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Pharmacokinetics and Pulmonary Distribution of Clarithromycin and Rifampicin after Concomitant and Consecutive Administration in Foals

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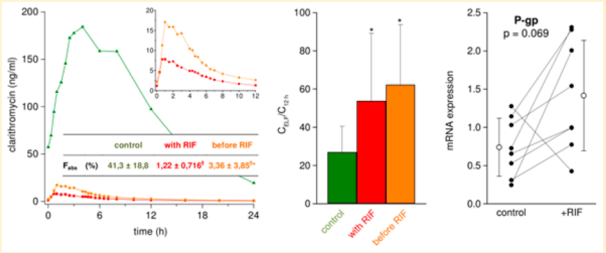
ABSTRACT: Drug interactions often result from multiple pharmacokinetic changes, such as after rifampicin (RIF) and clarithromycin (CLA) in the treatment of abscessing lung diseases. Comedication of RIF may interact with CLA disposition by either induction of presystemic elimination processes and/or inhibition of uptake mechanisms because it regulates gene transcription and modulates function of various CYP enzymes, multidrug efflux and uptake transporters for which CLA is a substrate. To distinguish the transcriptional changes from the modulating interaction components upon CLA absorption and pulmonary distribution, we initiated a repeated-dose study in 12 healthy foals with CLA (7.5 mg/kg, p.o., b.i.d.) in comedication with RIF (10 mg/kg, p.o., b.i.d.) given either concomitantly with CLA or consecutively 4 h after CLA. Affinity of CLA to human P-gp, MRP2, and MRP3 and to OCT1, OCT3, and PEPT1 was measured using Sf9-derived inside-out membrane vesicles and transfected HEK293 cells, respectively. *ABCB1* (P-gp) induction by RIF and affinity of CLA to equine P-gp were studied using primary equine hepatocytes. Absolute bioavailability of CLA was reduced from ∼40% to below 5% after comedication of RIF in both schedules of administration, and T_{max} occurred ∼2−3 h earlier. The loss of bioavailability was not associated with increased 14-hydroxyclarithromycin (14-OH-CLA) exposure. After consecutive dosing, absolute bioavailability and pulmonary penetration of CLA increased ∼2-fold compared to concomitant use. In vitro, CLA showed affinity to human and equine P-gp. Expression of *ABCB1* mRNA was upregulated by RIF in 7 of 8 duodenal biopsy specimens and in primary equine hepatocytes. In conclusion, the major undesired influence of RIF on oral absorption and pulmonary distribution of CLA is associated with induction of intestinal P-gp. Consecutive administration to avoid competition with its intestinal uptake transport results in significantly, although not clinically relevant, improved systemic exposure.



KEYWORDS: *clarithromycin, drug−drug interaction (DDI), foals, P-glycoprotein (P-gp), pharmacokinetics, Rhodococcus equi, rifampicin*

INTRODUCTION

Treatment of patients with ligands of the nuclear pregnane X receptor (PXR) as rifampicin (RIF), carbamazepine, or St. John's wort can significantly influence the efficacy of concomitantly given drugs (e.g., immunosuppressant, hormonal contraceptives, anticonvulsants, cardiac glycosides, oral anticoagulants) by induction of drug metabolizing enzymes and/or drug transporters. In particular, however, the clinical outcome of a drug−drug interaction (DDI) may result from multiple changes in a complex pharmacokinetic network.[1] One example is treatment with the PXR-ligand RIF, which is known to regulate gene transcription of cytochrome P450 (CYP) enzymes (e.g., *CYP3A4*), efflux carriers of the ATP-binding cassette (ABC) transporter family such as P-glycoprotein (P-gp), MRP2 or MRP3, and uptake carriers of the organic anion transporting polypeptide (OATP) family, e.g., OATP1A2.[2−4] Conversely, RIF is a potent inhibitor of P-gp and of intestinal and hepatic OATPs.[5−7] To date, it is unknown whether it modulates further intestinal uptake transporters, such as the organic cation

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transporter OCT1 and/or the peptide transporter PEPT1, or basolateral efflux pumps that may be functionally involved in oral absorption by translocation of the drugs toward portal venous blood (e.g., MRP3).[8] Taken together, RIF interacts with the drug absorption route either by upregulation of presystemic elimination mechanisms (e.g., intestinal and hepatic CYP3A4, intestinal P-gp, and MRP2) or functional absorption carriers (e.g., MPR3) and/or inhibition of intestinal and/or hepatic uptake carriers (e.g., OATP1B1, OATP2B1).

As an example for complex DDIs, we have recently evaluated the combination of RIF plus clarithromycin (CLA) for the treatment of foals with abscessing bronchopneumonia caused by lung infection with *Rhodococcus equi*[9,10] because this protocol was shown to be superior in efficacy to combinations with other macrolides.[11] CLA is a substrate for P-gp and CYP3A4 and might have affinity for MRP2.[12−17] Therefore, we expected that comedication of RIF (10 mg/kg b.i.d., 11 days) may result in major lowering of CLA bioavailability, though not by ∼90% as observed in nine healthy foals.[9,10] Exposure of 14-hydroxyclarithromycin (14-OH-CLA), which is formed by inducible CYP3A4, was not equivalently increased.[9,10,14] Even short-term comedication of 10 mg/kg RIF b.i.d. over 2.5 days (5 doses, 9 foals) lowered the relative bioavailability of CLA by ∼70%.[9,10] These observations indicated relevant PXR-regulated gene expression of *CYP3A4*, *ABCB1*, and *ABCC2* even after short-term RIF exposure. After long-term treatment, however, PXR-type induction was more pronounced. We concluded from these data that RIF may lower intestinal CLA absorption by PXR-type induction of its presystemic elimination (CYP3A4, P-gp) and, additionally, by competition with a to date unknown uptake transporter that substantially contributes to the overall absorption deficit. Data from in vitro and in vivo studies in rats suggested that intestinal OATPs might be involved.[18]

However, there were two limitations in our former pharmacokinetic studies. In the long-term study, RIF comedication was stopped 12 h before the last CLA dosing to avoid competitive interaction with CLA, i.e., the gene transcriptional effect may have been overestimated. Second, in the short-term study, the PXR-type enzyme induction could not be fully excluded as transcriptional changes may occur within just 2.5 days,[19] i.e., the hypothesized competitive effects might be overestimated.

The present study was designed according to the 2012 draft FDA guidance on DDI studies, where a delayed administration of a substrate for, i.e., OATP transporters, is recommended in the case of coadministration with rifampicin. Therefore, as a primary objective to distinguish the transcriptional from the competitive interaction potential of RIF, we initiated a DDI study with repeated-dose administration of CLA in comedication with RIF, which was swallowed either together with CLA (concomitant treatment) or 4 h after the CLA administration (consecutive treatment), i.e., after the expected T_{max} of CLA (randomized, crossover). It seems reasonable to assume that enzyme/transporter induction effects should not be affected by the treatment regime, whereas high intestinal RIF concentrations, as expected in the case of concomitant application of both drugs, are a prerequisite for uptake inhibition. As a second objective, we measured the in vitro affinity of CLA to the intestinal ABC transporters P-gp, MRP2, and MRP3 and to the uptake carriers OCT1, OCT3, and PEPT1. In our former study, we already confirmed that CLA is not a substrate for OATP1A2, OATP2B1, OATP1B1, or OATP1B3.[9]

EXPERIMENTAL SECTION

Chemicals and Reagents. Clarithromycin (CLA) for intravenous administration was purchased as a 500 mg powder from Martindale Pharma (ALTAMEDICS, Cologne, Germany). It was dissolved in sterile water (Aqua ad iniectabilia, B. Braun Melsungen AG, Germany) to produce an injectable solution.

