Title

Investigations of Hydrazine Cleavage of Eltrombopag in Humans

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Investigations of gut microbial degradation of eltrombopag

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Number of text pages: 19
Number of tables: 3
Number of figures: 6
Number of references: 24
Number of words in Abstract: 137
Number of words in Introduction: 347
Number of words in Discussion: 947

Nonstandard Abbreviations:
ITP           immune thrombocytopenia
PEG400       polyethylene glycol 400
LC-MRM       multiple reaction monitoring
Abstract

Following oral administration to humans, eltrombopag undergoes extensive cleavage of its hydrazine linkage to metabolites which are exclusively eliminated in urine. In vitro, the cleavage pathway was not detected in systems using the cytochrome P450 enzymes, renal or hepatic microsomes, or hepatocytes, but was readily evident following anaerobic incubation with rodent cecal contents or human fecal homogenate. Antibiotic treatment in vitro and in vivo inhibited eltrombopag cleavage, further indicating that cleavage is via gut microbes. Antibiotic treatment did not alter the systemic exposure of eltrombopag in mice. Oral and IV pharmacokinetic characterization in the mice with one of the cleavage products indicated that it was readily absorbed, conjugated and eliminated in urine, consistent with its fate following oral administration of eltrombopag. Variation in this microbial pathway, for example by antibiotic co-therapy, is unlikely to be clinically significant.
Introduction

Eltrombopag, a non-peptide thrombopoietin receptor agonist, has recently been approved for treatment of idiopathic thrombocytopenic purpura (ITP) (Bussel et al., 2007). It is a biphenyl hydrazone with its full chemical name as follows: 3’-{(2Z)-2-[1-(3,4-dimethylphenyl)-3-methyl-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene]hydrazino}-2’-hydroxy-3-biphenylcarboxylic acid. The pharmacokinetics, metabolism and excretion of eltrombopag were studied in healthy humans following oral administration of [14C]eltrombopag olamine (Deng et al, DMD/2011/040170). Briefly, following a single oral dose of [14C]eltrombopag olamine (75 mg, 100 μCi), eltrombopag predominated in circulation. Three acyl glucuronides and a mono-oxygenation product of eltrombopag were also circulating, each accounting for <10% of total plasma radioactivity. Approximately 59% of the dose was eliminated in feces, mostly as glutathione-related conjugates of eltrombopag (21%) or unchanged eltrombopag (20%). These glutathione conjugates were likely derived from biotransformation of the absorbed drug and then eliminated in bile. Approximately 31% of the dose was eliminated in the urine, the majority as two cleavage products M3 and M4 (together 20% of radio-dose, Figure 1), suggesting that the hydrazone linkage of eltrombopag was extensively cleaved in humans (Deng et al, DMD/2011/040170). Various in situ and in vitro systems such as isolated perfused animal (rat and rabbit) livers and kidneys, hepatocytes, microsomes, or expressed cytochrome P450 enzymes were investigated, but none produced notable cleavage products (European Public Assessment Report, http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/001110/WC500089964.pdf).
Mammalian intestines contain more than 500 different species of bacteria with up to $10^{11}$ bacterial cells/g of luminal contents (Guarner and Malagelada 2003). Gut microbes contribute to diverse mammalian physiological functions, including host defense against pathogens in the gut, contribution to immunity through generation of signal molecules/metabolites, development of intestinal wall structure, fermentation of non-digestible dietary fibers, and energy recycling by anaerobic metabolism of peptides and proteins (Nicholson et al, 2005). These intestinal bacteria perform a variety of metabolic reactions including hydrolysis, acetylation, reduction and decarboxylation (Ilett et al 1990; Sweeny et al, 2010). Many drugs are identified as substrates of intestinal microbial metabolism (Sousa et al 2008).

In this report, we described results from our investigations of gut microbial involvement in eltrombopag degradation. The potential for interaction of eltrombopag with antibiotics was also investigated in mice.
Materials and Methods

Chemicals and Reagents. [14C]Eltrombopag olamine (bismonoethanolamine salt, specific activity of 113 μCi/mg, radiochemical purity of 96%), unlabeled eltrombopag olamine (chemical purity of 99%), and a cleavage product of eltrombopag, SB-611855 (2’-hydroxy-3’-amino-3-biphenylcarboxylic acid), were all synthesized by Chemical Development, GlaxoSmithKline, UK. Chemicals and solvents of reagent or HPLC grade were purchased from standard commercial sources such as J. T. Baker Chemicals Co. (Phillipsburg, NJ) and Sigma-Aldrich (St. Louis, MO). Scintillation cocktails Ultima Gold, Permafluor, Ultima-Flo M, and Carbosorb CO2 absorbent were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). HPLC columns Luna C18 (2), 4.6 x 250 mm (analytical) 5μ were purchased from Phenomenex Inc. (Torrance, CA).

