Defining the role of gut bacteria in the metabolism of deleobuvir: *in vitro* and *in vivo* studies

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Nonstandard Abbreviations**:**

BLQ, below limit of quantification; CFU, colony forming unit; CYP450 , cytochrome P450;

DDI, drug-drug interaction; GI, gastrointestinal ; HLC, human liver cytosol; HLM , human liver

microsomes; LLOQ, lower limit of quantification; *m/z,* mass to charge ratio; NS5B, non-

structural protein 5B; pGF, pseudo-germ free; q8h, dose every 8 hours

Abstract

Deleobuvir is a potent inhibitor of the hepatitis C virus NS5B polymerase. In humans, deleobuvir underwent extensive reduction to form CD 6168. This metabolite was not formed *in vitro* in aerobic incubations with human liver microsomes or cytosol. Anaerobic incubations of deleobuvir with rat and human fecal homogenates produced CD 6168. Using these *in vitro* formation rates, a retrospective analysis was conducted to assess whether the fecal formation of CD 6168 could account for the *in vivo* levels of this metabolite. The formation of CD 6168 was also investigated using a pseudo-germ free (pGF) rat model, in which gut microbiota were largely eradicated by antibiotic treatment. Plasma exposure ($AUC_{0-\infty}$) of CD 6168 was approximately 9-fold lower in pGF rats (146 ± 64 ng·h/mL) compared to control rats ($1,312 \pm 649$ ng·h/mL). Similarly, in pGF rats lower levels of CD 6168 (1.5% of the deleobuvir dose) were excreted in feces compared to control rats (42% of the deleobuvir dose). In agreement with these findings, in pGF rats approximately all of the deleobuvir dose was excreted as deleobuvir into feces (105% of dose) whereas only 26% of the deleobuvir dose was excreted as deleobuvir in control rats. These differences in plasma and excretion profiles between pGF and control rats confirm the role of gut bacteria in the formation of CD 6168. These results underline the importance of evaluating metabolism by gut bacteria and highlight experimental approaches for nonclinical assessment of bacterial metabolism in drug development.

Introduction

The gastrointestinal (GI) tract of vertebrates harbors a complex microbial community that provides essential function for the host (Nordgard et al., 2005). The mucosal surface of the human gut is colonized by approximately 10^{14} bacteria (Suau et al., 1999) with 400 different species (Gorbach, 1996). The composition and distribution of gut bacteria demonstrate high intra- and inter-individual variability in humans and is susceptible to changes in composition with age, diet, GI transit time and disease state. The majority of gut microbiota colonize the colon, where there is very slow motility and low oxidation-reduction potential. This contributes to the fact that 99% of colonic microbiota are obligate anaerobes (Hao and Lee, 2004).

Gut bacteria are responsible for the biotransformation of many endogenous and exogenous molecules, usually involving their breakdown via hydrolysis, de-conjugation or reduction (Sousa et al., 2008). There are several examples of commercially available drugs that are metabolized by gut bacteria with extensive reviews provided by Hartiala (1973) and Sousa et al. (2008). Sousa et al. (2008) suggested that the recent increase in focus on metabolism mediated by gut bacteria correlates with the increase in drugs reaching the market with extended release formulations, or lower permeability and solubility. In addition, drug-drug interactions can be a concern, especially for drugs that affect the composition of the microbiota, which can potentially alter the metabolism of a concomitantly administered substrate for bacterial biotransformation. Drug-drug interactions can also be mediated through metabolites produced by gut bacteria. Sorivudine, an antiviral drug released in the Japanese market in 1993, was withdrawn due to a fatal drug-drug interaction between a gut metabolite of sorivudine and the anti-cancer drug 5-fluorouracil (Okuda et al., 1998).

There are a number of challenges in evaluating the involvement of gut bacteria in drug metabolism. A drug can be incubated *in vitro* with intestinal content, fecal samples or isolated microbes (O'Sullivan, 2000), but there are several shortcomings with these methods. For example, fecal samples may not accurately reflect the actual active proportion of microbes over the entire length of the gut *in vivo* (Finegold et al., 1983) and it has been suggested that only 25% of intestinal bacteria are cultivable (Bartosch et al., 2004). *In vivo* evaluation of gut bacterial metabolism in animals is feasible. However, the limitations of large interspecies differences in microbiota composition and distribution have to be taken into consideration (Rowland et al., 1986; Sousa et al., 2008). Pseudo-germ free (pGF) rats can be created by treatment with broad-spectrum antibiotics and have helped in elucidating the role of gut bacteria in the metabolism of drugs (Jin et al., 2010; Lee et al., 2012; Liu et al., 2012; Yoo et al., 2014).

Deleobuvir is a potent inhibitor of the hepatitis C virus (HCV) NS5B polymerase. In a human Phase Ia study in healthy male volunteers, deleobuvir was found to undergo extensive reduction to form a major circulating metabolite, CD 6168 (Fig. 1), which was confirmed later in a human ADME (absorption, distribution, metabolism and excretion) study (Chen et al., 2015). In ^{14}C-deleobuvir ADME studies in rats, CD 6168 was also found in the feces and accounted for approximately 43% of the administered dose (data on file, Boehringer Ingelheim Pharmaceuticals, Inc.). Interestingly, in bile-cannulated rats, CD 6168 represented only 3% of the radioactive dose recovered in the bile. This observation, together with the fact that CD 6168 is a reduction product of the parent molecule, suggested that gut bacteria might be involved in the formation of CD 6168.