For oral administration, CLA was purchased from 1 A Pharma GmbH (Oberhaching, Germany) in the form of 1 A Pharma 500 mg tablets and rifampicin (RIF) from RIEMSER Arzneimittel AG (Greifswald/Insel Riems, Germany) in the form of Eremfat 600 mg tablets.

For in vitro cell culture assays, the radiolabeled chemicals [^{3}H]-1-methyl-4-phenylpyridinium (MPP^+, 85 Ci/mmol), [^{3}H]-glycyl-sarcosine (Gly-Sar, 29.4 Ci/mmol), and [^{3}H]-clarithromycin (CLA, 80 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA), and unlabeled substrates were purchased from Sigma-Aldrich (Taufkirchen, Germany). The scintillation cocktail *Rotiszint ecoplus* was purchased from Roth (Karlsruhe, Germany).

Study Protocol. *Animals.* The study was performed after approval by the State Agency for Agriculture, Food Safety, and Fishery Mecklenburg-Vorpommern (reference code: 7221.3-1.1-080/12). Twelve healthy warmblood foals (7 males, 5 females, age 42−70 days, body weight 90−140 kg) were included after confirmation of good health by taking a medical history and thorough physical examination, including sonography of the lungs and routine clinical-chemical and hematological screenings. The animals were kept on a natural light rhythm in paddocks together with their mother and had free access to equine milk, hay, oats, and tap water during the whole pharmacokinetic study. All clinical examinations were done in individual stables with straw bedding. On pharmacokinetic study days, an indwelling cannula (Vygon, Aachen, Germany) was placed into a jugular vein for blood sampling (sample volume: 5−7 mL each).

Study Protocol. The drug interaction study with CLA and RIF was performed in four stages over a total study period of 37 days as shown in the flowchart in Figure 1. In the first stage,

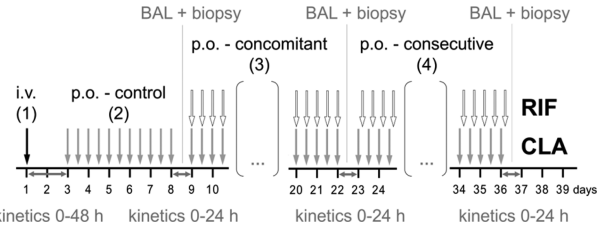

Figure 1. Flowchart of the study, which was performed in 4 stages. Intravenous (i.v.) administration (1) is only followed by blood sample collection (0−48 h). Each oral treatment (2−4) is followed by blood sample collection (0−24 h), bronchoalveolar lavage (BAL, 12 h after the last respective drug administration), and gastroduodenoscopy (biopsy).

pharmacokinetics of CLA after intravenous administration of 7.5 mg/kg was evaluated by venous blood sampling before the injection and after 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 4.33, 4.66, 5, 5.5, 6, 6.5, 7, 8, 10, 12, 16, 20, 24, 36, and 48 h. After that, 7.5 mg/kg of CLA was orally administered twice daily (b.i.d., 12 h intervals) for 34 days (up to the 36th study day).

The second stage of the study comprised 5 days of repeated dose administration with evaluation of the pharmacokinetics of CLA by blood sampling before and 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, and 24 h after the morning administration of CLA on

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the eighth study day. The evening dose of CLA was not given on that pharmacokinetic study day to properly measure CLA elimination.

After that, beginning with the ninth and 23rd study day, the foals received 10 mg/kg RIF b.i.d. either coadministered concomitantly with CLA or consecutively 4 h after the respective CLA dosing. These third and fourth stages of the study were performed according to a randomized crossover design. Pharmacokinetics of CLA and RIF were evaluated after the respective last administration in the morning of the 22nd and 36th study day by blood sampling before the last dosing and after 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 4.33, 4.66, 5, 5.5, 6, 6.5, 7, 8, 10, 12, 16, 20, and 24 h.

Twelve hours after the respective CLA administration in the morning of study days 8, 22, and 36, a gastro-duodenoscopy and a bronchoscopy with bronchoalveolar lavage (BAL) were performed to obtain biopsy specimens from the duodenal mucosa, samples from the epithelial lining fluid (ELF), and bronchoalveolar lavage cells (BALC) as described in a former study.[20] The biopsy specimens as well as aliquots of 5×10^6 BALC and lavage were immediately snap frozen using liquid nitrogen and stored at −80 °C until analysis. Plasma was obtained by centrifugation of the blood samples at 2,000*g* for 10 min and stored at −80 °C until analysis. For oral administrations, RIF and CLA tablets were suspended in 30 mL of tap water and slowly administered in the mouth of the foals using a syringe to ensure complete swallowing by the foals.

Quantitative Assays for Drugs and Biomarkers. *Clarithromycin and Rifampicin.* CLA, 14-hydroxyclarithromycin (14-OH-CLA), RIF, and 25-*O*-desacetyl rifampicin (DAc-RIF) were quantified in plasma, lavage fluid, and BALC using a validated LC-MS/MS method as recently described.[21] The lower limit of quantification for all matrices was 2.5 ng/mL. The within-day accuracy of the assay ranged from −5.5 to 10.9% for CLA, −12.0 to 7.8 for RIF, and −12.3 to 12.1 for DAc-RIF of the respective nominal concentrations, and precision was 0.5 to 8.5%, 0.5 to 12.8%, and 1.0 to 14.1% of means (coefficient of variation), respectively. Between-day accuracy was −6.8 to 5.2% for CLA, −4.6 to 5.5% for RIF, and −6.7 to 2.9% for DAc-RIF of the respective nominal concentrations, and precision was 0.5 to 9.5%, 2.1 to 8.9%, and 2.4 to 13.1% of the respective mean control values. CLA, 14-OH-CLA, RIF, and DAc-RIF concentrations in ELF were assessed by normalizing to the concentration ratio of urea in plasma over bronchoalveolar fluid and in BALC to a mean alveolar cell volume of 1.2 μL/10^6 cells in foals.[22,23] Urea was quantified using the kit LT-UR 0010 (Labortechnik Eberhard Lehmann, Berlin, Germany).

