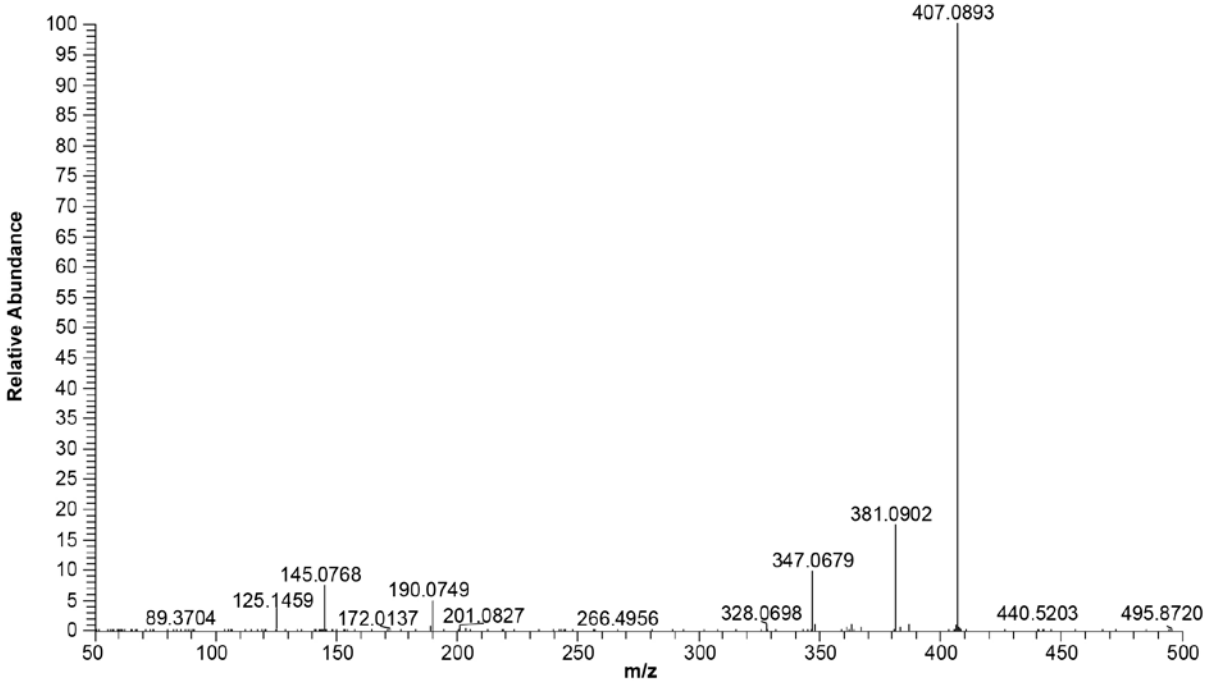
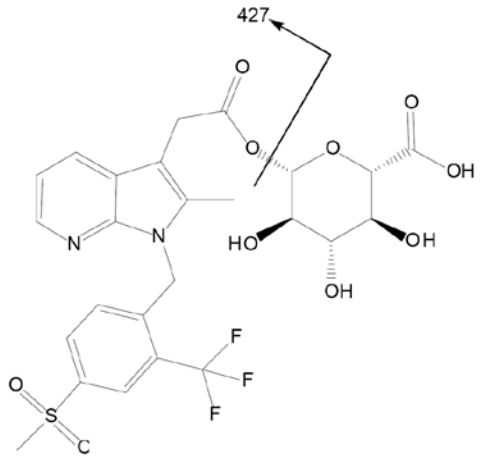
F: FTMS + cESI Full ms2 427.09@cid35.00 [50.00-500.00]



B)