In Vitro Incubations of [14C]Eltrombopag with Rodent Cecal or Human Fecal Contents.

Incubation with rodent cecal contents – The cecal sample harvest and incubation procedure was modified from an established method by Fouda et al (1997). One male Crl:CD(SD) rat (552 g in weight) (Charles River Laboratories, Inc., Raleigh, NC) was euthanized via CO2 asphyxiation followed by exsanguinations and transferred to a glove chamber (Glove Bag™ Inflatable Glove Chamber; Glas-Col Co, Terre Haute, IN) purged with nitrogen. The rat was dissected in a nitrogen atmosphere (chamber atmosphere of <5% oxygen). A piece of the large intestines (1 to 2.5 cm above the caecum to the first visible fecal pellet) was removed. Cecal contents were gently squeezed out through a small hole cut into the organ into a culture tube containing 20 mL pre-warmed (37°C) and nitrogen-gassed incubation media consisting of 0.5% glucose, 0.5% yeast extract and
0.5% bacteriological peptone in 0.1 M sodium phosphate buffer (pH 7.4). The tube was then sealed with Teflon tape, removed from the glove chamber and incubated at 37°C for 1 h. Following the pre-incubation, the cecal content suspension was returned to the glove chamber for preparation of drug incubations.

A stock solution of [14C]eltrombopag at 4 mM was prepared by dissolving 8.85 mg of [14C]eltrombopag olamine in 4 mL of dimethylsulfoxide (DMSO). A stock solution of unlabelled eltrombopag at 17.8 mM was prepared by dissolving 20.3 mg of eltrombopag olamine in 2 mL of DMSO. Aliquots of the stock solutions (8.7 µL of the [14C]eltrombopag stock and 29.8 µL of the unlabelled eltrombopag stock) were added to 4.96 mL of incubation media to achieve a final test solution concentration of approximately 113 μM. Inside the nitrogen-filled glove chamber, 1 ml of the test solution was added to culture tubes containing 1 mL of cecal suspension (final eltrombopag concentration of 56.5μM). All test tubes were sealed with Teflon tape, removed from the glove bag and incubated at 37°C for 24 h. Reactions were stopped by adding 2 mL of a freshly prepared mixture of acetonitrile:ethanol (80:20, v:v). Samples were then centrifuged at approximately 3,500 g_{av} at 4°C for 5 minutes. The resulting supernatant was analyzed by radio-HPLC and LC/MS. Control incubations without eltrombopag (no-drug control) were conducted in parallel using blank incubation media containing an appropriate amount of DMSO in place of the eltrombopag test solution without the test substance. Control incubations without cecal content suspension (no-cell control) were also conducted using the incubation media and test solution only.
Incubations with cecal contents harvested from five male CD1 mice (approximately 58 g each, Charles River Laboratories, Inc., Raleigh, NC) were conducted under identical conditions as described above, except that 10 mL of incubation media was used to initially collect the cecal contents.

**Incubation with human fecal contents** – A fresh fecal sample was collected from an anonymous donor at GlaxoSmithKline, Upper Merion, PA. To minimize atmospheric contact, the sample was immediately submerged in nitrogen-gassed 0.1 M sodium phosphate buffer (pH 7.4) upon collection, and homogenized under a stream of nitrogen gas. After homogenization, the fecal sample was transferred to the nitrogen-filled glove chamber where 10 mL of the homogenate was mixed into 20 mL of the initial incubation media. Identical incubations as described above were conducted with the fecal homogenate.

**Gut microbial viability assay** – Gut anaerobic microbial activity was examined by mixing 1 mL of each cecal/fecal content suspension with 1 mL of methylene blue (50 μg/mL) in incubation media at 37ºC for 24 h (LeBoffe and Pierce, 1996). Samples with viable microbial colonies remained colorless throughout the incubation (reduced methylene blue is colorless).

**In vitro antibiotic treatment** - To confirm the involvement of gut microbes in the cleavage of eltrombopag in vitro, antibiotics were added to the incubations with rat cecal contents. Cecal suspensions were pre-treated for 24 h with a mixture of bacitracin, neomycin and streptomycin in saline at approximately 10 mg/mL each (modified from...
Kinouchi et al, 1993). Another dose of antibiotics was added to the suspensions 4 h prior to sample incubation. No-cell controls were prepared with and without antibiotics. Microbial viability before and after antibiotic treatment was confirmed by methylene blue assay as described above.