Cholesterol. Cholesterol (CHOL) and 4β-hydroxycholesterol (4β-OH-CHOL) were quantified in equine plasma using LC-MS/MS. The analysis was performed with a PerkinElmer 200 series HPLC system (PerkinElmer instruments, Norwalk, USA) coupled to the API 4000 QTRAP mass spectrometer (AB Sciex, Darmstadt, Germany). In brief, 500 μL of plasma samples diluted with 7% HSA-saline (1:5, v/v for 4β-OH-CHOL, 1:5000, v/v for CHOL) were mixed with 10 μL of the internal standard solution of 100 μg/mL [26.26.26.27.27.27-$^2H_6$]-4β-OH-cholesterol (d6-CHOL), and the sterol esters were saponified with a sodium methoxide ethanol solution (2 M) at room temperature for 20 min. Then, the samples were extracted using *n*-hexane and centrifuged at 4,000*g* at room temperature for 5 min. The supernatant was evaporated to dryness under a gentle nitrogen stream at room temperature. The resulting remnant was reconstituted in 100 μL of the mobile phase and transferred to sample vials of which 30 μL were injected into the chromatographic system. Isocratic chromatography was performed on a Supelco Acsentis C8 column (2.1 × 100 mm; Sigma-Aldrich, Steinheim, USA) temporized at 50 °C using 10% ammonium acetate (5 mM, pH 3.8) and 90% acetonitrile as mobile phase with a flow rate of 300 μL/min. The MS/MS analysis was performed in positive ionization mode by monitoring the following *m*/*z* transitions: *m*/*z* 369.3/161.5, 369.3/147.1, and 369.3/95.2 for CHOL; *m*/*z* 385.2/367.4 and 385.2/109.1 for 4β-OH-CHOL; and *m*/*z* 374.4/249, 374.4/167, and 374.4/152.3 for d6-CHOL.

The method was validated between 5 and 250 ng/mL for 4β-OH-CHOL and between 50 and 1000 ng/mL for CHOL and was shown to possess sufficient precision (between-day: 5.7 to 13.1% for CHOL and 4.0 to 10.1% for 4β-OH-CHOL; within-day: 1.4 to 15.5% for CHOL and 3.4 to 7.7% for 4β-OH-CHOL) and accuracy (between-day: −4.4 to 2.0% for CHOL and −12.4 to −5.1% for 4β-OH-CHOL; within-day: −7.7 to 1.7% for CHOL and −13.2 to −3.1% for 4β-OH-CHOL) during sample analysis as recommended by current guidelines on bioanalytical method validation.

Cells and Cell Culture. Human embryonic kidney 293 (HEK293) cells were purchased from the European Collection of Cell Cultures (Salisbury, United Kingdom) and grown and transfected with human OCT1 or OCT3 as described previously.[24,25] MDCKII cells expressing human PEPT1 were a kind gift from Pfizer (Groton, Connecticut, U.S.A.). Equine hepatocytes plated on collagen-coated 24-well plates with a density of 10^6 cells/well were isolated from the liver of a male foal aged 6 months, which had to be sacrificed after lateral patellar luxation and severe arthrosis of both femoropatellar joints. Hepatocytes were cultured for 2 days using human hepatocyte maintenance medium (HHMM, PRIMACYT Cell Culture Technology GmbH, Schwerin, Germany) before further use.

Analysis of mRNA Expression. For studying P-gp induction by RIF, equine hepatocytes were cultured with HHMM containing 1, 5, 10, 25, or 50 μM RIF for 24 h. RNA was extracted from primary equine hepatocytes using peqGOLD RNAPure (Peqlab, Erlangen, Germany) and from intestinal biopsy specimens and BALCs using the NucleoSpin RNAII Kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. RNA content was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Darmstadt, Germany) as well as the Precision RT all-in-one mix (Primerdesign, Southampton, United Kingdom) for hepatocytes, biopsy specimens, and BALCs, respectively.

Quantitative real-time PCR was performed on a 7900HT qPCR system (Life Technologies, Darmstadt, Germany) using the TaqMan Gene Expression Master Mix (Life Technologies, Darmstadt, Germany) or the PrecisionFAST qPCR MasterMix (Primerdesign, Southampton, United Kingdom). The qPCR assays for equine *ABCB1*, *CYP3A89* (the equine homologue of *CYP3A4*[26]), and *ABCC2* were developed by Primerdesign (Southampton, United Kingdom) using the following reference sequences: *ABCB1*, XM_001492023 (primer sequence: 5′-AGGATGTTCTGTTGGTATTCTCA-3′ and 5′-GACACTT-TGGCTTTGGCATAG-3′); *CYP3A89*, NM_001101651 (primer sequence: 5′-AATCCCTCAGGTTATTCCCAATT-3′ and 5′-CCATCACCACTGTCCCTTTAG-3′); *ABCC2*, XM_001500707 (primer sequence: 5′-ACTTCAATGCCACC-

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AACTATCC-3′ and 5′-CACCTTGTGCTAATCCCAGAG-3′). qPCR assays for *SLCO2B1* (Ec03467681_m1), *18S*rRNA (Hs03003631_g1), and *GAPDH* (Ec03210916_gH) were purchased from Life Technologies (Darmstadt, Germany).

Gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method[27] using the *18S*rRNA or *GAPDH* expression values to calculate the ΔCt values. It has been confirmed by the supplier that the transporter assays and their respective controls have equal amplification efficiencies (±10%). All analyses were performed in duplicate, and Ct values ≥40 were excluded from further analysis.

In Vitro Transport Studies. Membrane transport of [^{3}H]-CLA mediated by human MRP2, MRP3, and P-gp was investigated using inside-out membrane vesicles prepared from Sf9 cells (Life Technologies, Darmstadt, Germany). The experiments were performed as described previously.[24,25] MRP2, MRP3, and P-gp vesicles were incubated with CLA concentrations between 3.16 and 1000 μM (6.7 μCi/ml) for 10 min.

Competition assays were carried out using radiolabeled MPP^+ for human OCT1 and OCT3 and Gly-Sar for human PEPT1 at a final concentration of 1 μM. The respective cells were incubated with CLA and the respective standard substrate (buffer: 142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.5 mM $CaCl_2 \times 2\ H_2O$, 1.2 mM $MgSO_4$, 5 mM glucose, and 12.5 mM HEPES, pH 7.3) for 1 min (OCT1, OCT3) or 5 min (PEPT1) and then washed thrice with ice-cold PBS followed by cell lysis using 800 μL of 0.2% SDS. Uptake of [^{3}H]-CLA by OCT1, OCT3, and PEPT1 was analyzed after incubation with CLA (final concentration: 100 μM, 0.5 μCi/ml) for 1 min (OCT1, OCT3) and 5 min (PEPT1).

For measuring CLA transport by equine P-gp, primary equine hepatocytes were loaded with [^{3}H]-CLA (10 μM, 2 μCi/ml). After 20 min, the cells were washed twice with PBS before incubation with P-gp inhibitors PSC833 (20 μM) and verapamil (50 μM) was initiated. Fifty microliters from each well were sampled for CLA quantification after 15, 30, 60, and 120 min.

All radiolabeled compounds were quantified using the scintillation cocktail Rotiszint ecoplus and the Wallac type 1409 scintillation beta-counter (LKB-Wallac, Turku, Finland).

Biometrical Evaluation. The pharmacokinetics of CLA and RIF at steady-state were evaluated as follows: Maximum (C_{max}) and minimum (C_{min}) plasma concentrations and the time of C_{max} (T_{max}) were taken from the plasma concentration−time curves. The area under the plasma concentration−time curve during an administration interval ($AUC_{0-12\ h}$) was calculated using the trapezoidal rule. Terminal elimination half-life ($T_{1/2}$) was estimated by log−linear regression analysis of the terminal slope. $AUC_{0-\infty}$ after intravenous administration was assessed using the measured data points from the time of administration until the last quantifiable concentration by the trapezoidal formula (AUC_{0-t}) and extrapolated to infinity. Total body clearance (CL_{tot}) was calculated by dose/$AUC_{0-\infty}$, bioavailability (F) by $CL_{tot} \times$ AUC/dose, and the volume of distribution (V_{ss}) by dose × AUMC/AUC^2. Mean ± standard deviation (SD) values are given. Differences between samples were evaluated using the nonparametric Wilcoxon test with $p \leq 0.05$ as the level of statistical significance. Information regarding statistical analysis of presented data is included in the respective picture or table caption.