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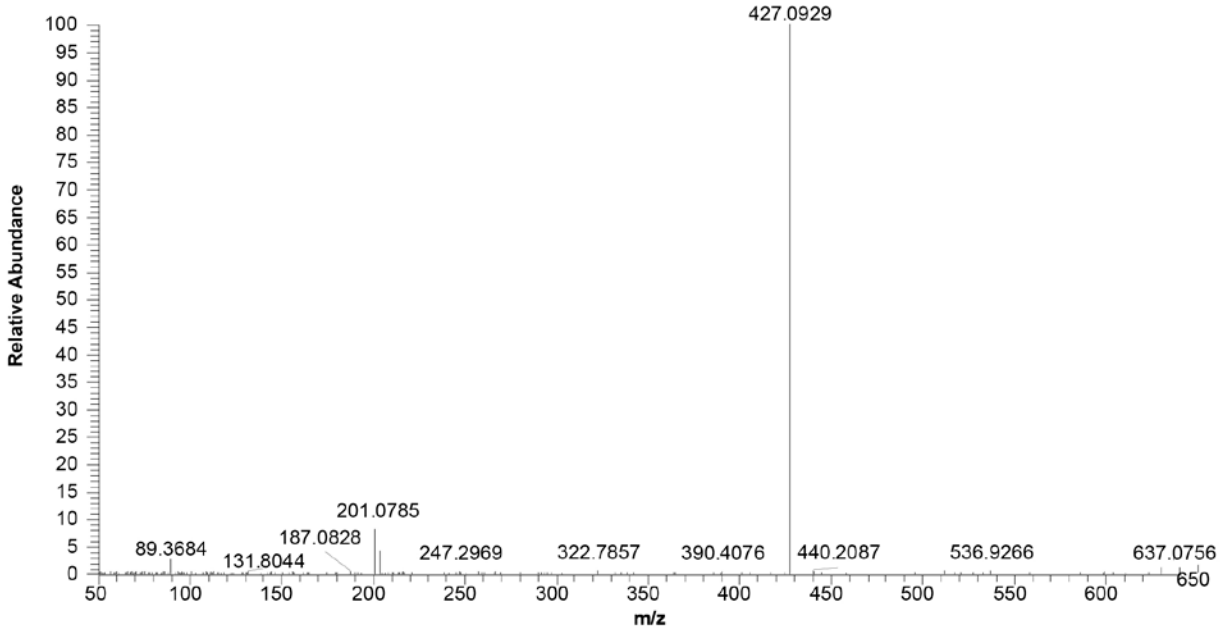
F: FTMS + c ESI Full ms2 603.13@cid35.00 [50.00-650.00]



C)

