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Effect of ritonavir on pharmacokinetics of antimalarial drug combinations in rats

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ABSTRACT

The current treatment for Human Immunodeficiency Virus (HIV) patients coinfected with malaria involves the coadministration of antimalarial and antiretroviral (ARV) drugs. The World Health Organization (WHO) recommends artemisinin-based therapy for malaria that usually consists of artemether, artesunate or dihydroartemisinin with non-artemisinin derivatives such as amodiaquine, lumefantrine and mefloquine. Protease inhibitors (PI) such as ritonavir contribute to the improved health of HIV-positive individuals, and the inclusion of ritonavir in antiretroviral regimens is common in clinical practice. Ritonavir is a potent inhibitor of human CYP3A4, which is the primary enzyme involved in the metabolism of many of artemisinin-based drugs, as well as amodiaquine and proguanil. Upon co-administration, ritonavir can potentially influence the metabolism and thus increase the systemic exposure of these drugs. In order to understand this pharmacokinetic (PK) drug interaction, the current work evaluated the effect of ritonavir (50 mg/kg orally) on the PK of antimalarial drug combinations in Sprague Dawley (SD) rats. When co-administered with ritonavir, the exposure (AUC) of the antimalarial drugs artemether, artesunate and proguanil was increased by approximately 3.5-fold. Correspondingly, peak plasma concentrations (C_{max}) of these drugs increased as well. There was no apparent influence of ritonavir on the PK of lumefantrine, amodiaquine and atovaquone. This study demonstrates the potential influence of ritonavir on the pharmacokinetics of at least some antimalarial drugs, likely a result of inhibition of CYP3A. Further evaluation of clinically relevant drug interaction in humans may be warranted to ensure safe and effective use of anti-malarial and anti-HIV drugs concomitantly.

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INTRODUCTION

Approximately 70% of the world's HIV-infected population lives in SSA (Sub Saharan Africa), where it is estimated that approximately 350 million people are exposed to infection with malaria (World Health Organization, 2016). In these regions, it is a common medical practice to administer anti-malarial combination drugs as well as anti-retroviral therapies concomitantly to treat both infections. As concurrent management of HIV and malaria infections necessitates the use of multiple drug combinations, there is a potential for drug interactions and overlapping toxicities (Dooley *et al.*, 2008). Evaluation of

potential drug interactions is critical to ensure safe and effective treatment options for both infections and to guide any dosage adjustments that may be necessary.

Even in developing countries, access to ARV's and ACT's (artemisinin-based combination therapy) is increasing; however, limited information is available on drug interactions involving these drug combinations (Giao and Vries, 2001). Therefore, studies evaluating the potential interactions between protease inhibitors and these anti-malarials are important. *In vitro* systems are routinely used to predict drug interactions, but the results may sometimes fail to predict the magnitude of *in vivo* interactions accurately since these are not dynamic systems and have limitations in representing physiology and tissue concentrations. Rat is a widely used animal species in drug discovery for early assessment of drug metabolism and pharmacokinetics; however, there are differences with regards to isoform composition, expression and catalytic activities of drug-metabolizing enzymes between rat and human. Cytochrome P450 (CYP) enzymes are involved in the majority of phase 1 oxidative metabolism of drugs. In humans, CYP3A4/5 is involved in the metabolism of nearly 70% of marketed drugs. CYP3A1, 3A2, 3A9, 3A18, 3A23 and 3A62 enzymes are the CYP3A isoforms present in rats, while in humans CYP3A4 and 3A5 are the primary CYP3A drug-metabolizing isoforms (Martignoni *et al.*, 2006). Rat CYP3A1/A2 are the main CYP3A enzymes in the liver that is analogous to human CYP3A4 and the majority of ACTs, amodiaquine, lumefantrine and proguanil metabolism is carried out by CYP3A4. Sprague Dawley rats have previously been used to study ritonavir-mediated drug interactions wherein the victim drug was primarily metabolized by CYP3A4 in humans (Neearti and Chenna, 2012). The *in vivo* rat screening model has been used to predict pharmacokinetic drug interactions using CYP3A4 substrates (Mandlekar *et al.*, 2007) and this model can provide insights into the likelihood of drug interactions involving ritonavir and anti-malarial drugs.