**Aerobic in vitro incubations** – An aliquot of the rat cecal content suspension was gently aerated with 95% O₂/5% CO₂ during the 1 h pre-incubation. Identical incubations as described above were conducted with this aerated cecal content suspension, except that incubations were performed under a 95% O₂/5% CO₂ atmosphere.

**Excretion and Pharmacokinetics of Eltrombopag in Mice Pretreated with Antibiotics.**

**Urinary excretion** – An excretion study was conducted with [¹⁴C]eltrombopag olamine in mice. Sixteen female CD-1 mice (23-32 g in weight, Charles River Laboratories, Inc,) were divided into four groups as described in Table 1. Mice in groups 1 and 2 were dosed by oral gavage twice daily (12 hours apart) for 5 days with the same antibiotic mixture previously described for in vitro experiments. Mice in groups 3 and 4 received saline only. On Day 4 after the first dose of antibiotics or saline, mice in groups 1 and 3 were dosed by oral gavage with [¹⁴C]eltrombopag at 10 mg/kg in 2% aqueous hydroxypropyl methylcellulose with 0.2% sodium lauryl sulfate. Urine samples were then collected from mice of groups 1 and 3 over dry ice for 24 hours. At the end of the study, cecal contents were collected from mice in each group and checked for gut microbial viability by incubations with [¹⁴C]eltrombopag (Groups 2 and 4) or methylene blue (all groups) as described above.
Urine samples were pooled per group upon collection and centrifuged at 15800 g_{av} at ambient temperature for 5 minutes to remove any particulates. Radioactivity present in the pooled urine samples was measured by liquid scintillation counting prior to and after centrifugation to ensure that no radioactivity had associated with the particulates. Metabolite profiles in the urine were determined by radio-HPLC and LC/MS.

**Pharmacokinetics** – The in vivo effect of antibiotic treatment on eltrombopag pharmacokinetics was studied in mice in a composite study design. Fifty-four female CD1 mice (26-32 g in weight, Charles River Laboratories, Inc.) were divided into three groups (18 mice per group). Group A received neither antibiotics nor saline (vehicle for antibiotic mixture) pretreatment. Group B was treated with the antibiotic mixture previously described while group C was treated with saline for 4 days as described above. On Day 4 (at approximately 1 hour post antibiotic/saline treatment for Groups B and C), the mice, fasted overnight, received an oral dose of eltrombopag at 10 mg/kg in 2% aqueous hydroxypropyl methylcellulose and 0.2% sodium lauryl sulfate. Food was returned at approximately 4 hours after dosing with eltrombopag. Mice in groups B and C continued the antibiotic/saline treatment for an additional 24 hours after eltrombopag administration. Terminal blood samples were collected via cardiac puncture from 3 mice per time point at 0 (pre-dose), 1, 2, 4, 8, and 24 hours following eltrombopag administration. Blood was collected into tubes containing K3 EDTA and immediately placed in wet ice. Plasma was prepared from blood within 1 hour of sample collection by centrifugation (15800 g_{av}) for 5 minutes at 4°C. Following blood collection, cecal
contents from the mice of 24 h sample collection were collected and incubated with methylene blue as described above to determine gut microbial activity.

**Pharmacokinetics of SB-611855 in Mice.** To further evaluate the disposition of eltrombopag after cleavage, the pharmacokinetics of a cleavage product, SB-611855, was studied in mice in a composite study design. The dose solution of SB-611855 was prepared at 1 mg/mL in 25% polyethylene glycol 400 (PEG 400) in saline containing 5 mM ammonium acetate (pH 4.0 with acetic acid). Twenty-nine male jugular vein cannulated or naive CD-1 mice (22-33 g from Charles River Laboratories, n=4 per time point unless stated otherwise) received SB-611855 either via an intravenous bolus through the jugular vein cannula (administered over 1 minute) at approximately 6 mg/kg or by oral gavage at approximately 15 mg/kg under fasted conditions. Terminal blood samples were collected via cardiac puncture into tubes containing EDTA at 5, 15, 30, 60, 120, 180, 240 (n=1), and 1440 minutes following dose administration. Plasma was prepared from blood by centrifugation as described previously. Concentrations of SB-611855 in the resulting plasma samples were analyzed by LC/MS using a method described below. Urine samples were also collected from three mice approximately 24 hours after intravenous or oral dose administration and analyzed for metabolites by LC/MS.