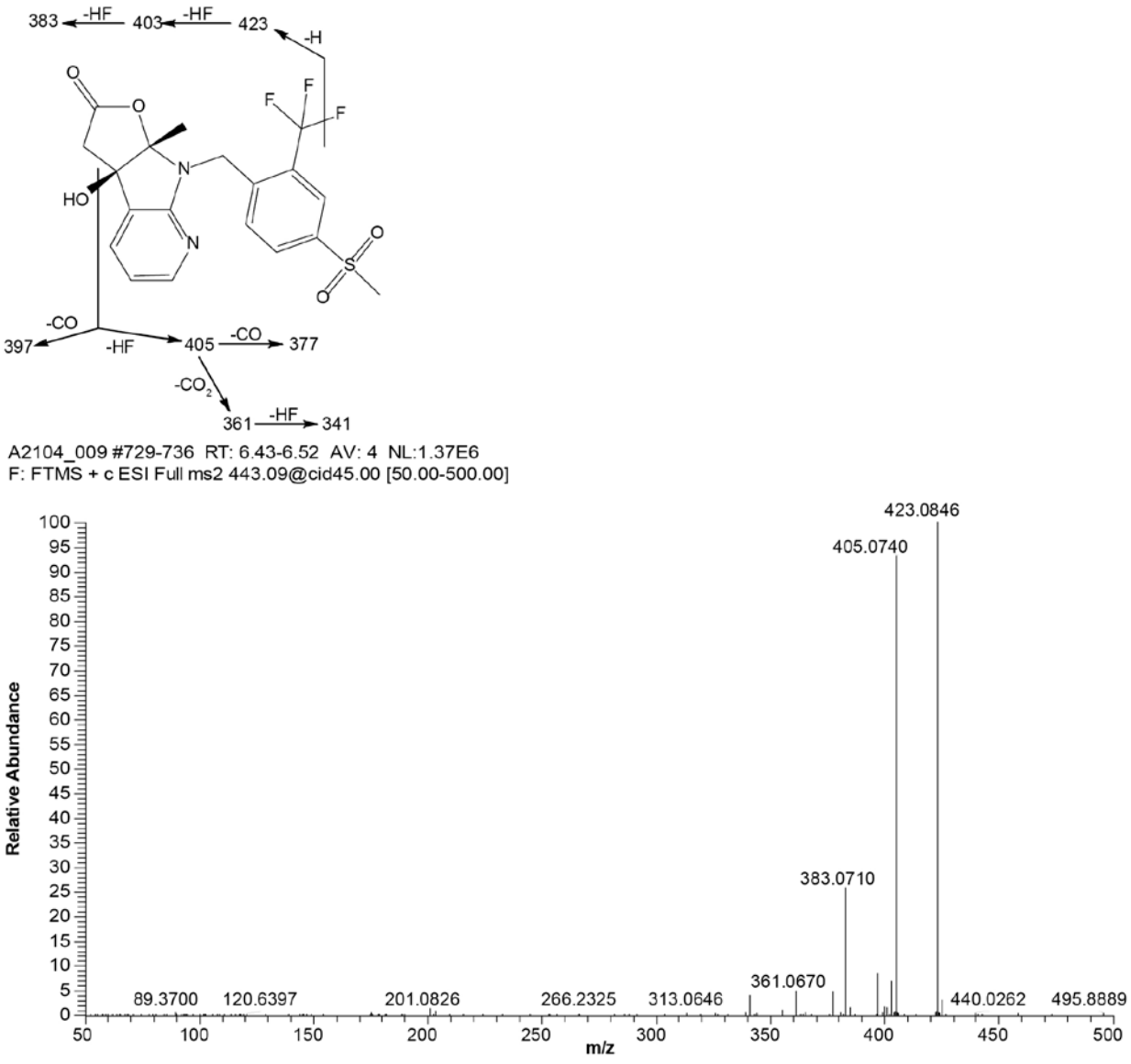


Figure S1. MS/MS spectra and proposed fragmentation of A) fevipiprant B) AG metabolite and C) lactone metabolite

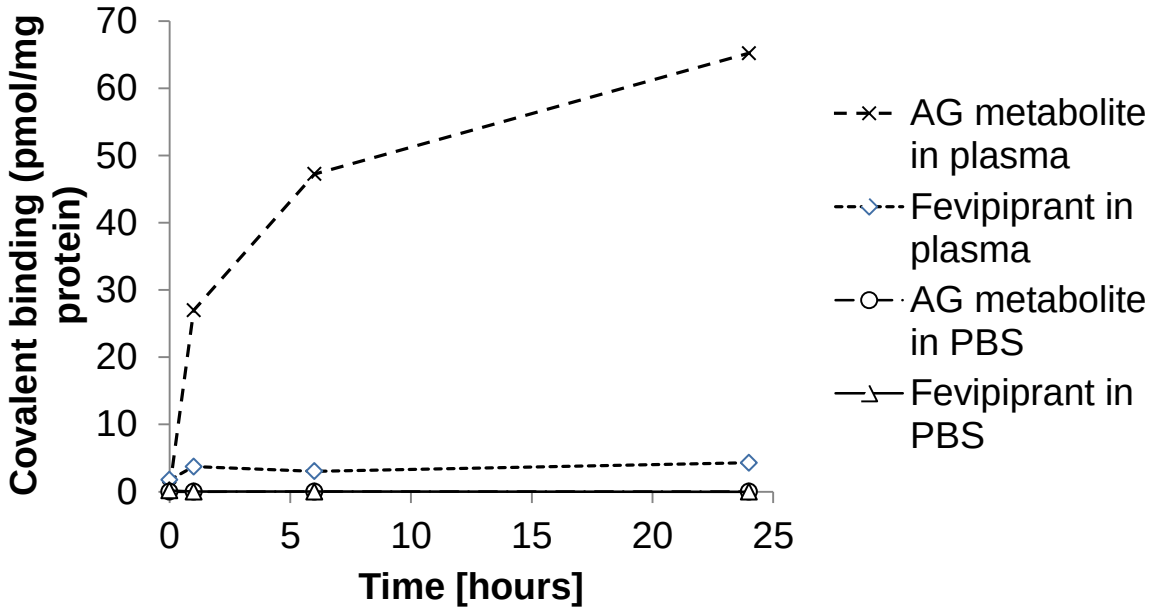


Figure S2. Covalent protein binding after incubation of [^{14}C]-fevipiprant (20 µM) or [^{14}C]-AG metabolite (20 µM) with 1:1 human plasma/PBS or PBS at 37 ºC, followed by protein precipitation, filtration and LSC quantification of protein-bound radioactivity. PBS, phosphate buffered saline

