Assessing Pleiotropic Effects of a Mixed-mode Perpetrator Drug

Rifampicin by Multiple Endogenous Biomarkers in Dogs

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Running title: Assessing Complex Drug-drug Interactions Using Biomarkers

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Abbreviations:

4β-HC, 4β-hydroxycholesterol; AUC, area under the plasma concentration-time curve; 
Cmax, the maximum plasma concentration; CP, coproporphyrin; DDI, drug-drug interaction; 
LC-MS/MS, liquid chromatography–tandem mass spectrometry; MDZ, midazolam; 
OATP, organic anion transporting polypeptides; PXR, pregnane X receptor; 
RIF, rifampicin; Tmax, time to reach the maximum concentration; T1/2, terminal half-life
Abstract

Rifampicin (RIF) is a mixed-mode perpetrator that produces pleiotropic effects on liver cytochrome P450 (CYP) enzymes and drug transporters. To assess the complex drug-drug interaction (DDI) liabilities of RIF in vivo, a known probe substrate midazolam (MDZ) along with multiple endogenous biomarkers were simultaneously monitored in beagle dogs before and after a 7-day treatment period by RIF at 20 mg/kg/day. Confirmed by the reduced MDZ plasma exposure and elevated 4β-hydroxycholesterol (4β-HC, biomarker of Cyp3a activities) level, Cyp3a was significantly induced after repeated RIF doses, and such induction persisted for 3 days after cessation of the RIF administration. On the other hand, increased plasma levels of coproporphyrin-I and III (CP-I and CP-III, biomarkers of organic anion transporting polypeptides 1b (Oatp1b) activities) were observed after the first dose of RIF. Plasma CPs started to decline as RIF exposure decreased, and they returned to baseline 3 days after cessation of the RIF administration. The data suggested the acute (inhibitory) and chronic (inductive) effects of RIF on Oatp1b and Cyp3a enzymes, respectively, and a 3-day washout period is deemed adequate to remove superimposed Oatp1b inhibition from Cyp3a induction. In addition, apparent self-induction of RIF was observed, as its terminal half-life was significantly altered after multiple doses. Overall, our investigation illustrated the need for appropriate timing of modulator dosing to differentiate between transporter inhibition and enzyme induction. As further indicated by the CP data, induction of Oatp1b activities was not likely after repeated RIF administration.
Significance Statement

Our investigation demonstrated the utility of endogenous biomarkers towards complex DDIs by RIF and successfully determined the optimal timing to differentiate between transporter inhibition and enzyme induction. Based on experimental evidence, Oatp1b inhibition following repeated RIF administration was unlikely, and apparent self-induction of RIF was observed.
Introduction

Liver metabolizing enzymes play a vital role in the biotransformation of many currently prescribed drugs (Wright et al., 2019), while drug transporters are membrane proteins responsible for translocating endogenous and exogenous molecules across membrane barriers (Liang et al., 2015). These enzymes and transporters often work together to mediate drug absorption, distribution, and elimination (Nigam et al., 2020). Both CYP enzymes and drug transporters can be critical players in DDIs, necessitating thorough assessment before human use. Unfortunately, some commonly prescribed drugs can act as perpetrators, affecting CYP enzymes and drug transporters in complex and sometimes perplexing ways after administration. For instance, RIF is a clinically relevant inducer that activates the pregnane X receptor (PXR) and simultaneously inhibits OATP1B, resulting in mixed-mode perpetrator effects (Chen and Raymond, 2006; Schilling et al., 2020). As exemplified by Zheng et al., when an intravenous dose of RIF is administered after a CYP induction period by oral RIF treatment, the inhibition of OATP1B can conceal the induction effect of liver CYP enzymes on the DDI involving glyburide, a dual substrate of CYP and OATP (Zheng et al., 2009). Another study by Reitman et al. suggested the need for multiple assessments of a probe substrate dosed at different stages of a clinical trial to distinguish the acute (inhibitory) and chronic (inductive) effects of RIF as a perpetrator (Reitman et al., 2011). Despite these clinical observations, recent comprehensive physiologically-based pharmacokinetic (PBPK) models have provided additional insights into RIF’s impact on the disposition of glyburide (Asaumi et al., 2018; Asaumi et al., 2019).
To evaluate the potential DDI liabilities of new molecular entities (NMEs) as perpetrators, concomitant administration of an index probe drug is often involved (Prueksaritanont et al., 2013). Since many endogenous metabolites are substrates of metabolizing enzymes and transporters, a complementary approach that monitors endogenous DDI biomarkers is gaining popularity. For example, the elevated plasma level of 4β-HC, a downstream product of the CYP3A-mediated cholesterol metabolism, is detected both in monkeys and humans after 14 days of RIF administration (Kanebratt et al., 2008; Li et al., 2014). Therefore, 4β-HC has been recognized as an endogenous biomarker of CYP3A activities. On the other hand, CP-I and CP-III are substrates of the OATP1B subfamily (Shen et al., 2016), and cumulative studies were reported to support CP-I and CP-III as sensitive and selective OATP1B biomarkers in humans and preclinical species (Shen et al., 2016; Lai, 2023). The use of these endogenous biomarkers for drug-metabolizing enzyme- and transporter-mediated DDIs is recommended by multiple research groups (Dong et al., 2017; Mariappan et al., 2017; Lee et al., 2021) and the International Transporter Consortium (ITC) (Chu et al., 2018).