RESULTS

CLA reached maximum plasma concentrations of 863−6,360 ng/mL after slow intravenous injection of 7.5 mg/kg of body weight. The drug was widely distributed with V_{ss} between 6.67 and 13.8 l/kg and was eliminated with half-lives between 4.59 and 6.85 h. Systemic exposure with its major metabolite 14-OH-CLA accounted for only 1.12−3.83% of the total CLA exposure.

After repeated oral administration at steady-state (7.5 mg/kg b.i.d., 5 days), CLA was slowly but erratically absorbed with bioavailability of 20.2−73.2% and C_{max} of 99.6−425 ng/mL after 1.00−8.02 h. The systemic exposure with 14-OH-CLA was higher as after intravenous dosing. Elimination half-lives, however, were not markedly different (Figure 2, Table 1).

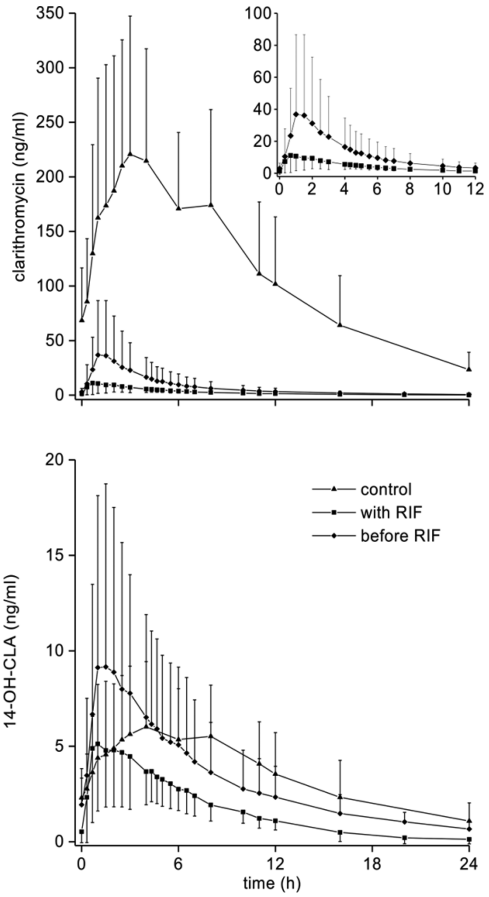

Figure 2. Plasma concentration−time curves of clarithromycin (CLA) and 14-hydroxyclarithromycin (14-OH-CLA) after oral repeated dosing of 7.5 mg/kg b.i.d. for 5 days (control) and after coadministration of RIF (10 mg/kg, b.i.d. for 13 days) orally either together with RIF (with RIF) or 4 h before RIF (before RIF). Mean ± SD values are given.

Both CLA and its major metabolite deeply penetrated into pulmonary compartments and reached manifold higher levels in the ELF (C_{ELF}/C_{12h} ratios: 11.6−54.9 (CLA), 2.05−16.3 (14-OH-CLA)) and BALC (C_{BALC}/C_{12h} ratios: 207−994 (CLA); 24.5−559 (14-OH-CLA)) than in plasma at the same time (Figure 3).

Chronic oral treatment with RIF (10 mg/kg, b.i.d., >13 days) caused an ∼5.8-fold increase of the 4β-OH-CHOL/CHOL plasma ratio (Figure 4A), a surrogate for PXR-type induction of drug metabolizing enzymes and transporter proteins.[28] In primary hepatocytes isolated from one foal, the mRNA expression levels of equine *ABCB1* and *CYP3A89* were upregulated by RIF (Figure 4B). In foals treated with RIF, *ABCB1* was markedly upregulated by ∼2.0-fold in 7 of our 8 foals; one subject showed an extreme opposite outcome (p = 0.069). *ABCC2*, which is known to be regulated via the same

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Table 1. Pharmacokinetic Characteristics of Clarithromycin (CLA) and 14-Hydroxyclarithromycin (14-OH-CLA) in Healthy Foals (n = 12) after Intravenous Injection (7.5 mg/kg, single dose; i.v. injection), Repeated Oral Administration (7.5 mg/kg b.i.d. for 5 days; control), and after Coadministration of Rifampicin (RIF, 10 mg/kg, b.i.d. for 13 days) Swallowed Either Together with RIF (with RIF) or 4 h before RIF (before RIF)

			steady-state after oral treatment		
		i.v. injection	control	with RIF	before RIF
CLA					
AUC[d]	(ng × h/mL)	5,020 ± 1,830	1,980 ± 903[a]	55.3 ± 32.7[a,b]	158 ± 181[a,b,c]
$AUC_{0-4\ h}$	(ng × h/mL)		709 ± 409	37.4 ± 25.4[b]	113 ± 137[b,c]
F_{abs}	(%)		41.3 ± 18.8	1.22 ± 0.72[b]	3.36 ± 3.85[b,c]
C_{max}	(ng/mL)	1,710 ± 1,580	270 ± 116[a]	13.5 ± 10.1[a,b]	40.8 ± 52.3[a,b,c]
C_{12h}	(ng/mL)		102 ± 61.5	1.40 ± 0.67[b]	3.33 ± 3.08[b,c]
T_{max}	(h)		4.21 ± 2.65	1.79 ± 1.87[b]	1.79 ± 0.81[b]
$T_{1/2}$	(h)	5.91 ± 0.76	5.62 ± 0.97	5.26 ± 2.24	6.14 ± 1.90
ELF_{12h}	(μg/mL)		2.77 ± 2.50	0.07 ± 0.05[b]	0.18 ± 0.16[b,c]
$BALC_{12h}$	(μg/mL)		47.0 ± 26.7	0.75 ± 0.85[b]	1.68 ± 1.76[b,c]
14-OH-CLA					
AUC[d]	(ng × h/mL)	96.7 ± 45.5	59.1 ± 31.4[a]	34.3 ± 15.5[b]	61.0 ± 50.3[c]
$AUC_{0-4\ h}$	(ng × h/mL)		19.0 ± 12.7	20.5 ± 11.4	35.6 ± 31.4
AUC[d]-ratio[e]	(%)	1.89 ± 0.75	3.20 ± 1.87[a]	39.1 ± 7.24[a,b]	33.7 ± 8.61[a,b,c]
$AUC_{0-4\ h}$-ratio[e]	(%)		2.98 ± 1.99	35.6 ± 7.14[b]	30.0 ± 8.15[b,c]
C_{max}	(ng/mL)	7.47 ± 3.07	7.20 ± 3.34	6.60 ± 3.34	10.5 ± 9.17
C_{12h}	(ng/mL)		3.55 ± 2.17	1.10 ± 0.48[b]	2.34 ± 1.61[c]
T_{max}	(h)	2.45 ± 1.19	4.37 ± 2.70[a]	2.43 ± 2.20[b]	2.17 ± 0.78[b]
$T_{1/2}$	(h)	8.51 ± 2.65	8.92 ± 4.48	6.22 ± 4.05	8.28 ± 2.66
ELF_{12h}	(ng/mL)		16.9 ± 7.68	9.27 ± 4.97[b]	14.7 ± 7.94
$BALC_{12h}$	(ng/mL)		301 ± 137	39.3 ± 21.5[b]	79.9 ± 73.9[b,c]

[a]Relative to i.v. injection. [b]Relative to control. [c]Relative to that with RIF, $p \leq 0.05$ (Wilcoxon). [d]$AUC_{0-\infty}$ after i.v.-injection; AUC_{0-12h} for steady-state data. [e]$AUC_{14\text{-OH-CLA}}/(AUC_{14\text{-OH-CLA}} + AUC_{CLA})$.

nuclear PXR-receptor mechanism as *ABCB1*, was significantly induced by ∼2.6-fold (p = 0.05). The transporter was upregulated in 6 subjects, and two subjects showed lower expression after RIF (Figure 5). In BALC, mRNA expression of SLCO2B1 tended to be lower after RIF induction (p = 0.074).