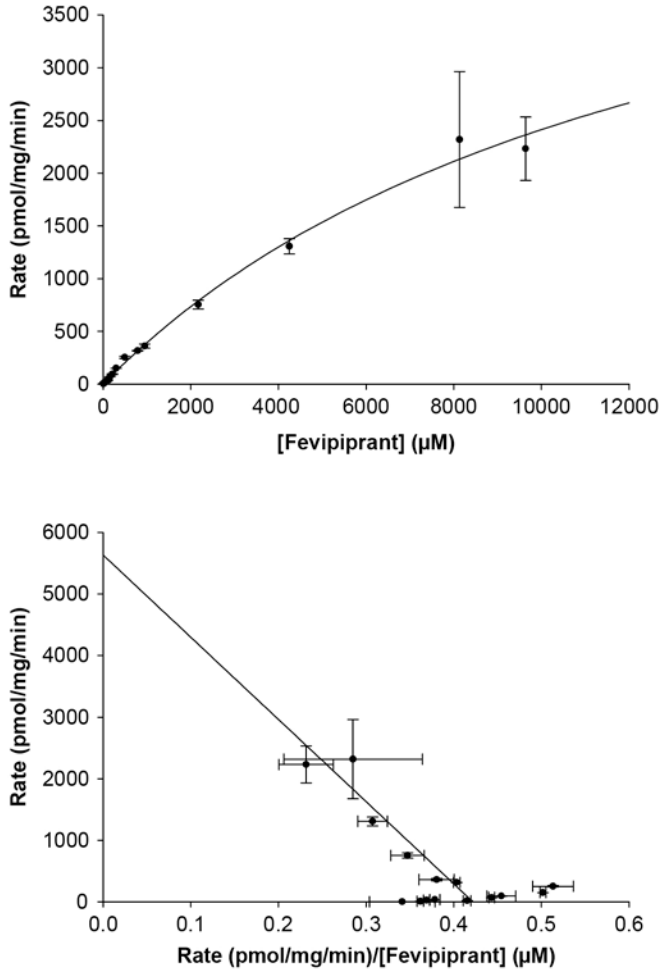


Figure S3. Enzyme kinetics of [^{14}C]-fevipiprant metabolism by recombinant UGT1A3, presented as reaction rate vs substrate concentration, and as reaction rate vs rate/substrate concentration. Reaction rates were measured by incubation of [^{14}C]-fevipiprant with recombinant UGT1A3 in TRIS buffer at 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.

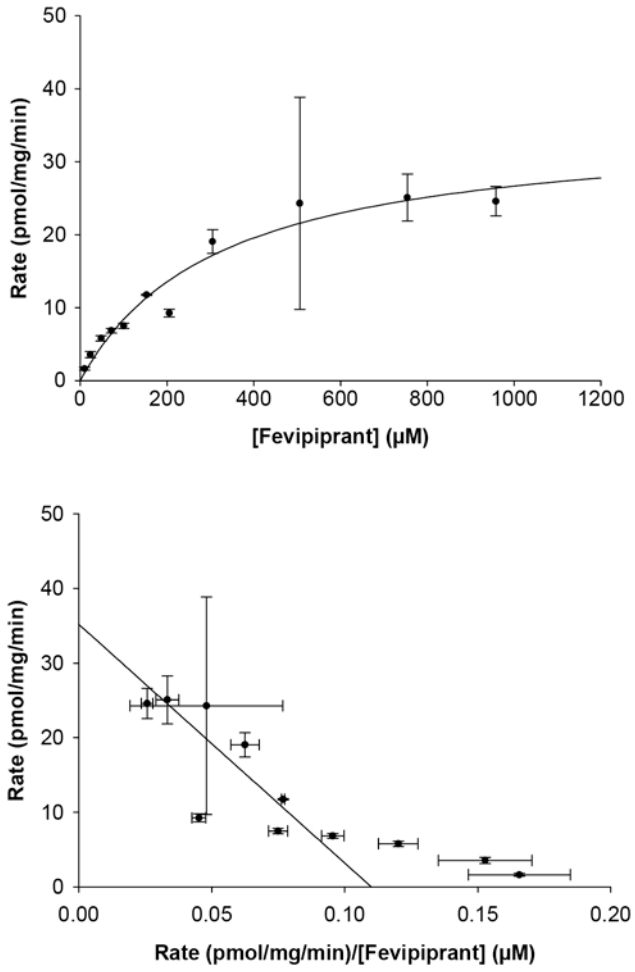


Figure S4. Enzyme kinetics of [^{14}C]-fevipiprant metabolism by recombinant UGT2B7 presented as reaction rate vs substrate concentration, and as reaction rate vs rate/substrate concentration.

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.

