Assessing Pleiotropic Effects of a Mixed-Mode Perpetrator Drug, Rifampicin, by Multiple Endogenous Biomarkers in Dogs

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ABSTRACT

Rifampicin (RIF) is a mixed-mode perpetrator that produces pleiotropic effects on liver cytochrome P450 enzymes and drug transporters. To assess the complex drug-drug interaction 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 per 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 (CP)-I and 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

This investigation demonstrated the utility of endogenous biomarkers towards complex drug-drug interactions by rifampicin (RIF) and successfully determined the optimal timing to differentiate between transporter inhibition and enzyme induction. Based on experimental evidence, Oatp1b induction following repeated RIF administration was unlikely, and apparent self-induction of RIF elimination was observed.

Introduction

Liver-metabolizing enzymes play a vital role in the biotransformation of many currently prescribed drugs (Wright et al., 2019), whereas 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 cytochrome P450 (CYP) enzymes and drug transporters can be critical players in drug-drug interactions (DDIs), necessitating thorough assessment before human use.

For instance, rifampicin (RIF) is a clinically relevant inducer that activates the pregnane X receptor (PXR) and simultaneously inhibits organic anion transporting polypeptide (OATP)-1B, resulting in mixed-mode perpetrator effects (Chen and Raymond, 2006; Schilling et al., 2020). As exemplified by Zheng et al. (2009), 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. Another study by Reitman et al. (2011) 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. Despite these clinical observations, recent comprehensive physiologically based pharmacokinetic models have provided additional insights into RIF’s impact on the disposition of glyburide (Asaumi et al., 2018, 2019).

To evaluate the potential DDI liabilities of new molecular entities 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-hydroxycholesterol (4β-HC), a downstream product of the CYP3A-mediated cholesterol metabolism, is detected both in monkeys and

ABBREVIATIONS: 4β-HC, 4β-hydroxycholesterol; AUC\(_{0-24}\), area under the plasma concentration-time curve over the firstlast 24-h dosing interval; AUC\(_{\text{last}}\), area under the curve from the time of dosing to the last measurable concentration; CP, coproporphyrin; DDI, drug-drug interaction; MDZ, midazolam; OATP, organic anion transporting polypeptide; CYP, cytochrome P450; PXR, pregnane X receptor; RIF, rifampicin.
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, coproporphyrin (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 (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 mice and rabbits are more responsive to the treatment of RIF (Strolin Benedetti and Dostert, 1994). Although dogs are frequently used as nonrodent 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., midazolam (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, chrysins, midazolam, 1'-hydroxymidazolam, 4'-hydroxy- midazolam, 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). Carbamazepine, 4β-hydroxycholesterol, and 4β-hydroxycholesterol-d6 were purchased from Cayman Chemical Company (Ann Arbor, MI). CP-I, CP-III, and CP-I-15N2 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, WI 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 and approved by the institution’s Animal Care and Use Committee. Male dogs (n = 3) were orally administered with RIF at 20 mg/kg per day for 7 days (days 2–8), whereas MDZ, a probe for CYP3A metabolism, was orally administered at 0.5 mg/kg on day 1, 9, and 11 (Fig. 1). Approximately 1 mL of blood was collected at 0 (pre-dose), 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours postdose in K3EDTA tubes on days 1, 2, 9, and 11. The collected blood samples were maintained on wet ice before centrifugation (within 1 hour) 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 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 Supplemental Table 1. Typically, calibration standards and quality control samples were prepared in either dog plasma or surrogate matrix (i.e., 1% bovine serum albumin). Calibration standards, quality control, 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).

Results

Pharmacokinetics of RIF. The concentration-time profile of RIF is shown in Fig. 1. Following the first dose on day 2, RIF was rapidly absorbed, with a time to reach the maximum concentration of 0.833 ± 1.01 hours and a plasma Cmax of 25.6 ± 10.8 µM. After the last dose on day 8, the plasma concentration declined in a monophasic manner to reach a Cmax of 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 hours) to day 9 (6.92 ± 0.0929 hours) (Supplemental Table 2).