As pharmacokinetic outcomes of PXR-induced transcriptional changes, absolute bioavailability of CLA lowered from values of ∼40% in noninduced foals to values below 5% after comedication of RIF in both regimes of administration (CLA with RIF, CLA before RIF). The absorption periods of CLA were apparently earlier terminated by RIF comedication as T_{max} occurred just after ∼2 h compared to ∼4−5 h in the control group without RIF. Accordingly, formation of the metabolite 14-OH-CLA also lasted up to only ∼2 h compared to ∼4−5 h in the absence of RIF. Contrary to the major influence of RIF on CLA absorption, elimination routes for CLA were obviously not markedly influenced as the elimination half-lives of CLA remained unchanged. The dramatic loss in bioavailability was not associated with an adequate increase of the AUC values of 14-OH-CLA; 14-OH-CLA exposure was even significantly lower after concomitant swallowing of CLA and RIF as compared to control. The metabolic ratios ($AUC_{14\text{-OH-CLA}}/(AUC_{14\text{-OH-CLA}} + AUC_{CLA})$), however, increased ∼15-fold after RIF-type enzyme induction (16.6 ± 9.97-times with RIF, 13.9 ± 7.58-times before RIF, $p \leq 0.05$), indicating a substantial increase in CYP3A4-mediated hydroxylation of the bioavailable CLA dose fraction. All changes in CLA disposition were significantly less expressed when RIF was administered 4 h after CLA (∼T_{max} of CLA) instead of giving both drugs concomitantly. Absolute bioavailability was more than 2-times higher when RIF was administered after T_{max} of CLA. Accordingly, C_{max}, C_{12h}, ELF_{12h}, and $BALC_{12h}$ were also significantly increased compared to the concomitant dosage schedule (Table 1, Figure 2).

Penetration of CLA and 14-OH-CLA in pulmonary compartments (ELF, BALC) was lowered after RIF comedication by >90% as expected parallel to the substantial changes in plasma exposure. Additionally, RIF exerted additional influence on the pulmonary distribution of CLA and 14-OH-CLA as the C_{ELF}/C_{12h} ratios increased nearly 2-fold ($p \leq 0.05$) whereas C_{BALC}/C_{12h} remained unchanged and, accordingly, C_{BALC}/C_{ELF} decreased. Concerning the pulmonary penetration of 14-OH-CLA, the C_{ELF}/C_{12h} ratio was not influenced, whereas C_{BALC}/C_{12h} and C_{BALC}/C_{ELF} were lowered. The schedule of swallowing CLA and RIF was not of significant impact; the slight numerical differences are obviously a reflection of the differences in CLA and 14-OH-CLA plasma exposure (Figure 3).

Basic data on pharmacokinetics and pulmonary distribution of RIF are given in Tables 2 and 3. Exposure of RIF and its metabolite DAc-RIF were significantly enhanced when administered 4 h after CLA (Figure 6) and therefore also reached higher levels in the ELF and in BALC than after concomitant treatment. RIF did not accumulate in pulmonary compartments.

By using inside-out membrane vesicles, the affinity of CLA could be confirmed to P-gp (K_m = 0.18 ± 0.07 mmol/L; V_{max} = 2.66 ± 0.36 nmol/mg × min) but not to MRP2 and MRP3 (Figure 7A). There was also no interaction between CLA and OCT1, and OCT3 and PEPT1, respectively, as evaluated in competition and uptake assays using cell models overexpressing the respective membrane transporter (data not shown). The cellular efflux of CLA in primary equine hepatocytes was significantly inhibited by the P-gp inhibitors PSC833 (20 μM) and verapamil (50 μM).

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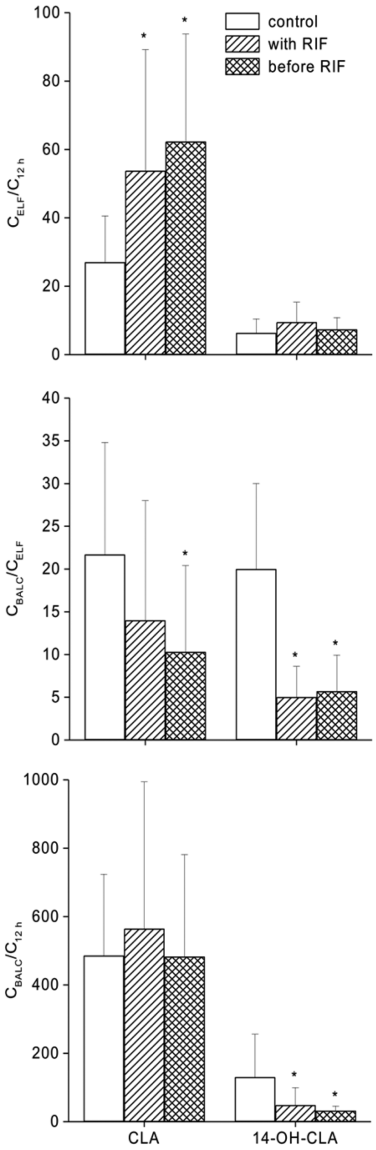

Figure 3. Penetration of clarithromycin (CLA) and 14-hydroxyclarithromycin (14-OH-CLA) in pulmonary compartments at steady state 12 h after the last administration of CLA. Mean ± SD values are given; $*p \leq 0.05$ vs control (Wilcoxon). C_{12h}, plasma concentration; C_{ELF}, concentration in the bronchial epithelial lining fluid; C_{BALC}, concentration in bronchoalveolar lavage cells.