**Qualitative HPLC Analysis.** Agilent 1100 LC systems were used. A typical LC system consisted of a model G1312A pump, a model G1329A autosampler and a β-RAM Model 2B radiometric detector with a 500 μL homogeneous liquid scintillant flow cell. Aliquots (20 – 800 μL) of samples were injected onto a Luna C18(2) analytical column (4.6 x 250 mm, 5μ, Phenomenex) and eluted at 1 mL/min with solvents A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid). The
following four step gradient was used: 0 to 30 min, 10% solvent B in solvent A; 30 to 60 min, 10% to 30% B in A; 60 to 90 min, 30% to 100% B in A; 90 to 100 min, 100% B. The gradient was then returned to 10% B in A and the column allowed to equilibrate at the starting conditions for 10 min before next injection. Urine and cecal/fecal content supernatants were analyzed with on-line radio-detection, during which the eluent was mixed with Ultima Flo M scintillant at a ratio of 1:3 by volume. The radioactivity data were collected at a rate of 1 point/second.

Mass Spectroscopic Analysis.

Qualitative analysis - Metabolite characterization was conducted by LC-MRM (multiple reaction monitoring) analysis. The LC conditions were similar to those used for metabolite profiling as described above. HPLC eluent was split at approximately 1:9 ratio between a mass spectrometer and a radiometric flow detector (Packard 515TR, PerkinElmer, Waltham, MA or β-RAM Model 3, IN/US Systems Inc., Tampa, FL). LC-MRM measurements were performed on a Waters Quattro Ultima Triple Quadrapole equipped with an ESI source or a Quattro Premier Triple Quadrapole equipped with an ESI source (Waters Corporation, Milford, MA). All instruments utilized a CTC PAL autosampler (LEAP Technologies, Carrboro, NC) for sample introduction. Data were acquired and processed using Masslynx software (version 4.1, Waters Corporation, Milford, MA).

Quantitative analysis of eltrombopag - Plasma samples were analyzed using a validated analytical method based on protein precipitation, followed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) analysis as
previously described (Williams et al, 2009). Briefly, eltrombopag was extracted from the plasma samples using a mixture of acetonitrile and 10 mM ammonium formate, pH 3 (90:10, by volume), containing $[^{13}\text{C}_4]eltrombopag$ as an internal standard. Extracts were then analyzed by HPLC/MS/MS using a TurboIonSpray interface with negative ion multiple reaction monitoring (m/z, 441 to 201 and 445 to 205). Plasma concentrations of eltrombopag were determined using a standard calibration curve constructed with standard solutions prepared with human plasma over the range of 10 to 2500 ng/mL. Using a 50 μL aliquot of plasma, the lower limit of quantification for the assay was 10.0 ng/mL and the higher limit of quantification was 2500 ng/mL.

**Quantitative analysis of SB-611855** – A similar method based on protein precipitation with acetonitrile followed by LC/MS analysis was used to measure concentrations of SB-611855 in plasma samples. The LC/MS analysis used a TurboIonSpray interface with negative ion multiple reaction monitoring (m/z, 228 to 184). The validated method demonstrated specificity and acceptable within-run bias and error (<15%) over a concentration range of 50 to 5000 ng/mL. The lower limit of quantitation was 50 ng/mL using 50 μL of mouse plasma.

**Pharmacokinetic Data Analysis.** Plasma concentrations versus time data of eltrombopag and SB-611855 following oral administration were analyzed by a noncompartmental method using WinNonLin™ software program (version 4.1, WinNonLin™ Enterprise, Pharsight Corporation, Cary, NC). The mean plasma concentrations for each analyte at mean sampling time points were calculated and used for the analysis. The area under the composite plasma concentration-time curve (AUC)
from the time of dosing to the last quantifiable time point (AUC\textsubscript{0-t}) was determined by WinNonLin\textsuperscript{TM} using the linear-logarithmic trapezoidal rule. The maximum concentration (C\textsubscript{max}) and time of C\textsubscript{max} observed (T\textsubscript{max}) were determined by WinNonLin\textsuperscript{TM} or by visual inspection of the data. The half-life of the terminal log-linear elimination phase was calculated as \(\ln(2)/k\textsubscript{el}\), where \(k\textsubscript{el}\) is the value of the slope of the log-linear elimination phase.