As aforementioned, RIF is a well-characterized inducer of CYP3A enzymes and an inhibitor of OATP1B transporters. However, the inductive effects of RIF are species-specific. For example, RIF does not induce metabolizing enzymes in rats and guinea pigs, whereas the mice and rabbits are more responsive to the treatment of RIF (Benedetti and Dostert, 1994). Although dogs are frequently used as non-rodent testing species in drug discovery, the pleiotropic effects of rifampin, as is the case with many investigational drugs, still need to be fully characterized. In addition, an appropriate washout period after repeated doses of RIF still needs to be determined to reliably
assess the CYP induction without superimposed OATP1B inhibition. In this regard, we conducted *in vivo* studies to illustrate the diverse effects of RIF on Cyp3a enzymes and Oatp1b transporters by simultaneously monitoring multiple plasma biomarkers in conjunction with administered probe substrate (i.e., MDZ) in beagle dogs. Here, we report the changes in biomarker concentrations resulting from RIF's acute and chronic effects and discuss insights into complex DDIs.

**Materials and Methods**

**Materials**

Rifampicin, chrysin, midazolam, 1-hydroxymidazolam, 4-hydroxymidazolam, ethanol, formic acid, bovine serum albumin (BSA), potassium hydroxide solution, hydrochloric acid solution, and hexane were purchased from Sigma-Aldrich (St. Louis, MO). Ethyl acetate, water, acetonitrile, methanol, and 2-propanol were purchased from Thermo Fisher Scientific (Waltham, MA). Carbutamide, 4β-hydroxycholesterol, and 4β-hydroxycholesterol-d7 were purchased from Cayman Chemical Company (Ann Arbor, MI). CP-I, CP-III, and CP-I-15N4 were purchased from Toronto Research Chemicals Inc. (North York, ON).

**Pharmacokinetic Studies in Beagle Dogs**

Purebred beagle dogs from the LabCorp stock colony maintained at Madison, MI were used for this study. The study was in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (NIH) and approved by the institution’s Animal Care and Use Committee. Male
dogs (n = 3) were orally administered with RIF at 20 mg/kg/day for 7 days (Days 2–8), while MDZ, a probe for Cyp3a metabolism, was orally administered at 0.5 mg/kg on Day 1, 9, and 11 (Figure 1). Approximately 1 mL of blood was collected at 0 (pre-dose), 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h post-dose in K_2EDTA tubes on Day 1, 2, 9, and 11. The collected blood samples were maintained on wet ice before centrifugation (within 1 h) to harvest the plasma. The resulting dog plasma samples were stored at -70 °C. The sample collection, centrifugation, and storage processes were protected from light as much as possible to prevent possible degradation of CP-I and CP-III.

