Synergy of mefloquine activity with atorvastatin, but not chloroquine and monodesethylamodiaquine, and association with the \textit{pfmdr1} gene

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Objectives: The aim of the study was to assess the \textit{in vitro} potentiating effects of atorvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, in combination with mefloquine, chloroquine or monodesethylamodiaquine against \textit{Plasmodium falciparum} and to evaluate whether the effects of atorvastatin could be associated with mutations or gene copy number in multidrug resistance (MDR)-like protein genes.

Methods: The susceptibilities of 21 parasite strains to combinations of atorvastatin with mefloquine, chloroquine or monodesethylamodiaquine were assessed using the \textit{in vitro} isotopic microtest. Genotypes and gene copy number were assessed for \textit{pfmdr1}, \textit{pfmdr2} and \textit{pfmrp} genes.

Results: Atorvastatin demonstrated synergistic effects in combination with mefloquine. The mefloquine IC\textsubscript{50} (50\% inhibitory concentration) was reduced by 7\%, 24\% and 37\% in the presence of atorvastatin at concentrations of 0.1, 0.5 and 1.0 \textit{mM}, respectively. The synergistic effect of atorvastatin on the response to mefloquine was significantly associated with \textit{pfmdr1} copy number. The concentration of atorvastatin that could reduce the IC\textsubscript{50} of mefloquine by 50\% was 2.4 ± 1.3 \textit{mM} for the 12 strains that contained one copy of \textit{pfmdr1} and 5.8 ± 2.1 \textit{mM} for the 9 strains that contained two copies or more. The synergistic effect of atorvastatin in combination with mefloquine was found to be significantly unrelated to mutations in \textit{pfmdr1}, \textit{pfmdr2} or \textit{pfmrp} genes.

Conclusions: The synergy of the effect of mefloquine at concentrations relevant to its achievable plasma concentrations in patients taking 80 mg of atorvastatin daily suggests that atorvastatin will be a good candidate in combination with mefloquine for malaria treatment.

Keywords: \textit{Plasmodium}, malaria, resistance, drugs
with chloroquine, monodesethylamodiaquine, mefloquine, quinine, lumefantrine, dihydroartemisinin, atovaquone or doxycycline, and IC_{50} (50% inhibitory concentration) values for atorvastatin were found to be unrelated to mutations occurring in transport protein genes involved in quinoline antimalarial drug resistance, such as pfcrt, pfmdr1, pfmrp and pfne-1. Atorvastatin, used alone at 20 mg/kg of body weight, failed to prevent death from cerebral malaria or to affect parasitaemia in infected mice. One possibility is that atorvastatin could act as an adjuvant therapy. Atorvastatin is an inhibitor of phosphoglycoprotein (Pgp), an efflux protein in cancer cells. Multidrug resistance (MDR)-like proteins are involved in mefloquine resistance in P. falciparum. The resistance to mefloquine was linked both to mutations in the pfmdr1 gene (P. falciparum multidrug resistance 1) encoding PfMrp and to increases in copy number of the wild-type gene. The objectives of this study were: (i) to assess the in vitro potentiating effects of atorvastatin in combination with mefloquine, chloroquine or monodesethylamodiaquine against 21 strains of P. falciparum from a wide panel of countries and with different susceptibility profiles; (ii) to evaluate whether the effects of atorvastatin could be associated with mutations in the pfmdr1 gene or with the gene copy number; and (iii) to evaluate whether the effects of atorvastatin could be associated with mutations or with copy number of other ABC transporter or MDR-like protein genes, such as pfmdr2 (P. falciparum multidrug resistance 2) (encoding PfMRD2) or pfmrp (P. falciparum multidrug resistance-associated protein) encoding PfMRP1.

