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Evaluation of metabolic stability of antimalarial and antiretroviral drugs

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ABSTRACT

The concomitant administration of antiretroviral drugs and antimalarial drugs is recommended for the treatment of HIV (Human Immunodeficiency Virus) patients coinfected with malaria resulting in drug-drug interactions (DDI) causing either lack of efficacy or toxicities. Drug metabolism is often the first step in understanding the DDI potential of either a new chemical entity or a combination of drugs. Protease inhibitor (PI) such as ritonavir is a potent CYP 3A4 inhibitor and may interact with these antimalarial drugs that are metabolized by CYP3A4 to cause metabolism-related DDI's. Hence the present study is an attempt to evaluate the potential for a drug-drug interaction between antimalarials and antiretrovirals through invitro metabolic stability studies. Metabolic stability of antimalarial and antiretroviral drugs alone and in combination with and without ritonavir and lopinavir was evaluated using human liver microsomes (HLM). The antimalarial drugs artemether, artesunate and amodiaquine were metabolically unstable alone (% metabolism $\geq$ 80%) and in combination with other antimalarial drugs in HLM. Lumefantrine, atovaquone and proguanil were metabolically stable (% metabolism $\leq$ 30%). Antiretroviral drug lopinavir was metabolically unstable while ritonavir was moderately stable. *In-vitro* intrinsic clearance of antimalarial drugs artemether, artesunate and amodiaquine decreased from 106.4, 290.6 and 230 ml/min/kg to 32, 44.8 and 49.5 ml/min/kg in the presence of ritonavir. However, there was no change in the *in-vitro* intrinsic clearance of lumefantrine, atovaquone and proguanil in the presence of ritonavir. Lopinavir did not alter the clearance of antimalarial drugs. This study suggests that ritonavir affected the clearance of a few antimalarial drugs in HLM probably by the inhibition of CYP3A4 and findings may need to be further evaluated in clinical studies.

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INTRODUCTION

The treatment of patients with a coinfection of Malaria and HIV is challenging with multiple concurrent medications, with a potential for drug interactions and overlapping toxicities. There is a significant risk of DDIs between HIV protease inhibitors and artemisinin-containing antimalarial regimens (Dooley *et al.*, 2008).

The changes in exposure to antiretrovirals (ARV) or antimalarials caused by a DDI may result in either development of resistance or drug toxicity.

There is a critical need for safe and effective treatment regimens for both infections. The in-vitro metabolism-based studies using HLM are used to guide possible preliminary drug interactions (FDA, 2017). The altered clearance provides insight into the likelihood of drug-drug interactions and the requirement for clinical studies.

Metabolic stability refers to the percentage of parent compound cleared over time in the presence of a metabolically active test system such as liver microsomes, liver S9, or hepatocytes. Invitro half-life (t1/2) and intrinsic clearance (CLint) are mainly utilized to express metabolic stability (Słoczyńska *et al.*, 2019).

It serves as a basic tool to determine the drug interaction potential of drugs. Drugs with either potent cytochrome inhibition or induction potential may influence the pharmacokinetic characteristics of coadministered drugs.

Drugs with CLint in HLM above 45 mL/min/kg are classified as high clearance compounds, drugs with CLint values between 15 and 45 mL/min/kg as intermediate clearance compounds, and agents with CLint below 15 mL/min/kg as low clearance compounds (Mcnaney *et al.*, 2008).

In HIV treatment, PIs (ritonavir) are used in highly active antiretroviral therapy regimen (HAART). Ritonavir is one of the antiretroviral drugs from the PI class used to treat HIV infection and AIDS. Ritonavir inhibits cytochrome P450 3A4 (CYP3A4) and is metabolized by CYP3A4 and CYP2D6.

Among available protease inhibitors, Ritonavir carries the highest risk of causing drug interactions due to inhibition of cytochrome P450 activity (Moltke *et al.*, 1998).

In view of the influence of ritonavir on CYP3A activity, its presence in antiretroviral therapy may influence the pharmacokinetics of antimalarials, particularly because artemisinin-based combination therapy (ACT's) are metabolized by CYP3A4 (Khoo *et al.*, 2005). In vitro metabolism-based experiments has been used to predict pharmacokinetic interactions involving CYP3A substrates.

The aim of the present investigation was to evaluate metabolic stability of antimalarial, antiretroviral drugs alone, their combinations and its influence on DDI potential of coadministered drugs.

MATERIALS AND METHODS

Artemether, artesunate, lumefantrine, amodiaquine was provided as a gift sample from IPCA (The Indian Pharmaceutical Combine Association Limited), Mumbai India. Glenmark Pharmaceuticals Limited provided proguanil and atovaquone. Ritonavir and lopinavir was provided as a gift sample from Aurobindo Pharma, Hyderabad, India.

Pooled human liver microsomes (HLM) was purchased from XenoTech, USA. Nicotinamide Dinucleotide adenine phosphate reduced (NADPH) was procured from SRL Laboratories, Mumbai, India. Magnesium Chloride, Dimethyl Sulphoxide (DMSO) and Phosphate buffer of Sigma, India were used. 2mL Deep well plates of Axygen, Mexico was used.