Bioanalysis of RIF, MDZ, 1-hydroxymidazolam, 4-hydroxymidazolam and Endogenous Biomarkers

Concentrations of RIF, MDZ, 1-hydroxymidazolam, 4-hydroxymidazolam, 4β-HC, CP-I, and CP-III were measured through liquid chromatography-tandem mass spectrometry (LC-MS/MS) using developed or previously published methods with modifications (Link et al., 2007; Kim et al., 2010; Shen et al., 2018a). The bioanalytical methods and conditions are shown in Table S1. Typically, calibration standards (STDs) and quality control (QC) samples were prepared in either dog plasma or surrogate matrix (i.e., 1% BSA). STDs, QC, and study samples were first purified using supported liquid extraction (for 4β-HC) or protein precipitation (for other analytes) and then injected into a Sciex Triple Quad 6500+ mass spectrometer (Redwood City, CA) coupled with a Shimadzu LC-30 pump system (Columbia, MD). A linear gradient separation was carried out with mobile phase A containing water with 0.1% formic acid and mobile phase B containing acetonitrile with 0.1% formic acid. Analytes of interest and the corresponding internal standards were monitored using multiple reaction monitoring.
(MRM) under positive ion mode. Universal peak integration was performed in Analyst 1.7 (Sciex, Redwood City, CA), and a linear regression with a weighting factor of 1/x^2 was conducted to construct the calibration curve.

Pharmacokinetic Analysis and Statistical Analysis

Acquired concentration-time data were processed using Phoenix WinNonLin (version 8.4, Certara, Princeton, NJ) to obtain pharmacokinetic parameters for analytes of interest on Day 1, 2, 9 and 11. Paired two-tail t-test was performed in Prism 8 (GraphPad, San Diego, CA). Paired one-way analysis of variance (ANOVA) was performed in Prism 8 followed by restricting the false discovery rate (FDR) < 0.05 for each pairwise comparison post hoc.

Results

Pharmacokinetics of RIF

The concentration-time profile of RIF is shown in Figure 1. Following the first dose on Day 2, RIF was rapidly absorbed with time to reach the maximum concentration (T_{max}) of 0.833 ± 1.01 h and the plasma C_{max} = 25.6 ± 10.8 µM. After the last dose on Day 8, the plasma concentration declined in a monophasic manner to reach the C_{min} = 0.0173 ± 0.00320 µM at the end of Day 11. In addition, the apparent terminal half-life of RIF was significantly shortened from Day 2 (8.26 ± 0.199 h) to Day 9 (6.92 ± 0.0929 h) (Table S2).

Plasma Exposure of MDZ, 1-hydroxymidazolam and 4-hydroxymidazolam
The concentration-time profiles of MDZ and its two Cyp3a-mediated metabolites, 1-hydroxymidazolam and 4-hydroxymidazolam, are shown in Figure 2. The plasma exposure (AUC_{last}) of MDZ significantly decreased by 7.7-fold on Day 9 compared with Day 1 (Table 1). On Day 11, such a reduction was still significant (6-fold). Correspondingly, the apparent terminal half-life of MDZ reduced from 0.906 ± 0.158 h (Day 1) to 0.532 ± 0.196 h (Day 9) and 0.675 ± 0.124 h (Day 11). On the other hand, an insignificant change of the 1-hydroxymidazolam and 4-hydroxymidazolam plasma exposure was observed on Day 9 and Day 11 compared with Day 1. However, the metabolites/parent ratio (defined below) significantly increased on Day 9 but not on Day 11.

\[
\text{Metabolite/parent ratio} = \frac{AUC_{last,1\text{-hydroxymidazolam}} + AUC_{last,4\text{-hydroxymidazolam}}}{AUC_{last,midazolam}}
\]

Change of Endogenous Biomarkers

The concentration-time profile of 4β-HC is shown in Figure 3. On Day 1, the baseline concentration of 4β-HC was 88.0 ± 1.38 nM (Table 2). The plasma C_{max} slightly increased by 1.2-fold (statistically insignificant) after the first RIF dose. On Day 9, a 1.6-fold increase \((p < 0.05)\) was observed, which persistently retained statistical significance on Day 11 (by 1.5-fold). Correspondingly, the AUC_{0–24h} presented a similar trend and comparable degree of elevation compared with C_{max} (i.e., 1.3-fold on Day 9 and Day 11, \(p < 0.05\)). The concentration-time profiles of CP-I and CP-III are shown in Figure 4. The baseline concentrations of CP-I and CP-III on Day 1 were 1.62 ± 0.666 and 3.93 ± 0.780 nM, respectively (Table 3). Following the first RIF dose on Day 2, the C_{max} of CPs
significantly increased by 5.9 and 7-fold for CP-I and CP-III, respectively, compared with Day 1. On Day 9, the $C_{\text{max}}$ of CP-I but not CP-III remained significantly elevated (by 4.6-fold, $p < 0.05$) compared with Day 1. On Day 11, both CPs returned to the baseline level. Similarly, the $\text{AUC}_{0-24h}$ of CPs were significantly elevated on Day 2 with less degree of change (i.e., increased by 3.6 and 4.8-fold for CP-I and CP-III, respectively) compared with $C_{\text{max}}$ and returned to baseline on Day 9.