Materials and methods

P. falciparum cultures

In total, 21 parasite strains (familiar laboratory strains or strains obtained from isolates after growth in culture for an extended period of time) from a wide range of countries (Brazil, Cambodia, Cameroon, Djibouti, French Guyana, the Gambia, Honduras, Indochina, Niger, Republic of Comoros, Republic of the Congo, Senegal, Sierra Leone, Sudan and Uganda) were maintained in culture in RPMI 1640 (Invitrogen, Paisley, UK), supplemented with 10% human serum (Abcys S.A. Paris, France), and buffered with 25 mM HEPES and 25 mM NaHCO₃. Parasites were grown in 10% O₂, 5% CO₂ and 85% N₂ at 37 °C, with a human blood type A+ blood under controlled atmospheric conditions. Immediately after incubation, plates were frozen and then thawed to lyse the erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter GF/B; Perkin-Elmer) and washed using a cell harvester (Filter-Mate Cell Harvester; Perkin-Elmer). Filter microplates were dried and 25 μL of scintillation cocktail (Microscint O; Perkin-Elmer) was placed in each well. Radioactivity incorporated into nucleotides by the parasites was measured with a scintillation counter (Top Count; Perkin-Elmer).

The IC_{50} was assessed by determining the drug concentration corresponding to incorporation of 50% of the tritiated hypoxanthine by the parasite in the drug-free control wells. The IC_{50} value was determined by non-linear regression analysis of log-based dose–response curves (Rismost™, Packard, Meriden, USA).

To evaluate atorvastatin modulation of quinoline resistance, isobolograms were constructed by plotting a pair of fractional IC_{50}s for each combination of atorvastatin and mefloquine, chloroquine or monodesethylamodiaquine and for both parasite strains. The atorvastatin fractional IC_{50} was calculated by dividing the fixed concentration by the IC_{50} of the tested drug alone, and then plotted on the horizontal axis. The corresponding mefloquine, chloroquine or monodesethylamodiaquine fractional IC_{50} was calculated by dividing the mefloquine, chloroquine or monodesethylamodiaquine IC_{50} combined with fixed concentrations of atorvastatin, and then plotted on the vertical axis. Points lying above the straight diagonal line (corresponding to the points where there is no interaction between the drugs) are antagonistic, and points below the straight diagonal line are considered to be synergistic.

In addition, three concentrations of atorvastatin (0.1, 0.5 and 1 μM), which were relevant to atorvastatin plasma concentrations achievable in patients taking 80 mg of atorvastatin daily, were reanalysed separately.

In addition, we calculated the concentration of atorvastatin that could reduce the IC_{50} of mefloquine by 50% when used alone, [atorvastatin]_{mefloquine}.

Nucleic acid extraction

Total genomic DNA of each strain was isolated using the E.Z.N.A. Blood DNA kit (Omega Bio-Tek, GA, USA) extraction method.

Drugs

Atorvastatin calcium salt was purchased from Molekula (UK). Mefloquine was purchased from Sigma (St Louis, MO, USA). Monodesethylamodiaquine was obtained from the WHO (Geneva, Switzerland). Atorvastatin was dissolved in DMSO 1% (v/v) in RPMI. Two-fold serial dilutions, with final concentrations ranging from 0.01 to 200 μM, were prepared in 1% DMSO in RPMI and distributed into Falcon 96-well plates just before use. Mefloquine was first dissolved in methanol and then diluted in water to obtain concentrations ranging from 5 to 3200 nM. Monodesethylamodiaquine was first dissolved in methanol and then diluted in water to obtain final concentrations ranging from 0.063 to 1000 nM.

In vitro assay

To assess the synergy of atorvastatin with quinoline drugs, 25 μL/well of atorvastatin and 200 μL/well of the suspension of synchronized parasitized red blood cells (final parasitaemia, 0.5%; final haematocrit, 1.5%) were distributed in 96-well plates pre-dosed with mefloquine, chloroquine or monodesethylamodiaquine. Parasite growth was assessed by adding 1 μCi of tritiated hypoxanthine with a specific activity of 14.1 Ci/mmol (Perkin-Elmer, Courtaboeuf, France) to each well at time zero. The plates were then incubated for 48 h in controlled atmospheric conditions. Immediately after incubation, plates were frozen and then thawed to lyse the erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter GF/B; Perkin-Elmer) and washed using a cell harvester (Filter-Mate Cell Harvester; Perkin-Elmer). Filter microplates were dried and 25 μL of scintillation cocktail (Microscint O; Perkin-Elmer) was placed in each well. Radioactivity incorporated into nucleotides by the parasites was measured with a scintillation counter (Top Count; Perkin-Elmer).