As previously mentioned, methodologies to evaluate gut bacteria metabolism are associated with several caveats. Therefore, the studies reported herein use a combination of *in vitro*

anaerobic incubations with rat and human feces and an *in vivo* antibiotic-treated (pseudo-germ free) rat model to confirm the role of gut bacteria in the formation of CD 6168 from deleobuvir. In addition, since *in vitro* studies for gut bacterial metabolism generally only provide a qualitative answer for the extent of metabolism observed *in vivo*, we have attempted to use a scaling approach to provide a more quantitative result. The advantages and limitations of these approaches are discussed.

Materials and Methods

Chemicals, Reagents and Other Materials

Deleobuvir, CD 6168, $^{13}C_6$-deleobuvir (label on the benzimidazole ring), $^{13}C_6$-CD 6168 (label on the benzimidazole ring) were synthesized at Boehringer Ingelheim Pharmaceuticals, Inc (Ridgefield, CT). Hesperidin, hesperetin, streptomycin, neomycin, D-(+)-glucose, NADH and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). d_3-Hesperetin was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Pooled human liver microsomes and human liver cytosol were purchased from BD Corning Life Science (Tewksbury, MA). Blank rat plasma was obtained from Bioreclamation (Westbury, NY). β-Glucuronidase (140 U/mL) was purchased from Roche Applied Science (Penzberg, Upper Bavaria, Germany). All other reagents and solvents were of analytical grade or higher purity and were obtained from commercial suppliers.

Incubation of deleobuvir with human liver microsomes (HLM) *or human liver cytosol* (HLC)

Deleobuvir (0.1, 1, 10 μM) was incubated with HLM or HLC at protein concentrations of 1 mg/mL in 50 mM of potassium phosphate buffer (pH 7.4) at 37 °C. After 5 min pre-incubation,

reaction was initiated by the addition of 2 mM NADPH, NADH or buffer. Reactions were

terminated at 0, 5, 10, 15, 30, 60, 90 and 120 min by removing an aliquot of incubate and adding

quench solution containing 40% acetonitrile with $^{13}C_6$-deleobuvir and $^{13}C_6$-CD 6168 as internal

standards. Samples were analyzed by LC-MS/MS, monitoring selective ions for deleobuvir and

CD 6168.

Incubation of deleobuvir with rat or human feces

Individual fecal samples from three untreated male rats and also two healthy male human

volunteers were collected and immediately transferred to an anaerobic chamber with oxygen

<5 ppm atmosphere (Coy Laboratory Products, Inc., Grass Lake, MI). All of the *in vitro*

processes up to analysis by LC-MS/MS were carried out under anaerobic conditions. Fecal

samples were mixed with Dulbecco's phosphate-buffered saline containing 20 mM of glucose to

obtain a concentration of 0.05 g/mL (weight of fecal sample/volume of buffer). Samples were

then homogenized and centrifuged at 500 rpm for 5 min at 4 ^{o}C to remove debris. Processed

aliquots were pre-incubated for 5 min at 37 ℃ and reactions were initiated by the addition of

deleobuvir (100 µM, final concentration). Reactions were terminated at 0, 5, 10, 20, 30, 60, 90

and 120 min by removing an aliquot of incubate and adding quench solution containing 80%

acetonitrile with $^{13}C_6$-CD 6168, as an internal standard. Sealed samples were removed from the

anaerobic chamber and analyzed by LC-MS/MS for levels of CD 6168.

Pseudo-germ free rat study

This pharmacokinetic study was conducted in accordance with guidelines from the

Institutional Animal Care and Use Committee. Twenty male Sprague Dawley rats,

approximately 320-380 g, were used for this study. Rats were fasted overnight until 4 h after

dosing with water available *ad libitum.* A cross-over design was used. Briefly, drug was

administered to control rats (non-antibiotic treated). Then, after a 1 week washout period, antibiotic treatment began. Rats received streptomycin sulfate and neomycin sulfate at a dosage of 100 mg/kg, via gavage twice daily for six days. Drug was again administered 24 h after final antibiotic administration to these pseudo-germ free rats (pGF rats).

Drug administration: Deleobuvir was administered as a single oral dose of 10 mg/kg (25% PEG 400, 3% Tris and 72% water) to 10 rats. In parallel, a positive control was used to validate the *in vivo* methodology. Hesperidin, which is a known substrate for gut bacterial metabolism (Garg et al, 2001), was administered orally as a single dose of 50 mg/kg to 10 rats. Hesperidin dosing solution contained 47% PEG 400, 3% DMSO and 50% water.