The total body clearance (CL), the volume of distribution of SB-611855 at steady-state (V\textsubscript{ss}), and the mean residence time (MRT) were calculated after intravenous administration of SB-611855. The plasma concentrations versus time data of SB-611855 were analyzed by an open two-compartmental method using WinNonLin\textsuperscript{TM}, with elimination from the central compartment. During the modeling, the weight of 1/Y\textsubscript{hat} was used to better fit the experimental data, where Y\textsubscript{hat} is the predicted concentration. The volume of distribution of the central compartment (V\textsubscript{1}) and the rate constants for SB-611855 transfer between compartments and for elimination (k\textsubscript{12}, k\textsubscript{21}, and k\textsubscript{10}) are used to calculate the pharmacokinetic parameters in the equation \(C_t = Ae^{-\alpha t} + Be^{-\beta t}\), where A and B are the extrapolated zero intercepts, and \(\alpha\) and \(\beta\) are the slopes representing the apparent first order distribution and elimination rate constants, respectively. The oral bioavailability of SB-611855, expressed as a percentage, was estimated by dividing the dose-normalized AUC value after oral dose by that after the intravenous dose.
Results

In Vitro Cleavage by Gut Microbes. Under anaerobic conditions, eltrombopag was converted predominantly to a cleavage product (M8) in rodent cecal contents, and to a mixture of M8 and SB-611855 in human fecal contents (Figure 2). As illustrated in Figure 1, M8 is an N-acetyl conjugate of SB-611855. Under aerobic conditions, minimal eltrombopag cleavage was observed (Figure 3). Pretreatment of the cecal contents with antibiotics completely prevented eltrombopag from cleavage, as demonstrated by incubations of \(^{14}\text{C}\)eltrombopag with rat cecal contents (Figure 3). These results suggest a reductive cleavage of the hydrazine linkage of eltrombopag by the gut microbes, leading to formation of SB-611855. Cleavage products derived from the phenylpyrazole moiety which lacked the \(^{14}\text{C}\) label were not radio-chromatographically detectable.

Effect of Antibiotics on Urinary Excretion. After treatment of female mice with a mixture of antibiotics (bacitracin, neomycin and streptomycin) for five days (total daily antibiotic dose of 1200 mg/kg), cecal microbial activity was diminished as confirmed by the methylene blue assay and the lack of in vitro eltrombopag cleavage in incubations with the resulting cecal contents (Figure 4). Following the oral administration of \(^{14}\text{C}\)eltrombopag to the antibiotic-treated mice, 1% of the administered dose was excreted in the urine within 24 hours in comparison to 5% of the dose in saline-treated mice over the same period (Table 1). Previous excretion and metabolism studies indicated that elimination of cleavage products of eltrombopag was via urine exclusively in humans and rodents, the majority of which occurred within 24 hours after oral administration (Deng et al, DMD/2011/040170). As expected, radio-HPLC and LC/MS analysis confirmed that the predominant radio-components in urine from saline-treated
mice were the conjugated cleavage product M8 and a glucuronide conjugate of M8 (M3) (Figure 5). Unconjugated SB-611855 was not observed in mouse urine.

**Effect of Antibiotics on Systemic Exposure.** Following five days of antibiotic pre-treatment, mouse cecal contents lacked bacterial activity as indicated by the methylene blue assay. Key pharmacokinetic parameters of eltrombopag calculated are summarized in Table 2. For all three groups, the peak plasma concentrations were achieved within 1 hour post dose, and remained quantifiable for 24 hours. The systemic exposures (AUC$_{0-24h}$) and C$_{max}$ of eltrombopag in the naive and saline treated mice were similar, with value ranges of 93.9-109 μg.h/mL and 21.2-21.5 μg/mL, respectively, suggesting that two daily gavages of saline over the course of 5 days had no notable impact on eltrombopag pharmacokinetics in mice. The C$_{max}$ value of eltrombopag in the antibiotic treated mice was also comparable (19.3 μg/mL) to the untreated group (21.5 μg/mL). The (AUC$_{0-24h}$) in the antibiotic treated mice was slightly lower at 70.3 μg/mL.

**Pharmacokinetics of SB-611855.** The pharmacokinetics of SB-611855, a cleavage product observed in incubation of eltrombopag with human feces, was studied following single intravenous and oral administration to mice at 6 and 15 mg/kg, respectively. The concentration-time profiles for plasma SB-611855 are depicted in Figure 6. Considerable variability in plasma SB-611855 concentrations was observed following both routes of administration. Following intravenous bolus administration, the plasma SB-611855 declined biexponentially, with a distribution half-life (T1/2 - α) of 3.7 min and a terminal half-life (T1/2 - β) of 59 min. The coefficient of variation values (CV%) for the model parameters (V1, k10, k12 and k21) were 11, 8.8, 50, and 87%, respectively. As
illustrated in Figure 6, this two-compartment model well predicted the observed concentration-time profile (solid line versus open circles). The fast distribution phase defined the total plasma clearance of SB-611855 in mice. The mean residence time (MRT) for SB-611855 was 11 min. The systemic exposure (AUC) of SB-611855 dropped by greater than 90% within 30 min following the IV bolus dose. Following oral administration, SB-611855 was readily absorbed. Its plasma concentration reached the highest (9.9 μg/mL) at 5 min, the first post-dose sampling, but decreased to or below limit of quantitation (50 ng/mL) within 3 hours post dose (Figure 6). SB-611855 plasma decay also appeared biexponential following oral administration, but a noncompartmental model was used to estimate pharmacokinetic parameters since the concentration-time profile did not capture the absorption phase of SB-611855. The mean residence time was 26 min. The absolute oral bioavailability was approximately 29%.