The HPLC grade methanol, acetonitrile and ammonium acetate were obtained from Merck Chemicals, Mumbai, India. The drug analysis was carried out using LC-MS system (ABSCIEX 3200, CA, USA), HPLC system Shimadzu (CA, USA), HPLC Gemini column C18, 5 μ column 4.6 $\times$ 50 mm internal diameter Phenomenex (CA, USA), and Eppendorf centrifuge (Chennai, Tamilnadu, India) were used for the study.

Methods

Metabolic Stability experiment in HLM

The HLM used in this study comprised of 440 pmoles/mg of total P450 and 429 pmoles/mg cyt. b5. The NADPH cytochrome c reductase activity is 156 nmol/mg protein/min. It consists of CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, 4A11, FMO, UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 UGT2B7 and UGT2B17 where the enzyme activity of CYP2C8, 3A4, and 2D6 was 2350, 4070, and 301 pmoles/(mg.min) respectively. The volume of the enzyme per vial was 0.5 ml, and the protein content was 20 mg/ml in 250 mM sucrose.

The metabolic stability assay protocol was performed considering the drug solution at 1uM, protein concentration at 1mg/ml and 2.4mM of NADPH. The drugs and different drug combination evaluated are as per Table 1.

First, the metabolic stability of individual drugs were evaluated, followed by anti-malarial drug combinations. The effect of lopinavir and ritonavir on the metabolic stability of antimalarial drug combinations was evaluated. Hepatic intrinsic clearance of anti-malarial drug combinations alone and in the presence of lopinavir and ritonavir was also determined (Obach, 1999).

There were samples of 0min, 60 min and the positive control with low and high clearance drugs. All the incubations were performed in triplicates. All the components used in the reaction mixture are shown in Table 2.

The drug solution in DMSO was prepared at 5 mM. The buffer used was 0.1 M potassium phosphate pH

Table 1: Drugs evaluated for Metabolic Stability and Intrinsic Clearance

S.No	Drugs	Drug combinations 1	Drug combinations 2	Drug combinations 3
1	Artemether	Artemether+ Lumefantrine	Artemether+ Lumefantrine+ Ritonavir	Artemether+ Lumefantrine+ Lopinavir
2	Lumefantrine	Artesunate+ Amodiaquine	Artesunate+ Amodiaquine+ Ritonavir	Artesunate+ Amodiaquine+ Lopinavir
3	Artesunate	Atovaquone+ Proguanil	Atovaqune+ Proguanil+ Ritonavir	Atovaqune+ Proguanil+ Lopinavir
4	Amodiaquine	–	–	–
5	Atovaquone	–	–	–
6	Proguanil	–	–	–
7	Ritonavir	–	–	–
8	Lopinavir	–	–	–

Table 2: Components of Reaction Mixture for Metabolic Stability Study

Components	Test/PC/well	Buffer Control/well	Final Concentration
Protein-Buffer mix	235uL	NA	0.94mg/ml
PBS Buffer	NA	235 uL	0.1M
Drug/control	2.5uL	2.5 uL	1uM
NADPH	12.5uL	12.5 uL	2.4mM

PC=Positive control;NA=Not Applicable

Table 3: Components of Reaction Mixture for Intrinsic Clearance

Components	Test/PC/well	Buffer control/well	Final Concentration
Protein-Buffer Mix (1.06mg/ml)	710 uL	NA	1.0mg/ml
PBS Buffer	NA	710 uL	0.1M
Drug or drug mixture (0.3mM)	2.5 uL	2.5 uL	1uM
NADPH (48mM)	37.5 uL	37.5 uL	2.4mM

PC=Positive control

Table 4: Details of gradient elution mode of separation

Time (min)	Mobile phase B (%)	Mobile phase A (%)	Flow rate
0.01	90	10	0.7mL/min
1	10	90	0.7mL/min
2.5	10	90	0.7mL/min
2.6	90	10	0.7mL/min
3	90	10	0.7mL/min

Table 5: Details of gradient elution used for Atovaquone

Time (min)	Mobile phase B (%)	Mobile phase A (%)	Flow rate
0.01	90	10	0.7mL/min
1	10	90	0.7mL/min
3.5	10	90	0.7mL/min
3.6	90	10	0.7mL/min
4	90	10	0.7mL/min

Table 6: Summary of analytical parameters

Analyte	MRM (Q1)	MRM (Q3)	Ionisation mode	Flow rate (ml/min)	Run time (min)	Injection Volume(uL)
Artesunate*	402.080	267.200	Positive	0.7	3	5
Amodiaquine	356.155	283.200	Positive	0.7	3	5
Lumefantrine	528.084	510.200	Positive	0.7	3	5
Proguanil	254.090	170.200	Positive	0.7	3	5
Ritonavir	721.228	140.200	Positive	0.7	3	5
Lopinavir	629.504	155.300	Positive	0.7	3	5
Artemether*	316.234	267.300	Positive	0.6	2	15
Atovaquone	365.062	337.100	Negative	0.7	4	5
Losartan**	423.100	207.100	Positive	0.7	3	5

MRM: Multiple reaction monitoring; * NH4 adduct; ** Internal Standard

7.4. The protein buffer mix was prepared by spiking HLM (20mg/ml) into a buffer to obtain 1mg/ml. The reaction mixture was prepared by first adding protein buffer mixture followed by the addition of drug into 2mL deep well plate.

