Metabolism of fostamatinib, the oral methylene phosphate prodrug of the spleen tyrosine kinase (Syk) inhibitor R406 in humans: Contribution of hepatic and gut bacterial processes to the overall biotransformation.

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Running Title Page

a) Running Title: Gut bacterial involvement in R406 metabolism

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d) Non standard abbreviations: Syk, spleen tyrosine kinase; SECO, secoisolaricirresinol; MTBE, t-Butyl methyl ether; BHI, brain heart infusion media; ACR, American College of Rheumatology; RA, rheumatoid arthritis; ITP, idiopathic thrombocytopenia purpura; R788, fostamatinib, N4-(2,2-Dimethyl-4-[(dihydrogenphosphonoxy)methyl]-3-oxo-5-pyrid[1,4]oxazin-6-yl)-5-fluoro-N2-(3,4,5-trimethoxyphenyl)-2,4-pyrimidinediamine disodium hexahydrate; R406, N4-(2,2-Dimethyl-3-oxo-4-pyrid[1,4]oxazin-6-yl)-5-
fluoro-N2-(3,4,5-trimethoxyphenyl)-2,4-pyrimidinediamine; lactam N-glucuronide of R406, N4-((2, 2-Dimethyl-4-(1-β-D-glucopyranuronosyl)-3-oxo-5-pyrido[1,4]oxazin-6-yl])-5-fluoro-N2-(3,4,5-trimethoxyphenyl)-2,4-pyrimidinediamine; 3,5-benzene diol metabolite of R406, N2-(3, 5-Dihydroxyphenyl)-N4-(2, 2-dimethyl-3-oxo-5-pyrido[1,4]oxazin-6-yl)-5-fluoro-2, 4-pyrimidinediamine; R529, N4-(2, 2-Dimethyl-3-oxo-5-pyrido[1,4]oxazin-6-yl)-5-fluoro-N2-(4-hydroxy-3, 5-dimethoxyphenyl)-2, 4-pyrimidinediamine.
Abstract

The metabolism of the Syk inhibitor R406 and its oral prodrug R788 were determined in vitro and in humans. R788 was rapidly converted to R406 by human intestinal microsomes and only low levels of R788 were observed in plasma of human subjects following oral administration of $^{14}$C-R788. R406 was the major drug-related compound in plasma from human subjects, and only low levels of metabolites were observed in plasma. The plasma metabolites of R406 were identified as a sulfate conjugate and glucuronide conjugate of the para O-demethylated metabolite of R406 (R529), and a direct N-glucuronide conjugate of R406. Elimination of drug related material into the urine accounted for 19% of the administered dose, and the major metabolite in urine from all human subjects was the lactam N-glucuronide of R406. On average 80% of the total drug was recovered in feces. Two drug related peaks were observed, one peak was identified as R406, while the other peak was identified as a unique 3,5-benzene diol metabolite of R406. The 3,5-benzene diol metabolite appeared to result from the subsequent O-demethylations and dehydroxylation of R529 by anaerobic gut bacteria, as only R529 was converted to this metabolite following the in vitro incubation with human fecal samples. These data indicate that the major fecal metabolite of R406 observed in humans is a product of a hepatic CYP-450 mediated O-demethylation and subsequent O-demethylations and dehydroxylation by gut bacteria.
Introduction

Fc receptor signaling is an important process in the immunological response for macrophages, neutrophils and mast cells (Turner, et al., 2000). Activation of the Fc receptor results in the degranulation and gene transcription of cytokines that play a critical role in inflammation. Binding of ligands to the Fc receptor results in activation of intracellular immunoreceptor tyrosine-based activation motifs (ITAMS) that interact with the non receptor Spleen Tyrosine Kinase (Syk) for downstream signaling. Because of its critical role in the Fc receptor signal transduction, inhibition of Syk represents a potential target for the treatment of autoimmune diseases such as rheumatoid arthritis (RA) and idiopathic thrombocytopenia purpura (ITP) (Wong, et al., 2004; Singh and Masuda, 2007).

R406 is a small molecule that is a relatively selective Syk inhibitor and a potent inhibitor of IgE and IgG-mediated activation of Fc receptor signaling (Braselmann et al., 2006). In a rodent collagen-induced arthritis model R406 was shown to produce significant improvement in histopathology, clinical scores, and joint radiography (Pine et al., 2007). R406 exhibits low aqueous solubility, which limits the potential for solid dosage form development. Previous reports suggested that use of phosphate prodrugs can produce extensive improvement in bioavailability when compared with the parent compound (Vierling and Greiner, 2003; Furefine et al., 2004). Therefore, the methylene phosphate prodrug of R406, R788 (fostamatinib), was synthesized and tested in preclinical and clinical studies. When tested in animals and in human volunteers, R788 in a simple suspension formulation resulted in systemic exposure of R406 that is comparable, or
better than R406 administration using a solution formulation. In RA patients, oral administration of R788 tablets resulted in R406 exposure that is sufficient to demonstrate significant improvement of ACR scores in two large clinical trials (Weinblatt et al., 2008, Weinblatt et al., 2009). R788 has also shown promise in the treatment of ITP (Podolanczuk et al., 2009).