Plasma Exposure of MDZ, 1'-Hydroxymidazolam, and 4'-Hydroxy- midazolam. The concentration-time profiles of MDZ and its two CYP3A-mediated metabolites, 1'-hydroxymidazolam and 4'-hydroxymidazolam, are shown in Fig. 2. The area under the curve from the time of dosing to the last measurable concentration (AUClast of MDZ) significantly decreased by 77.7-fold on day 9 compared with day 1 (Table 1). On day 11, such a reduction was still significant (sixfold). Correspondingly, the apparent terminal half-life of MDZ reduced from 0.906 ± 0.158 hours (day 1) to 0.532 ± 0.196 hours (day 9) and 0.675 ± 0.124 hours (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 metabolite/parent ratio (defined below) significantly increased on day 9 but not on day 11.

Metabolite/parent ratio =

\[
\frac{AUC_{\text{last, 1'-hydroxymidazolam}} + AUC_{\text{last, 4'-hydroxymidazolam}}}{AUC_{\text{last, midazolam}}}
\]

Change of Endogenous Biomarkers. The concentration-time profile of 4β-HC is shown in Fig. 3. On day 1, the baseline concentration

![Fig. 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 days 2–8. T1/2, terminal half-life.](image-url)
of 4β-HC was 88.0 ± 1.38 nM (Table 2). The plasma Cmax 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 area under the plasma concentration-time curve over the first 24-h dosing interval (AUC0–24h) presented a similar trend and comparable degree of elevation compared with Cmax (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 Fig. 4. The baseline concentrations of CP-I and CP-III on day 1 were 88.0 ± 1.38 nM (Table 2). The plasma Cmax slightly increased by 1.2-fold (statistically insignificant) after the first RIF dose.

### TABLE 1

<table>
<thead>
<tr>
<th>Day</th>
<th>Midazolam</th>
<th>Fold Change*</th>
<th>1’-Hydroxymidazolam</th>
<th>Fold Change*</th>
<th>4-Hydroxymidazolam</th>
<th>Fold Change*</th>
<th>Metabolite/Parent</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</td>
<td>-0.7</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</td>
</tr>
<tr>
<td>11</td>
<td>12.5 ± 5.44</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>

FDR, false discovery rate; NA, not applicable.
*Fold change calculation is based on day 1 data.
†Metabolite/parent = AUCmetabolite/AUCparent
‡Paired one-way ANOVA pairwise comparison P < 0.05 with FDR < 0.05 compared with day 1 data.

**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 AUClast on day 9, CYP3A was significantly induced at the end of the 7-day induction period (days 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 per 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 interindividual variation, the change of 1’-hydroxymidazolam and 4-hydroxymidazolam exposure on day 9 and day 11 was not statistically significant compared with day 1. Given that both metabolites are subjected to undergo further O-glucuronidation.

### TABLE 2

<table>
<thead>
<tr>
<th>Day</th>
<th>Cmax (nM)</th>
<th>Fold Change*</th>
<th>AUC0–24h (nM·h)</th>
<th>Fold Change*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>88.0 ± 1.38</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</td>
<td>1.6</td>
<td>2979 ± 331</td>
<td>1.3</td>
</tr>
<tr>
<td>11</td>
<td>130 ± 20.0</td>
<td>1.5</td>
<td>2878 ± 416</td>
<td>1.3</td>
</tr>
</tbody>
</table>

FDR, false discovery rate; NA, not applicable.
*Fold change calculation is based on day 1 data.
†Average concentration from 0–24 h.
‡Paired one-way ANOVA pairwise comparison P < 0.05 with FDR < 0.05 compared with day 1 data.
mediated by phase II enzymes, including uridine diphosphate glucuronosyltransferases (UGTs) 2B4 and 2B7 (Seo et al., 2010), and that 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, differences in 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 levels.