The plate was preincubated at 37°C for 5min. Then the reaction was initiated by the addition of cofactor NADPH followed by incubation of reaction mixture at 37 °C for 1 hr at 120 rpm in an incubator shaker. At the end of incubation, the reaction was terminated by the addition of 500uL of quenching solvent, which was 1:4 of Methanol and ACN containing Losartan as the internal standard.

For 0 min control sample, ice-cold quenching solvent was added to the protein buffer mixture containing drug followed by the addition of NADPH and was prepared in a separate plate. The contents of both 0 min and 60 min plates were shaken for 5 min and then centrifuged at 2500 rpm for 40 min at 4°C.

The supernatant was subjected for LC-MS/MS analysis. In the metabolic stability experiments, Tolbutamide and Diltiazem at 1uM concentration were used as high and low-to-moderately stable positive controls respectively.

For intrinsic clearance, 100ul of the samples at predefined time intervals of 0min, 15min, 30min, 45min and 60min were aliquoted and added to a plate containing 500μL of quenching solvent containing 5μM of IS mixture. Samples were mixed for 5 min and then centrifuged at 2500 rpm for 40 min.

After centrifugation, a plate containing the supernatant was submitted for LC/MS/MS analysis. Quinidine at 1uM concentration was used as a positive control for intrinsic clearance experiments. All the incubations were performed in duplicates. The details of the incubation mixture is provided in Table 3.

The composition of the buffer control sample is the same as the test item reaction mixture except that it does not have protein or HLM. This sample serves as the control to check on the stability of the drug in a buffer and to ensure that loss of drug is due to metabolism and not due to crashing of the drug in a buffer.

Instrumentation

Liquid chromatography-mass spectrometry assay (LC-MS/MS) for the quantitation of artemether, artesunate, amodiaquine, lumefantrine, atovaquone and proguanil was performed on an Shimadzu LC 20 AD system (Kyoto, Japan) coupled to an API 3200 Q trap mass spectrometer (Applied Biosystems/MDS Sciex, US) via a Turbo Ion Spray ionization (ESI) interface. The fragmentation transitions for the multiple-reaction monitoring (MRM) of drugs are as per Table 6.

Chromatographic separation was achieved on a Gemini C18 column (50 $\times$ mm i.d, 5 μm; Phenomenex, CA, USA). The chromatography was performed at 40°C. The 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.

Qualitative Estimation of Antimalarial drugs, Lopinavir and Ritonavir in metabolic stability samples and intrinsic clearance samples

The area of analyte and its corresponding internal standard were acquired using LC-MS/MS method. Positive ion multiple reaction monitoring was used for the mass spectrometric detection of all drugs except for atovaquone. The details of multiple reaction monitoring (MRM), run time, ionization mode, flow rate and injection volume used for various drugs are provided in Table 6.

The ammonium adducts of artemether and arte-

Table 7: Metabolic Stability of Antimalarial and Antiretroviral Drugs Alone in HLM

Drug	% Metabolism (HLM)	% Metabolism (Buffer)
*Artemether	80.1 ± 1.7	1.8 ± 0.4
*Artesunate	100 ± 0	0.4 ± 0.1
Atovaquone	26.3 ± 7.71	-22.4 ± 18.6
Lumefantrine	7.0 ± 2.3	4 ± 0.5
Amodiaquine	98.0 ± 0.5	-2.9 ± 0.3
Proguanil	7.0 ± 3.45	-1.6 ± 0.5
Ritonavir	76.2 ± 0.3	-4 ±1.8
Lopinavir	92.2 ± 3.8	-1± 3.0

*Ammonium adduct was monitored. Values are mean ± SD

Table 8: Metabolic Stability of Antimalarial Drug Combinations in HLM

Drug Combination	Drug	% Metabolism (HLM)	% Metabolism (Buffer)
Artemether-Lumefantrine	Artemether*	82.9 ± 1.4	-4.5 ± 5.6
	Lumefantrine	12.6 ± 9.2	4.6 ± 1.7
Artesunate-Amodiaquine	Artesunate*	100.0 ± 0	4.0 ± 2.8
	Amodiaquine	96.9 ± 1.9	-6.4 ± 4.3
Atovaquone-Proguanil	Atovaquone	11.9 ± 4.1	1.1 ± 0.2
	Proguanil	-9.2 ± 2.6	-9.2 ± 6.9

*Ammonium adduct was monitored. Values are mean ± SD

Table 9: Effect of Ritonavir and Lopinavir on Metabolic Stability of Antimalarial Drugs

Drug combination	Compound	% Metabolism in presence of Ritonavir	% Metabolism in presence of Lopinavir
Artemether-Lumefantrine	Artemether*	55.7 ± 5.0	74.5 ± 5.8
	Lumefantrine	-2.8 ± 9.8	13.7 ± 5.5
	Ritonavir/Lopinavir	85.2 ± 1.2	99.0 ± 0.1
Artesunate-Amodiaquine	Artesunate*	83.4 ± 2.6	100 ± 0
	Amodiaquine	79.9 ± 1.8	100 ± 0
	Ritonavir/Lopinavir	87.1 ± 3.5	98.6 ± 0.1
Atovaquone-Proguanil	Atovaquone	5.3 ± 2.8	19.6 ± 2.1
	Proguanil	11.8 ± 1.3	4.3 ± 2.6
	Ritonavir/Lopinavir	83.0 ± 2.3	99.3 ± 0.1

*Ammonium adduct was monitored. Values are mean ± SD

sunate showed higher intensity in response and were considered for monitoring of the analyte response.