**Discussion**

In this work, *in vivo* studies were conducted to investigate and characterize the pleiotropic effects of RIF in dogs. As indicated by the significant and substantial reduction of MDZ $\text{AUC}_{\text{last}}$ on Day 9, Cyp3a was significantly induced at the end of the 7-day induction period (Day 2–8). Cyp3a remained significantly induced 3 days after cessation of the RIF administration in dogs, which was in line with a previous publication reporting a $>2$-week washout time in humans following a 28-day RIF induction period at 600 mg/day (Reitman et al., 2011). In addition, the metabolite/parent ratio of MDZ on Day 9 was significantly increased (i.e., by 8.2-fold) compared with Day 1 (Table 1), agreeing with similar observations in monkeys after a 6-day RIF induction period at 15 mg/kg/day (i.e., increased by 1.6-fold) (Kim et al., 2010) and in humans following a 5-day RIF induction period at 600 mg/day (i.e., increased by 9.5-fold) (Backman et al., 1998). Together, these results suggest that in addition to cynomolgus monkeys and humans, beagle dogs also respond in a similar fashion to these ligands (i.e., PXR agonists).
Due to the relatively large inter-individual variation, the change of 1-hydroxymidazolam and 4-hydroxymidazolam exposure on Day 9 and Day 11 was not statistically significant compared to Day 1. Given that both metabolites are subjected to undergo further $O$-glucuronidation mediated by phase II enzymes, including uridine diphosphate glucuronosyltransferases (UGTs) 2B4 and 2B7 (Seo et al., 2010), and there is ample in vitro experimental evidence to suggest that UGT2B4 expression is upregulated after RIF treatment (Gufford et al., 2018; Dyavar et al., 2020), it is likely that these phase II enzymes may also be induced in dogs. As a result, individual expression of phase II enzymes (Zhou et al., 2003) and their induction following RIF treatment (Soars et al., 2004) may contribute to the inter-subject variation of plasma 1-hydroxymidazolam and 4-hydroxymidazolam.

As a reported endogenous biomarker of Cyp3a activities, the plasma 4β-HC was monitored. Notably, the ratio of 4β-HC/cholesterol may not be a sensitive biomarker to access CYP3A activities reliably (Ma et al., 2013) and, therefore, was not evaluated in our study. The baseline concentration of 4β-HC in dogs (88.0 ± 1.38 nM) is lower than in cynomolgus monkeys (197 nM) but comparable to humans (74.3 nM) (Diczfalusy et al., 2011; Li et al., 2014). In line with the MDZ data, plasma $C_{\text{max}}$ and $AUC_{0-24h}$ of 4β-HC were significantly increased at the end of the RIF induction period by 1.6 and 1.3-fold, respectively. However, the fold change of 4β-HC concentration was less pronounced than MDZ but comparable to that observed in human subjects (i.e., 1.5-fold) receiving RIF at 20 mg/day for 14 days (Kanebratt et al., 2008). The 4β-HC level was also significantly high on Day 11, suggesting that enzyme induction remains. In fact, once the CYP proteins are induced, they follow a normal rate of protein degradation, with a
half-life of 36-140 hours (Grimm et al., 2009), to return to their baseline level. This aligns with previous studies that reported a washout period of over 2 weeks for complete recovery from rifampin induction in humans (Reitman et al., 2011). Although the rifampin induction effect was detected after the 7-day washout period or longer, the inductive effect was significantly attenuated (Reitman et al., 2011). A shorter washout period is necessary to detect the true effects of enzyme induction, which supports our conclusion.