Urine samples collected over the 24 hour period post dose of SB-611855 were analyzed for metabolites by LC/MRM. Following oral dose, SB-611855 and its conjugated products M3 and M8 were observed in the urine. Following the IV dose, products M3 and M8, and a glucuronide of M8 (metabolite M24, exact structure unknown) were detected. SB-611855 itself was not observed in the urine following intravenous administration.

**Structural Characterization of Metabolites.** A summary of HPLC retention times and MRM transitions for metabolites are depicted in Table 3. SB-611855 has been characterized by NMR analysis in a previous study (Deng et al, re-submitted on 19 April 2011). Metabolite M3 had a retention time of 55.2 minutes, consistent with previously
reported data (Deng et al, DMD/2011/040170). Metabolite M3 was detected by negative ion LC/MRM utilizing the transition 446→270; this transition was 176 daltons, suggesting that M3 was a glucuronide, consistent with previously reported data. Metabolite M8 had a retention time of 64.9 minutes. This metabolite was detected by negative ion LC/MRM utilizing the transition m/z 270→184, and determined to be identical to a synthetic standard, based on chromatographic RT utilizing the MRM transition, m/z 270→184. Metabolite M24 had a retention time of 43.8 minutes. M24 was detected in the urine by negative ion LC/MRM utilizing the transition 446→270. As with M3, the observed neutral loss of 176 daltons was indicative of a glucuronide conjugate.
Discussion

Following an oral administration of [14C]eltrombopag to six healthy men, about 31% of dose was eliminated in urine, the majority as cleavage products of eltrombopag (Deng et al, DMD/2011/040170). Unchanged eltrombopag was not observed in urine. Earlier investigations on the hydrazine linkage cleavage of eltrombopag were not conclusive. Incubation of eltrombopag with hepatocytes, human liver/kidney microsomes or expressed cytochrome P450 enzymes did not generate any cleavage products. Isolated rat and rabbit livers/kidneys perfused with eltrombopag also did not yield any observable cleavage (European Public Assessment Report, http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/001110/WC500089964.pdf).

The origin and disposition of eltrombopag cleavage products are elucidated by this investigation. First, in vitro experiments demonstrated that gut microbes from mice, rats and humans all readily cleaved the hydrazine linkage of eltrombopag under anaerobic conditions to SB-611855 and/or its secondary metabolite, N-acetyl SB-611855. In vivo studies then confirmed that the biphenyl moiety of eltrombopag (SB-611855) was readily absorbed (T_{max} of 5 minutes after oral dose to mice), cleared from circulation (T_{1/2} of 3.7 min), conjugated, and ultimately eliminated as metabolite M3 (in addition to M8, a glucuronide of M3), the predominant metabolite found in human urine following oral administration of eltrombopag (Deng et al, DMD/2011/040170). Although similar studies have not been conducted on the phenylpyrazole moiety of eltrombopag due to its chemical instability, a stable glucuronide conjugate derived from this moiety (product M4) was detected in human urine following oral administration of eltrombopag (Deng et al, DMD/2011/040170).
al, DMD/2011/040170). Thus, after gut microbial cleavage, the phenylpyrazole portion of eltrombopag likely followed a similar disposition pattern to that of the biphenyl moiety did (absorption, conjugation and renal elimination).

The rapid absorption and plasma clearance of SB-611855 observed in mice is likely the reason no circulating cleavage products have ever been detected in animal or human eltrombopag metabolism studies. In those studies, the plasma sampling for metabolic profiling was sparse (typically at 4, 12, 24, and 48 hours post dose only), and, therefore, any quantifiable cleavage products in circulation would be easily missed. As demonstrated in mice, plasma exposure of SB-611855 decreased by >90% within 30 min after absorption, with concentrations close to or below the lower quantitation limit of 50 ng/mL by 3 hour post-dose.