The chromatographic separation of artesunate, amodiaquine, lumefantrine, ritonavir and lopinavir was achieved with gradient mode of separation with mobile phase delivered at a flow rate of 0.7 mL/min as shown in Table 4. Atovaquone was separated using a gradient mode of elution, as shown in Table 5.

Isocratic mode of separation was used for the elution of artemether at a flow rate of 0.6 mL/min with 20%: 80% of Mobile phase B and A respectively for 2min.

Data Analysis

Area ratio of drug to internal standard was determined for each drug from the LC-MS method. Percent of drug remaining of each drug and its combination was calculated from the area ratio of a drug at 60 min and 0min sample using the below formula.

% drug Remaining: 100-(area ratio of test drug at 60 min/area ratio of 0min sample*100)

% Metabolism: 100 - % drug remaining

To determine hepatic clearance value, first Log-linear plots of the percentage of drug remaining ver-

Table 10: Effect of Ritonavir on the intrinsic clearance of antimalarial drugs

Drug Combination	Drugs	% Metabolism	In vitro Clintr (ml/min/kg)	CLhep-pred (ml/min/kg	% Metabolism	In vitro Clintr (ml/min/kg	CLhep-pred (ml/min/kg)
		Without Ritonavir			With Ritonavir		
Artemether-Lumefantrine	*Artemether	86.2	106.4	17.5	77.3	32.0	12.7
	Lumefantrine	-4.8	-0.79	-0.82	-3.3	-5.6	-7.6
	Ritonavir	NA	NA	NA	79.7	33.1	12.8
Artesunate-Amodiaquine	*Artesunate	100	290.6	19.6	81	44.8	14.3
	Amodiaquine	100	230.0	19.2	82	49.5	14.7
	Ritonavir	NA	NA	NA	79.7	34.2	13.0
Atovaquone-Proguanil	Atovaquone	-15.4	-4.7	-6.1	-6.0	-8.4	-6.0
	Proguanil	-12.7	-0.46	-0.47	-0.6	-0.7	-0.6
	Ritonavir	NA	NA	NA	79.5	34.2	13.0
Quinidine	Quinidine	74.4	29.5	12.3			

NA: Not Applicable. *Ammonium adduct was monitored

Table 11: Effect of Lopinavir on the intrinsic clearance of antimalarial drugs

Drug combination	Drug	% Metabolism	In vitro Clintr (ml/min/kg)	CLhep-pred (ml/min/kg)	% Metabolism	In vitro Clintr (ml/min/kg)	CLhep-pred (ml/min/ kg)
		Without Lopinavir			With Lopinavir		
Artemether-Lumefantrine	*Artemether	86.2	106.4	17.5	86.32	84.5	16.8
	Lumefantrine	-4.8	-0.79	-0.82	-22.7	-3.5	-4.2
	Lopinavir	NA	NA	NA	100	NC	NC
Artesunate-Amodiaquine	*Artesunate	100	290.6	19.6	96.7	235.5	19.3
	Amodiaquine	100	230.0	19.2	93.5	231.9	19.3
	Lopinavir	NA	NA	NA	100	NC	NC
Atovaquone-Proguanil	Atovaquone	-15.4	-4.7	-6.1	-4.7	-8.3	-13.8
	Proguanil	-12.7	-0.46	-0.47	-4.4	-6.2	-8.8
	Lopinavir	NA	NA	NA	100	NC	NC
Quinidine	Quinidine	74.4	29.5	12.3			

NC= Not Calculable. * Ammonium adduct was monitored. NA Not Applicable

sus time were plotted, and the slope of the curve was calculated by linear regression of the log-linear curve. Parameters such as *invitro* intrinsic clearance (CL_{int}) and predicted *in-vivo* hepatic clearance (CL Hepatic) of each drug is calculated using the below-mentioned equations.

Elimination rate constant (k) = - (slope), Half-life ($t_{1/2}$) in min = 0.693/k

The *invitro* hepatic clearance (CL_{int}) (mL/min/kg) of each drug was estimated using invitro data and the equation as mentioned below

$$CL_{int} = \frac{0.693}{t_{1/2}} \times \frac{mg\ microsome}{g\ liver} \times \frac{Liver\ weight\ (g)}{Body\ weight\ (kg)}$$

Where 49 is the total liver protein content in mg per gram of liver and 26gms is the average liver weight per kg body weight of a human (Projean *et al.*, 2003).

The *in vivo* hepatic clearance predicted (mL/min/kg) of each drug was estimated as

CL Hepatic predicted = (CL_{int} *21)/ (CL_{int} +21)

Where 21 refers to human liver blood flow in mL per min per kg body weight of human and CL_{int} refers to intrinsic clearance. The percent metabolism values and CL_{int} were expressed as mean ± SD. The % metabolism and CL_{int} of drugs and its combinations are provided in Tables 7, 8 and 9.