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In addition, we calculated the concentration of atorvastatin that could reduce the IC_{50} of mefloquine by 50% when used alone, [atorvastatin]_{mefloquine}.

pfmdr1 single nucleotide polymorphisms (SNPs)

pfmdr1 (PF13150w) was amplified by PCR using the following primer pairs: 5′-AGA GAA AAA AGA TGG TAA CCT CAG-3′ and 5′-ACC ACA AAC ATA AAT TAA CGG-3′ to amplify codons 86 and 184; and 5′-CAG GAA GCA TTT TAT AAT ATG C-3′ and 5′-CGT TTA ACA TCT TCC AAT GTT GCA-3′ to amplify codons 1034, 1042 and 1246. The reaction mixture consisted of ~200 ng of genomic DNA, 0.5 μM of forward and reverse primers, buffer (50 mM KCl/10 mM Tris, pH 8.3), 2.5 mM MgCl₂, 200 μM deoxynucleotide triphosphate (dNTP) and 0.3 U of Taq DNA polymerase (Eurogentec) in a final volume of 50 μL. The thermal cycler (T3 Biometra) was programmed as follows: an initial 94 °C for 2 min; followed by
40 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1 min; and a final 15 min extension step at 72°C. The amplified fragments were sequenced as previously described. Sequences were analysed with the software BioEdit Sequence Alignment Editor 7.0.9.0.

**pfmdr2 SNPs**

pfmdr2 (PF14_0455) was amplified by PCR using the following primer pairs: 5′-ATG GAT GTA TCA AAT TAC TAG TAT TT-3′ and 5′-CTA CCA TAT CCT TTG TTT AAA AAT GAT GC-3′; 5′-CTT CTG ATG TTC AGG AGG AAA ATA-3′ and 5′-GCT ATA TCA TGA TAT ACA TCA TTC ATC-3′; 5′-TGA TTA TTC CGG CAA CAA TAG-3′ and 5′-TAT AAC TGA GCC GAT TTA ACA GC-3′; and 5′-CTC TTG TAG GTC ATG CCT GTG-3′ and 5′-CTA TTT TTT GTT GAT CAT ATT ATC A-3′. The reaction mixture consisted of ~200 ng of genomic DNA, 0.5 μM of forward and reverse primers, 250 μM dNTPs and 0.3 μL of Taq DNA polymerase in a final volume of 50 μL. The thermal cycling (T3 Biometra®) was programmed as follows: 94°C for 2 min for the first cycle and 30 s for subsequent cycles; 52°C for 1 min for the first cycle and 1 min for subsequent cycles; and 72°C for 1 min and 30 s for all cycles, for a total of 40 cycles, followed by a 15 min extension step at 72°C. The amplified fragments were sequenced as previously described. Sequence analyses were performed with the software BioEdit Sequence Alignment Editor 7.0.9.0.

**pfmrp SNPs**

PCR amplification followed by sequencing was used to detect SNPs in *pfmrp* (PFA0590w) at positions 191 and 437. The primers used for amplification and sequencing were: pfmrp-501F, 5′-TTT CAA AGT ATT CAG TGG GT-3′; and pfmrp-1409R, 5′-GCC ATA ATT AAT GAT GTA AA-3′.