In HIV treatment, protease inhibitors (PI) are used in highly active antiretroviral therapy regimen (HAART). Ritonavir is an antiretroviral drug from the PI class used to treat HIV infection and AIDS. Ritonavir is metabolized primarily by CYP3A4 and CYP2D6; however, it is also an inhibitor of CYP3A4 (Eagling *et al.*, 1997). The modulation of CYP3A4 by ritonavir is complex and involves competitive inhibition, mechanism-based inhibition, as well as induction (Rock *et al.*, 2014). Additionally, ritonavir is also an inhibitor of P-glycoprotein (Pgp). Amongst the available protease inhibitors, ritonavir potentially has the highest likelihood of causing drug interactions (Moltke *et al.*, 1998). In view of the impact of ritonavir on CYP3A activity, its use in combined antiretroviral-antimalarial therapy may impact the pharmacokinetics of antimalarials metabolized by CYP3A4, particularly the ACT's (Khoo *et al.*, 2005). The aim of the present investigation was to study the influence of ritonavir on the pharmacokinetics of orally administered antimalarial drug combinations in rats.

MATERIALS AND METHODS

Artemether, artesunate, lumefantrine, amodiaquine were provided as gift samples from IPCA (The Indian Pharmaceutical Combine Association Limited), Mumbai. Proguanil and atovaquone were obtained from Glenmark Pharmaceuticals Limited, ritonavir and lopinavir were provided as gift samples from Aurobindo Pharma, Hyderabad. HPLC grade methanol, acetonitrile and ammonium acetate were procured from Merck Chemicals, Mumbai.

Pharmacokinetic drug interaction study in normal rats

Male Sprague Dawley rats of 7-8 weeks of age weighing between 180-210 g were used for the studies. The rats were obtained from the animal unit, Glenmark Pharmaceuticals Limited (Navi Mumbai, India) on the day of the study where they were housed under standard laboratory conditions at an ambient temp of 25 $\pm 2^{\circ}$C, a relative humidity of 40-70%, with a 12-hour light/12-hour dark cycle. They were fed a standard pellet diet (Altromin, Germany) and water *ad libitum*. Prior approval for conducting the experiments in rats was obtained from Glenmark Institutional Animal Ethics Committee (GRC/NCE/PK/OSD/R/2016/01)

Rats were randomized into six groups, with six animals in each group. All animals within a given cage (n=3) were identified with temporary ink during the acclimatization period, before randomization and grouping. Cages were identified by a cage-card containing information including the study number, group, animal number and treatment identification. Human oral therapeutic doses were converted to rat-equivalent doses based on body surface area (FDA, 2005) by multiplying the recommended human dose in mg/kg for each drug by 6.2 to derive the dose of a drug in mg/kg to be administered to the rat. For these calculations, a default human body weight of 60 kg and a rat bodyweight of 200 g were used for all calculations. This approach was used for all of the evaluated drugs except for artesunate and artemether, where higher doses (approximately 3-fold higher) wer administered to obtain quantifiable

plasma concentrations due to sensitivity limitations of the LC-MS/MS assay. Six animals in each group were orally administered a single dose of the drug combinations and doses listed in Table 1.

Drug administration and sample collection

The required amount of drugs (antimalarial and ritonavir) were weighed and placed in two separate mortars, the required amount of Tween 80 (1.5%) was added as a wetting agent and triturated well until the mixture was wet and then 0.5% of methylcellulose suspension was added via gravimetric dilution and the mixture triturated to uniformity. The resulting suspension was administered to the respective groups of animals via oral gavage (Diehl *et al.*, 2001). Individual doses were calculated from each animal's body weight recorded just prior to drug administration. Ritonavir was administered 2hr prior to antimalarial drug administration to coincide with the expected ritonavir T_{max} (Kempf *et al.*, 1997) to maximize the potential for competitive inhibition. Blood samples were collected by retro-orbital puncture at 0.08, 0.25, 0.5, 1, 1.5, 2.5, 3, 4, 5, 24, 48, 96, 120 h in heparinized collection tubes. Plasma was separated by centrifugation at 4000 rpm for 10 min and stored at -80 °C until liquid chromatography/mass spectrometry (LC/MS-MS) analysis.

Analysis of drug concentrations

Stock solutions of antimalarial drugs were prepared by dissolving each of the accurately weighed reference compounds in methanol. For calibration standard and quality control sample preparation, 2.5 μL of antimalarial drug working solutions was spiked in 47.5 μL of blank plasma, 25 μL of IS (internal standard) (2 μg/mL) was added and vortex mixed. For the study samples, a 50 μL aliquot was mixed with 25 μL of IS (2 μg/mL). Lopinavir was used as the IS for artesunate, artemether, lumefantrine, amodiaquine, proguanil and losartan for atovaquone (Table 2).

Calibration standards, quality control and study samples were then extracted with 0.75 mL of ethyl acetate. Samples were vortexed and centrifuged at 15000 rpm for 3 min, and 0.6 mL of supernatant was harvested and further evaporated under N_2 gas to dryness. The residue was reconstituted with 300 μL of mobile phase A (90% ACN, 10% 2mM ammonium acetate pH 3.5) and then samples were injected on LC/MS/MS for analysis.