As a reported endogenous biomarker of CYP3A activities, the plasma 4'β-HC was monitored. Notably, the ratio of 4'β-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 4'β-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 Cmax and AUC0–24h of 4'β-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 4'β-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 4'β-HC level was also significantly high on day 11, suggesting that enzyme induction remained. In fact, once the CYP proteins are induced, they follow a normal rate of protein degradation, with a half-life of 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 the concentration at 24 hours after dose administration (C24h) of day 9 through the end of day 11, were comparable to 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 Cmax at 6 hours on day 2, approximately 4 hours later than the time to reach the maximum concentration 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 Cmax 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 the concentration at 24 hours after dose administration (C24h) 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 in vitro human gene induction data as well as in vivo data in monkeys, showing that the plasma concentrations of pitavastatin (a substrate of OATP1B1), CP-I, and CP-III were not significantly altered after 7 days of RIF administration (18 mg/kg per day) (Niu et al., 2019). A separate 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 Cmax at 6 hours on day 2, approximately 4 hours later than the time to reach the maximum concentration 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 Cmax 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 the concentration at 24 hours after dose administration (C24h) 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 in vitro human gene induction data as well as in vivo data in monkeys, showing that the plasma concentrations of pitavastatin (a substrate of OATP1B1), CP-I, and CP-III were not significantly altered after 7 days of RIF administration (18 mg/kg per day) (Niu et al., 2019). A separate washout period is necessary to detect the true effects of enzyme induction, which supports our conclusion.

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TABLE 3

<table>
<thead>
<tr>
<th>Day</th>
<th>CP-I $C_{\text{max}}$ (nM)</th>
<th>Fold Change$^a$</th>
<th>CP-I AUC0–24h (nM·h)</th>
<th>Fold Change$^a$</th>
<th>CP-III $C_{\text{max}}$ (nM)</th>
<th>Fold Change$^a$</th>
<th>CP-III AUC0–24h (nM·h)</th>
<th>Fold Change$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.62 ± 0.666$^b$</td>
<td>NA</td>
<td>34.3 ± 12.5</td>
<td>NA</td>
<td>3.93 ± 0.780$^b$</td>
<td>NA</td>
<td>85.5 ± 13.6</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>9.50 ± 4.59$^c$</td>
<td>5.9</td>
<td>125 ± 44.1$^c$</td>
<td>3.6</td>
<td>27.6 ± 10.8$^c$</td>
<td>7.0</td>
<td>413 ± 151$^c$</td>
<td>4.8</td>
</tr>
<tr>
<td>9</td>
<td>7.38 ± 2.90$^c$</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>

FDR, false discovery rate; NA, not applicable.
$^a$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.
human study found that subjects possessed similar predose 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 coadministered 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$_{\text{max}}$ and AUC$_{0-24h}$ of CP-I decreased following the letermovir and RIF coadministration, and the authors concluded potential OATP1B induction. However, letermovir is an OATP1B substrate and clinically relevant inhibitor. When letermovir is orally dosed daily at 480 mg, it can cause significant OATP1B inhibition DDIs, resulting in a 3.29-fold AUC increase of the OATP1B probe substrate atorvastatin (letermovir drug label, https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/209939 orgi1s000,209940orgi1s000blf.pdf). Furthermore, the “predose” CP-I level at the beginning of the letermovir-RIF coadministration remained seemingly unchanged compared with that after the coadministration, indicating that induction of OATP1B did not occur (Robbins 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 (from Nett 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% Oatp) (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 OATP1B2) (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 (Karligren 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 (Supplemental Table 2), indicating self-induction of elimination. 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 (Accocella, 1978; Accocella 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 Oatp1b4 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 Oatp1b1 inhibition. Additionally, experimental evidence was provided against the hypothesis of Oatp1b1 induction following PXR activation with a repeated RIF dose regimen. Our results further advocate the usefulness of endogenous biomarkers toward complex enzyme- and transporter-mediated DDIs at the early stage of drug development.

Acknowledgments

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Data Availability

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, Ma, Mok, Murray, Lai.

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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</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>39</td>
</tr>
<tr>
<td>4β-HC</td>
<td>4β-HC-d7</td>
<td>SLE</td>
<td>APCI</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-15N&lt;sub&gt;4&lt;/sub&gt;</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-15N&lt;sub&gt;4&lt;/sub&gt;</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