Little is known regarding the metabolism and disposition of R788 in humans. Therefore, \textit{in vitro} studies using human liver and intestinal microsomes were conducted to evaluate the pathways of R788/R406 metabolism. In addition, a $^{14}$C-radiolabelled mass balance study in healthy male subjects was performed in an effort to gain understanding of the metabolic pathways and disposition of the R788/R406 in humans. The findings from these studies are summarized in this manuscript. A metabolic scheme for R788/R406 in humans, including a unique contribution of anaerobic gut bacteria to the overall metabolism, is proposed.
Material and Methods

Materials.

R788 (fostamatinib, N4-(2,2-Dimethyl-4-[(dihydrogenphosphonoxy)methyl]-3-oxo-5-
pyrid[1,4]oxazin-6-yl)-5-fluoro-N2-(3,4,5-trimethoxyphenyl)-2,4-pyrimidinediamine
disodium hexahydrate) was synthesized by DSM fine chemicals (Linz, Austria). R406
(N4-(2,2-Dimethyl-3-oxo-4-pyrid[1,4]oxazin-6-yl)-5-fluoro-N2-(3,4,5-
trimethoxyphenyl)-2,4-pyrimidinediamine), the para-O-demethylated metabolite of
R406 (R529), the lactam N-glucuronide of R406, the putative 3,4-benzene diol metabolite
of R406 and the 3,5-benzene diol metabolite of R406 were synthesized by Rigel. 14C-
R788 (55.2 mCi/mmol) was prepared by Aptuit (Lenexa, KS). The 14C was located in the
2-C position of the pyrimidine moiety of R406.

t-Butyl methyl ether (MTBE), alkaline phosphatase from bovine intestinal mucosa,
alamethacin, ketoconazole, furafylline, quinidine, sulfaphenazole and L-cysteine were
obtained from Sigma Chemical Co (St Louis, MO). Bacto™ brain heart infusion (BHI)
media was obtained from Becton Dickinson and Company (Columbia, MD). AnaeroGen
compact paper sachets, and anaerobic indicator strips were obtained from Oxoid
(Basingstoke, England). Pouch-bags were obtained from Remel (Lenexa, KS). Human
hepatic and intestinal microsomes were obtained from Xenotech (Lenexa, KS). Expressed
CYP450 isoforms and (S)-(+)N-3-benzylnirvanol were obtained from BD Biosciences
(San Jose, CA). All other chemicals were the highest grade available.
In vitro metabolism of R406 and R788

The stability of R406 was examined using hepatic human microsomes and expressed human CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Reactions were performed at 37°C in plastic vials at a final volume of 502 μL. The reaction mixture consisted of 445 μL of 0.1 M phosphate buffer (pH 7.4), 5 μL of hepatic microsomes (0.1 mg protein) or expressed enzymes (20 pmol), 2 μL of R406 (final concentration 1 μM) in DMSO, and 50 μL of NADPH (final concentration 1 mM). Metabolic reactions were initiated by the addition of the NADPH. For microsomal glucuronidation studies, 0.4 mg/mL human liver microsomes, 0.025 mg of alamethicin, 10 mM MgCl₂, 100 mM sodium phosphate buffer (pH 7.5), 10 μM of R406 and 5 mM UDPGA were incubated in a total volume of 200 μl. Before addition of the UDPGA, the microsomes were activated by alamethicin for 15 min on ice. Metabolic reactions were stopped at 0, 15, 30 and 50 min by removing 50 μL of the reaction mixture and adding this to 100 μL of a stop solution consisting of acetonitrile: DMSO: ethanol: 100 mM ammonium acetate: 0.5 μM verapamil internal standard (7.5:0.6:0.6:2.5:1 v/v). For the hepatic microsomal NADPH-dependent metabolism studies, the effect of the chemical inhibitors ketoconazole (1 μM), furafylline (10 μM), quinidine (10 μM), sulfaphenazole (10 μM) and 3-N-benzyl nirvanol (5 μM) was also examined. R406 concentrations were determined using a Sciex QTrap mass spectrometer in the MRM mode. Separations were preformed using a Dash-18 (Thermo) C18 column (20 mm x 2.1 mm, 3 μm) and a Shimadzu SCL-10A VP HPLC system. Flow rate was 0.25 mL/min. Mobile phase A was 10 mM ammonium acetate (pH 6.5) in water and mobile phase B was 100% acetonitrile. Initial conditions were 10%
mobile phase B, and after 0.75 min a linear gradient to 90% mobile phase B was achieved at 2.5 min and held at these conditions until 3.5 mins.