Antibiotic treatment reduced urinary elimination of cleavage products of eltrombopag by five fold in mice, but such treatment did not noticeably affect the systemic exposures of eltrombopag in this species. One explanation was that eltrombopag absorption took place in the small intestines while the microbial cleavage of unabsorbed or secreted eltrombopag occurred in the large intestines. Another explanation was that gut microbial cleavage represented a small pathway in eltrombopag disposition in mice (15% of dose in urine, presumably all as cleavage products) (European Public Assessment Report, http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/001110/WC500089964.pdf). The antibiotic effect may have been too subtle to be accurately assessed in our mouse PK study (n=4 only, not statistically powered). Gut microbial cleavage was only slightly more prominent in
humans (30% of administered dose, Deng et al, DMD/2011/040170) than mice, thus, antibiotic treatment is unlikely to notably alter eltrombopag exposures in humans, either. Interestingly, however, low levels of digoxin (16% of dose) were metabolized by gut microbes in humans (Greenwood et al, 1975) and notable reduction of urinary/fecal elimination of metabolites of digoxin has been observed in patients upon antibiotic treatment (erythromycin or tetracycline for 5 days) (Lindenbaum et al, 1981), but the serum concentrations of digoxin increased by two fold in that study (Lindenbaum et al, 1981). Digoxin disposition has been known to be complicated. In addition to gut bacterial metabolism, direct secretion of digoxin to gut lumen by P-glycoprotein has also played a role (Mayer et al, 1996). Erythromycin is a known Pgp inhibitor (Bjornsson et al, 2003 and Eriksson et al, 2006). Thus, the increase in serum digoxin concentrations observed by Lindenbaum et al could have resulted, at least in part, from inhibition of P-gp-mediated direct and/or biliary secretion of digoxin.

Animals, including humans, contain trillions of microbes in their gastrointestinal tract. Humans tend to have the highest gut bacterial concentrations (Rowland 1988), which may explain the higher amounts of eltrombopag cleavage products observed in man (20% of dose) than in rodents (< 10%) following oral administration (European Public Assessment Report, http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/001110/WC500089964.pdf). A single human gut could contain more than 400 bacterial species, the majority of which are anaerobic (Goldin, 1990). Human gut microbial populations and their activities in humans have been known to differ among individuals (Smith 1965; Rowland 1986 and Scheline 1973). Thus, it is not surprising to observe that the extent of urinary elimination of eltrombopag cleavage
products varied notably between six subjects in the human radiolabel study, ranging from 23% to 45% of the dose (Deng et al, DMD/2011/040170). It has long been recognized that gut microbes contribute to the metabolism of numerous drugs (Sousa et al, 2008). One of the best characterized examples was reductive deactivation of the cardiac glycoside digoxin by *Eubacterium lentum*, a common anaerobe in the colon (Dobkin et al, 1983). Gut microbial cleavage of a hydrazone/hydrazone has been rare, however.

Antila et al (1999) suggested that the hydrazone linkage of levosimendan was reductively cleaved by microbes in lower gastrointestinal tract in dogs. A cleavage product of levosimendan (OR-1855) was then absorbed and further metabolized to a pharmacologically active component in humans (Antila et al, 2004).

In conclusion, human and animal gut anaerobic bacteria readily cleaved eltrombopag. A cleavage product, SB-611855, was readily absorbed in mice, conjugated and eliminated in urine as the same urinary metabolite of orally administered eltrombopag in humans. Antibiotic treatment sharply reduced urinary elimination of eltrombopag related material, but had no notable impact on eltrombopag plasma exposures in mice. Antibiotic co-administration unlikely leads to significant changes in eltrombopag exposures in humans.
Acknowledgements:

We acknowledge Dr. Ken Lawrie for the synthesis and purification of [14C]eltrombopag olamine; Dr. Neil Whittall for synthesis of [12C]eltrombopag olamine; Dr. Stephen Etridge for the synthesis of SB-611855; Dr. Steve Castellino and Dr. Mary Wire for reviewing the manuscript; Mr. John Ulicne for formatting figures.
Authorship Contribution

Participated in research design: Deng, Rogers, Sychterz, Talley, Qian, Ho, Shi, Chen, Gorycki

Conducted experiments: Deng, Rogers, Sychterz, Talley, Qian, Bershas, Shi

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Performed data analysis: Deng, Rogers, Sychterz, Talley, Qian, Bershas, Ho, Shi, Chen, Serabjit-Singh, Gorycki

Wrote or contributed to the writing of the manuscript: Deng, Rogers, Sychterz, Talley, Qian, Bershas, Ho, Shi, Chen, Serabjit-Singh, Gorycki
References


Illett KF, Tee LBG, Reeves PT and Minchin RF (1990) Metabolism of drugs and other xenobiotics in the gut lumen and wall. *Pharmacol Ther* **46**:67-93


Footnotes

This work was supported by GlaxoSmithKline.