CP-I and CP-III are well-investigated biomarkers of OATP1B activities assessed in our study. Compared with previously reported endogenous levels in other species (Shen et al., 2018a; Takehara et al., 2019), the baseline concentration of CP-I in dogs (1.62 nM) is lower than in monkeys (7.4 nM) but higher than in humans (0.72 nM). For CP-III, the endogenous concentration in dogs (3.93 nM) is higher than in monkeys (1.4 nM) and humans (0.14 nM). Interestingly, both CPs reached C_{max} at 6 h on Day 2, approximately 4 h later than the T_{max} of RIF (first dose). Such “lag” time is not uncommon and is also seen in other studies investigating biomarkers of renal transporter inhibition in monkeys (Shen et al., 2018b; Liu et al., 2023). At the end of the RIF induction period on Day 9, the C_{max} of both CP-I and CP-III remained high in the plasma (4.6-fold and 3.4-fold, respectively). Still, the changes of CP-III were not statistically significant due to high inter-individual variation. Plasma CP levels returned to the baseline on Day 11, following the RIF exposure getting low (< 100 nM) to undetectable levels in the plasma. Our data suggested that the Oatp1b inhibition following RIF administration can last for about 2 days; therefore, the washout period (i.e., 3 days) was set to eliminate the masking effect of Oatp1b inhibition while...
assessing the Cyp3a induction on Day 11. It is worth noting that the plasma concentrations of CPs starting at C_{24h} of Day 9 through the end of Day 11 were comparable to the baseline (Day 1), indicating that the Oatp1b functions may not be induced through PXR activation. The results are consistent with \textit{in vitro} human gene induction data, as well as \textit{in vivo} data in monkeys showing that the plasma concentrations of pitavastatin (a substrate of OATP1B), CP-I, and CP-III were not significantly altered after 7 days of RIF administration (18 mg/kg/day) (Niu et al., 2019). A separate human study found that subjects possessed similar pre-dose CP-I levels after multiple-dose RIF administration compared with single-dose administration, indicating that OATP1B is not induced (Kunze et al., 2018). However, a recent report compared the CP-I concentrations in healthy volunteers co-administered with RIF (600 mg/day) and letermovir (480 mg/day) for 14 days with a “baseline” measured in subjects after continuously receiving letermovir at 480 mg/day for 14 days (Robbins et al., 2022). It was found that the plasma C_{max} and AUC_{0-24h} of CP-I decreased following the letermovir and RIF co-administration, and the authors concluded potential OATP1B induction. However, letermovir is an OATP1B substrate and clinically relevant inhibitor. When letermovir is dosed daily PO at 480 mg, it can cause significant OATP1B inhibition DDIs, resulting in a 3.29-fold AUC increase of OATP1B probe substrate atorvastatin (letermovir drug label, https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/209939orig1s000,209940orig1s000lbl.pdf). Furthermore, the “predose” CP-I level at the beginning of the letermovir-RIF co-administration remained seemingly unchanged compared with that after the co-administration, indicating that induction of OATP1B did not occur (Robbins et al., 2022).
et al., 2022). Since MRP2 is also involved in the biliary CP elimination (Kunze et al., 2018), the effect of rifampin as an inducer in the intestine (Fromm et al., 2000) and/or as an inhibitor in the liver (Neyt et al., 2013) on Mrp2 needs to be characterized in future studies.