DISCUSSION

The treatment of foals with abscessing bronchopneumonia by RIF in combination with CLA was found to be superior in efficacy protocols.[11] Contrary to that retrospective observation, we have now repeatedly shown that comedication of RIF leads to critical lowering of the CLA plasma concentrations with respect to the MIC_{90} for equine pathogens, including *Rhodococcus equi*.[9,10,29] In our present study, serum concentrations of CLA fall below the MIC_{90} of 0.5 μg/mL both after concomitant and consecutive RIF administration. This observation is most likely caused by the lower absolute bioavailability of the administered dosage form used in the present study. Direct chemical interference between the drugs can be excluded as pharmacokinetics of RIF are nearly unaffected. The deficit can also not be plausibly explained by metabolic "first-pass" elimination as exposure with 14-OH-CLA nearly remained unchanged, whereas

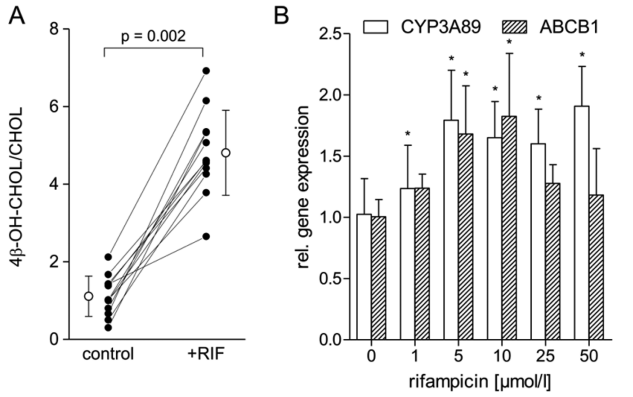

Figure 4. (A) Plasma ratios of 4β-hydroxycholesterol (4β-OH-CHOL) over cholesterol (CHOL) after monotherapy with 7.5 mg clarithromycin b.i.d. for 5 days (control) and after enzyme induction with 10 mg/kg rifampicin b.i.d. for ≥13 days (+RIF: mean of with RIF and before RIF). (B) Relative mRNA expression (reference gene: *GAPDH*) of *CYP3A89* and *ABCB1* (P-gp) in equine hepatocytes after incubation with 1, 5, 10, 25, and 50 μM rifampicin for 24 h. Mean ± SD values are given; $*p \leq 0.05$ vs DMSO control (one-way ANOVA with Dunnett's multiple comparisons test).

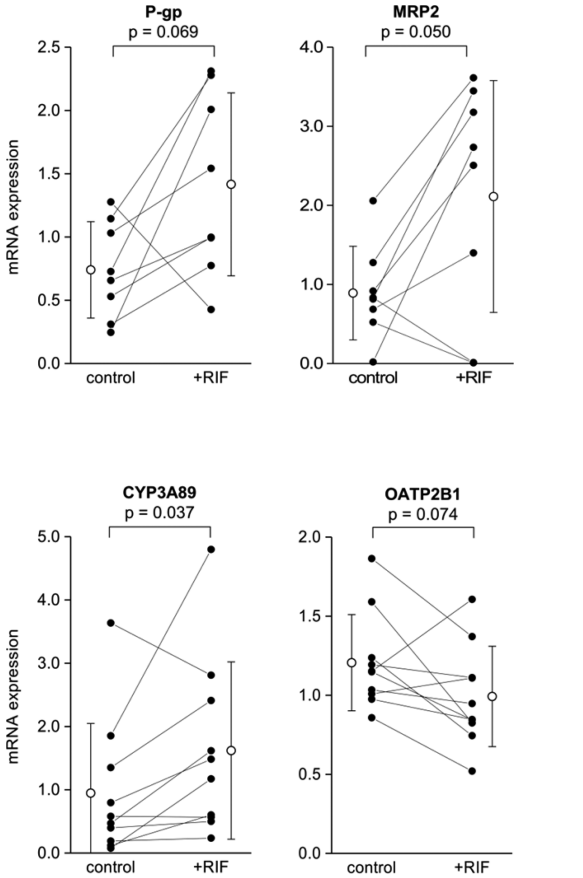

Figure 5. Relative mRNA expression (reference gene: *18S*) of *ABCB1* (P-gp) and *ABCC2* (MRP2) in duodenal biopsy specimens (above) and of *CYP3A89* and *SLCO2B1* in bronchoalveolar lavage cells (below) before (control) and after PXR-type induction with 10 mg/kg rifampicin b.i.d for ≥13 days (+RIF: mean of with RIF and before RIF).

the ^{14}C(R)-hydroxylation rate of the bioavailable CLA ($AUC_{14\text{-OH-CLA}}/(AUC_{14\text{-OH-CLA}} + AUC_{CLA})$), a surrogate for *CYP3A4* induction, was increased more than 10-fold. Finally, RIF

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Table 2. Pharmacokinetic Characteristics of Rifampicin (RIF) and 25-*O*-Desacetyl Rifampicin (DAc-RIF) after Repeated Dosing of 10 mg/kg Rifampicin b.i.d. for 13 Days Swallowed Together with Clarithromycin (CLA, with CLA) or 4 h after CLA (after CLA)

		with CLA	after CLA
RIF			
AUC	(μg × h/mL)	77.0 ± 13.3	110 ± 36.3[a]
C_{max}	(μg/mL)	8.20 ± 1.35	11.4 ± 4.06[a]
C_{12h}	(μg/mL)	5.39 ± 1.03	9.98 ± 3.33[a]
T_{max}	(h)	3.89 ± 2.57	4.17 ± 2.32
$T_{1/2}$	(h)	11.5 ± 3.02	6.95 ± 1.77[a]
ELF_{12h}	(μg/mL)	3.12 ± 1.47	6.65 ± 3.13[a]
$BALC_{12h}$	(μg/mL)	2.10 ± 0.85	3.56 ± 1.83[a]
DAc-RIF			
AUC	(μg × h/mL)	1.01 ± 0.51	2.41 ± 1.76[a]
AUC-ratio	(%)	1.39 ± 0.76	2.06 ± 1.04[a]
C_{max}	(μg/mL)	0.11 ± 0.05	0.26 ± 0.19[a]
C_{12h}	(μg/mL)	0.08 ± 0.05	0.25 ± 0.19[a]
T_{max}	(h)	6.12 ± 3.64	6.19 ± 1.76
$T_{1/2}$	(h)	5.49 ± 1.13	5.40 ± 0.94
ELF_{12h}	(μg/mL)	0.09 ± 0.04	0.16 ± 0.09[a]
$BALC_{12h}$	(μg/mL)	0.38 ± 0.20	0.60 ± 0.31

[a]Relative to the with CLA value, $p \leq 0.05$ (Wilcoxon).

Table 3. Ratios of Rifampicin (RIF) and 25-*O*-Desacetyl Rifampicin (DAc-RIF) 12 h after the Respective Last Administration of Clarithromycin (CLA, 7.5 mg/kg BW b.i.d. for 13 days) in Epithelial Lining Fluid (ELF) to Plasma, Bronchoalveolar Lavage Cells (BALC) to Plasma, and BALC to ELF after Swallowing of Rifampicin (RIF, 10 mg/kg BW b.i.d. for 13 Days) Either Together with CLA (with CLA) or 4 h after CLA (after CLA)

	with CLA	after CLA
RIF		
ELF/plasma	0.60 ± 0.30	0.68 ± 0.23
BALC/plasma	0.39 ± 0.14	0.34 ± 0.09
BALC/ELF	0.79 ± 0.44	0.61 ± 0.38
DAc-RIF		
ELF/plasma	1.21 ± 0.70	0.90 ± 0.56
BALC/plasma	5.47 ± 2.74	3.62 ± 2.87[a]
BALC/ELF	5.32 ± 3.35	4.15 ± 2.17

[a]Relative to the with CLA value, $p \leq 0.05$ (Wilcoxon).

did not influence systemic elimination of CLA as the terminal half-life remained unchanged. Small changes in elimination rate, however, cannot be excluded as distribution volume of CLA might have been increased after RIF comedication, e.g., by relative accumulation in the ELF.