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Legends for Figures

Figure 1  $^{14}$C-Eltrombopag and major cleavage products.

Figure 2  HPLC radiochromatograms following 24 hour anaerobic incubations of $^{14}$C-el trombopag with rodent cecal or human fecal contents. The samples were subjected to LC/MS analysis with radiometric detection.

Figure 3  HPLC radiochromatograms following 24 hour anaerobic incubations of $^{14}$C-el trombopag with rat cecal contents with antibiotic pretreatment or under aerobic conditions.

Figure 4  HPLC radiochromatograms following 24 hour anaerobic incubations of $^{14}$C-el trombopag with cecal contents obtained from female mice without or with antibiotic treatment for five days. The samples were subjected to LC/MS analysis with radiometric detection.

Figure 5  HPLC radiochromatograms of pooled urine samples following an oral administration of $^{14}$C-el trombopag to female mice without or with antibiotic treatment for five days. The samples were also analyzed by LC/MS with radiometric detection.

Figure 6  Individual and mean SB-611855 plasma concentration versus time profiles following an intravenous (top) or oral administration (bottom) of SB-611855 in solution to female mice at 6.3 mg/kg and 15 mg/kg, respectively. Concentrations <LLQ (50 ng/mL) were considered zero in mean value calculations and were not plotted.
Table 1 Administration and urinary elimination of drug related materials following an oral administration of $[^{14}\text{C}]$eltrombopag to female mice with or without antibiotic treatment

<table>
<thead>
<tr>
<th>Group (n=4)</th>
<th>Antibiotic Treatment (mg/kga)</th>
<th>$[^{14}\text{C}]$eltrombopag Administered (mg/kg)</th>
<th>% Administered Dose Eliminated in Urineb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1200</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1200</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>

a. Total daily dose of antibiotics; mice were dosed twice daily (on Days 1-4; once on Day 5) with a mixture of bacitracin, neomycin and streptomycin at 200 mg/kg each.

b. Data are mean values

NA = Not available
Table 2  Key pharmacokinetic parameters (mean ± standard deviation, where applicable) of eltrombopag following an oral administration of eltrombopag olamine at 10 mg/kg to female mice without or with antibiotic treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>AUC_{0-t}^a (μg h/mL)</th>
<th>C_{max} (μg/mL)</th>
<th>T_{max} (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Naive mice with eltrombopag</td>
<td>93.9 ± 24.4</td>
<td>21.5 ± 4.3</td>
<td>1.01</td>
</tr>
<tr>
<td>B</td>
<td>Antibiotic treated mice with eltrombopag</td>
<td>70.3 ± 11.8</td>
<td>19.3 ± 2.2</td>
<td>1.04</td>
</tr>
<tr>
<td>C</td>
<td>Vehicle treated mice with eltrombopag</td>
<td>109 ± 15.8</td>
<td>21.2 ± 5.8</td>
<td>1.02</td>
</tr>
</tbody>
</table>

a. AUC_{0-t} refers to the area from time 0 to the last quantifiable concentration.
Table 3  
Selected HPLC retention times and MRM transitions for SB-611855 and its metabolites

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>( t_R ) (min)</th>
<th>( m/z )</th>
<th>[M-H]:</th>
<th>MRM transitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB-611855</td>
<td>35.2</td>
<td>228</td>
<td></td>
<td>MRM 228→184</td>
</tr>
<tr>
<td>M24</td>
<td>43.8</td>
<td>446</td>
<td></td>
<td>MRM 446→270</td>
</tr>
<tr>
<td>M3</td>
<td>55.2</td>
<td>446</td>
<td></td>
<td>MRM 446→270</td>
</tr>
<tr>
<td>M8</td>
<td>64.9</td>
<td>270</td>
<td></td>
<td>MRM 270→184</td>
</tr>
</tbody>
</table>
Figure 1

P ([14C]eltrombopag)

SB-611855

M3

M4

M8
Figure 3

- Anaerobic Incubation + Antibiotics
- Aerobic Incubation
Figure 5

![Graph showing CPM vs. Retention Time for samples with and without antibiotic treatment.](image-url)
Figure 6

Intravenous

- **Individual Observed**
- **Mean Observed**
- **Predicted Data**

Concentration (ng/mL) vs. Time (min)

Oral

- **Mean Observed**
- **Individual Observed**

Concentration (ng/mL) vs. Time (min)