Collection and bioanalysis of plasma and fecal samples: Blood samples were collected from a tail vein using capillary microsampling technique at pre-dose, and 1, 2, 4, 6, 8, and 24 hours after dosing. Briefly, a 50 µL minivette (Sarstedt AG& Co; Hofstraße, Nümbrecht, Germany) coated with K_3EDTA was placed at the hub and filled to volume and dispensed into 0.25 mL microcentrifuge tubes (VWR; Radnor, PA). Samples were centrifuged at 4 °C. An exact 20 µL volume of plasma from each sample was dispensed into a microtube and stored at -20 °C until analysis.

Plasma protein was precipitated by the addition of 95% acetonitrile containing $^{13}C_6$-deleobuvir and $^{13}C_6$-CD 6168 (deleobuvir subgroup) as internal standards (1:1 plasma to organic, v/v) or 70% methanol with d_3-hesperetin (hesperidin subgroup) as internal standard (1:4 plasma to organic, v/v). After centrifugation, aliquots were analyzed by LC-MS/MS for hesperidin, hesperetin, deleobuvir, or CD 6168 levels. It has been previously reported that hesperetin is further metabolized to hesperetin glucuronide (Jin et al., 2010), thus plasma samples were hydrolyzed with β-glucuronidase prior to analysis. The protocol was adapted from Matsumoto et

al. (2004). Briefly, plasma samples were diluted 4-fold with 50 mM potassium phosphate buffer

(pH 7.2) containing β-glucuronidase (1.6 U). Samples were then incubated at 55°C for 60 min

and the reaction was terminated by the addition of quench solution containing internal standard.

Analytes were quantified using calibration curves, which were processed similarly to plasma

samples.

Rats were housed in metabolism cages prior to drug administration. During ^{14}C-ADME study in

rats, large majority of the radioactive dose (84%) was recovered in feces within 48 h. Hence,

feces from individual rats were collected in sterile vials at pre-dose, 24 and 48 h. Samples were

homogenized following the addition of 50% isopropanol/water (v/v) at 3 mL/g. Approximately

1 g of fecal homogenate was extracted with 1% formic acid containing [13]C_6-deleobuvir and [13]C_6-

CD 6168 (deleobuvir subgroup) or 70% methanol with d_3-hesperetin (hesperidin subgroup) in

acetonitrile. Fecal pellets were further extracted twice without internal standard. Extracts were

combined and evaporated to dryness under a nitrogen stream at room temperature. Samples were

reconstituted with 0.1% acetic acid, 40% water in acetonitrile. Aliquots were analyzed by LC-

MS/MS for hesperidin, hesperetin, deleobuvir and CD 6168 levels.

LC-MS/MS and chromatographic conditions

For deleobuvir and CD 6168, an SIL5000 autosampler (Shimadzu, Columbia, MD) and LC-

10AD vp Binary Pump (Shimadzu) connected to a Sciex 4000 Q Trap mass spectrometer

(Applied Biosystems/Sciex, Thornhill, Ontario, Canada) were used. Samples were separated on a

Gemini C_{18} column (50 × 2.0 mm) (Phenomenex, Torrance, CA) with a 5 μm particle size.

Mobile phase compositions were water/acetonitrile/acetic acid for mobile phase A: (95:5:0.2

v/v/v) and B: (5:95:0.2 v/v/v) to provide a gradient. A 2 min gradient was used at a flow rate of

0.35 mL/min starting at 25% mobile phase B for 0.2 min and then linearly increasing to 100% by

2 min. Multiple reaction monitoring analysis was performed in positive ionization mode for

deleobuvir (*m/z* 655 →384), CD 6168 (*m/z* 657→257), $^{13}C_6$-deleobuvir (*m/z* 661 →384) and

$^{13}C_6$-CD 6168 (*m/z* 663 →263).

For hesperidin and hesperetin, an Acquity UPLC (Waters Corporation, Milford, MA)

connected to a Sciex 5500 Q Trap mass spectrometer (Applied Biosystems/Sciex) was used.   An

Acquity C_{18} column (Waters Corporation) (50 × 2.1 mm) was used with 1.7 µm particle size.

Mobile phase composition was water/acetonitrile/acetic acid for A: (95:5:0.1 v/v/v) and B:

(5:95:0.1 v/v/v). A 0.8-min gradient was used at a flow rate of 0.7 mL/min with mobile phase B

starting at 15% and then linearly increasing to 99% by 0.8 min. Multiple reaction monitoring

analysis was performed in positive ionization mode for hesperidin (*m/z* 609 →301), hesperetin

(*m/z* 301→164) and d$_3$-hesperetin (*m/z* 304→164).

Data analysis

In vitro-in vivo correlation. The extent of *in vivo* formation of CD 6168 by gut bacteria was

extrapolated from *in vitro* incubations based on the following process: *In vitro* formation rate

(nmol of metabolite formed/mL/min) was determined based on the slope of metabolite formation

over the initial linear range. The metabolite formation rate was then normalized by the fecal

density in the incubation (g of feces/mL of incubation) to obtain the formation rate with a unit of

nmol of metabolite formed/ g of feces/min. The total amount of metabolite formed in the gut

(Metabolite $_{gut}$) was estimated using equation 1.

$$\text{Metabolite}_{gut} = \textit{formation rate} \times \textit{weight of feces or cecum content} \times \textit{colonic transit time} \qquad \text{Eq.1}$$

For rats, the weight of cecal content was 2.9 g and for human the weight of feces was 110 g

(Rowland et al., 1986). The average colonic transit time was 6 h for rats (de Zwart et al., 1999)

and 24 h for humans (Wilson, 2000).

