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In vitro interaction of lumefantrine and piperaquine by atorvastatin against *Plasmodium falciparum*

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Abstract

Background: There is an urgent need for the discovery of new anti-malarial drugs and combination therapy. A combinatorial approach protects each drug from the development of resistance and reduces generally the overall transmission rate of malaria. Statins, the inhibitors of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase and a family of lipid-lowering drugs, have *in vitro* anti-malarial properties, and more specially atorvastatin. However, atorvastatin has a short elimination half-life (14 hours) and an efficient combination of anti-malarial drugs must associate a drug with a short elimination half-life and a drug with a long elimination half-life. The objective of the present work was to identify new potential partners among standard new anti-malarial drugs with long elimination half-life, such as lumefantrine, piperaquine, pyronaridine and atovaquone, to improve the *in vitro* activity of atorvastatin against different *Plasmodium falciparum* strains to treat uncomplicated malaria.

Methods: *In vitro* interaction of atorvastatin in combination with lumefantrine, piperaquine, pyronaridine and atovaquone was assessed against 13 *P. falciparum* strains by isotopic test.

Results: Atorvastatin showed additive effects with pyronaridine, piperaquine and lumefantrine. Atorvastatin increased the *in vitro* activity of lumefantrine and piperaquine at concentrations expected in clinical observations. The average IC_{50} values of lumefantrine decreased significantly from 31.9 nM to 20.5 nM (a decrease of 35.7%) in combination with 1 μM of atorvastatin.

Conclusions: Even though *in vitro* data indicate that atorvastatin improved the activity of lumefantrine and piperaquine, the same may not necessarily be true *in vivo*. Piperaquine, a new drug with long terminal elimination half-life, is currently a very promising anti-malarial drug.

Keywords: Malaria, *Plasmodium falciparum*, Anti-malarial, Resistance, *In vitro*, statin

Background

Over the past 20 years, many strains of *Plasmodium falciparum* have become resistant to chloroquine and other anti-malarial drugs [1]. In 2002, the World Health Organization (WHO) recommended that artemisinin-based combination therapy (ACT) be used to treat all cases of uncomplicated malaria and that artesunate should be used as the first-line treatment for severe malaria in

2011. Several recent studies have reported clinical failures or extended parasite clearance times in Cambodia [2-4].

There is an urgent need for the discovery of new anti-malarial drugs and combination therapy. A combinatorial approach protects each drug from the development of resistance and reduces generally the overall transmission rate of malaria [5].

Statins, the inhibitors of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CoA reductase) and a family of lipid-lowering drugs, have *in vitro* anti-malarial properties [6,7]. Moreover, atorvastatin improved the *in vitro* activity of mefloquine [8], quinine [9], dihydroartemisinin [10] and Proveblue®, a methylene blue preparation that complies with the European Pharmacopoeia

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and contains limited organic impurities and heavy metals of recognized toxicity [11], at the plasma concentrations expected in clinical observations in patients taking 80 mg of atorvastatin daily (0.1 to 0.5 μM) [12]. However, atorvastatin used alone failed to prevent death from cerebral malaria or to affect the parasitaemia of infected mice [13-15]. Atorvastatin combined with mefloquine (a long half-life drug) led to a significant delay in mouse death and had an effect on the onset of cerebral malaria symptoms [16]. However, the mice died of severe anaemic malaria. The combination of dihydroartemisinin and atorvastatin was effective as a therapeutic scheme for improving mouse survival but less effective for cytokine modulation, which is associated with protection against cerebral malaria [17]. In a therapeutic intraperitonal-scheme, the combination of atorvastatin and dihydroartemisinin *versus* dihydroartemisinin alone resulted in a significant delay in mouse death and had an effect on the onset of cerebral malaria symptoms and on the level of parasitaemia. The experimental conditions did not prevent death. Atorvastatin failed to improve *in vivo* efficacy of quinine in cerebral malaria [17]. Atorvastatin has a short elimination half-life (14 hours) compared to proguanil (24 hours), atovaquone (31–73 hours), chloroquine (two to three days), lumefantrine (four to six days), pyronaridine (six days), mefloquine (six to 41 days), or piperaquine (22 days). A combination of anti-malarial drugs must associate a drug with a short elimination half-life and a drug with a long elimination half-life. However, this is a debatable concept because the long half-life drug is exposed for a significant period of time to select parasite resistance.

The objective of the present work was to identify new potential partners among standard new anti-malarial drugs with long elimination half-life, such as lumefantrine, piperaquine, pyronaridine, and atovaquone, to improve the *in vitro* activity of atorvastatin against different *P. falciparum* strains to treat uncomplicated malaria.