RESULTS AND DISCUSSION

Metabolic Stability of Antimalarial and Antiretroviral drugs in HLM

It was observed that antimalarial drugs artemether, artesunate and amodiaquine is metabolically unstable alone where >80% of drug disappears in the reaction mixture and also in combination with other antimalarial drugs in HLM. Atovaquone, lumefantrine and proguanil were metabolically stable ($\leq$ 30%).

Among the antiretroviral drugs, ritonavir was moderately stable while lopinavir was unstable. *In vitro* metabolism was measured by the disappearance of the drug at 1uM, in incubations with NADPH-fortified human liver microsomes in comparison to 0min sample as depicted in Table 7 and Table 8.

The results from buffer samples indicate that the disappearance of a drug from protein incubated sample is not due to inadequate solubility of drugs but due to hepatic metabolism. The percent metabolism of positive controls tolbutamide and diltiazem was 8.9% and 82.7% respectively, demonstrating the validity of the experiment.

Metabolic Stability of Antimalarial drugs in HLM in the presence of Lopinavir and Ritonavir

The percent metabolism of artemether, artesunate and amodiaquine decreased from 82.9%, 100% and 96.9% to 55.7%, 83.4% and 79.9% in the presence of ritonavir. However, in the presence of lopinavir, the percent metabolism of artemether reduced to 74.5% from 82.9%, but no change was observed with artesunate and amodiaquine.

There was no change in the metabolism of lumefantrine, atovaquone and proguanil in the presence of ritonavir and lopinavir Table 9.

The antimalarial drugs did not seem to affect the metabolism of ritonavir and lopinavir. The percent metabolism of positive controls tolbutamide and diltiazem was 7.1% and 87% respectively, demonstrating the validity of the experiment.

Effect of Ritonavir on the intrinsic clearance of antimalarial drugs

The *in vitro* intrinsic clearance and predicted *in vivo* hepatic clearance of artemether reduced by 3.3 fold and 1.4 fold respectively in the presence of ritonavir. For artesunate and amodiaquine, the *in vitro* intrinsic clearance decreased by 6.5 fold and 4.6 fold respectively while the *in vivo* hepatic clearance decreased by approximately 1.5 fold and 1.3 fold respectively in the presence of ritonavir.

There was no change observed in *in vitro* intrinsic clearance and predicted *in vivo* hepatic clearance of lumefantrine, atovaquone and proguanil in the presence of ritonavir as shown in Table 10.

The clearance of ritonavir remained unchanged in the presence of antimalarial drugs. The clearance of positive control qunidine was 29.5 ml/min/kg validating the performance of the experiment and comparable to reported results as mentioned in the literature (Wood *et al.*, 2017). The log percent of drug remaining was plotted against time, as shown in Figure 1 to estimate intrinsic clearance values.

Effect of Lopinavir on the intrinsic clearance of antimalarial drugs

In the presence of lopinavir, the *in vitro* intrinsic clearance and predicted *in vivo* hepatic clearance of artemether and artesunate reduced approximately only by 1.3 fold and 1.2 fold respectively. Lopinavir did not change *in vitro* intrinsic clearance and predicted *in vivo* hepatic clearance of lumefantrine, amodiaquine, atovaquone and proguanil, as shown in Table 11.

The clearance of lopinavir remained unchanged in the presence of antimalarial drugs. The clearance of positive control qunidine was 29.5 ml/min/kg validating the performance of the experiment and comparable to reported results as mentioned in the literature (Wood *et al.*, 2017). The log percent of drug remaining was plotted against time, as shown in Figure 2 to estimate intrinsic clearance values.

Artemisinin-based combination treatment (ACT) is now being widely used as the first-line treatment for *Plasmodium falciparum* malaria throughout the world (Dondorp *et al.*, 2009).

Artemisinin and or its derivatives such as artemether, artesunate and arteether are short-acting antimalarials with a short half-life and hence in general combined with one or two long-acting antimalarial drugs like amodiaquine, mefloquine, sulfadoxine, pyrimethamine or lumefantrine (Kerb *et al.*, 2009).

Atovaquone co-formulated with proguanil is used for prophylaxis and treatment of malaria. As per WHO recommendation, the ACTs and antiretroviral drugs (ARV) are used in the treatment of patients coinfected with HIV/AIDS and malaria (World Health Organization, 2016).

Artemether is metabolized by CYP3A4 and CYP3A5 to its more active metabolite dihydroartemisinin (Aweeka and German, 2008). In vitro data provides evidence for CYP2A6 as the major metabolizing enzyme for artesunate to active dihydroartemisinin (Li *et al.*, 2003).

The antimalarial activity of artemether and artesunate is mainly due to dihydroartemisinin. Lumefantirne is also mainly metabolised by CYP3A4 in HLM (Djimdé and Lefèvre, 2009).