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Results

In vitro metabolism of deleobuvir by HLM or HLC

Deleobuvir (0.1, 1, 10 µM) was stable with both HLM and HLC up to 120 min of incubation, in the presence and absence of NADPH and NADH (data not shown). No quantifiable levels of CD 6168 were detected (LLOQ of 7.8 nM, which would represent 0.078% conversion of 10µM deleobuvir).

In vitro metabolism of deleobuvir with rat or human fecal homogenates

Preliminary studies were conducted to establish linearity with respect to time and deleobuvir concentration up to 100 µM for the formation of CD 6168 (data not shown). Incubation of deleobuvir (100 µM) with rat fecal homogenate generated CD 6168 at a rate of 3.18 ± 2.05 nmol/min/g of fecal content (average of 3 animals; Fig. 2A). With human fecal homogenate, under similar incubation conditions, the formation rates of CD 6168 were 1.95 nmol/min/g and 0.184 nmol/min/g of fecal content for subject I and subject II, respectively (Fig. 2B).

Validation of pseudo-germ free rat model

The mean plasma concentration-time profiles of total hesperetin (hesperetin and hydrolyzed hesperetin-glucuronide), after administration of 50 mg/kg of hesperidin to control and pGF rats, are illustrated in Fig. 3. As shown in Table 1, t_{max} values ranged from 6-8 h for control rats and 4-8 h for pGF rats. The $AUC_{0-\infty}$ values of hesperetin were 5-fold higher in control (3,454 ± 760 ng·h/mL) compared to pGF rats (685 ± 368 ng·h/mL).

The cumulative amounts of hesperidin and hesperetin excreted in feces up to 48 h post-dose are shown in Fig. 4. Slightly higher levels of hesperidin were excreted by pGF rats (12 ± 5% of dose) compared to control rats (7.8 ± 3.4 % of dose). This finding was also reflected in the lower

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levels of hesperetin excreted by pGF rats (0.9 ±0.3% of parent dose) compared to control rats

(2.4 ± 1.6 % of parent dose). The excretion of both hesperidin and hesperetin in pGF rats was

statistically significant (P<0.05) compared to control.

Metabolism of deleobuvir in pseudo-germ free rats

The mean plasma concentration-time profiles of deleobuvir and CD 6168 are presented in

Fig. 5A and 5B, respectively, following the administration of 10 mg/kg deleobuvir to control and

pGF rats. Pharmacokinetic parameters are summarized in Table 2. Deleobuvir was rapidly

absorbed in both control and pGF rats, with t_{max} values of 1 h for both groups. Comparable

plasma exposure ($AUC_{0-\infty}$) was observed for deleobuvir in control rats (63,438 ±

18,181 ng·h/mL) and pGF rats (66,683 ± 21, 253 ng·h/mL). In addition, although plasma levels

of CD 6168 were relatively low compared to the parent (representing 0.7% of parent C_{max} and

2.1% of parent AUC), levels of CD 6168 were much higher in control rats (C_{max} of 114 ±

65 ng/ml and AUC of 1, 312 ± 649 ng·h/mL) compared to pGF rats (C_{max} of 15.5± 7.7 ng/ml and

AUC of 146 ± 64 ng·h/mL). The cumulative amounts of deleobuvir and CD 6168 excreted in

feces over 48 h in both control and pGF rats are presented in Fig. 6A(deleobuvir) and Fig. 6B

(CD 6168). Substantially higher levels of deleobuvir were excreted in feces of pGF rats (105 ±

21% of dose) compared to control rats (26 ± 15% of dose). In addition, CD 6168 accounted for

1.5 ± 1.3 % of the deleobuvir dose in pGF rats, compared to 42 ± 8% in control rats.

In vitro to in vivo extrapolation based on fecal incubations

Based on *in vitro* formation rates and the assumptions inherent in these calculations (as

outlined in Methods and Discussion), the total amount of CD 6168 estimated to be formed from

deleobuvir (using Eq. 1) was 2.18 ± 1.40 mg in rats and 201 mg (subject I) and 19.0 mg (subject

II) in humans. These numbers were compared to *in vivo* levels of CD 6168 excreted into feces,

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which are summarized in Table 3. For rats, the amount of CD 6168 excreted into feces was 1.5 ±

0.3 mg (data derived from control rats in the pseudo-germ free rat study). For human, the

amount of CD 6168 excreted into feces was 280 mg (35% of a 800 mg dose) (Chen et al., 2015).

Discussion

The overall role of the gut microbiome in health and disease is increasingly being appreciated

(Owyang and Wu, 2014; Ursell et al., 2014; Yip and Chen, 2015; Klaassen and Cui 2015), as is

the contribution of gut bacteria to the metabolism of drugs (Sousa et al., 2008). Our own

experience with BILR 355 (Li et al., 2012) and deleobuvir, reported here, has also underlined an

important role of gut bacteria in the pre-systemic conversion of parent drug.