Ritonavir and antimalarial drug concentrations from plasma samples were measured using an LC-MS/MS method (Mishra *et al.*, 2012; Xing *et al.*, 2011) using a Shimadzu LC 20 AD system (Kyoto, Japan) coupled to an API 3200 Q trap mass spectrometer (Applied Biosystems/MDS Sciex, US) with a Turbo Ion Spray ionization (ESI) interface. The fragmentation transitions for the multiple-reaction monitoring (MRM) are as per Table 2. Chromatographic separation was achieved on a Gemini C18 column (50 $\times$ mm i.d, 5 μm; Phenomenex, CA, USA) at a column temperature of 40°C. Mobile phase A consisted of acetonitrile and 2mM ammonium acetate (pH 3.5) (90:10, v/v) and mobile phase B 0.1% (v/v) formic acid, delivered isocratically at a flow rate of 0.7 mL/min for quantitation assays of artesunate, artemether, lumefantrine, ritonavir, atovaquone and proguanil and 0.6 mL/min for amodiaquine.

Positive ion multiple reaction monitoring was utilized, and the details of multiple reaction monitoring (MRM) used for the various drugs are presented in Table 2. During the development of the analytical methods for quantitation of artemether and artesunate, the ammonium adducts of these two drugs exhibited higher intensity in response than the protonated molecular ion $[M+H]^+$ and thus were utilized for quantification. Calibration curves were plotted considering the area ratio of analyte to internal standard versus the concentration of an analyte, and fit utilizing a weighted ($1/x^2$) least-squares linear regression model. The concentration of study samples were interpolated from the calibration curve using the Analyst software version 2.0.

Pharmacokinetic and statistical analysis

The plasma concentration profiles versus nominal sampling times of each experimental drug were analyzed by non-compartmental analysis (NCA) using Phoenix WinNonlin® (Version 8.1). The peak plasma concentration (C_{max}) and time-to-peak concentration (T_{max}) were obtained from the individual animal plasma concentration-time raw data. The half-life of plasma drug elimination ($T_{1/2}$) was the ratio of 0.693 to the slope obtained by log-linear regression of the terminal phase of the drug plasma profile, and a minimum of three points were used for the determination of k_{el} (elimination rate constant). The area under the concentration-time curve to last quantifiable concentration (AUC_{0-t}) and area under the concentration-time curve to infinite time ($AUC_{0-\infty}$) were calculated by the linear/log trapezoidal rule. The oral plasma clearance (CL/F) was calculated from the dose/AUC. The apparent oral volume of distribution (Vz/F) was calculated using dose, AUC and k_{el}. The pharmacokinetic parameters were expressed as mean $\pm$ SD. The PK parameters of antimalarial drug concentrations alone (control group) were compared to antimalarial drug concen-

Table 1: ***Experimental Design***

Group No.	Drug combinations	Dose (mg/kg)	Dose Conc (mg/mL)	Dose Volume (mL/kg)
1	Artemether+Lumefantrine	100+85	20/17	5
2	Artemether+Lumefantrine+Ritonavir	100+85+50	20/17/10	5
3	Artesunate+Amodiaquine	120+95	24/19	5
4	Artesunate+Amodiaquine+ Ritonavir	120+95+50	24/19/10	5
5	Atovaquone+Proguanil	45+20	10/5	5
6	Atovaquone+Proguanil+ Ritonavir	45+20+50	10/5/10	5

Table 2: ***Summary of analytical parameters***

Drug	Internal Standard	MRM (Drug)	MRM (IS)	Ionisation mode
Artemether*	Lopinavir	316.234/267.3	629.504/155.3	Positive
Lumefantrine	Lopinavir	528.084/510.20	629.504/155.3	Positive
Artesunate*	Lopinavir	402.08/267.2	629.504/155.3	Positive
Amodiaquine	Lopinavir	356.155/283.2	629.504/155.3	Positive
Atovaquone	Losartan	365.062/171	421.14/126.9	Negative
Proguanil	Lopinavir	254.09/170.2	629.504/155.3	Positive

*MRM: Multiple reaction monitoring; * NH4 Adducts*

Table 3: ***Summary of LC-MS/MS method***

Drug	Range Calibration curve (ng/ml)	Retention time (analyte)	Retention time IS
Artemether	5.03 - 5026.6	1.68	1.61
Artesunate	2.20 - 2095.1	0.77	0.85
Lumefantrine	10.13 - 9643.7	0.35	0.73
Amodiaquine	10.62 - 10112.1	0.34	1.41
Atovaquone	10.76 – 5126.0	2.71	1.55
Proguanil	10.61 - 10108.2	0.29	0.75
Ritonavir	10.24 - 9751.6	0.70	0.75