LC/MS analysis with UV (254 nm) detection was utilized to determine the pattern of the NADPH-dependent R406 metabolites formed in human hepatic microsomes. The microsomal incubation conditions were similar to those described above, but with a final concentration of R406 at 10 µM. Samples from the *in vitro* glucuronidation assay were also examined. Reactions were terminated after 15 mins for the NADPH-dependent reaction and at 50 mins for the glucuronidation assay by removing 50 µL of the reaction mixture and adding this to an equal volume of acetonitrile. After centrifugation, the supernatants were analyzed using a Shimadzu SCL-10A VP HPLC system equipped with an Agilent 1100 series diode array detector and Sciex QTrap mass spectrometer. A Betasil (Thermo) C18 column (150 mm x 1 mm, 3 um) was used for the analysis. Mobile phase A was 1% ammonium acetate in water while mobile phase B was 100% acetonitrile. Initial conditions were 5% B and a linear gradient to 75% B was performed over 35 mins, and held at these conditions for 10 mins. Flow rate was 0.20 mL/min.

R788 (10 µM) stability in human intestinal microsomes (0.5 mg/mL) or alkaline phosphatase (42 units/mL) was examined at 37°C in a 0.2 M Tris buffer (pH 7.4) containing 0.05 M MgCl₂. Reactions were terminated at 0 min, 15, and 30 mins by removing 50 µL and adding to an equal volume of acetonitrile. Analyses were performed by LC/MS with UV (254 nm) detection as described above.
Human Mass Balance Study

The human mass balance study was performed at the Covance Clinical Research Unit, Inc. (Madison, WI). Six healthy male subjects (19-35 yrs old) with a body weight between 63.8 and 85.3 kg participated in the study according to an IRB approved protocol. Participants were housed in the clinical unit until the end of the study (up to 8 days). The target dose of $^{14}$C-R788 (150 mg, 100 µCi) was administered orally as a solution in 0.01 N citrate buffer (pH 6) using a syringe. Subjects were fasted for 10 hrs before R788 administration.

Blood samples were obtained pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 32, 48, 72, 96, 120, 144, and 168 hrs post-dose, and then at 24-hr intervals until discharged. Blood samples were collected into lavender top Vacutainer® tubes containing K$_2$EDTA and were kept on ice until plasma was prepared by centrifugation. Urine samples were obtained pre-dose, 0-4, 4-8, 8-12, 12-24 hrs and at 24-hr intervals until the subjects were discharged. Feces were obtained pre-dose and at 24 hr intervals until the subjects were discharged. Blood, plasma and urine samples were stored at -20°C until analyzed. Feces were kept at -70°C until analyzed.

Analysis of Samples obtained from the Human Mass balance study

Radioanalysis was performed at Covance Laboratories (Madison, WI). Duplicate samples of plasma and urine (0.2ml) were combusted in a Model 307 sample Oxidizer (Packard) and the $^{14}$CO2 was trapped in a mixture of perma Fluor and Carbo-Sorb. Fecal samples were diluted with an equal weight of methanol, and frozen overnight. The fecal mixtures
were thawed and then homogenized using a polytron PT 10/35 equipped with a 36 mm generator. Duplicate weighed aliquots (approximately 0.2 g) were combusted. All combusted samples were analyzed for radioactivity in Model 2900TR liquid scintillation counters (Packard Instrument Company) for at least 5 minutes or 100,000 counts.

Metabolite profiles were determined for extracts of the plasma, urine and fecal samples. Two mL of the plasma samples from each subject collected between 0.5 and 24 hours post dose were pooled by time point, and 5 mL of each pooled plasma sample was extracted twice with acetonitrile (1:3, v:v). The combined supernatants were evaporated to dryness and reconstituted in 0.01 M ammonium acetate in 50% acetonitrile. Urine samples were pooled by subject to generate a single 0- to 72-hour sample. Samples were centrifuged prior to analysis. Fecal samples (4 g) collected between 0-24 hrs from each subject were extracted twice with methanol. The combined fecal extracts were evaporated to dryness and reconstituted in 0.01 M ammonium acetate in 50% acetonitrile:MeOH (1:0.1, v:v). Duplicate aliquots of the urine and extracts of plasma and feces were analyzed by liquid scintillation counting to determine extraction recoveries. Recovery of radioactivity was 71.4 to 111.0% from plasma, 63.8 to 77.6% from feces, and 94.1 to 104.0% for urine.