It is worth noting that precautions must be taken to translate RIF DDI findings directly to other investigational drugs and so to their human DDIs, as reported quantitative proteomics results suggested a difference in liver transporter compositions between humans (29% OATPs) and dogs (69% Oatps) (Wang et al., 2015). Moreover, the major isoforms of hepatic Oatps in dogs (Oatp1b4 and Oatp2b1) are different than those in humans (OATP1B1, OATP1B3, and OATP2B1) (Wang et al., 2015). Given that current in vivo DDI data are still lacking for Oatp1b4 in dogs, in vitro results showed that it possesses 69% and 72% homology to human OATP1B1 and OATP1B3, respectively (Gui and Hagenbuch, 2010), and it shares similar substrate specificities with OATP1B1/3 (Wilby et al., 2011). On the other hand, although CP-III but not CP-I is a substrate of OATP2B1 (Bednarczyk and Boiselle, 2016), the Oatp2b1-mediated disposition of CP-III was not likely affected because RIF is not a potent inhibitor of OATP2B1 (Karlgren et al., 2012). Indeed, a comparable degree of $C_{\text{max}}$ and AUC fold change was observed between CP-I and CP-III after RIF administration (Table 3). Nevertheless, although future investigation is warranted to understand the effect of species-dependent OATP expression on CP disposition, the elevated CP levels observed in humans, monkeys, and dogs indicate pan-OATP1B inhibitory effects by RIF.
In addition to monitoring endogenous biomarkers, the concentration of RIF was also monitored. Compared with a single dose on Day 2, the terminal half-life of RIF significantly reduced by 16.2% on Day 9 after repeated doses (Table S2), indicating self-induction. This observation is in line with earlier publications reporting a decreased serum concentration and half-life accompanied by increased metabolic derivatives in the bile after multiple RIF doses compared with a single dose in humans (Acocella, 1978; Acocella et al., 1978) and monkeys (Niu et al., 2019). Although debates remain on whether CYP3A directly mediates the metabolism of RIF (Nakajima et al., 2011; Kunze et al., 2018; Rodrigues et al., 2020), our data demonstrated the induction of RIF elimination pathways following multiple doses. The weaker inhibition of Oatp1b due to reduced RIF exposure results in lower plasma CP levels during repeating RIF treatment (Table 3). Therefore, RIF concentration should be measured when conducting DDI studies involving repeated RIF administration.

In summary, we provided an approach to tackle complex in vivo DDI through interrogation of multiple endogenous biomarkers simultaneously. Our data shed light on the pleiotropic effects RIF produced as a mixed-mode perpetrator on Cyp3a enzymes as a chronic inducer and liver Oatp1b transporters as an acute inhibitor. A washout period of 3 days was determined adequate in preclinical and likely in clinical settings to assess Cyp3a induction while avoiding masking effects from Oatp1b inhibition. Additionally, experimental evidence was provided against the hypothesis of Oatp1b induction following PXR activation with a repeated RIF dose regimen. Our results further advocate the usefulness of endogenous biomarkers towards complex enzyme and transporter-mediated DDIs at the early stage of drug development.
Acknowledgments

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Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper.

Authorship Contributions

Participated in research design: Liu, Ma, Mok, Murray, Subramanian, Lai

Conducted experiments: Liu, Ma

Performed data analysis: Liu, Ma, Mok, Lai

Wrote or contributed to the writing of the manuscript: Liu, Murray, Mok, Ma, Lai
References


Footnotes

RL, BM, and MM contribute equally to this work.

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Figure Legends

Figure 1. (Top) Dose regimen and sample collection schedule of the 11-day in-life pharmacokinetic study in dogs (n = 3). Solid sample vials denote dates for blood sample collection. (Bottom) Concentration-time profile of RIF in dog plasma. The shaded area represents the RIF treatment period from Day 2–8. RIF: rifampicin. MDZ: midazolam. T\(_{1/2}\): terminal half-life.

Figure 2. Concentration-time profiles of (A) the Cyp3a probe substrate midazolam, and its two Cyp3a-mediated metabolites, (B) 1-hydroxymidazolam, and (C) 4-hydroxymidazolam on Day 1 (diamond), 9 (square) and 11 (triangle) in dog plasma (n = 3).

Figure 3. Concentration-time profile of the Cyp3a endogenous biomarker, 4β-hydroxycholesterol (4β-HC), on Day 1 (diamond), 2 (circle), 9 (square), and 11 (triangle) in dog plasma (n = 3).

Figure 4. Concentration-time profiles of the Oatp1b endogenous biomarkers, (A) coproporphyrin-I (CP-I) and (B) coproporphyrin-III (CP-III), on Day 1 (diamond), 2 (circle), 9 (square), and 11 (triangle) in dog plasma (n = 3).
Tables

Table 1. Measured plasma exposure of midazolam and its two Cyp3a-mediated metabolites