For deleobuvir, the substantial presence of a reduced metabolite, CD 6168, in rat feces but

not in bile in a rat ^{14}C-ADME study, suggested that CD 6168 was likely formed by gut bacteria.

While reductive reactions can be carried out by other drug metabolizing enzymes, including

cytochrome P450 (Guengerich, 2001), these are relatively rare. In addition, *in vitro* incubations

of deleobuvir with human liver microsomes and cytosol did not generate CD 6168. Anaerobic

incubations with deleobuvir, using rat and human fecal homogenates, demonstrated substantial

formation of CD 6168. The enzymes responsible for reduction can be sensitive to the presence

of oxygen and as such it was important to adopt appropriate *in vitro* conditions. An anaerobic

chamber was instrumental to these studies (Finegold et al., 1983; Kang et al., 2013).

To assess whether the plasma levels of CD 6168 could arise from bacterial biotransformation,

the *in vitro* formation rate of CD 6168 was used to extrapolate the amount of CD 6168 which

could be formed *in vivo*, with a number of assumptions (see below), and which was compared to

levels of CD 6168 in the human ^{14}C-ADME and rat studies (Table 3). It should be noted that the

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in vitro deleobuvir concentrations (100 µM) were limited by solubility and *in vivo* gut

concentrations could be higher. An 800 mg dose of deleobuvir in humans could theoretically

achieve gut concentrations of almost 5,000 µM (800 mg in 250 mL). In rats, a 10 mg/kg dose

would translate to ~1,700 µM (~3.5 mg in 3.2 mL gut volume; McConnel et al., 2008). In rats,

the average total amount of CD 6168 formed was estimated to be 2.18 ± 1.40 mg based on *in*

vitro incubations, compared to 1.5 ± 0.3 mg excreted in feces (Table 3), which is only a 1.5-fold

difference between predicted and observed amounts. In humans, based on *in vitro* incubations,

202 mg (subject I) and 19.1 mg (subject II) of CD 6168 were estimated to be formed *in vivo*. In

the human ^{14}C-ADME study (Chen et al., 2015), a single oral dose of ^{14}C-deleobuvir (800 mg)

resulted in 280 mg of CD 6168 being recovered in feces (35% of the dose of deleobuvir). There

was negligible excretion in urine (<1% of dose). In human feces, significant amounts (~70% of

CD 6168) of oxidative metabolites that may originate from CD 6168 were also found (Chen et al,

2015). Such secondary metabolism was significantly lower in rats (data on file). As such, in

humans, the actual amount of CD 6168 being generated could be up to 1.7-fold higher than

nominally found in the feces. Anaerobic *in vitro* fecal incubations will only generate CD 6168

since the oxidative enzymes that can generate secondary metabolites of CD 6168 will not be

active. The secondary metabolism of CD 6168 *in vivo* likely contributes to the underestimation

of the total amount of CD 6168 predicted from *in vitro* experiments.

There are several caveats associated with *in vitro-in vivo* extrapolation (IVIVE) of gut

bacterial metabolism. There is a large variability associated with the cecal content/fecal weight

and GI transit time (Kelsay et al., 1978; Klaassen and Cui 2015). In addition, the composition of

gut bacteria in fecal homogenate may not reflect the abundance and distribution of bacteria in the

GI tract (Finegold et al., 1983). An alternative approach is to consider bacterial load (CFU/g of

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feces) and the total number of bacteria in the GI tract. However, these numbers are not readily available and at best are approximations. This highlights the necessity to better characterize the gut microbiome. In this study, the two volunteers generated very different levels of CD 6168, which emphasizes a challenge with this approach. Clearly, due to inherent variability in gut bacterial metabolism, several human fecal donor samples should be tested for a more robust measure of IVIVE. Interspecies differences in CD 6168 oxidation adds additional challenges for a cross species IVIVE using a single approach. However, for both human and rat, the goal was to determine whether bacterial biotransformation could possibly account for large circulating levels of CD 6168. Considering the limitations of this IVIVE, we believe that this goal was achieved, although generating accurate IVIVE for such mechanisms needs further work in the field of microbiome.

A similar methodology was used to investigate the metabolism of BILR 355 by gut bacteria, forming BILR 402 (Li et al., 2012). The *in vitro* formation rate of BILR 402 in human fecal homogenates (9.2 nmol/min/g of feces), with the approach outlined here, was used to estimate the *in vivo* formation of BILR 402. The turnover of BILR 355 to BILR 402 was fast at 0.430 mg/min and confirmed the observation that BILR 355 was present in only trace amounts in feces *in vivo* and the majority of fecal radioactivity was accounted for by BILR 402 and its down-stream metabolites (Li et al., 2012).

A pseudo-germ free (pGF) rat model was also investigated to assess the importance of gut bacterial biotransformation in the disposition of deleobuvir. There can be substantial species differences in specificity and substrate selectivity for drug metabolizing enzymes between rats and humans (Martignoni et al., 2006). Similarly, species differences in gut microbiota (Rowland et al., 1986) make inter-species extrapolations difficult. Germ-free rats have been used to

evaluate the role of bacterial microbiota in the metabolism of several compounds, including

hesperidin, acetaminophen and mangiferin (Jin et al., 2010; Lee et al., 2012; Liu et al., 2012).