Table 4: ***Mean pharmacokinetic parameters of artemether-lumefantrine combination following its oral administration of 100 (artemether) and 85 (lumefantrine) mg/kg with and without ritonavir (50 mg/kg) in SD rats***

Mean PK Parameters	Unit	Artemether Alone	Artemether with Ritonavir	Lumefantrine Alone	Lumefantrine with Ritonavir
Tmax*	(hr)	0.17 (0.08-1.5)	0.25 (0.08-0.25)	4 (4-5)	4 (3-4)
Cmax	(ng/mL)	545 ± 196.03	1980 ± 620.6[a]	25164 ± 7232	21321 ± 7025
AUClast	(hr*ng/mL)	819 ± 238.89	2756 ± 683.1[a]	334127±73850	453030 ± 164046
AUC 0-INF	(hr*ng/mL)	849.5±238.89	2813 ± 701[a]	399120±74690	456136 ± 163954
Tlast	(hr)	4.33 ± 0.52	5 ± 0	120 ± 0	120 ± 0
CL/F	(ml/min/kg)	2089.2±541.2	629.5 ± 180.3[a]	4.4 ± 1.0	3.5 ± 1.2
HL_Lambda_z	(hr)	0.8 ± 0.35	0.9 ± 0.54	21.6 ± 9.64	20.5 ± 7.29
Vz_F	(L/kg)	161.6 ± 59.2	40.4 ± 24.2[a]	8.0 ± 3.1	5.9 ± 2.3

* Median value (range); Data are expressed as mean ± SD in (n =6) rats; a=Significant, P<0.05

Table 5: ***Mean pharmacokinetic parameters of artesunate-amodiaquine combination following oral administration at 120 (artesunate) and 95 (amodiaquine) mg/kg with and without ritonavir (50 mg/kg) in rats***

Mean PK Parameters	Unit	Artesunate Alone	Artesunate with Ritonavir	Amodiaquine Alone	Amodiaquine with Ritonavir
Tmax*	(hr)	0.08 (0.08-0.08)	0.08 (0.08-0.25)	0.25 (0.25-24)	4.5 (2-24)
Cmax	(ng/mL)	1255±738.04	3437±2500.6[a]	230 ± 108.24	205 ± 71.4
AUClast	(hr*ng/mL)	261 ± 112.23	840 ± 272.6[a]	2289±607.42	2863 ± 800
AUC 0-INF	(hr*kg/mL)	299.41 ± 93.7	847.9 ± 271[a]	NC	NC
Tlast	(hr)	3 ± 0.41	4 ± 0.41	24 ± 0	24 ± 0
CL/F	(ml/min/kg)	7361.2±2776.6	2694.3± 1324.7[a]	NC	NC
HL_Lambda_z	(hr)	0.65 ± 0.22	0.62 ± 0.23	NC	NC
Vz_F	(L/kg)	456.3 ± 320.2	150 ± 93.2 [a]	NC	NC

* Median value (range); Data are expressed as mean ± SD in (n=6) rats; NC= Not calculable; a= Significant, P<0.05

Table 6: ***Mean Pharmacokinetic parameters of atovaquone-proguanil combination following its oral administration at 45 (atovaquone) and 20 (proguanil) mg/kg with and without ritonavir (50 mg/kg) in rats***

Mean PK Parameters	Unit	Atovaquone Alone	Atovaquone with Ritonavir	Proguanil Alone	Proguanil with Ritonavir
Tmax*	(hr)	24 (24-48)	24 (24-24)	0.7(0.7-2)	2 (0.7-2)
Cmax	(ng/mL)	26722±5737.2	26002±4521.8	36 ± 9.02	52 ± 9.7[a]
AUClast	(hr*ng/mL)	1686776±382102	1138297±381200	116±30.9	396 ± 37.5[a]
AUC0-INF	(hr*ng/mL)	1603477±32545	1191958±379602	142.10±16.8	420.6 ± 46.8[a]
Tlast	(hr)	120 ± 0	120 ± 0	4 ± 0	6 ± 13.8
CL/F	(ml/min/kg)	0.5 ± 0.01	0.67±0.18	2362.4±280	799.4 ± 93.1[a]
HL_Lambda_z	(hr)	29 ± 1.08	26 ± 4.75	4 ± 1.24	10 ± 4.20[a]
Vz_F	(L/kg)	1.2 ± 0.02	1.5 ± 0.6	740.6±340.0	668.2 ± 227.7

* Median value (range); Data are expressed as mean ± SD in (n=6) rats; a=Significant, P<0.05

trations in the presence of ritonavir. Statistical significance was determined using an unpaired t-test (Graphpad Prism, version 3, San Diego, CA, USA). Results were considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

LC-MS/MS analytical method

A linear-fit was used for the calibration curves across the evaluated concentration range for all antimalarial drugs and ritonavir. There were no interfering peaks observed in blank plasma chromatograms at the retention times of ritonavir or any of the antimalarial drugs indicating the selectivity of the present method. Details of the calibration curves and retention times of the drugs under evaluation and IS are provided in Table 3. The correlation coefficients were > 0.9 for all calibration curves. Quality control (QC) samples were prepared at low, medium and high concentrations to assess accuracy and precision. The overall accuracy of QC samples was in the range of ± 20 %.