<table>
<thead>
<tr>
<th>Day</th>
<th>Midazolam $\text{AUC}_{\text{last}}$ (nM∙h)</th>
<th>Fold change$^a$</th>
<th>1-hydroxymidazolam $\text{AUC}_{\text{last}}$ (nM∙h)</th>
<th>Fold change$^a$</th>
<th>4-hydroxymidazolam $\text{AUC}_{\text{last}}$ (nM∙h)</th>
<th>Fold change$^a$</th>
<th>Metabolite/parent$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72.1 ± 40.4</td>
<td>NA</td>
<td>94.0 ± 83.8</td>
<td>NA</td>
<td>53.5 ± 31.9</td>
<td>NA</td>
<td>1.89 ± 0.462</td>
</tr>
<tr>
<td>9</td>
<td>9.38 ± 3.63$^c$</td>
<td>-7.7</td>
<td>14.9 ± 9.06</td>
<td>-6.3</td>
<td>133 ± 76.2</td>
<td>2.5</td>
<td>15.5 ± 4.44$^c$</td>
</tr>
<tr>
<td>11</td>
<td>12.5 ± 5.44$^c$</td>
<td>-6.0</td>
<td>14.1 ± 9.36</td>
<td>-6.7</td>
<td>35.0 ± 12.1</td>
<td>-1.5</td>
<td>4.38 ± 1.90</td>
</tr>
</tbody>
</table>

$^a$Fold change calculation is based on Day 1 data

$^b$Metabolite/parent = \frac{\text{AUC}_{\text{last,1-hydroxymidazolam}} + \text{AUC}_{\text{last,4-hydroxymidazolam}}}{\text{AUC}_{\text{last,midazolam}}}

$^c$Paired one-way ANOVA pairwise comparison $p < 0.05$ with FDR $< 0.05$ compared with Day 1 data
Table 2. Measured maximum plasma concentration ($C_{\text{max}}$) and area under the concentration-time curve ($\text{AUC}_{0-24\text{h}}$) of 4β-hydroxycholesterol in dog plasma

<table>
<thead>
<tr>
<th>Day</th>
<th>$C_{\text{max}}$ (nM)</th>
<th>Fold change$^a$</th>
<th>AUC$_{0-24\text{h}}$ (nM·h)</th>
<th>Fold change$^b$</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>88.0 ± 1.38$^b$</td>
<td>NA</td>
<td>2240 ± 167</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>103 ± 26.4</td>
<td>1.2</td>
<td>1985 ± 188</td>
<td>0.9</td>
</tr>
<tr>
<td>9</td>
<td>139 ± 14.0$^c$</td>
<td>1.6</td>
<td>2979 ± 331$^c$</td>
<td>1.3</td>
</tr>
<tr>
<td>11</td>
<td>130 ± 20.0$^c$</td>
<td>1.5</td>
<td>2878 ± 416$^c$</td>
<td>1.3</td>
</tr>
</tbody>
</table>

$^a$Fold change calculation is based on Day 1 data

$^b$Average concentration from 0–24 h

$^c$Paired one-way ANOVA pairwise comparison $p < 0.05$ with FDR < 0.05 compared with Day 1 data

Table 3. Measured maximum plasma concentration ($C_{\text{max}}$) and area under the concentration-time curve ($\text{AUC}_{0-24\text{h}}$) of CP-I and CP-III in dog plasma
<table>
<thead>
<tr>
<th>Day</th>
<th>CP-I C&lt;sub&gt;max&lt;/sub&gt; (nM)</th>
<th>Fold</th>
<th>CP-I AUC&lt;sub&gt;0–24h&lt;/sub&gt; (nM-h)</th>
<th>Fold</th>
<th>CP-III C&lt;sub&gt;max&lt;/sub&gt; (nM)</th>
<th>Fold</th>
<th>CP-III AUC&lt;sub&gt;0–24h&lt;/sub&gt; (nM-h)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.62 ± 0.666&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA</td>
<td>34.3 ± 12.5</td>
<td>NA</td>
<td>3.93 ± 0.780&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA</td>
<td>85.5 ± 13.6</td>
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</tr>
<tr>
<td>2</td>
<td>9.50 ± 4.59&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.9</td>
<td>125 ± 44.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.6</td>
<td>27.6 ± 10.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.0</td>
<td>413 ± 151&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.8</td>
</tr>
<tr>
<td>9</td>
<td>7.38 ± 2.90&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.6</td>
<td>60.2 ± 42.2</td>
<td>1.8</td>
<td>13.4 ± 4.50</td>
<td>3.4</td>
<td>146 ± 64.9</td>
<td>1.7</td>
</tr>
<tr>
<td>11</td>
<td>1.86 ± 0.388</td>
<td>1.1</td>
<td>22.2 ± 2.14</td>
<td>-1.5</td>
<td>4.18 ± 0.783</td>
<td>1.1</td>
<td>74.0 ± 1.42</td>
<td>-1.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fold change calculation is based on Day 1 data  
<sup>b</sup>Average concentration from 0–24 h  
<sup>c</sup>Paired one-way ANOVA pairwise comparison p < 0.05 with FDR < 0.05 compared with Day 1 data
Figure 2