There are significant challenges in the maintenance and use of germ-free animals (Gordon and

Pesti, 1971). Pre-treatment of animals with antibiotics can provide a temporary, almost complete

microbe free animal model (Sousa et al., 2008; Kang et al., 2013). This simpler pGF rat model

was validated in our studies using a known probe for gut bacterial metabolism, hesperidin. The

pharmacokinetic parameters obtained for hesperetin in this study (Table 1) were comparable to

data reported by Jin et al. (2010). The pGF rats excreted significantly higher levels of hesperidin

compared to control rats, consistent with the lack of conversion of hesperidin to hesperetin by

gut bacteria (Fig. 4). Similarly, the plasma exposure of the metabolite, hesperetin, was

significantly higher in control compared to pGF rats (Fig. 3). The flavonoid backbone of

hesperetin is further degraded by gut bacteria into numerous phenolic and carboxylic acid

products (Garg et al, 2001) which explains the low levels of hesperetin in feces of pGF and

control rats (<3% of dose).

Although formation of CD 6168 in rats, compared to humans, is a relatively less abundant

metabolic pathway, these findings in pGF rats support a key role of gut bacteria in the formation

of CD 6168 from deleobuvir. CD 6168 exposure levels were significantly lower in pGF rats

with an average $AUC_{0-\infty}$ of 146 ± 64 ng·h/mL (Fig. 5B and Table 2), compared to control rats,

$1,312 \pm 649$ ng·h/mL. About 1.5 mg (42 % of dose) of CD 6168 was excreted in feces of control

rats, but a mere 0.06 mg (1.51 % of dose) was found in feces of pGF rats. Conversely,

significantly higher levels of deleobuvir were excreted into feces in pGF rats (105% of dose)

compared to control rats (26 % of dose), confirming the role of gut bacteria in the

biotransformation of deleobuvir. For deleobuvir, the plasma exposure levels were similar in both

rat groups but significantly different in amount excreted into feces. The amount of deleobuvir

excreted into feces is a combination of unabsorbed drug (60% bioavailability) with the amount

excreted into bile as deleobuvir and deleobuvir-glucuronide.

Deleobuvir, as well as CD 6168, were rapidly absorbed with a t_{max} of approximately 3 hours.

Both exhibited similar pharmacokinetics and high variability (Chen et al, 2015), but there was no

clear inverse correlation of deleobuvir and CD 6168 exposure *in vivo*. This suggests that while

the variability in gut bacteria between humans may account for some of the variability in plasma

exposure, other aspects of differential clearance between the two compounds, i.e. further

metabolism of CD 6168 versus direct glucuronidation of deleobuvir may also play a role.

Overall, using appropriate *in vitro* and *in vivo* tools of gut bacterial metabolism offers

experimentally viable approaches to identify a role for gut bacteria in the metabolism of a drug.

Attempts at IVIVE, taking the rate of metabolite formation in feces and scaling to the amount of

gut bacterial metabolite formed *in vivo*, may offer a way to assess the contribution by gut

bacteria to the overall biotransformation, with awareness of inter-individual variability and the

complex nature of the GI tract.

Acknowledgments:

Authorship contribution

Participated in research design: McCabe, Sane, Tweedie, Johnstone and Li

Conducted experiments: Whitcher-Johnstone, Xu, McCabe, King, Keith-Luzzi

Wrote or contributed to the writing of the manuscript: McCabe, Sane, Tweedie and Li

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Figure Legends

Fig. 1. Chemical structures of deleobuvir and CD 6168

Fig. 2. Formation of CD 6168 over time (A): in rat fecal samples, average of 3 male rats,

$r^2=0.99$; (B): in human fecal samples, closed circles for subject I, $r^2=0.97$ and open circles for

subject II, $r^2=0.90$.

Fig. 3. Mean (SD) plasma concentration-time profiles of hesperetin after oral administration of

50 mg/kg of hesperidin to control rats (closed circles) and pGF rats (open circles).

Fig. 4. Percent of dose excreted in feces as hesperidin and hesperetin for 48 h after oral

administration (50mg/kg) of hesperidin to control (vertical lines) and pGF (checkers) rats.

Unpaired t-test, where (*) denotes p<0.05 comparing pGF versus control rats.

Fig. 5. Mean (SD) plasma concentration-time profiles of deleobuvir (A) and CD 6168 (B) after

oral administration (10 mg/kg) of deleobuvir. Solid circles indicate control rats and open circles

indicate pGF rats.

Fig. 6. Percent of dose excreted in feces as (A) deleobuvir and (B) CD 6168 over 48 h after

dosing with deleobuvir (10 mg/kg) to pGF rats (checkers) and control rats (vertical lines).

Unpaired t-test, where (****) denotes p<0.0001 comparing pGF versus control rats.