Pharmacokinetic interactions in SD rats

Artemether-Lumefantrine (AL)

The pharmacokinetic parameters AUC, C_{max}, Vz/F and CL/F for artemether, were altered significantly following a single treatment of ritonavir as compared to artemether-lumefantrine treatment alone (control group). Compared to the control group, ritonavir increased the artemether C_{max} by 3.6-fold, AUC by 3.3-fold, and decreased artemether clearance by 3.3-fold and volume of distribution by 4-fold. The $T_{1/2}$ of artemether was comparable with and without ritonavir.

Unlike that seen for artemether, ritonavir co-administration had no impact on lumefantrine pharmacokinetics when administered as an artemether-lumefantrine combination. Mean plasma concentra-

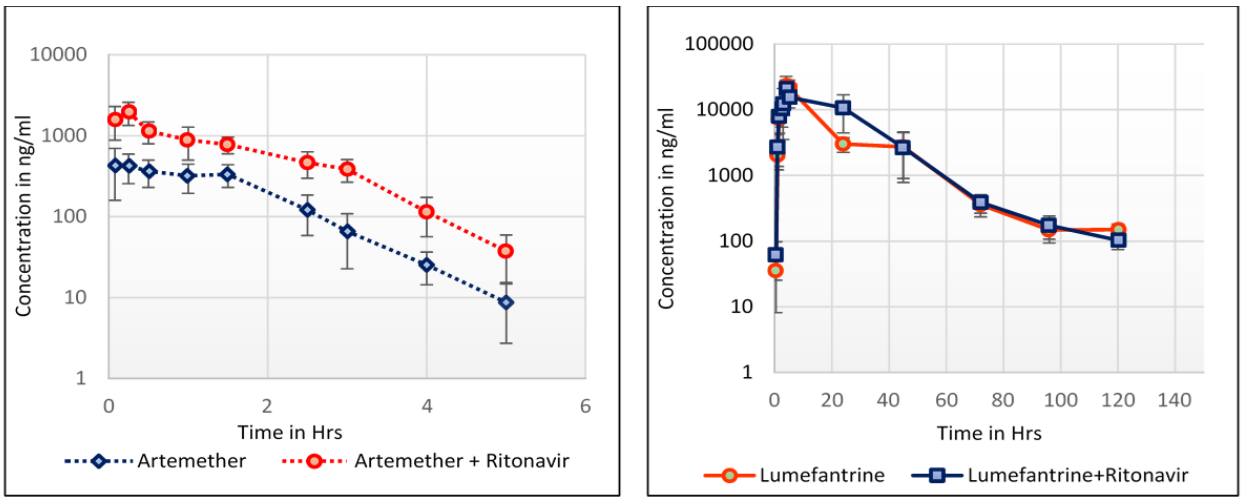

Figure 1: ***Mean plasma concentration-time curves of artemether and lumefantrine combination following oral administration of 100 (artemether) and 85 (lumefantrine) mg/kg with and without ritonavir (50 mg/kg) in rats. Data are expressed as mean ± SD in (n = 6)rats***