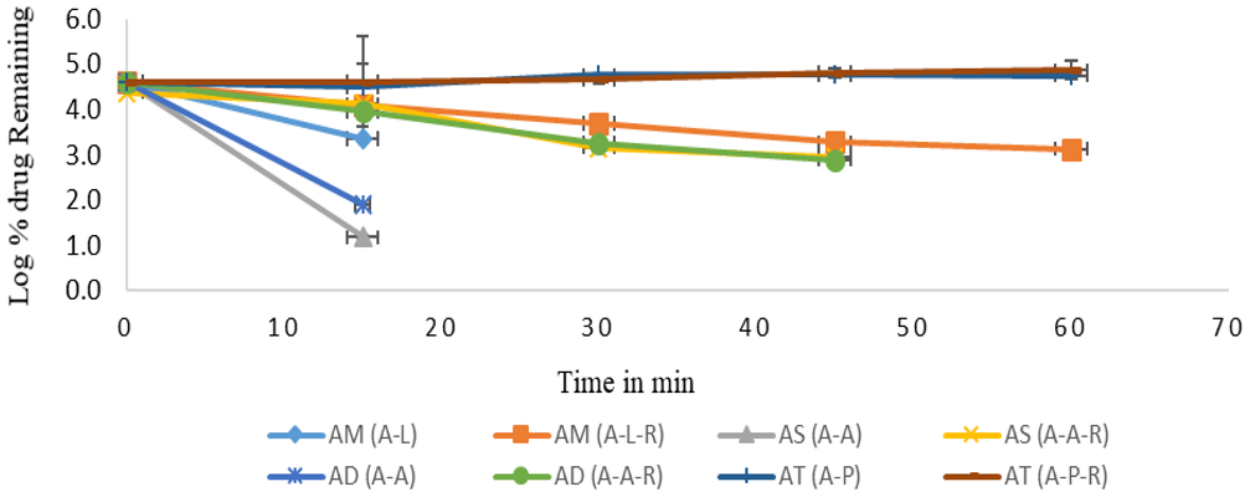

Figure 1: Plot of log percentage of drug remaining vs time of Antimalarial drugs in presence and absence of Ritonavir. A-L= Artemether+Lumefantrine; A-A=Artesunate+Amodiaquine; A-P= Atovaqone+Proguanil; R=Ritonavir; AM=Artemether;AS=Artesunate; AD=Amodiaquine; AT=Atovaquone

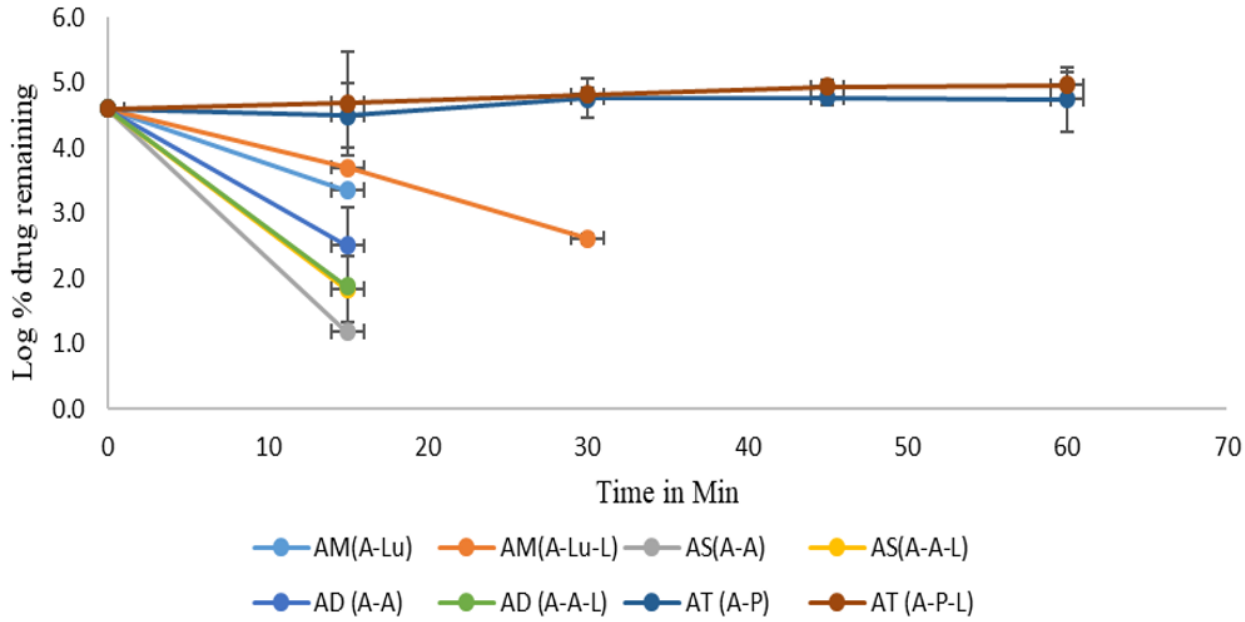

Figure 2: Plot of log percentage of drug remaining vs time of Antimalarialdrugs in presence and absence of Lopinavir. A-Lu= Artemether+ Lumefantrine;A-A= Artesunate+ Amodiaquine; A-P= Atovaquone+ Proguanil; L=Lopinavir; AM=Artemether;AS=Artesunate; AD=Amodiaquine; AT=Atovaquone

Amodiaquine metabolism to N-desethylamodiaquine involves CYP3A4, CYP2C8, CYP2C9 and CYP2D6. This metabolite is considered to determine the pharmacological activity of amodiaquine (Zhang *et al.*, 2017).

Atovaquone combination with proguanil is used for prophylaxis and treatment of malaria where Proguanil is metabolized by CYP3A4/2C19 and elimination of atovaquone does not require metabolism (Nixon *et al.*, 2013).