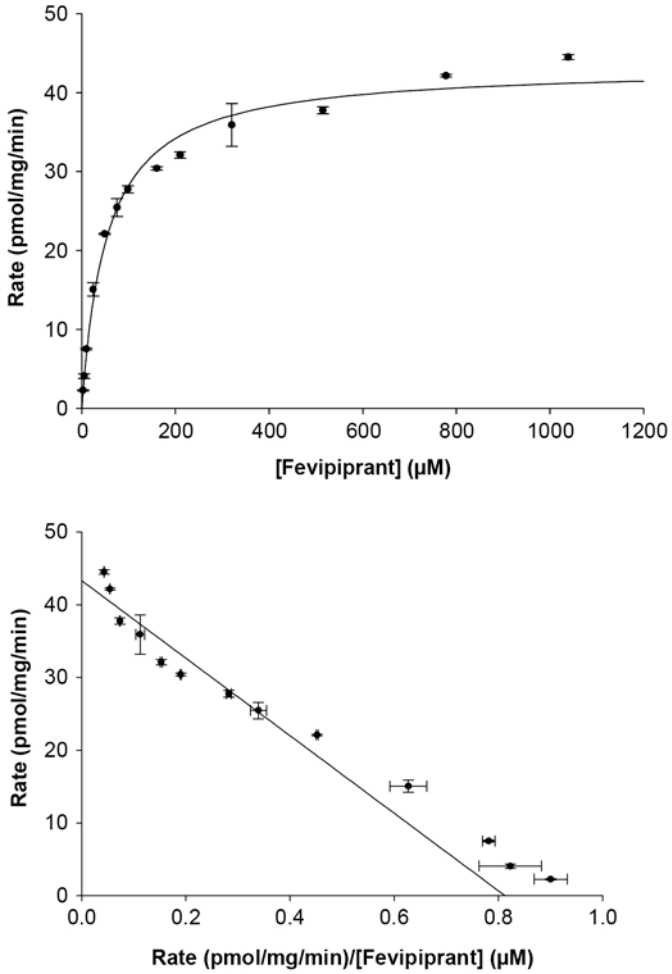


Figure S5. Enzyme kinetics of [14C]-fevipiprant metabolism by recombinant UGT2B17 presented as reaction rate vs substrate concentration, and as reaction rate vs rate/substrate concentration. Reaction rates were measured by incubation of [^{14}C]-fevipiprant with recombinant UGT2B17 in TRIS buffer at 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.