Radioactivity profiles of urine, plasma and feces were performed using a Hewlett-Packard HP1000 series HPLC system equipped with a Waters Nova-Pak C18, 3.9 x 300 mm column (4 μm) and a Phenomenex C18, 3 x 4mm guard column. Columns were
heated to 30°C. Mobile phase A consisted of 0.02M ammonium acetate in water (pH 7) and mobile phase B was acetonitrile. Initial conditions were 3% B, and after 3 min the contribution of the mobile phase B was linearly increased to 20% (20 min), 24% (30 min), 55% (30 min) and 80% (43 min), where it was held for 5 min. Flow rate was 1 mL/min. Fractions were collected at 10-second intervals and analyzed by TopCount solid scintillation counting.

Selected samples of human plasma, urine, and feces were analyzed by LC/MS using a Shimadzu/ Prominence HPLC system consisting of SIL-20AC HT (10°C) autosampler, a CBM-20A controller, two LC-20AD pumps, and a CTO-20AC column oven (30°C). Mass spectrometry was performed using a Thermo Fisher Scientific LTQ Orbitrap XL with electrospray ionization in the positive mode and Xcalibur 2.0 software. Separations were performed using a Waters Nova-Pak C18 column (3.9 x 300 mm, 4 µm) equipped with a Phenomenex C18 guard column (3 x 4 mm). Mobile phase A was 20 mM ammonium acetate and mobile phase B was acetonitrile. Flow rate was 1 mL/min. Initial conditions were 97% mobile phase A and the percentage of mobile phase B was increased linearly to 20% (20 mins), 24% (30 mins), 55% (38 min), and 80% (43 min) where it was held for 5 mins.

**Purification and NMR analysis of major fecal metabolite**

Twenty grams of human feces were mixed with an equal volume of water. Twice the volume of MTBE was added for liquid-liquid extraction (LLE). After 15 minutes of shaking, the mixture was centrifuged at 750 rpm for 5 minutes in a Beckman Allegra 6
centrifuge. The MTBE solvent layer was transferred to glass tubes and dried down under nitrogen using a TurboVap LV evaporator (Zymark). After repeating the LLE 4 times, all of the MTBE extracts were dried down and resuspended with 200 µL of H₂O/acetonitrile/formic acid (70:30:0.1). The major fecal metabolite was purified by an isocratic HPLC method on an Agilent 1100 HPLC system. Solvent A was HPLC water with 5% acetonitrile and 10 mM ammonium acetate. Solvent B was 100% acetonitrile. The isocratic method was run at 30% B. An Alltima C8 HPLC column (100 x 4.6 mm) was used with flow rate of 1.0 mL/minute. The elution time for this metabolite was approximately 6 minutes. The collected fractions were dried down in the TurboVap evaporator and then resuspended in deuterated DMSO for NMR analysis. NMR analysis was run on a Varian Unity-300 NMR instrument. Due to the low amount of purified metabolite, the ¹H signal was collected for 3 days.

**In vitro fecal incubation studies**

Human fecal incubations were performed using a sterile BHI media (3.7 g /100 mL water) containing 5 mg L-cysteine /100 mL. Under aseptic conditions, 2.8 mL of the BHI media was added to a 6-well flat bottom plate (Costar 3516). R788 (100 µM), R406 (100 µM), R529 (100 µM) or the N-glucuronide of R406 (50 µM) were added in 10 µL of DMSO. Fresh human fecal material (6 gm) was placed into a 50 mL falcon tube and 20 mL of the BHI media was added. The fecal mixture was vortexed for 2 minutes until homogenous. The human fecal mixture (0.2 mL) was added to the plate, after which the plate was placed into a pouch-bag. Two AnaeroGen compact paper sachets were added
along with two Oxoid anaerobic indicator strips. The excess air was expelled from the pouch-bag, the bag was sealed using a Hualion model FS-205 impulse sealer and placed onto a Lab-line 3-D rotator in a 37°C Yamato IC600 incubator. The samples were rotated continuously during the incubation. After approximately 36 hours, the samples were removed, the pouch-bag was opened, and 0.5 mL of sample from each well was added to an equal volume of acetonitrile. The samples were vortexed, centrifuged, and then analyzed by LC/MS with UV detection as described above for the R406 \textit{in vitro} metabolism studies.
Results