Methods

Strains of *Plasmodium falciparum*

Thirteen parasite strains or clones from a wide panel of countries (Cambodia, Cameroon, Gabon, the Gambia, Indochina, Republic of Comoros, Republic of the Congo, Senegal, Sierra Leone, and Uganda) were maintained in culture in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 10% human serum (Abcys SA, Paris, France) and buffered with 25 mM HEPES and 25 mM $NaHCO_3$. Parasites were grown in type A^+ human red blood cells under controlled atmospheric conditions (10% O_2, 5% CO_2, and 85% N_2) at 37°C with a humidity of 95%. All strains were synchronized twice with sorbitol before use [18]. Clonality was verified using PCR genotyping of polymorphic genetic markers, msp1, msp2, and microsatellite loci [19,20]. The potentiation evaluation of each strain

was assessed in three independent experiments, as previously described [21].

Drugs

Stock solutions were prepared by dilution in methanol for piperaquine, pyronaridine and atovaquone; and in ethanol for lumefantrine. Final concentrations ranged from 0.05 to 318 nM for lumefantrine, 0.1 to 10,000 nM for atovaquone, 0.16 to 100 nM for pyronaridine and 0.78 to 994 nM for piperaquine. A total of 25 μL of stock solution of each concentration was distributed in duplicate or triplicate into Falcon 96-well flat bottom plates. Plates were dried overnight. Atorvastatin stock solution was prepared extemporely with 1% DMSO (v/v) in RPMI and diluted in sterile distilled water for final concentrations included between 0.006 and 32 μM. Aliquots of 25 μl of atorvastatin solution were distributed into Falcon 96-well plates pre-dosed with anti-malarial agents.

In vitro assay

The 50% inhibitory concentration (IC_{50}), i.e., the drug concentration corresponding to 50% of the uptake of 3H-hypoxanthine by the parasites in drug-free control wells, was determined by non-linear regression analysis of log-dose/response curves. Data were expressed as the geometric mean IC_{50}, and 95% confidence intervals (95% CIs) were calculated. Each anti-malarial drug was serial diluted and combined with a static concentration of atorvastatin to obtain IC_{50} for each anti-malarial drug. Ten various atorvastatin concentrations were used to construct the isobolograms. Isobolograms were constructed by plotting a pair of fractional IC_{50}s for each combination of anti-malarial treatment and atorvastatin. Fractional IC_{50}s were calculated by dividing the atorvastatin fixed concentration by its IC_{50} alone, and these data were plotted on the horizontal axis. The corresponding fractional IC_{50}s of each classical anti-malarial treatment were calculated by dividing the combined IC_{50}s of each classical anti-malarial treatment with the atorvastatin by the IC_{50} of the anti-malarial treatment alone, and these data were plotted on the vertical axis. A straight diagonal isobologram indicates an additive effect. Curves above or below the diagonal indicate antagonistic or synergistic effects, respectively. Results close to the diagonal are considered to be additive.

Statistical analysis

The effects of atorvastatin at concentrations ranging from 0.1 to 1 μM, without intrinsic effects, were analysed with respect to the lumefantrine, piperaquine, pyronaridine, and atovaquone activity. These concentrations were relevant to atorvastatin plasma concentrations that are achievable in patients taking 80 mg of atorvastatin daily (0.1 to 0.5 μM). Statistical analysis was performed using R

software® (version 2.10.1). The global Friedman test was used to compare the medians of different matched groups, and the Wilcoxon test was used to compare the medians of two matched under-groups.

Results

Atorvastatin showed additive effects with piperaquine, lumefantrine and pyronaridine (Figure 1).

Static concentration of atorvastatin affected significantly the *in vitro* activity of lumefantrine and piperaquine. The average IC_{50} values of lumefantrine decreased significantly from 31.9 nM to 28.1 nM (a decrease of 11%) in combination with 0.12 μM of atorvastatin, 26.3 nM (a decrease of 17.6%) in combination with 0.25 μM of atorvastatin, 24.6 nM (a decrease of 24.8%) in combination with 0.5 μM of atorvastatin and 20.5 nM (a decrease of 35.7%) in combination with 1 μM of atorvastatin, at atorvastatin plasma concentrations expected from clinical observations in patients taking 80 mg of atorvastatin daily (Table 1).

The average IC_{50} values of piperaquine decreased significantly from 61.1 nM to 52.5 nM (a decrease of 14.1%) in combination with 0.5 μM of atorvastatin and 51.2 nM (a decrease of 16.2%) in combination with 1 μM of atorvastatin.