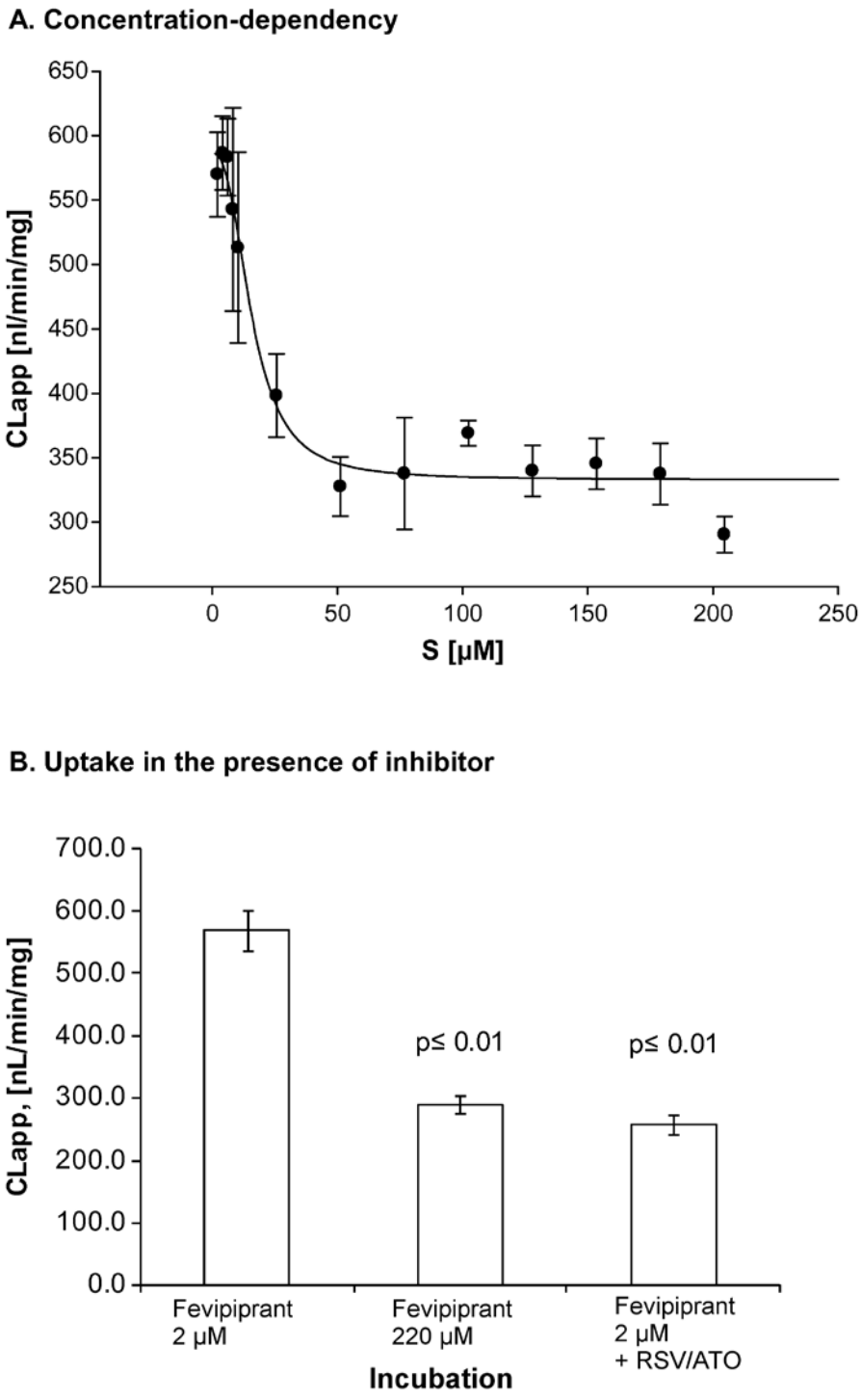


Figure S6 A, B. Uptake of fevipiprant by OATP1B3-transporter expressing HEK293 cells presented as CLapp for various substrate concentrations and in the presence and absence of inhibitors of OATP1B3. CLapp was calculated from the amount of radioactivity present in

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.

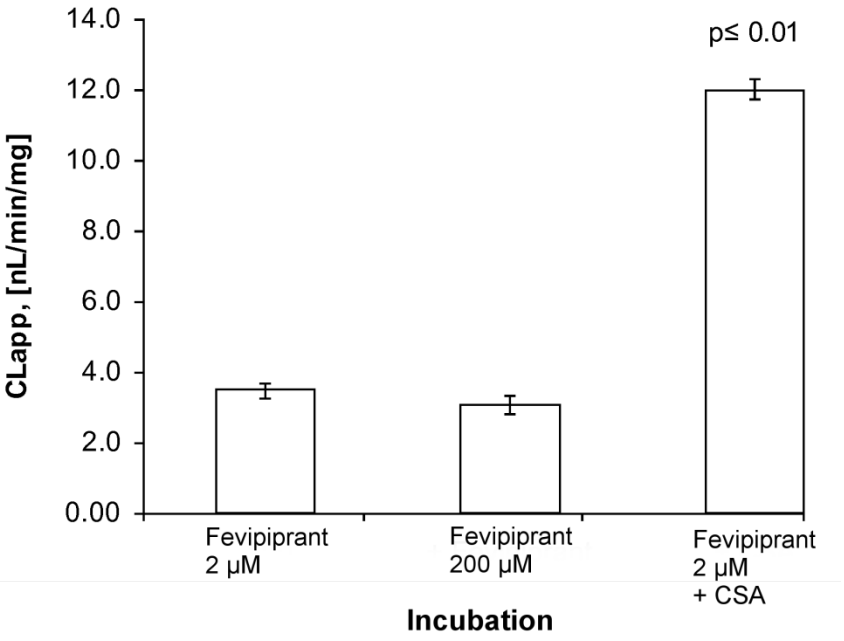


Figure S7. Uptake of fevipiprant by MDR1-transporter expressing LLCPK1 cells presented as CLapp for two substrate concentrations and in the presence and absence of an inhibitor of MDR1. CLapp was calculated from the amount of radioactivity present in MDR1-expressing LLCPK1 cells after incubation with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