**Copy number of pfmdr1**

pfmdr1 copy number was estimated by TaqMan real-time PCR (7900HT Fast Real-Time PCR system, Applied Biosystems, Courtaboeuf, France) relative to the single copy gene, β-tubulin (PF10_0084). The following oligonucleotide primers and probes as previously reported were used with slight modifications:16 5′-TGC ATAT A AA AAG CTC ACG AGA AA A-3′, 5′-TGG TGT CTT CGA GAC GCT GAT CGA AGA GCC TTT GGT TTT AAT TAC CCA ACA CAA TTA-3′ and 5′-VIC-TTT AAT AAC CCT GAT TTT CAA AGT ATT CAG TGG GT-3′ for pfmdr1; and 5′-TGG TGT CTT CGA GAC GCT GAT CGA AGA GCC TTT GGT TTT AAT TAC CCA ACA CAA TTA-3′ and 5′-TCT TTT GGC ATC ATC CTC C-3′ for pfmrp (Eurogentec, Angers, France). Individual PCRs were carried out using 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM forward primer, 900 nM reverse primer, 250 nM Taqman probe and 5 μL of template DNA in a final volume of 25 μL. The reaction mixtures were prepared at 4°C in a 96-well optical reaction plate (Applied Biosystems) covered with optical adhesive covers (Applied Biosystems). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min and 50 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was assayed in triplicate, and analysed with the SDS software 2.2.1 (Applied Biosystems). PCR efficiencies of all primer pairs were evaluated on a 2× dilution series of *P. falciparum* 3D7 genomic DNA and found to be sufficient close to obviate the need for any correction factor. Therefore, the ΔΔCt method of relative quantification was used and adopted to estimate copy numbers of the *pfmdr1* gene where ΔΔCt= (Ct pfmdr1 – Ct β-tubulin)sample – (Ct pfmdr1 – Ct β-tubulin)calibrator.26,27 Genomic DNA extracted from the *P. falciparum* 3D7 strain, which has a single copy of each gene, was used as a calibrator, whereas β-tubulin served as the control housekeeping gene in all experiments.

**pfmdr2 copy number** was estimated as previously described for *pfmdr1*. The following primer pair and probe, designed by using Primer Express v2.0 (Applied Biosystems), were used to amplify *pfmdr2*: 5′-AAT TAC CCA ACA CCA CCA TTA CAT ACA-3′ and 5′-TCT CTC CTT TTG AAT CAT AGA AGC GTA AA-3′; and 5′-VIC-ACA TAA AAC CAC GAT CCA CAA GTG CTC TTG TAG GTC TAMRA-3′.

**Statistical analysis**

The differences in mefloquine IC50 between atorvastatin concentration groups have first been tested using analysis of variance (ANOVA) for repeated measures to take into account the fact that observations made within each strain were not independent. Using the most conservative correction for interdependence between observations (i.e. Box’s conservative epsilon), the differences in mefloquine IC50 were tested for concentrations relevant to atorvastatin plasma concentrations achievable in patients taking 80 mg of atorvastatin daily (0.1, 0.5 and 1 μM). Using a random effect linear regression approach, the regression coefficients for the log-transformed mefloquine IC50 indicated the significance of the mean fold change in mefloquine IC50 when adding atorvastatin concentrations of 0.1, 0.5 and 1.0 μM. The Kruskal–Wallis test or the Mann–Whitney U test were used when appropriate to compare equality of populations for each mutation. To account for multiple comparisons, the results of these tests were considered to be statistically significant only when P<0.005 (0.05 divided by the number of tests = 10), applying the Bonferroni correction.

**Results**

While atorvastatin demonstrated antagonistic effects in combination with chloroquine and monodesethylamodiaquine against the 21 *P. falciparum* strains (Figure 1), atorvastatin demonstrated synergistic effects in combination with mefloquine (Figure 2). The differences in mefloquine IC50 between atorvastatin concentrations groups have first been tested using ANOVA for repeated measures to take into account the fact that observations made within each strain were not independent. Using the most conservative correction for interdependence between observations (i.e. Box’s conservative epsilon), the differences in mefloquine IC50 were highly significant (P<0.001) when adding atorvastatin at 0.5 and 1.0 μM, concentrations relevant to atorvastatin plasma concentrations achievable in patients taking 80 mg of atorvastatin daily (Table 1). Using a random effect linear regression approach, the regression coefficients for the log-transformed mefloquine IC50 indicated that the mean fold change in mefloquine IC50 when adding atorvastatin concentrations of 0.5 and 1.0 μM (0.76 and 0.63, respectively) also highly significant (P<0.001). The mefloquine IC50 was reduced by 7% (0% to 15%; 95% confidence interval (CI) 0%–14%), 24% (4% to 39%; 95% CI 17%–30%) and 37% (12% to 60%; 95% CI 31%–42%) in the presence of atorvastatin at concentrations of 0.1, 0.5 and 1.0 μM, respectively. These reductions were significant (P<0.001) for 0.5 and 1.0 μM atorvastatin.