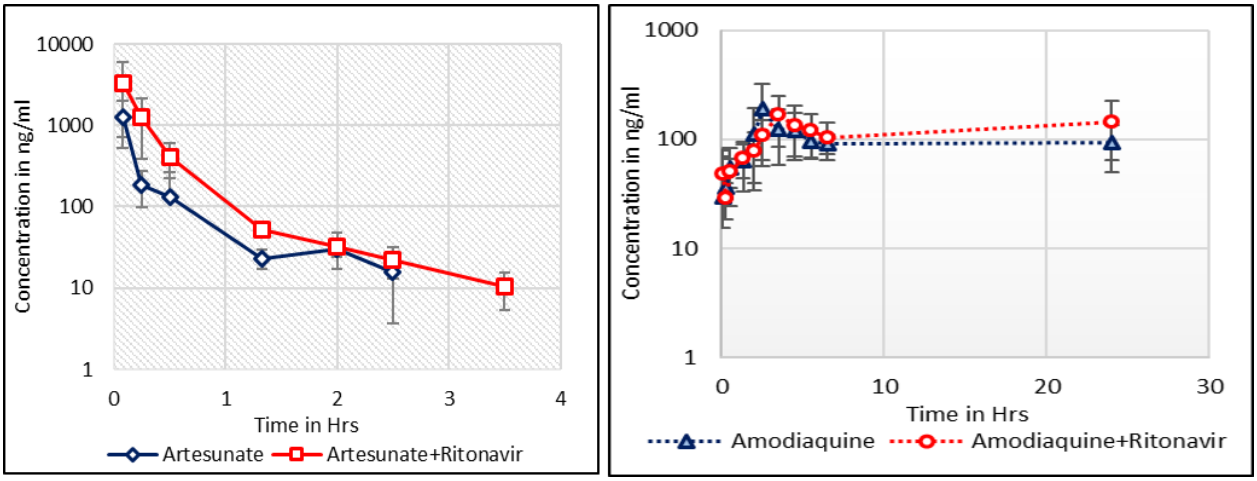

Figure 2: ***Mean plasma concentration-time curves after oral administration of artesunate and amodiaquine combination at 120 (artesunate) and 95 (amodiaquine) mg/kg with and without ritonavir (50 mg/kg) in rats. Data are expressed as mean ± SD in (n = 6) rats.***

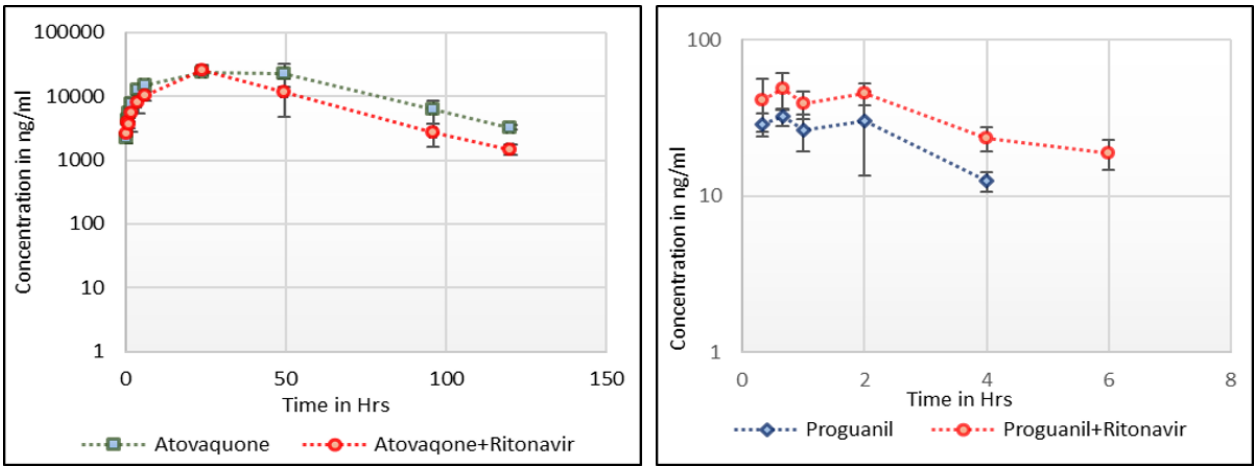

Figure 3: ***Mean plasma concentration-time curves of atovaquone and proguanil following oral administration at 45 (atovaquone) and 20 (proguanil) mg/kg with and without ritonavir (50 mg/kg) in rats. Data are expressed as mean ± SD in (n = 6) rats.***

tion versus time profiles for artemether and lumefantrine in the absence and presence of ritonavir are presented in Figure 1, and the mean pharmacokinetic parameters are presented in Table 4.

Artesunate-Amodiaquine (AA)

Statistically significant increases in AUC, $C_{max,}$ Vz/F and CL/F were observed for artesunate PK (administered as Artesunate-Amodiaquine) when co-administered with ritonavir in compared to the group that did not receive ritonavir. Compared to the control group (artesunate-amodiaquine without ritonavir), ritonavir increased artesunate C_{max} by 2.7-fold, AUC by 2.8-fold and decreased artesunate apparent clearance and apparent volume of distribution by 2.7-fold and 3-fold, respectively. There was no change in half-life of artesunate with and without ritonavir. However, amodiaquine pharmacokinetics did not change in the presence of ritonavir. The percentage of amodiaquine AUC extrapolated was greater than 20% for amodiaquine with and without ritonavir, preventing accurate estimation of the elimination phase. Although PK sample collection was performed up to 120 hours post-dose, measurable concentrations were not detected beyond the 24 hour time point indicating a potential sharp decline between the 24 and 48-hr time period. The terminal half-life and related parameters, therefore, could not be estimated accurately. Mean plasma concentration profiles of artesunate and amodiaquine in the absence and presence of ritonavir are depicted in Figure 2 and mean pharmacokinetic parameters are presented in Table 5.