Atorvastatin did not significantly affect the IC_{50} values of pyronaridine or atovaquone.

Discussion

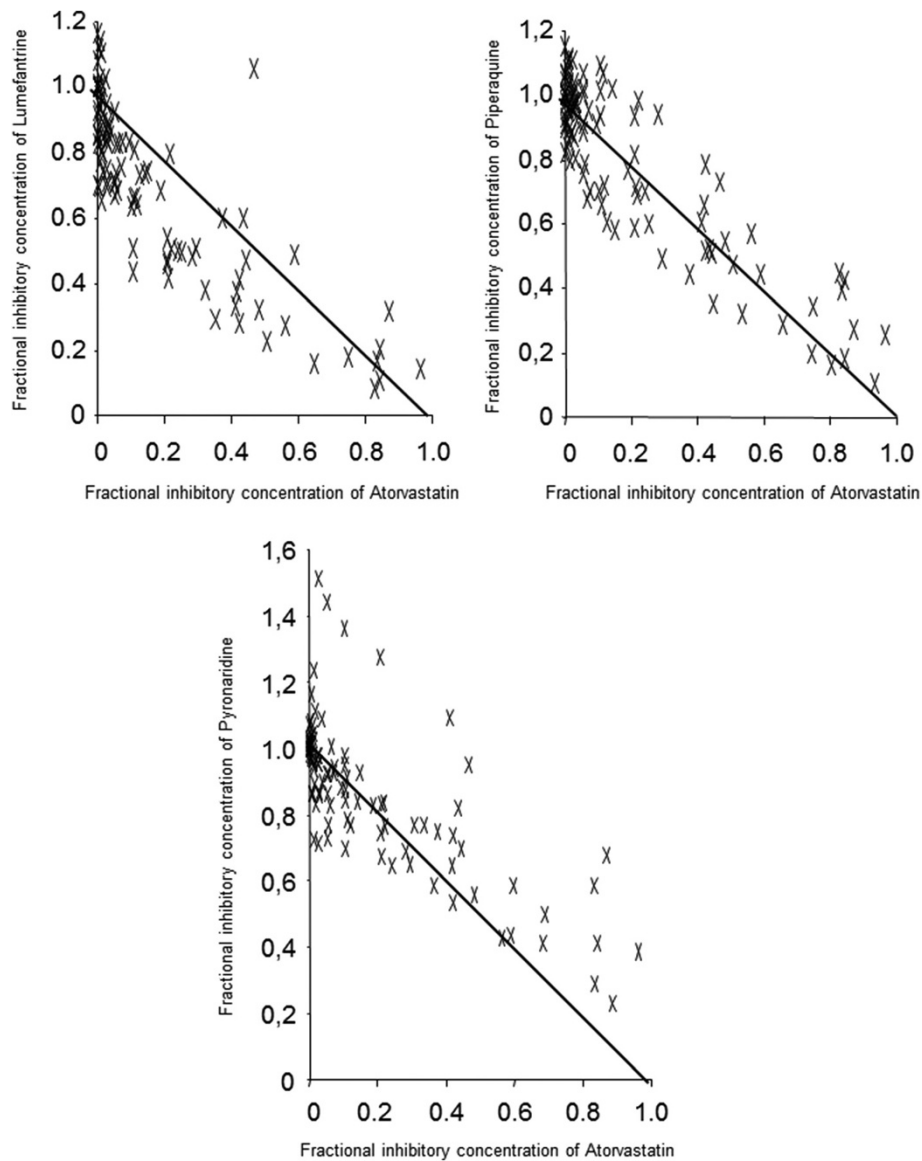Atorvastatin improved strongly the *in vitro* activity of dihydroartemisinin [10], mefloquine [8], quinine [9], and

Figure 1 In vitro combinations of atorvastatin with lumefantrine, piperaquine and pyronaridine against 13 *Plasmodium falciparum* strains.