Tables

Table 1. Pharmacokinetic parameters of hesperetin in plasma after oral administration of

50 mg/kg hesperidin to control and pGF rats

Group	t_{max} (h)	C_{max} (ng/mL)	$AUC_{0-\infty}$ (ng·h/mL)
	Hesperetin		
Control (in-house)	6-8[a]	314 ± 58	$3,454 \pm 760$
Control (Jin et al., 2010)	8	580 ± 310	$6,300 \pm 1,700$
pGF (in house)	4-6[a]	55.1 ± 31.0	685 ± 368
pGF (Jin et al., 2010)	6.5	200 ± 50	$2,300 \pm 2,000$

[a] expressed as range

Table 2. Pharmacokinetic parameters of deleobuvir and CD 6168 in plasma after oral

administration of 10 mg/kg deleobuvir to control and pGF rats

Group	Analyte	t_{max} (h)	$t_{1/2}$ (h)	C_{max} (ng/mL)	$AUC_{0\to\infty}$ (ng·h/mL)
Control	Deleobuvir	1	4^a	15,784 ± 4,533	63,438 ± 18,181
pGF		1	5±1	18,718 ± 9,840	66,683 ± 21,253
Control	CD 6168	$6\text{-}8^b$	ND	114 ± 65	1,312 ± 649
pGF		ND	ND	15.5 ± 7.7	146 ± 64

ND: not determined

[a] SD lower than 1

[b] expressed as range

Table 3. Estimation of the CD 6168 formation levels *in vivo* from in *vitro* anaerobic fecal incubations in rats and humans

Species	Rate of formation (nmol/min/g)	Estimated formation of CD 6168 (mg) based on *in vitro* data [a]	For comparison (*in vivo*)	
			Deleobuvir dose (mg)	Amount of CD 6168 formed (mg)
Rat	3.18 ± 2.05^{b}	2.18 ± 1.40	3.6	1.5
Human	1.95 (subject I) 0.184 (subject II)	202 (Subject I) 19.1 (Subject II)	800	280^{c}

[a] Using first IVIVE approach
[b] n=3
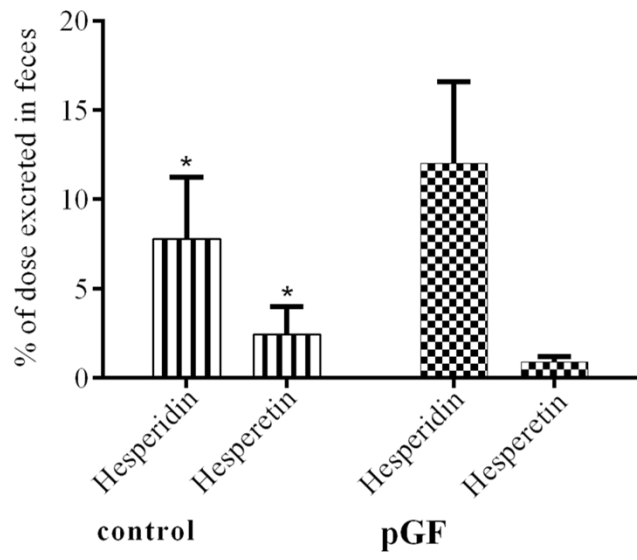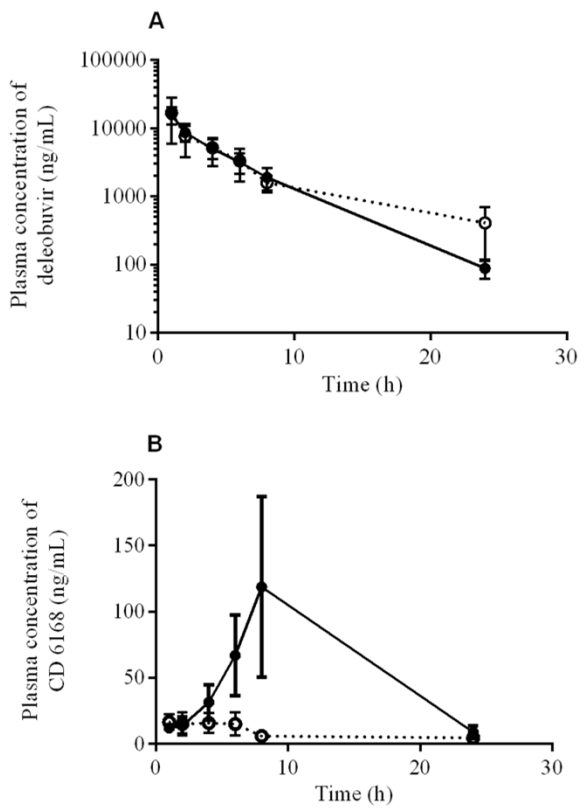[c] Secondary metabolism not accounted for
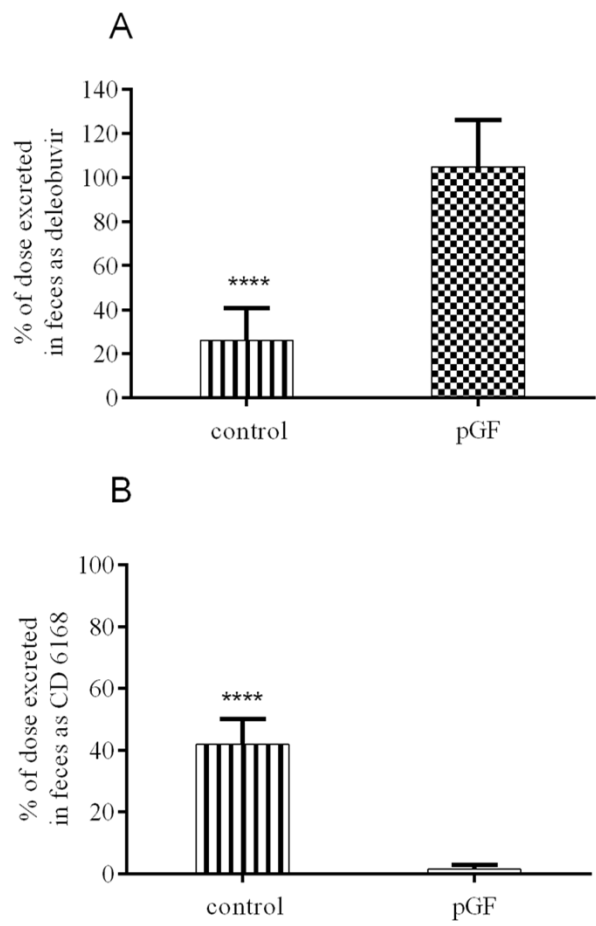
Deleobuvir