Atovaquone-Proguanil (AP)

Proguanil pharmacokinetic parameters, including AUC, $C_{max,}$ $T_{1/2}$ and CL/F, were altered significantly after a single treatment of ritonavir in rats when compared to the control group (atovaquone-proguanil without ritonavir). Compared to the control group, ritonavir increased proguanil C_{max} by 1.4-fold, half-life by 2.5-fold, and $AUC_{0-\infty}$ by 3.4-fold and decreased proguanil clearance by 3-fold. In contrast to the observations with artemether and artesunate, proguanil volume of distribution was not altered by ritonavir.

Atovaquone pharmacokinetics were not altered by the coadministration of ritonavir. Mean plasma concentration-time profiles for atovaquone and proguanil in the absence and presence of ritonavir are presented in Figure 3 and mean pharmacokinetic parameters are presented inTable 6.

It is a well-established practice to prescribe artemisinin-based combination treatment (ACT) and ARVs for treating patients with a coinfection of HIV/AIDS and malaria as per WHO recommendations. The concomitant administration of antiretroviral drugs and antimalarial drugs can cause drug-drug interactions that may result in either lack of efficacy or development of toxicity (Giao and Vries, 2001). Artemether is commonly co-formulated with either lumefantrine or halofantrine to prevent the emergence of malarial resistance. Artemether is metabolized by CYP3A4 to its more active metabolite dihydroartemisinin. Lumefantrine and halofantrine are also metabolized by CYP3A4 (Djimdé and Lefèvre, 2009). Artesunate is commonly co-formulated with amodiaquine, a 4-aminoquinoline drug that is active against drug-resistant P. falciparum. Artesunate is primarily metabolized by CYP2A6, with a minor contribution from CYP3A4. Amodiaquine metabolism involves CYP3A4, CYP2C8, CYP2C9 and CYP2D6 (Zhang *et al.*, 2017). Atovaquone co-formulated with proguanil is also used for prophylaxis and treatment of malaria. Proguanil is metabolized by CYP2C19, whereas atovaquone does not undergo metabolism as part of its elimination (Nixon *et al.*, 2013).

The protease inhibitor ritonavir is a potent CYP3A4 and P-glycoprotein (Pgp) inhibitor and may interact with antimalarial drugs that are metabolized by CYP3A4, resulting in significant concentration-related toxicities (Sulkowski, 2004). At the present time, comprehensive data on pharmacokinetic interactions between antiretroviral drugs and antimalarial drugs are lacking. As the concomitant use of ARVs and ACTs increases, it becomes imperative to define potential interactions between antimalarial ACTs and ARVs.

CYP3A4 is the most abundant CYP isoform in the human liver (as well as the intestine) and is responsible for the metabolism of many different drugs (Furge and Guengerich, 2006) including several antimalarial drugs. Several studies have reported that ritonavir is a potent inhibitor of CYP3A4 (Kirby *et al.*, 2011). The CYP3A inhibitory activity of ritonavir in rats has been reported with repaglinide, which is primarily metabolised by CYP3A4 in humans (Goud *et al.*, 2016). Particularly pertinent to the current work, a pharmacokinetic interaction between the antimalarial quinine and ritonavir following concurrent administration has been reported, with a marked elevation in the plasma levels of quinine and a pronounced decrease in plasma concentrations of the major metabolite 3-hydroxyquinine, which is primarily produced from quinine by CYP3A4 (Soyinka *et al.*, 2010). Accordingly, the present study was designed to evaluate the ability of ritonavir to alter CYP3A activity in rats.

Results from the current study with the artemether-lumefantrine (AL) combination indicate that artemether is rapidly absorbed after oral administration, with median T_{max} of 0.17h. The plasma concentrations of artemether increased in rats upon single-dose oral administration of AL with ritonavir in comparison to the AL alone control group. Artemether undergoes extensive first-pass metabolism (Teja-Isavadharm *et al.*, 1996) and ritonavir inhibited the CYP3A-mediated biotransformation resulting in increased C_{max} (3.6 fold) and $AUC_{0-\infty}$ (3.3 fold). These findings suggest that ritonavir may have increased the bioavailability of artemether through an effect on the first-pass metabolism. With a single dose of ritonavir, both the apparent volume of distribution and the clearance decreased by approximately the same magnitude (~ 4fold) resulting in no change in half-life of artemether. As there was no change in half-life, it can be inferred that the observed changes in exposure may not be related to inhibition of post-absorptive metabolism by ritonavir. While the exact reason is unknown, it is possible that the increased C_{max} and exposure of artemether when dosed with ritonavir result from an overall increase in the oral bioavailability/fraction absorbed of artemether (Duan, 2010), which may be due to one or more of several factors including local inhibition of gut CYP enzymes, possible inhibition of efflux transporters, or a local formulation interaction that influences fraction absorbed. Though ritonavir has been demonstrated to also inhibit transporters likely P-glycoprotein, this is purely conjectured in the current work as this was not addressed directly. In contrast, there was no change in lumefantrine pharmacokinetics when co-administered with ritonavir despite the fact that lumefantrine is metabolised by CYP3A4. These results seem contrary to expectation and need further investigation (Figure 1 and Table 4). However, lumefantrine has been reported to undergo a relatively low first-pass effect and is absorbed and cleared slowly, suggesting that any CYP 3A4 inhibition by single-dose ritonavir would be minor. In line with the above explanation, it must be noted that a clinical drug-drug interaction study between ketoconazole (a strong inhibitor of CYP3A4 and lumefantrine resulted in only a modest increase in lumefantrine exposure (Djimdé and Lefèvre, 2009).