Table 1 Inhibitory activities of lumefantrine, piperaquine, pyronaridine and atovaquone alone and in combination with atorvastatin against 13 strains of *Plasmodium falciparum*

Anti-malarial drugs	Lumefantrine		Piperaquine		Pyronaridine		Atovaquone	
	IC_{50} mean (nM)	P-value	IC_{50} mean (nM)	P-value	IC_{50} mean (nM)	P-value	IC_{50} mean (μM)	P-value
Alone	31.9 ± 15.1		61.1 ± 15.5		30.3 ± 9.6		3.79 ± 2.0	
+ 0.12 μM Atorvastatin	28.1 ± 15.1	0.0068	59.5 ± 19.7	NS	28.9 ± 9.2	NS	3.35 ± 1.5	NS
+ 0.25 μM Atorvastatin	26.3 ± 12.4	0.0019	61.7 ± 18.7	NS	29.1 ± 10.1	NS	3.35 ± 1.6	NS
+ 0.5 μM Atorvastatin	24.6 ± 11.7	0.0019	52.5 ± 17.5	0.0146	28.4 ± 10.2	NS	3.23 ± 1.3	NS
+ 1 μM Atorvastatin	20.5 ± 11.0	0.0009	51.2 ± 19.2	0.0244	26.9 ± 10.0	NS	2.93 ± 1.3	NS

NS if P-value > 0.05.

methylene blue [11] and less strongly those lumefantrine and piperaquine. Atorvastatin demonstrated antagonistic effects with anti-malarial drugs whose resistance involves the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*), such as chloroquine, monodesethylamodiaquine [9]. In addition, atorvastatin didn't affect significantly the *in vitro* activity of pyronaridine whose resistance seems to interact also with *pfcrt* (unpublished observations). A decrease in *P. falciparum* susceptibility to lumefantrine, mefloquine or dihydroartemisinin is associated with polymorphisms in the genes encoding the multidrug resistance (MDR)-like proteins, such as Pgh1, encoded by *pfmdr1* (*P. falciparum* multidrug resistance 1), or in copy numbers of this gene [22-25]. Atorvastatin is an inhibitor of the human phosphoglycoprotein (PgP), an efflux protein involved in cancer cells [26-28]. Pgh1 could be a target for atorvastatin in *P. falciparum* parasites. However, *in vitro* responses to piperaquine seem to be not associated with polymorphisms in *pfcrt* gene [29] and in *pfmdr1* [30] or copy number of *pfmdr1* [31], but associated with repeat polymorphisms in the low-complexity regions of a *P. falciparum* ABC transporter, *pfmdr6* [32]. Atorvastatin seems to improve the *in vitro* activity of anti-malarial drugs whose resistance involves preferentially ABC transporters such as *pfmdr1* and *pfmdr6* and don't affect or reduce the activity of drugs whose resistance involves preferentially *pfcrt*.

Atorvastatin given with mefloquine and dihydroartemisinin prevents cerebral malaria in a mouse model [16,17]. However, the mice that did not die of cerebral malaria died of severe anaemic malaria. Atorvastatin strongly protects endothelial cells against *P. falciparum*-induced collateral damage, cell apoptosis and endothelial barrier permeabilization [33]. Atorvastatin can be used to reduce *P. falciparum*cyto-adherence to endothelial cells; cyto-adherence and the inflammatory burst are the key events of pathogenesis in severe human malaria. In mice with *Plasmodium berghei* ANKA cerebral malaria, lovastatin reduces pro-inflammatory cytokines in the brain and prevents cognitive impairment [34].

Another hypothesis that could explain differences *in vitro* activity and *in vivo* efficacy is the role the cytochrome P450 enzyme. Most of the anti-malarial drugs are metabolised by the cytochrome P450 enzyme. Chloroquine, quinine, mefloquine, amodiaquine, lumefantrine, piperaquine, artemisinin derivatives and atorvastatin are metabolised by CYP3A4/A5 [35,36]. Artesunate and artemisinin are also metabolised by CYP2A6 and CYP2B6 [35]. Dihydroartemisinin is metabolised by UGT1A9 and UGT2B7 [35]. In addition, chloroquine and amodiaquine are metabolised by CYP2C8 [35]. Atorvastatin induces the activity of CYP2C8 and that of CYP3A4 but to a lesser way [37]. By increasing the P450 activity, atorvastatin could increase the catabolism of some anti-malarial drugs and reduce or sometimes increase (proguanil) the efficacy of the drug.

Even though *in vitro* data indicate that atorvastatin improved the activity of lumefantrine and piperaquine, the same may not necessarily be true *in vivo*. Piperaquine, a new drug with long terminal elimination half-life, is currently a very promising anti-malarial drug. These observations support calls for an *in vivo* evaluation of combination of atorvastatin with lumefantrine and piperaquine in a mouse model to treat uncomplicated malaria.

Competing interests
All authors declare that they have no competing interests.

Authors' contributions
JD, HS, RA, and EB carried out the *in vitro* tests. BP conceived and coordinated the study. JD and BP analysed the data. JD and BP drafted the manuscript. All the authors read and approved the final manuscript.

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