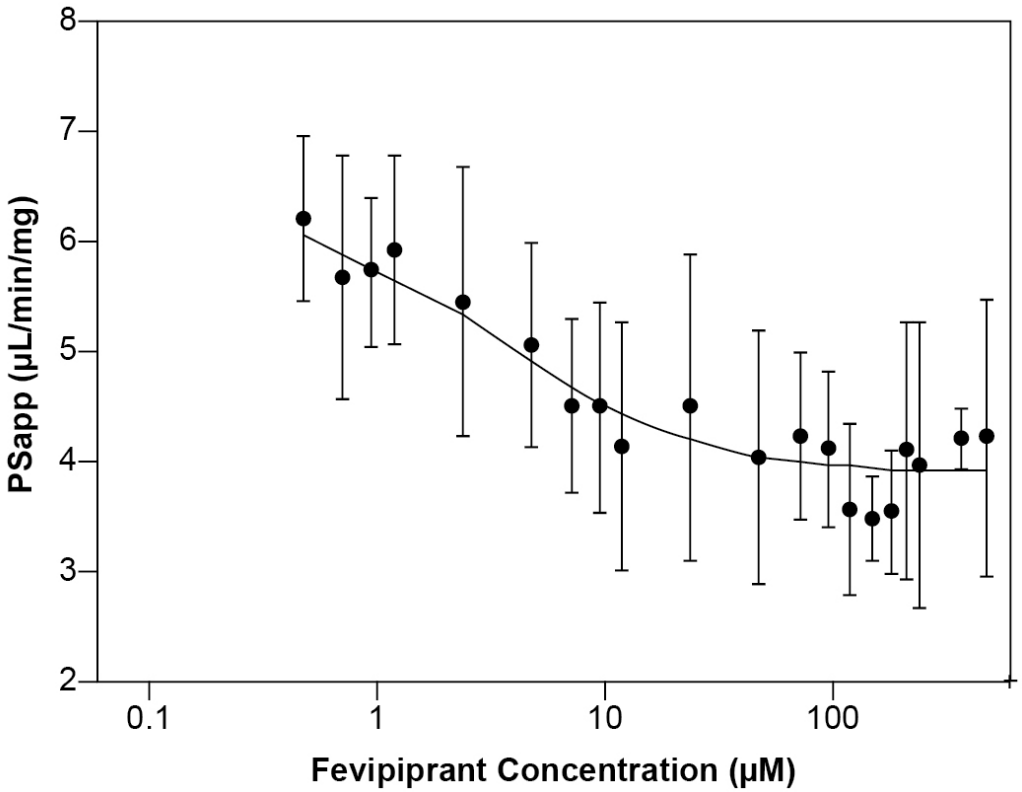


Figure S8. Uptake of fevipiprant by OAT3 transporter expressing HEK293 cells presented as PSapp for various substrate concentrations. PSapp was calculated from the amount of radioactivity present in OAT3-expressing HEK293 cells after incubation with [^{14}C]-fevipiprant at 37ºC for 3 minutes, by fitting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