In vitro metabolism

R788 was completely hydrolyzed to R406 by human intestinal microsomes within 15 mins (Figure 1). Complete hydrolysis to R406 was also achieved with alkaline phosphatase (not shown). With human hepatic microsomes and NADPH, a time-dependent decrease in the R406 peak area was observed (T_{1/2} 24 mins). One major metabolite of R406 was observed by UV detection (Figure 2), and the protonated molecular ion of this peak (m/z 457) was 14 mass units less than R406. Based on co-elution with an authentic standard, the metabolite was identified as the para O-demethylated product of R406 (R529). A smaller metabolite peak, representing the meta-O-demethylated metabolite (RT 35.78 min) was also observed in the microsomal studies. Among the P450 inhibitors tested, ketoconazole was the only inhibitor that markedly (>90 %) reduced the formation of this metabolite. In addition, expressed CYP3A4 metabolized R406 to the greatest extent of any of the expressed isoforms examined. No appreciable loss of R406 peak area was observed following incubation with human hepatic microsomes and UDPGA. However, three distinct direct glucuronide conjugates of R406 could be observed by LC/MS (m/z 647) (Figure 3).

Plasma radioactivity time course

The time course of plasma radioactivity in subjects who received a single oral dose of \textsuperscript{14}C-R788 is shown in Figure 4. Peak plasma radioactivity levels were observed at 1 hr after dosing in all individuals and steadily declined thereafter. Cmax for plasma
radioactivity levels ranged from 1770 to 2630 dpm/mL, while the plasma half-life ranged from 10.8 to 15.7 hrs. Subject 4 had the shortest plasma half life and the lowest plasma radioactivity exposure (10493 dpm*hr/mL) of all of the individuals in the study. Subject 2 had the longest half-live and the highest exposure (25343 dpm*hr/mL) of all of the subjects.

**Plasma metabolites**

The major peak observed in plasma at all time points was identified by LC/MS as R406, based on the protonated molecular ion at m/z 471. Representative radioactivity profiles for the pooled plasma obtained at 0.5 hr and 4 hr after dosing are shown in Figure 5. The prodrug R788 (m/z 581) was also observed in plasma, with higher levels being observed in the early time points samples. All other radioactive peaks in plasma were minor at every time point examined. The plasma metabolites were identified by LC/MS as the O-glucuronide conjugate R529 (m/z 633) based on the 176 amu loss to generate a product ion at m/z 457, a sulfate conjugate of R529 (m/z 537) based on the 80 amu loss to a product ion at 457 m/z and direct N-glucuronides of R406 (m/z 647) based on the 176 amu loss generate a product ion at m/z 471.

**Profile of metabolites in urine and feces**

Overall mean recovery of radioactivity from the human subjects was 99.3%. The majority of the dose (80%) was recovered in feces within 96 hrs, while urinary recovery (19.3%) was nearly complete within 72 hrs (Table 1). One major radioactive peak in urine, accounting for greater than 75.2 % of the total urinary radioactivity, was observed in the 0-72 hr sample of all patients. A representative chromatogram for urine is shown in
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Figure 6. The major urinary metabolite was identified by LC/MS as an N-glucuronide of R406 (m/z 647) based on the characteristic loss of 176 mass units to the m/z 471 ion of R406. This N-glucuronide conjugate of R406 observed in urine co-eluted with the major R406 N-glucuronide that was formed in hepatic microsomes, and was identified as the lactam N-glucuronide of R406 based on co-elution with the chemically synthesized standard.

Two major radioactive peaks were observed in the 0 to 120 hr pooled feces from all subjects, but the relative amount of each of these peaks varied between individuals (Figure 7). Subject 4 had a greater proportion (11.6 to 1) of the earlier (35 min) eluting radioactive peak, while subject 2 had a greater proportion (5.5 to 1) of the later eluting radioactive peak (40 min). Three of the additional subjects had a greater proportion (1.7-3.9 to 1) of the later eluting peak, while the remaining subject had more (1.9 to 1) of the earlier eluting peak. The later eluting peak was identified by LC/MS as R406 (m/z 471). The protonated molecular ion of the earlier eluting peak was observed at m/z 413, which was 58 daltons lower than R406. The accurate mass measurement (413.13659) suggested a formula of C$_{19}$H$_{18}$FN$_{6}$O$_{4}$$^+$ (loss of C$_{3}$H$_{6}$O from R406) with a mass error of ± 0.5ppm. The mass spectrum of the fecal metabolite showed product ions at m/z 393 (loss of hydrogen fluoride), 385 (loss of carbon monoxide), 375 (loss of hydrogen fluoride and water), and 365 (loss of hydrogen fluoride and carbon monoxide). The metabolite was purified by HPLC for structural elucidation by NMR. $^1$H NMR analysis of the purified metabolite did not show any singlet in the aliphatic region corresponding to OCH$_3$ signals, indicative of the loss of CH$_3$ groups present on the trimethoxyphenyl ring of R406.
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(Figure 8). Although the spectra showed multiple peaks down field of δ 2.0, a characteristic peak singlet resonance observed at δ 1.49 in the aliphatic region corresponding to gem-dimethyl signals, as observed for R406, indicated conservation of the 6,6-bicyclic system of R406 in the metabolite. The protons observed at δ 6.59 and 5.80 of the metabolite are attributable to chemical shifts of the aromatic ring protons of the aryl ring. There was no pattern of splitting observed for these two protons, thereby precluding the formation of 3,4-benzene diol as the ortho aromatic protons are expected to show a coupling constant at least >7 Hz. The mass spectrum of the chemically synthesized 3,5-benzene diol metabolite was similar to that of the purified metabolite (Figure 9). Therefore, based on the NMR data, the HPLC co-elution with an authentic chemically synthesized standard (data not shown) and the MS/MS data the fecal metabolite was identified as the 3,5-benzene diol metabolite of R406.