Concentrations of atorvastatin that reduced the IC50 of mefloquine by 50% when used alone, [atorvastatin]mefloquine, are presented in Table 2. The decrease in the mefloquine IC50 in the presence of atorvastatin was not significantly correlated with the mefloquine IC50 (r=0.26, P=0.6772) or atorvastatin IC50 (r=0.20, P=0.2330).
The mean IC$_{50}$ for atorvastatin used alone was determined for each strain, and the copy number and mutations identified in the \textit{pfmdr1}, \textit{pfmdr2} and \textit{pfmrp} genes are presented in Table 2. The following mutations were identified for at least one strain: \textit{pfmdr1} N86Y, Y184F, S1034C, N1042D and D1246Y; \textit{pfmdr2} S208N and F423Y; and \textit{pfmrp} H191Y and S437A. The copy number of \textit{pfmdr1} ranged from one to three. Only one copy of \textit{pfmdr2} was found in all of the 21 \textit{P. falciparum} strains.

The synergistic effect of atorvastatin on the mefloquine response was significantly associated ($P=0.0022$) with the copy number of the \textit{pfmdr1} gene (Table 3). The mean IC$_{50}$ for [atorvastatin]$_{\text{mefloquine}}$ was $2.4 \pm 1.3 \, \mu\text{M}$ for the 12 strains with one copy of \textit{pfmdr1}, $5.5 \pm 2.1 \, \mu\text{M}$ for the 8 strains with two copies and $7.8 \, \mu\text{M}$ for the only strain with three copies ($5.8 \pm 2.1 \, \mu\text{M}$ for the 9 strains with two copies and more of \textit{pfmdr1}).

**Discussion**

Mefloquine is currently one of the recommended chemoprophylactic regimens for travellers visiting malaria-endemic areas of south-east Asia, Africa and South America. Mefloquine is recommended by the French Consensus conference for chemoprophylaxis in countries with a high prevalence of resistance to chloroquine or multiresistance (group 3 countries).\textsuperscript{28} In addition, the combination of artemisinine and mefloquine has been evaluated and used mainly in south-east Asia and South America for malaria treatment.\textsuperscript{29} Despite its efficacy against chloroquine-resistant strains, the emergence of mefloquine resistance has been documented during the last two decades in south-east Asia. Mefloquine–artemisinin combination therapy is beginning to fail in southern Cambodia and Thailand.\textsuperscript{30–32} Furthermore, failures of antimalarial prophylaxis based on mefloquine were observed in Africa.\textsuperscript{33}

A large amount of scientific effort is directed toward elucidation of the mechanisms underlying resistance to antimalarial drugs with the hope of restoring/improving the efficacy of existing drugs and of developing new drugs that can bypass resistance mechanisms. One of the strategies for reducing the prevalence of malaria is the combinatorial use of drugs. The use of combinations protects each drug from the development of resistance and reduces the overall transmission of malaria.\textsuperscript{34}
**In vitro** synergy of atorvastatin and mefloquine

**Table 1.** *In vitro* susceptibility of *P. falciparum* strains to atorvastatin alone, mefloquine alone and the combination of mefloquine + atorvastatin at concentrations of 0.1, 0.5 and 1.0 μM