CD 6168
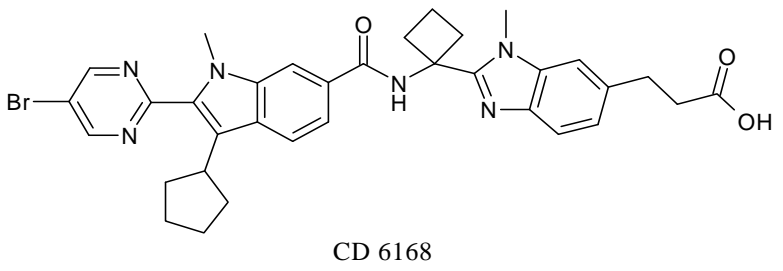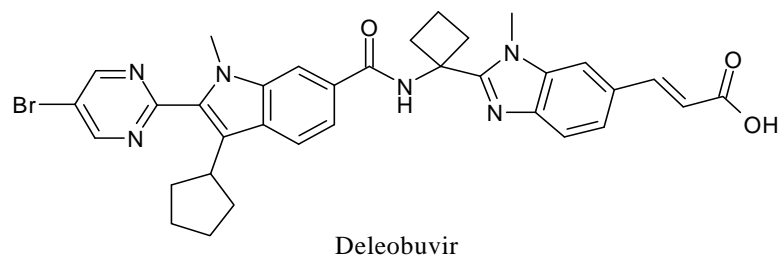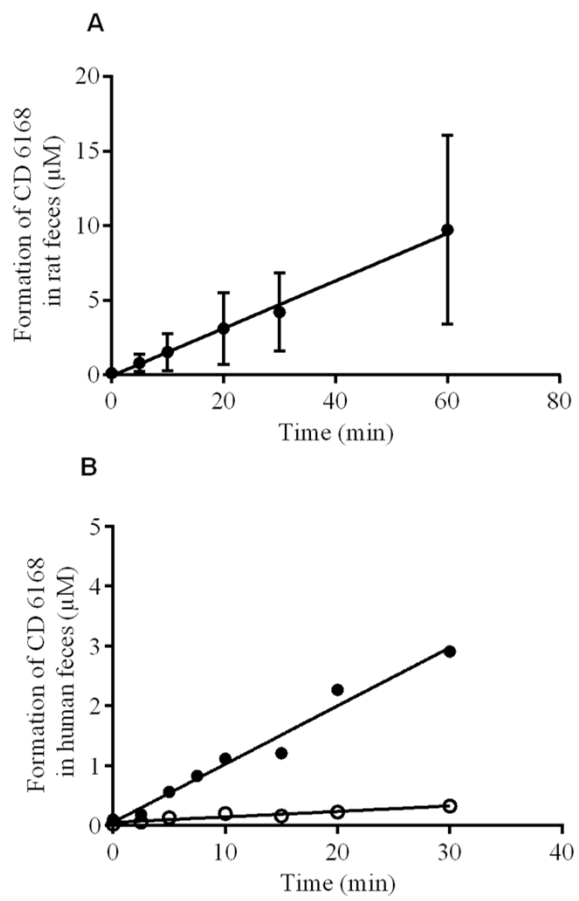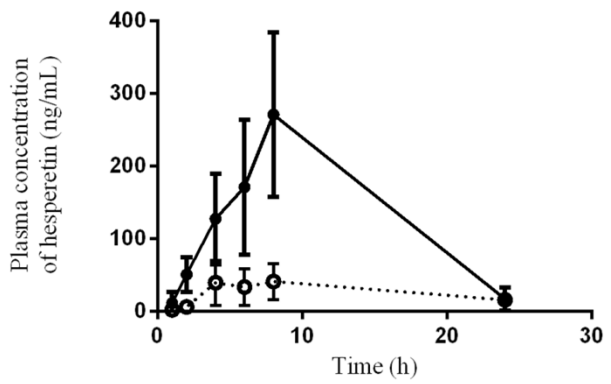
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6