It has been reported that ritonavir is a potent inhibitor of CYP3A4, which may play a role in the metabolism of antimalarial drugs that are metabolised by CYP 3A4 (Kirby *et al.*, 2011).

One of the principal cytochrome P450 isoforms involved in the metabolism of ACT's is CYP3A4. CYP3A4 is the most abundant isoform of cytochrome P450 in the human liver and is responsible for the metabolism of many different drugs (Furge and Guengerich, 2006).

The fixed-dose combination of lopinavir and ritonavir in the name of Kaletra is widely used in the treatment of HIV/AIDS and is the only protease inhibitor coformulation approved by FDA in both children and adults (Croxtall and Perry, 2010). CYP3A4 is the primary enzyme involved in lopinavir bioactivation (Li *et al.*, 2012).

The concomitant administration of antiretroviral drugs and antimalarial drugs results in drug-drug interactions causing either lack of efficacy or toxicities (Giao and Vries, 2001).

Ritonavir being a potent CYP3A4 inhibitor, may interact with these antimalarial drugs that are majorly metabolized by CYP3A4 to cause significant concentration-related toxicities (Sulkowski, 2004).

Hence the present study is an attempt to evaluate the effect of ritonavir and lopinavir on the metabolism of antimalarial drugs by estimating the changes in metabolic stability, *in vitro* intrinsic clearance and predicted *in vivo* human hepatic clearance in HLM.

The result from the metabolic stability studies indicates that artemether, artesunate and amodiaquine is metabolically unstable either alone or in combination with other antimalarial drugs in HLM.

There was a decrease in percent metabolism of artemether, artesunate and amodiaquine from 82.9%, 100% and 96.9% to 55.7%, 83.4% and 79.9% in the presence of ritonavir respectively (Table 9).

However, in the presence of lopinavir, there was no change in percent metabolism in any of the tested drugs except for artemether where there was a modest reduction from 82.9% to 74.5% (Table 9).

This supports the fact that ritonavir being potent CYP 3A4 inhibitor is able to inhibit the CYP 3A4 mediated metabolism of the antimalarial drugs artemether, artesunate and amodiaquine to result in decreased metabolism and increased plasma concentrations. But this may impact efficacy since the metabolites of these antimalarial drugs are considered responsible for their antimalarial activity.

The antimalarial drugs atovaquone, lumefantrine and proguanil alone were metabolically stable in HLM and continue to show no change in percent metabolism either in combination with other antimalarial drugs or ritonavir or lopinavir.

This finding suggests that enzymes other than CYP's may be involved in their metabolism or the elimination route is other than metabolism like atovaquone. No change in lumefantrine metabolic stability in the presence of ritonavir despite being metabolised by CYP3A4 is probably due to the fact that lumefantrine is absorbed and cleared more slowly.

This study has demonstrated decrease in the *in vitro* intrinsic clearance values of artemether, artesunate and amodiaquine by 3.3 fold, 6.5 fold and 4.6 fold respectively in the presence of ritonavir. This supports the objective of the study that Ritonavir being potent CYP 3A4 inhibitor alters the microsomal stability of antimalarial drugs majorly metabolised by CYP 3A4.

However, the decrease in predicted hepatic clearance was modest (less than 1.5 fold) with these antimalarial drugs in the presence of ritonavir. The correlation of *in vitro* intrinsic clearance to predicted *in vivo* hepatic clearance was poor for metabolically unstable drugs.

There was no major change in *vitro* clearance of lumefantrine, atovaquone and proguanil in the presence of ritonavir same as metabolic stability results (Table 10 and Figure 1). Lopinavir did not influence the *in vitro* clearance or the *in vivo* hepatic clearance of antimalarial drugs suggestive of not being an inhibitor of CYP enzymes (Table 11 and Figure 2).

In general, among the evaluated combination of anti-malarial drugs, the metabolism of artemether, artesunate and amodiaquine mediated majorly by CYP3A4 was decreased by ritonavir. The decreased *in vitro* intrinsic clearance of these drugs due to CYP inhibition by ritonavir may lead to an increase in exposure of drugs and decrease in exposure of their active metabolites with impact on efficacy.

CONCLUSION

In this study, ritonavir decreased percent metabolism and *in vitro* intrinsic clearance of artemether, artesunate and amodiaquine in HLM. The decreased metabolic clearance of these drugs might be mainly due to inhibition of the CYP3A4-mediated metabolism by ritonavir. The decreased clearance of artemether, artesunate and amodiaquine results in decreased concentrations of their active metabolites and may influence antimalarial activity. The current study has raised awareness of potential drug interactions by concomitant administration of artemether-lumefantrine, artesunate-amodiaquine with ritonavir in humans. The significance of these findings needs to be further evaluated in 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 manuscript writing. Co-authors have reviewed the experimental work and manuscript.

REFERENCES

Aweeka, F. T., German, P. I. 2008. Clinical Pharmacology of Artemisinin-Based Combination Therapies. *Clinical Pharmacokinetics*, 47(2):91–102.

Croxtall, J. D., Perry, C. M. 2010. Lopinavir/Ritonavir. *Adis Drug Evaluation*, 70(14):1885–1915.

Djimdé, A., Lefèvre, G. 2009. Understanding the pharmacokinetics of Coartem®. *Malaria Journal*, 8(S1).