So far, there is convincing evidence that induction of intestinal efflux contributes to a major extent to the major undesired DDI: First, CLA has affinity for human and equine P-gp in vitro as shown by our transport experiments using inside-out membrane vesicles and primary equine hepatocytes as well as by literature data.[12,15] Second, affinity to MRP2 could not be confirmed in our in vitro study. Third, RIF is known to regulate gene transcription of human multidrug efflux transporters of the ABC-transporter family via the nuclear PXR-receptor pathway.[30−32] As a surrogate for RIF-mediated PXR-type enzyme induction,[28] the metabolic ratio of 4β-OH-CHOL/CHOL was, as expected, manifold elevated by RIF. In addition, we confirmed that RIF can induce

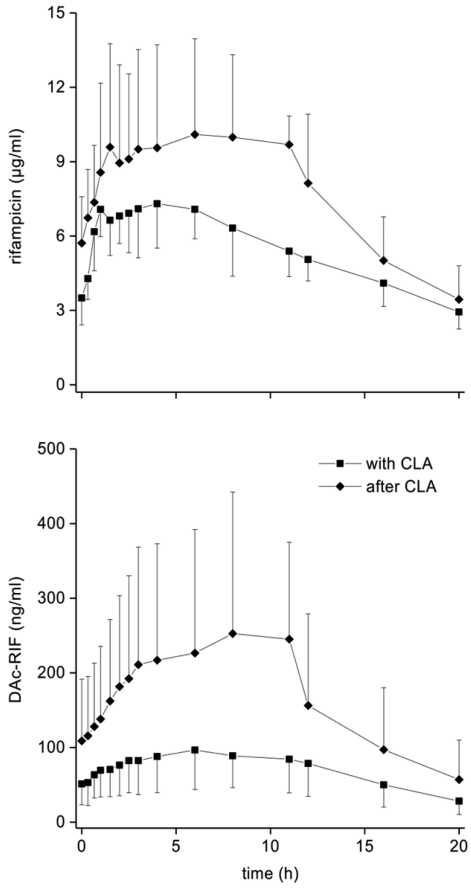

Figure 6. Plasma concentration−time curves of rifampicin (RIF) and 25-*O*-desacetyl rifampicin (DAc-RIF) after repeated dosing of 10 mg/kg rifampicin b.i.d. for 13 days swallowed together with clarithromycin (with CLA) or 4 h after CLA (after CLA). Mean ± SD values are given.

mRNA expression of *ABCB1* and *CYP3A89* in primary equine hepatocytes. Fourthly, equine duodenal mRNA expression of *ABCB1* (and *ABCC2*) was ∼2−3-fold upregulated in most foals, and finally, the relative absorption deficit of >90% for CLA was extremely strong[9,10] as compared to the results of DDI studies with PXR ligands and other probe drugs for intestinal P-gp in men. Thus, RIF, carbamazepine, and St. John's wort induced human duodenal *ABCB1* mRNA expression up to 8.3-fold and, in turn, lowered bioavailability of the nonmetabolized probe drugs digoxin and talinolol absolutely by ∼10−20% and relative to pretreatment data only by ∼15−35%.[33−38] Relative bioavailability of the nonmetabolized fexofenadine was only decreased by ∼45% after RIF.[39]

Therefore, as an alternative method for how RIF may have lowered CLA absorption, we evaluated inhibition of (an) intestinal uptake transporter(s). The involvement of an intestinal uptake carrier in CLA absorption has been already concluded from the results of a former short-term, "non-inductive" interaction study with five doses of 10 mg/kg RIF over 2.5 days. The ∼70% absorption deficit in that study, however, clearly overestimates the function of the uptake carrier because substantial upregulation of PXR-regulated proteins could not be avoided by the shortest treatment necessary to achieve pharmacokinetic steady-state conditions.[9] Maximum upregulation of *ABCB1* gene transcription by RIF-PXR signaling occurs just within ∼6 h, and protein synthesis is only slightly delayed (∼12 h).[19,40] In line with that, re-evaluation of the previous data

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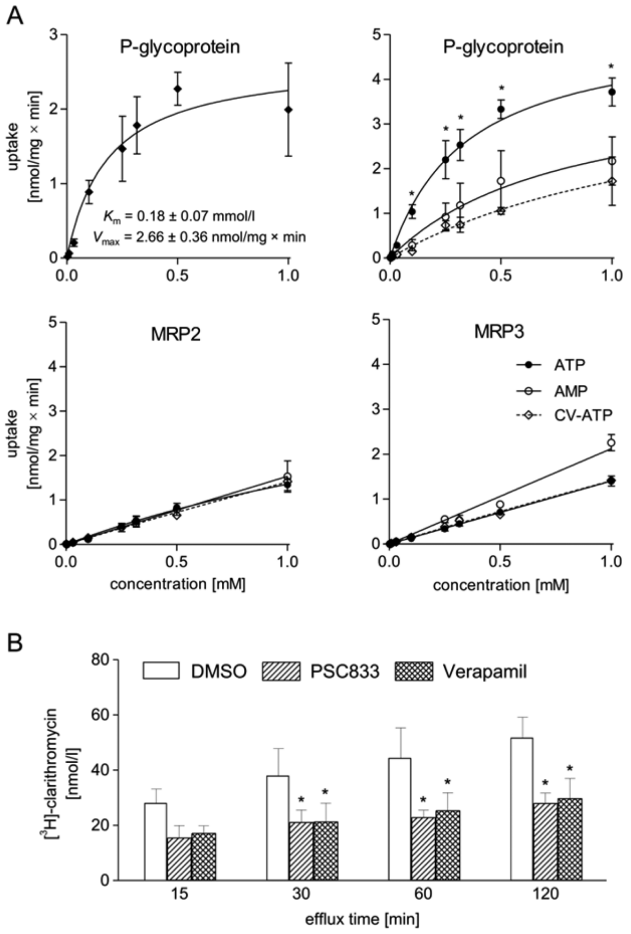

Figure 7. (A) Uptake of [^{3}H]-clarithromycin (CLA) in inside-out membrane vesicles overexpressing human P-glycoprotein (P-gp), MRP2, MRP3, or control (CV) in the presence and absence of ATP. MRP2, MRP3, and P-gp vesicles were incubated for 10 min with CLA concentrations between 3.16 and 1000 μM (6.7 μCi/ml). The difference curve for P-gp (ATP vs CV-ATP) with kinetic data for the Michaelis–Menten plot are given in the upper left diagram. Mean ± SD values are given; *$p \leq 0.05$ vs CV-ATP (two-way ANOVA with Bonferroni post-test). (B) Efflux transport of [^{3}H]-CLA out of equine hepatocytes. The cells were preincubated with CLA (10 μM, 2 μCi/ml) for 20 min before efflux was measured after 15, 30, 60, and 120 min in the absence (DMSO) or presence of the P-gp-inhibitors PSC833 (20 μM) and verapamil (50 μM). Mean ± SD values are given.; *$p \leq 0.05$ vs DMSO (two-way ANOVA with Bonferroni post-test).

has shown that the metabolic ratio for CLA ($AUC_{14\text{-OH-CLA}}$/($AUC_{14\text{-OH-CLA}}$ + AUC_{CLA})) was ~2.5-fold increased (compared to ~14–17-fold in the present study), a clear surrogate for significant PXR-type induction after just a short-term treatment of 2.5 days.[9]