Formation of fecal metabolite in vitro

The unusual 3,5-benzene diol metabolite of R406 was only observed in feces, and therefore the role for gut microflora in the formation of this metabolite was examined. R406, R529, R788 and the N-glucuronide of R406 were incubated with a human fecal sample under anaerobic conditions to determine if any of these compounds could be converted to the 3,5-benzene diol metabolite. After a 36 hr incubation, the only compound that was converted to the 3,5-benzene diol metabolite was R529 (Figure 10). R406 was stable under these incubation conditions, while R788 and the lactam N-glucuronide of R406 were both converted to R406.
Discussion.

Because of its poor pharmaceutical properties, R406 is orally administered as the methylene phosphate prodrug R788. This oral prodrug was designed to be cleaved to R406 by alkaline phosphatase(s) that are present on the apical brush border membranes of the intestinal enterocytes (Fleisher et al., 1985), after which the more hydrophobic R406 would be readily absorbed. The R788 prodrug appears to perform as designed, as only low levels of R788 were observed in plasma of the human subjects. Moreover, drug-related material was rapidly absorbed and R406 was the major radioactive peak in plasma at all time points. Although R406 was extensively metabolized prior to elimination, only low levels of circulating R406 metabolites were observed in plasma, indicating the rapid clearance of these metabolites. The plasma metabolites were identified as the glucuronide and sulfate conjugates of the para-\(O\)-demethylated metabolite (R529) and direct \(N\)-glucuronides of R406. Overall, these metabolites represented less than 3% of total radioactivity in plasma.

The rapid \(O\)-demethylation of R406 in human hepatic microsomes together with the absence of significant R406 disappearance in the presence of UDPGA suggested that oxidative metabolism of R406 may have predominated *in vivo*. However, glucuronidation of R406 was evident *in vivo*, as the lactam \(N\)-glucuronide of R406 was the major metabolite in urine. The lactam \(N\)-glucuronide in urine represented 14.9% of the administered dose. However, the contribution of \(N\)-glucuronidation to the metabolism of R406 *in vivo* may be higher as the biliary secreted \(N\)-glucuronide conjugate may not be
observed in feces due to hydrolysis to R406 by gut bacteria. The underestimation of the in vivo glucuronidation of R406 may be due in part to the poor solubility of R406 limiting the concentration (10 µM) used in the microsomal studies, as the Km’s of many substrates that undergo microsomal glucuronidation are reported in the high µM range (Williams et al., 2004). However, other studies using hepatic microsomes have also underestimated the extent of glucuronidation in vivo (Mistry and Houston, 1987; Miners et al., 2004), and it had been suggested that microsomes are not a useful model for predicting the clearance of drugs that undergo glucuronidation (Entrakul et al., 2005).