With the artesunate-amodiaquine (AA) combination, the pharmacokinetics of artesunate were also influenced by ritonavir. This study has demonstrated a significant increase in C_{max} (2.7 fold) nd $AUC_{0-\infty}$ (2.8 fold) of artesunate and decrease in clearance (2.7 fold) and volume of distribution (3 fold) in comparison to control group indicating the influence of ritonavir on metabolism CYP3A enzyme in rats. As with artemether, artesunate kinetics were significantly altered by ritonavir (Figure 2 and Table 5). Similarly, C_{max} and AUC were increased while Vd/F and CL/F were decreased with no change in half-life. Artesunate like Artemether undergoes extensive first-pass metabolism (Saunders *et al.*, 2012). Again, it appears that ritonavir may have altered the bioavailability of artesunate through an effect on the first-pass and gut metabolism, as was observed with artemether. However, in this case, the increase in plasma concentration is not likely due to inhibition of Pgp by ritonavir since artesunate is not a substrate of Pgp (Senarathna *et al.*, 2016). Since a percentage of AUC extrapolated was greater than 20% for amodiaquine with and without ritonavir reliable PK parameters for amodiaquine could not be derived, and thus, the influence of ritonavir on the pharmacokinetics of amodiaquine could not be established.

The pharmacokinetics of proguanil in the atovaquone-proguanil (AP) drug combination were also altered by ritonavir. There was a significant increase in $AUC_{0-\infty}$ (3 fold), $T_{1/2}$ (2.5 fold) and C_{max} (1.4 fold) of proguanil and decrease in clearance (3 fold) compared to control group (Figure 3 and Table 6). In contrast to artesunate and artemether, the half-life of proguanil was increased, but no change was observed with the volume of distribution. The increase in exposure may be attributable to decreased metabolic clearance due to CYP3A inhibition by ritonavir. No changes were observed in the pharmacokinetics of atovaquone in the presence of ritonavir. This supports reports that atovaquone undergoes limited metabolism in humans.

For artesunate and artemether, an increase in C_{max} and AUC $_{0-\infty}$ were observed without any associated changes in half-life when coadministered with ritonavir. It is possible that the PK changes for artesunate and artemether are not related to decreasing in metabolic clearance due to CYP3A inhibition by ritonavir but due to increase in the fraction absorbed/bioavailability due to potential inhibition of the first-pass metabolism through gut CYP3A inhibition or inhibition of efflux transporters by ritonavir or a formulation interaction. It must also be noted that in this study, the effect of only single dose of ritonavir was evaluated. Ritonavir has complex modulation of CYP3A and is known to be a mechanism-based inhibitor, an effect that takes time to manifest as well as reverse. Additionally, ritonavir is also an inducer of CYP3A4, and the net effect of the inhibition and induction can have different implications

upon repeat dosing. Despite the limitations in the experimental design and results, it is evident that potential first pass metabolic interactions between anti-malarial drugs and ritonavir are possible and need further investigation to assess the possible effects of ritonavir-mediated time-dependent inhibition and transporter interactions.

CONCLUSIONS

In this study, ritonavir enhanced the exposure of artemether, artesunate and proguanil in rats. The enhanced exposure of artemether and artesunate is due to an increase of bioavailability of these drugs either through an effect on first-pass metabolism or inhibition of efflux transporters by ritonavir. However, the increase in exposure of proguanil is most likely due to inhibition of the CYP3A mediated hepatic metabolism by ritonavir. The decreased metabolism of these drugs by ritonavir will result in decreased plasma exposure of their active metabolites that may influence antimalarial activity. The current study has raised awareness of potential drug interactions by concomitant administration of artemether-lumefantrine, artesunate-amodiaquine and atovaquone-proguanil with ritonavir in rats. The significance of these findings need to be further evaluated in human clinical studies.

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Author contribution statement

I hereby declare that the corresponding author had designed and performed the experiments, analyzed the data and wrote the manuscript. Co-authors have reviewed the experimental work and manuscript.

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