In general, however, the issue is much more complex as RIF on its own can inhibit uptake carriers such as OAT1/3 or OATPs[6,7,41] and the efflux transporters P-gp and MRP2.[42−44] Therefore, in our current crossover study, we administered RIF and CLA either with a dosing interval of 4 h in-between (at approximately the T_{max} of CLA) to measure the "undisturbed" inducing effects of RIF alone, and in the alternative study period, we gave both drugs concomitantly to evaluate the additional inhibitory effects of the inducer. In the scenario that RIF inhibits efflux carriers such as P-gp, we would expect that CLA is better absorbed if RIF is simultaneously present at the absorbing site along the gastrointestinal tract (concomitant administration). Contrary to the assumptions, however, concomitant dosing resulted even in a lower absorption than observed for administration with the 4 h interval. Therefore, RIF must have modulated the intestinal uptake of CLA, which explains the "excess" absorption deficit in addition to the deficit caused by induction of intestinal P-gp. That modulation, however, only contributes a minor extent to the overall absorption deficit, by our estimation, less than 5%. Unfortunately, we could not confirm by in vitro experiments that CLA is a substrate for any of the intestinal uptake transporters known to be relevant in drug absorption, including OATP2B1, OCT1, OCT3, and PEPT1. Also, MRP3 that might be involved in basolateral efflux to the portal venous blood[45,46] was not a transporter for CLA. Further research is needed to discover the intestinal uptake mechanism for macrolide antibiotics.

A second major result in our DDI study was that CLA penetrates better in the ELF after comedication of RIF independent of the time point of administration (Figure 3). In noninduced foals, we have repeatedly shown that CLA manifold accumulates in the bronchial ELF,[9,10] most likely caused by active, ATP-consuming efflux via P-gp,[12,15] which was localized to the apical membrane of bronchial epithelial cells.[47−50] Additional enrichment in the ELF after RIF, however, cannot be plausibly explained by *ABCB1* induction. Although RIF reaches PXR-regulating in vitro concentrations of ~4–8 μg/mL (5–10 μM) in plasma and in the ELF,[9,10,30,51−53] *ABCB1* mRNA expression was not affected in BALCs ($p > 0.5$) as already shown in our previous study demonstrating unaltered or even reduced *ABCB1* mRNA levels in BALCs and bronchial epithelial cells.[10] Relative accumulation of CLA in the ELF in the presence of RIF can also be explained by P-gp-mediated CLA transport, assuming higher transporter efficiency at lower CLA concentrations or inhibition of an apical bronchial uptake carrier that mediates bronchial rediffusion of CLA. Future research is needed to evaluate the carrier that might be the same as that involved in intestinal absorption of CLA.

As a third major finding, penetration of RIF into pulmonary compartments was not impaired by the dosing schedule of CLA. The drug and its microbiologically acting metabolite DAc-RIF[54] reached even significantly higher exposure in the ELF and the BALCs when administered 4 h after CLA. Obviously, consecutive dosing avoids some inhibitory effects of CLA on RIF absorption and metabolism as RIF exposure (AUC) and the metabolic ratio ($AUC_{DAc\text{-}RIF}$/(AUC_{RIF} + $AUC_{DAc\text{-}RIF}$)) were significantly increased in the absence of CLA during the absorption of RIF. This likely reflects the DDI of CLA with RIF with a to date unknown intestinal uptake mechanism for both drugs and/or can be explained by the fact that RIF is an OATP1B1 substrate whereas CLA is an inhibitor. In any case, however, the concentrations of unbound RIF in plasma and in all pulmonary compartments at steady state were in excess of the MIC_{90} for equine pathogens as β-hemolytic *Streptococci* (<0.5 μg/mL), *Staphylococcus spp.* (1 μg/mL), *Pasteurella spp.* (1 μg/mL), and *Rhodococcus equi* (<0.5 μg/mL).[29] Conversely, the undesired DDI with respect to the microbiologically active concentrations of CLA in equine blood and lung could not be avoided by consecutive dosing of the drugs. The average concentrations of CLA in the ELF, which were in the absence of RIF (~2.8 μg/mL), clearly above the MIC_{90} for *Rhodococcus equi* and other most common equine pathogens, dropped down to inactive levels in the presence of RIF (0.07 μg/mL and lower).

In conclusion, the major undesired influence of RIF on the disposition of CLA in foals is mainly caused by the absorption

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deficit due to intestinal *ABCB1* induction. In contrast, accumulation of CLA in the lungs seems to be independent of P-gp interactions. Consecutive administration to avoid competition with intestinal uptake results in significantly, although not clinically relevant, improved systemic exposure.

LIMITATIONS OF THE STUDY

First, we are aware of the low statistical power in our small sample of healthy foals that could be included in the invasive part of the study in an ethically and economically reasonable manner. We are also aware of the potential experimental limitations of endoscopic tissue sampling in the environment of a breeding establishment. *ABCB1* was markedly upregulated in 7 subjects; one subject showed an extreme opposite outcome. *ABCC2*, which is known to be regulated via the same nuclear PXR-receptor mechanism, however, was induced in 6 subjects, whereas 2 subjects showed lower expression after RIF. We concluded that RIF can regulate equine intestinal *ABCB1* in vivo, as both *ABCB1* and *ABCC2* were upregulated in most of the foals that were included in our clinical drug interaction study. The uncertainty as caused by the statistical limitations of the descriptive clinical study was further minimized by additional in vitro data confirming mRNA upregulation of equine *ABCB1* in primary hepatocytes. Second, the foals in the present study were treated with CLA tablets of lower absolute bioavailability compared to the CLA suspension administered in our previous studies.[9,10] Therefore, absolute data on absorption and distribution of CLA could not be directly compared with the results of our previous studies. Third, all in vitro assays, except the primary equine hepatocytes, were performed with human multidrug transporters. Therefore, these data can be translated to the in vivo situation in foals under consideration of the known species-related differences. However, horses exhibit higher protein homology to the respective human drug transporter analogues than rodents. For example, equine OATP1A2 and -2B1 are also highly conserved (mRNA homology compared to human genes: OATB1A2, 88%; OATP2B1, 84%).[9,10] P-gp, MRP1, MRP2, and BCRP, at least, are regulated at a transcriptional level.[55,56]

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ABCB1, P-glycoprotein (P-gp); *ABCC2*, multidrug resistance protein 2 (MRP2); ATP, adenosine triphosphate; AUC, area under the plasma concentration−time curve; AUMC, area under the first moment curve; BAC, bronchoalveolar cells; BALC, bronchoalveolar lavage cells; b.i.d, bis in die/twice a day; CHOL, cholesterol; CLA, clarithromycin; CV, control vesicle; DAc-RIF, 25-*O*-desacetyl rifampicin; DDI, drug−drug interaction; ELF, epithelial lining fluid; Gly-Sar, glycyl-sarcosine; MIC_{90}, minimal inhibitory concentration required to inhibit 90% of growth; MPP^+, 1-methyl-4-phenylpyridinium; OATP, organic anion transporting polypeptide; PEPT1, peptide transporter 1; PXR, pregnane X receptor; RIF, rifampicin; 14-OH-CLA, 14-hydroxyclarithromycin; 4β-OH-CHOL, 4β-hydroxycholesterol

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