The majority of radioactivity (80%) was eliminated in feces and two major radioactive peaks were observed. One of these radioactive peaks was R406, which may have resulted either from the hydrolysis of unabsorbed R788 or from the hydrolysis of the R406 N-glucuronide secreted into bile, as both of these compounds were shown to be converted to R406 following incubation with human feces. The other major fecal radioactive peak was identified as the 3,5-benzene diol metabolite of R406. This unique metabolite was only present in feces, and was conceivably formed by the removal of the 3 methoxy groups of R406 and the subsequent dehydroxylation of the para hydroxyl group. A role for gut bacteria in the formation of this metabolite was examined as anaerobic gut bacteria have been shown to carry out the O-demethylation and dehydroxylation of the dietary lignans (Eeckhaut et al., 2008; Adlercreutz, 2007). Secoisolaricirresinol (SECO) is the most widely studied plant lignan, and depends on conversion by colonic bacteria into the biologically active mammalian lignans enterodiol and enterolactone (Wang et al., 2001; Clavel et al., 2005). Multiple strains of anaerobic bacteria are responsible for this
metabolism, with the \( O \)-demethylation by acetogenic bacteria being reported to occur prior to the dehydroxylation process (Heider and Fuchs, 1997). In contrast to the aerobic environment, where the \( O \)-demethylation reactions are catalyzed by monooxygenases, in the anaerobic gut environment the \( O \)-demethylation reactions are mediated by corrinoid-dependent methyl transferases with tetrahydrofolate serving as ultimate methyl acceptor (Berman and Frazer, 1992). Following \( O \)-demethylation, the plant lignans are dehydroxylated by a reductive process by additional strains of anaerobic bacteria (Wang et al., 2001).

Since R788, R406, the direct \( N \)-glucuronide or conjugates of R529 may all present in the intestinal tract, any of these compounds could have been the precursor to the 3, 5-benzene diol metabolite. However, following \textit{in vitro} incubations with human fecal bacteria, only R529 was converted to the 3,5-benzene diol metabolite. Since R406 did not undergo \( O \)-demethylation by gut bacteria, the presence of the free para-hydroxy group in R529, like that in the plant lignan SECO, may be one structural requirement for meta-\( O \)-demethylation of R529 to ensue. In support of this supposition, in the sequential \( O \)-demethylation of 3,4,5-trimethoxybenzoate by \textit{Eubacterium limosum} the initial removal of the para-methoxy group was shown to be necessary for the meta \( O \)-demethylations to proceed (Cocaig et al., 1991).

The presence of R529 in the gut most likely resulted from the hepatic \( O \)-demethylation of R406 and subsequent elimination of the R529 conjugates into bile. Once in the gut, the conjugates could be hydrolyzed back to R529, which was then converted to the 3,5-benzene diol metabolite by gut bacteria. In support of this hypothesis, recent studies in
the monkey have shown that R529 conjugates are the major metabolites of R406 in bile and that incubation of these biliary metabolites with monkey feces lead to the conversion of the R529 conjugates to the 3,5-benzene diol metabolite (Sweeny et al.). As observed in human, the 3,5-benzene diol metabolite was also the major metabolite of R788 in the monkey feces.

The identification of the 3,5-benzene diol metabolite of R406 demonstrates a novel sequence of drug metabolism in which an O-demethylation mediated by a hepatic monooxygenase is followed by subsequent O-demethylations and dehydroxylation by anaerobic gut bacteria. These results indicate that gut bacteria are capable of modifying hepatic derived drug metabolites by pathways other than the commonly recognized reductive (Zachariah and Juchau, 1974) and hydrolytic pathways (Christopher et al., 2008).

The fecal levels of the 3,5-benzene diol metabolite varied markedly amongst subjects. Subject 4 had very high levels of the 3,5-benzene diol metabolite, while low levels of this metabolite were observed in Subject 2. One possible explanation for the variation could be differences in gut bacterial populations between subjects, as the activity of the gut microflora has been suggested to be a major determinant of the human lignan levels in plasma (Lampe and Chang, 2007). However, as no intact R529 was observed in the feces from any of the subjects, the differences in the fecal 3,5-bezene diol metabolite levels probably did not reflect variations in the colonic bacterial populations. More likely, the variation in the 3,5-benzene diol levels in feces represents differences in formation of
R529, which from the *in vitro* phenotyping experiments is mediated by CYP3A4. Higher levels of CYP3A4 in subject 4 may possibly be responsible for the more rapid clearance of R406 and the higher portion of the 3,5-benzene diol metabolite in feces from this individual.

In summary, the metabolic fate of R788 was delineated in the human mass balance study (Scheme 1) and additional *in vitro* studies. The prodrug R788 was most likely cleaved by intestinal alkaline phosphates to R406, which was the major drug related product observed in plasma. R406 underwent both direct glucuronidation, and a CYP3A4-mediated para O-demethylation to R529. R529 was most likely conjugated by sulfation and glucuronidation, and these conjugates were secreted into bile. Following secretion into the gut these conjugates were hydrolyzed to R529, which was subsequently O-demethylated and dehydroxylated by anaerobic gut bacteria to the 3,5-benzene diol metabolite, which was the major metabolite fecal of R788 in humans. Overall, the formation of the unique 3,5-benzene diol metabolite resulted from a sequential process involving a hepatic monooxygenase and subsequent metabolism by multiple strains of anaerobic gut bacteria.
Acknowledgements