Dondorp, A. M., Nosten, F., Yi, P., Das, D., Phyo, A. P., Tarning, J., White, N. J. 2009. Artemisinin Resistance in Plasmodium falciparum Malaria. *New England Journal of Medicine*, 361(5):455–467.

Dooley, K. E., Flexner, C., Andrade, A. S. 2008. Drug Interactions Involving Combination Antiretroviral Therapy and Other Anti-Infective Agents: Repercussions for Resource-Limited Countries. *The Journal of Infectious Diseases*, 198(7):948–961.

FDA 2017. Department of Health and Human Services Food and Drug Administration Center for Evaluation and Research (CDER). U.S. In Guidance for Industry: In Vitro Metabolism- Mediated Drug-Drug and Transporter- Interaction Studies. Draft Guidance.

Furge, L. L., Guengerich, F. P. 2006. Cytochrome P450 enzymes in drug metabolism and chemical toxicology: An introduction. *Biochemistry and Molecular Biology Education*, 34(2):66–74.

Giao, P., Vries, P. J. D. 2001. Pharmacokinetic Interactions of Antimalarial Agents. *Clinical Pharmacokinetics*, 40(5):343–373.

Kerb, R., Fux, R., Mörike, K., Kremsner, P. G., Gil, J. P., Gleiter, C. H., Schwab, M. 2009. Pharmacogenetics of antimalarial drugs: effect on metabolism and transport. The Lancet Infectious Diseases. 9:760–774.

Khoo, S., Back, D., Winstanley, P. 2005. The potential for interactions between antimalarial and antiretroviral drugs. *AIDS*, 19(10):995–1005.

Kirby, B. J., Collier, A. C., Kharasch, E. D., Whittington, D., Thummel, K. E., Unadkat, J. D. 2011. Complex Drug Interactions of HIV Protease Inhibitors 1: Inactivation, Induction, and Inhibition of Cytochrome P450 3A by Ritonavir or Nelfinavir. *Drug Metabolism and Disposition*, 39(6):1070–1078.

Li, F., Lu, J., Ma, X. 2012. CPY3A4-Mediated Lopinavir Bioactivation and Its Inhibition by Ritonavir. *Drug Metabolism and Disposition*, 40(1):18–24.

Li, X. Q., Bjrkman, A., Andersson, T. B., Gustafsson, L. L., Masimirembwa, C. M. 2003. Identification of human cytochrome P 450 s that metabolise anti-parasitic drugs and predictions of in vivo drug hepatic clearance from in vitro data. *European Journal of Clinical Pharmacology*, 59(5-6):429–442.

Mcnaney, C. A., Drexler, D. M., Hnatyshyn, S. Y., Zvyaga, T. A., Knipe, J. O., Belcastro, J. V., Sanders, M. 2008. An Automated Liquid Chromatography-Mass Spectrometry Process to Determine Metabolic Stability Half-Life and Intrinsic Clearance of Drug Candidates by Substrate Depletion. ASSAY and Drug Development Technologies. 6:121–129.

Moltke, L. L. V., Greenblatt, D. J., Grassi, J. M., Granda, B. W., Duan, S. X., Fogelman, S. M., Shader, R. I. 1998. Protease Inhibitors as Inhibitors of Human Cytochromes P450: High Risk Associated with Ritonavir. 38:106–111.

Nixon, G. L., Moss, D. M., Shone, A. E., Lalloo, D. G., Fisher, N., O'neill, P. M., Biagini, G. A. 2013. Antimalarial pharmacology and therapeutics of atovaquone. *Journal of Antimicrobial Chemotherapy*, 68(5):977–985.

Obach, R. S. 1999. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metabolism & Disposition*, 27(11):1350–1359.

Projean, D., Baune, B., Farinotti, R., Flinois, J. P., Beaune, P., Taburet, A. M., Ducharme, J. 2003. In vitro metabolism of chloroquine: identification of cyp2c8, cyp3a4, and cyp2d6 as the main isoforms catalyzing n-desethylchloroquine formation. *Drug Metabolism and Disposition*, 31(6):748–754.

Słoczyńska, K., Gunia-Krzyżak, A., Koczurkiewicz, P., Wójcik-Pszczoła, K., Żelaszczyk, D., Popiół, J., Pękala, E. 2019. Metabolic stability and its role in the discovery of new chemical entities. *Acta Pharmaceutica*, 69(3):345–361.

Sulkowski, M. S. 2004. Drug-Induced Liver Injury Associated with Antiretroviral Therapy that Includes HIV-1 Protease Inhibitors. *Clinical Infectious Diseases*, 38(Supplement_2):90–97.

Wood, F. L., Houston, J. B., Hallifax, D. 2017. Clearance Prediction Methodology Needs Fundamental Improvement: Trends Common to Rat

and Human Hepatocytes/Microsomes and Implications for Experimental Methodology. *Drug Metabolism and Disposition*, 45(11):1178–1188.

World Health Organization 2016. Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: Recommendations for a Public Health Approach. volume 2, Geneva. Geneva: World Health Organization.

Zhang, Y., Vermeulen, N. P. E., Commandeur, J. N. M. 2017. Characterization of human cytochrome P450 mediated bioactivation of amodiaquine and its major metabolite N-desethylamodiaquine. *British Journal of Clinical Pharmacology*, 83(3):572–583.