A. Midazolam
B. 1-hydroxymidazolam
C. 4-hydroxymidazolam
Figure 3

Plasma Concentration of 4β HC (nM) versus Time (h)

- • Day 1
- ○ Day 2
- □ Day 9
- △ Day 11
Assessing Pleiotropic Effects of a Mixed-mode Perpetrator Drug
Rifampicin by Multiple Endogenous Biomarkers in Dogs

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**Supplemental Data**

**Table S1.** Experimental methods and conditions for LC-MS/MS analyses

<table>
<thead>
<tr>
<th>Analyte</th>
<th>IS</th>
<th>Extraction method</th>
<th>Ionization method</th>
<th>Column</th>
<th>Flow rate (mL/min)</th>
<th>MRM transition (IS)</th>
<th>CE (IS, V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rif</td>
<td>Chrysin</td>
<td>PP</td>
<td>ESI</td>
<td>Waters XSelect HSS T3, 2.1 x 50 mm, 2.5 µm</td>
<td>0.8</td>
<td>823.2→791.3 (255.1→153.0)</td>
<td>30</td>
</tr>
<tr>
<td>MDZ</td>
<td>Carbutamide</td>
<td>PP</td>
<td>ESI</td>
<td>Waters XSelect HSS T3, 2.1 x 50 mm, 2.5 µm</td>
<td>0.8</td>
<td>326.0→291.1 (272.1→156.0)</td>
<td>29</td>
</tr>
<tr>
<td>1-hydroxymidazolam</td>
<td>Carbutamide</td>
<td>PP</td>
<td>ESI</td>
<td>Waters XSelect HSS T3, 2.1 x 50 mm, 2.5 µm</td>
<td>0.8</td>
<td>342.0→324.2 (272.1→156.0)</td>
<td>30</td>
</tr>
<tr>
<td>4-hydroxymidazolam</td>
<td>Carbutamide</td>
<td>PP</td>
<td>ESI</td>
<td>Waters XSelect HSS T3, 2.1 x 50 mm, 2.5 µm</td>
<td>0.8</td>
<td>342.0→297.0 (272.1→156.0)</td>
<td>30</td>
</tr>
<tr>
<td>4β-HC 4β-HC-d7</td>
<td>SLE</td>
<td>APCI</td>
<td></td>
<td>Waters Acquity BEH C18 2.1 x 100 mm, 1.7 µm</td>
<td>0.4</td>
<td>385.2→109.1 (392.2→97.0)</td>
<td>31</td>
</tr>
<tr>
<td>CP-I</td>
<td>CP-I-15N₄</td>
<td>SLE</td>
<td>ESI</td>
<td>ACE 3 C18-PFP 1 x 50 mm, 3 µm</td>
<td>0.1</td>
<td>655.4→596.3 (659.4→600.2)</td>
<td>75</td>
</tr>
<tr>
<td>CP-III</td>
<td>CP-I-15N₄</td>
<td>SLE</td>
<td>ESI</td>
<td>ACE 3 C18-PFP 1 x 50 mm, 3 µm</td>
<td>0.1</td>
<td>655.4→596.3 (659.4→600.2)</td>
<td>75</td>
</tr>
</tbody>
</table>

IS: internal standard, PP: protein precipitation, SLE: supported liquid extraction, ESI: electrospray ionization, APCI: atmospheric pressure chemical ionization, CE: collisional energy.

**Table S2.** The apparent terminal half-life of rifampicin in individual animals
<table>
<thead>
<tr>
<th>Day</th>
<th>Dog 1 (h)</th>
<th>Dog 2 (h)</th>
<th>Dog 3 (h)</th>
<th>Average (h)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8.38</td>
<td>8.37</td>
<td>8.03</td>
<td>8.26</td>
<td>0.20</td>
</tr>
<tr>
<td>9</td>
<td>7.03</td>
<td>6.86</td>
<td>6.88</td>
<td>6.92*</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Paired two-tail t-test p < 0.05 compared with Day 2 data
SD: standard deviation