<table>
<thead>
<tr>
<th>Strains</th>
<th>IC₅₀ MQ (nM)</th>
<th>IC₅₀ A VA alone</th>
<th>IC₅₀ MQ alone</th>
<th>IC₅₀ MQ + 0.1 μM A VA</th>
<th>IC₅₀ MQ + 0.5 μM A VA</th>
<th>IC₅₀ MQ + 1.0 μM A VA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMT 10354</td>
<td>38 ± 5</td>
<td>5.5 ± 0.5</td>
<td>3.9 ± 0.3</td>
<td>Y</td>
<td>F</td>
<td>S</td>
</tr>
<tr>
<td>W2</td>
<td>34 ± 6</td>
<td>5.7 ± 0.4</td>
<td>7.1 ± 0.5</td>
<td>Y</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>IMT 16332</td>
<td>24 ± 5</td>
<td>5.7 ± 0.5</td>
<td>1.9 ± 0.2</td>
<td>Y</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>D6</td>
<td>65 ± 9</td>
<td>5.8 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>N</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>IMT Bres</td>
<td>36 ± 6</td>
<td>5.8 ± 0.4</td>
<td>8.9 ± 1.4</td>
<td>Y</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>IMT 31</td>
<td>48 ± 5</td>
<td>5.9 ± 0.5</td>
<td>0.7 ± 0.2</td>
<td>N</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>FCM29</td>
<td>30 ± 7</td>
<td>6.4 ± 0.4</td>
<td>1.9 ± 0.3</td>
<td>Y</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>IMT K4</td>
<td>27 ± 5</td>
<td>6.8 ± 0.3</td>
<td>3.2 ± 0.5</td>
<td>N</td>
<td>Y</td>
<td>C</td>
</tr>
<tr>
<td>IMT 10500</td>
<td>38 ± 9</td>
<td>6.8 ± 0.6</td>
<td>2.2 ± 0.4</td>
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<td>Y</td>
<td>S</td>
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<tr>
<td>IMT 10336</td>
<td>34 ± 6</td>
<td>7.0 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>N</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>IMT K14</td>
<td>31 ± 4</td>
<td>7.2 ± 0.4</td>
<td>1.9 ± 0.3</td>
<td>N</td>
<td>F</td>
<td>C</td>
</tr>
<tr>
<td>IMT Guy</td>
<td>33 ± 5</td>
<td>7.3 ± 0.4</td>
<td>2.9 ± 0.4</td>
<td>N</td>
<td>F</td>
<td>S</td>
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<tr>
<td>IMT 9881</td>
<td>37 ± 8</td>
<td>7.4 ± 0.4</td>
<td>0.4 ± 0.2</td>
<td>N</td>
<td>F</td>
<td>D</td>
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<tr>
<td>IMT Vol</td>
<td>32 ± 7</td>
<td>7.9 ± 0.7</td>
<td>6.3 ± 1.3</td>
<td>Y</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>PA</td>
<td>36 ± 4</td>
<td>8.1 ± 0.3</td>
<td>7.8 ± 1.6</td>
<td>Y</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>106/1</td>
<td>26 ± 5</td>
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<td>5.8 ± 0.9</td>
<td>Y</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>IMT L1</td>
<td>37 ± 5</td>
<td>8.3 ± 0.1</td>
<td>4.9 ± 0.6</td>
<td>Y</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>FCR3</td>
<td>35 ± 7</td>
<td>8.5 ± 1.0</td>
<td>5.8 ± 0.8</td>
<td>Y</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>IMT K2</td>
<td>33 ± 5</td>
<td>8.7 ± 0.9</td>
<td>3.3 ± 0.7</td>
<td>N</td>
<td>F</td>
<td>C</td>
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<td>3D7</td>
<td>61 ± 11</td>
<td>9.2 ± 0.5</td>
<td>3.4 ± 0.5</td>
<td>N</td>
<td>Y</td>
<td>S</td>
</tr>
<tr>
<td>HB3</td>
<td>36 ± 6</td>
<td>11.0 ± 0.5</td>
<td>4.8 ± 0.5</td>
<td>N</td>
<td>F</td>
<td>S</td>
</tr>
</tbody>
</table>

**Table 2.** *In vitro* susceptibility to atorvastatin, the concentration of atorvastatin that decreased the mefloquine IC₅₀ by half ([A VA MQ], pfmdr1, pfmdr2 and pfmrp polymorphisms and the copy number of pfmdr1 and pfmdr2 genes in 21 *P. falciparum* strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>MQ IC₅₀ (nM)</th>
<th>A VA IC₅₀ (μM)</th>
<th>[A VA] MIC (μM)</th>
<th>Amino acid encoded by the pfmdr1 codon</th>
<th>Amino acid encoded by the pfmdr2 codon</th>
<th>pfmrp codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td>Amino acid</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>encoded by</td>
<td></td>
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A VA, atorvastatin; MQ, mefloquine. 
Values are means of mean IC₅₀ of three experiments for each strain. 
Bold font indicates point mutations.