The expertise of the Covance employees, especially Dr. Mohammad Bashir, Dr. Jie Ding, and Ms. Claudine Oelke, in analyzing samples from the human mass balance study is greatly appreciated. We also thank members of the Rigel clinical department, Theresa Musser and Aaron Siek, for their involvement in the study.
References


Fleisher D, Stewart BH and Amidon GL (1985) Design of prodrugs for improved gastrointestinal absorption by intestinal enzyme targeting. in Methods of Enzymology,


Scheme Legend

1. Proposed scheme of R788 metabolism in humans.
Figure Legends

1. Hydrolysis of R788 to R406 by human intestinal microsomes. The top panel shows the UV (254 nm) chromatogram of the 0 min sample, while the bottom panel shows a sample obtained following a 15 min incubation at 37°C.

2. UV profile of sample obtained 15 min following incubation of R406 (10 µM) with human hepatic microsomes and NADPH. The mass spectra for R406 and the para-O-demethylated metabolite of R406 (R529) are shown in the middle and bottom panels, respectively.

3. HPLC profile of sample obtained 50 min following incubation of R406 with human hepatic microsomes and UDPGA using selective ion monitoring (m/z 647) to detect the formation of three distinct direct N-glucuronides of R406.

4. Time course of plasma radioactivity in human subjects following oral administration of 14C-788.

5. Plasma radioactivity profiles of the pooled 0.5 hr and 4 hr plasma samples from human subjects orally administered 14C-788.

6. Representative profile of radioactivity in urine (0-72 hr) from a human subject orally administered 14C-788.

7. Profile of radioactivity in the 0-48 hr fecal sample of Subject 4 and Subject 2 obtained following the oral administration of 14C-788.

8. 1H-NMR of the major human fecal metabolite R406 and chemically synthesized standard. The numbers represent the assignment of protons in the 3,5-benzene diol metabolite.

9. MS/MS spectra of purified M413 and synthesized 3,5 benzene diol metabolite.
10. HPLC UV profile of the R529 samples incubated for 36 hrs under anaerobic conditions in the absence and presence of fresh human feces.
Table 1  Mean (±SD) percent of radioactive dose recovered in urine and feces at specified time intervals after administration of [14C]-R788 to six healthy male subjects

<table>
<thead>
<tr>
<th>Collection Interval (hrs)</th>
<th>0-24</th>
<th>24-48</th>
<th>48-72</th>
<th>72-96</th>
<th>96-120</th>
<th>120-144</th>
<th>144-168</th>
<th>168-192</th>
<th>0-192</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>13.73 ± 2.55</td>
<td>13.75 ± 0.82</td>
<td>1.21 ± 0.36</td>
<td>0.39 ± 0.20</td>
<td>0.13 ± 0.11</td>
<td>0.04 ± 0.07</td>
<td>0.00</td>
<td>0.00</td>
<td>19.5 ± 3.50</td>
</tr>
<tr>
<td>Feces</td>
<td>0.06 ± 0.11</td>
<td>17.40 ± 17.6</td>
<td>27.46 ± 15.7</td>
<td>26.70 ± 24.30</td>
<td>4.02 ± 2.89</td>
<td>3.09 ± 5.37</td>
<td>1.27 ± 2.49</td>
<td>0.37</td>
<td>80.0 ± 4.70</td>
</tr>
<tr>
<td>Total</td>
<td>13.79 ± 2.20</td>
<td>21.16 ± 17.87</td>
<td>28.67 ± 15.65</td>
<td>27.08 ± 24.35</td>
<td>4.14 ± 2.85</td>
<td>3.136 ± 5.41</td>
<td>1.27 ± 2.49</td>
<td>0.37</td>
<td>99.3 ± 1.93</td>
</tr>
</tbody>
</table>
Figure 1

UV absorbance

0 min

R788

15 min

R406

Time (min)
Figure 2

[Graphs showing UV absorbance, m/z intensity, and mass spectra with peaks labeled R406, R529, 471.4, and 457.1]
Figure 4

Plasma radioactivity (dpm/mL) vs. Time (hrs) for Subjects 1 to 6.
Figure 5

The figures illustrate the chromatographic analysis of metabolites over time. The top graph shows the CPM (Counts Per Minute) against time (in minutes) at 0.5 hours. The peaks are labeled as follows:

- N-glucuronide R406
- R529-sulfate
- R529-O-glucuronide
- R788

The bottom graph is similar but at 4.0 hours, with the same labeled peaks.

The CPM values are shown on the y-axis, ranging from 0 to 280 for the 0.5 hour graph and from 0 to 160 for the 4.0 hour graph. The time axis ranges from 0 to 50 minutes.