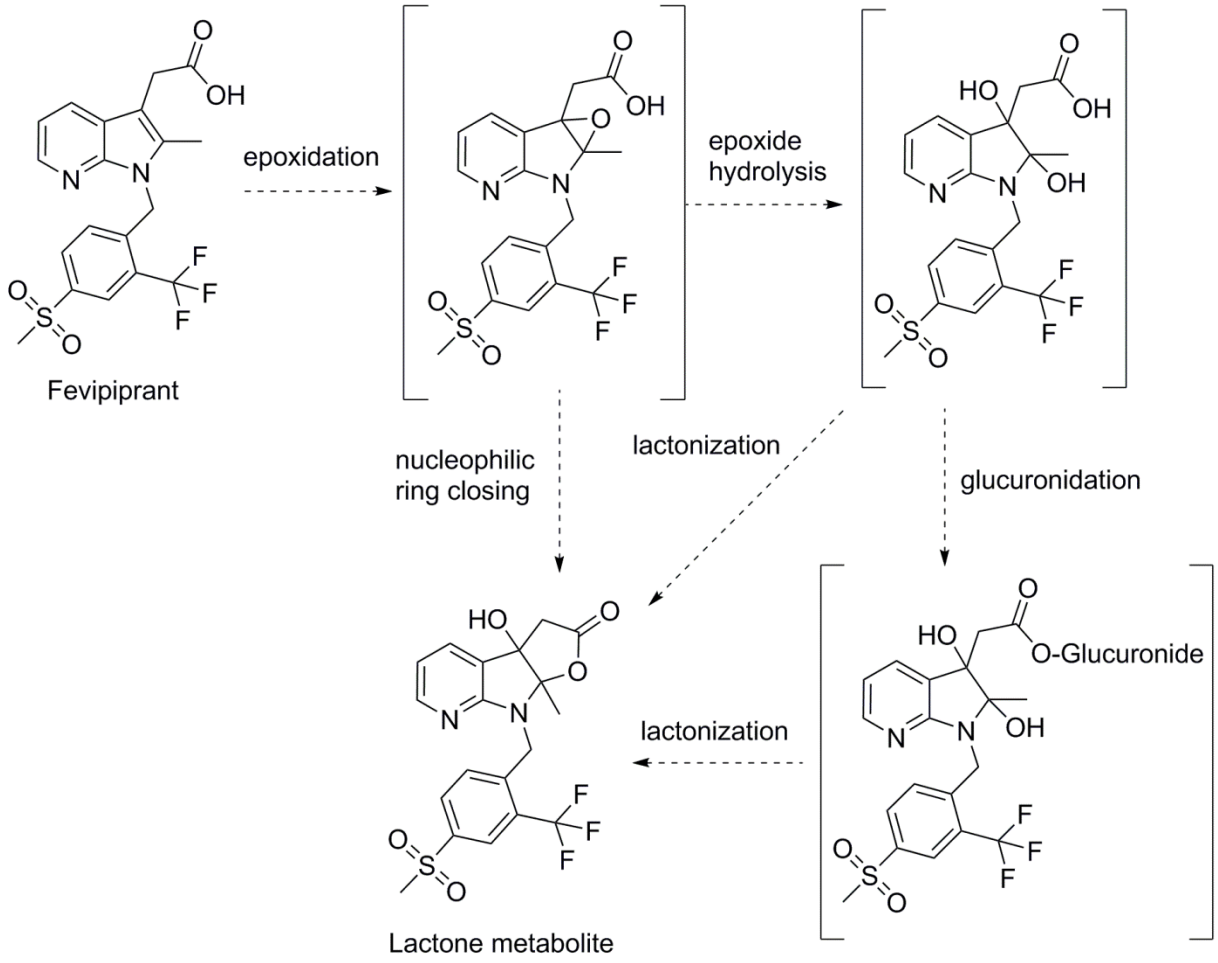


Figure S9. Proposed possible pathways of lactone metabolite formation

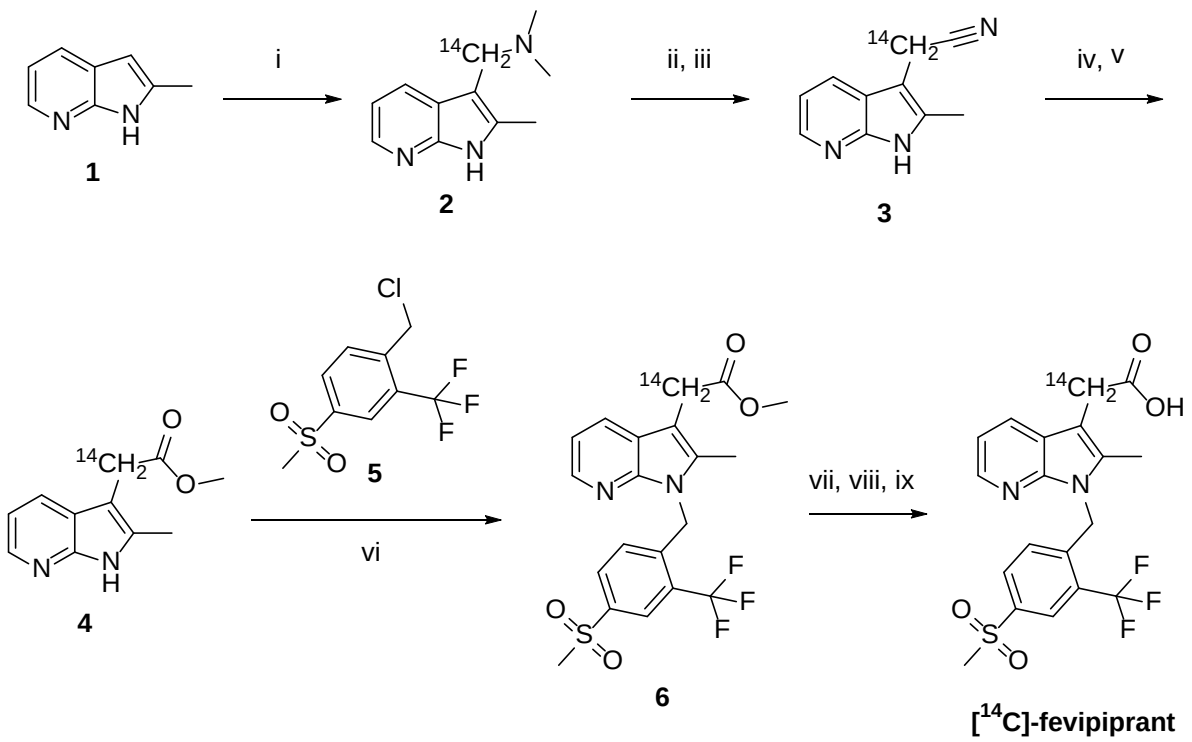


Figure S10. Synthetic scheme for [^{14}C]-fevipiprant. i) ($^{14}CH_2O$)n, H_2O, $Me_2NH.HCl$; ii) MeI, EtOH, 25°C; iii) NaCN, DMF, 100°C; iv) HCl, 100°C; v) MeOH, H_2SO_4, 100°C; vi) 5, $CsCO_3$, acetone, reflux; vii 12% NaOH, rt; viii) HCl; ix) crystallization EtOH/H_2O.

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.