Atorvastatin, a HMG-CoA reductase inhibitor, reduced in vitro growth of *P. falciparum* in the micromolar range. A generally agreed upon level of efficacy would be in the low or middle nanomolar range. However, if the mechanisms of action of such a compound are sufficiently new and different from those of the commonly used antimalarial drugs, this compound could warrant further investigation. The absence of in vitro cross-resistance between atorvastatin and mefloquine suggests that atorvastatin could be a good potential partner for mefloquine. These different considerations and the capacity of atorvastatin to inhibit human Pgh1 led us to evaluate atorvastatin in combination with mefloquine.

Atorvastatin improved the *in vitro* activity of mefloquine at concentrations relevant to atorvastatin plasma concentrations achievable in patients taking 80 mg of atorvastatin daily. Furthermore, the doses of atorvastatin administered to humans could be increased to 120 mg daily with only limited additional side effects. A dose of 120 mg of atorvastatin increased the susceptibility to atorvastatin, the concentration of atorvastatin that decreased the mefloquine IC₅₀ by half ([A VA MQ], pfmdr1, pfmdr2 and pfmrp polymorphisms and the copy number of pfmdr1 and pfmdr2 genes in 21 *P. falciparum* strains.

In *Cerebral malaria, malaria* shared common pathophysiological features with
The synergistic effect of atorvastatin in combination with mefloquine was found to be unrelated to mutations in the MDR-like protein genes. However, the synergy of the mefloquine response with atorvastatin was associated with pfmdr1 gene copy number. The atorvastatin concentration required to reduce the mefloquine response by half was higher in strains with more than two copies. These data are promising since mefloquine–artesunate combination therapy is beginning to fail in southern Cambodia and Thailand. Nevertheless, in the current study, the mefloquine IC50 of the isolates from Asia were reduced from 14% to 60% when compared with healthy volunteers (110.5 versus 5.9 ng/mL).37

One of our future objectives is to identify the modification of the proteome in parasites in contact with atorvastatin. Atorvastatin is an HMG-CoA reductase inhibitor. Nevertheless, the presence of an HMG-CoA homologue was not revealed by BLASTX comparison of the P. falciparum genome with other protozoal HMG-CoA protein sequences. Parasites treated with mevastatin show depressed biosynthesis of dolichol and isoprenoid pyrophosphate. Nevertheless, mevastatin decreases the viability of cells by inhibiting proteasome activity. One of our future objectives is to identify the modification of the P. falciparum proteome in parasites in contact with atorvastatin. The in vitro data on mefloquine support a prospective evaluation in animal models.

The different features in drug uptake and/or mode of action of atorvastatin compared with other antimalarial drugs and the synergy of the effect of mefloquine at concentrations relevant to atorvastatin plasma concentrations achievable in patients taking 80 mg of atorvastatin daily suggest that atorvastatin may be an alternative to maximize the efficacy and longevity of the P. falciparum treatment. Data on synergy of atorvastatin with artemisinin derivatives are contradictory. Wrong and Davis showed that the transporter Pgh1 was not involved in chloroquine resistance transporter, which belongs to the drug and metabolite transporter (DMT) superfamily, was the transport protein involved in chloroquine and monodesethylamodiaquine resistance. Hence, the hypothesis of inhibition of Pgh in human cells by atorvastatin,9–11 the association between pfmdr1 copy numbers and synergy of mefloquine effects by atorvastatin was a valid hypothesis. The transporter PfCRT was not involved in chloroquine or monodesethylamodiaquine resistance.44,45 PFCT (P. falciparum chloroquine resistance transporter), which belongs to the drug and metabolite transporter (DMT) superfamily, was the transport protein involved in chloroquine and monodesethylamodiaquine resistance.44,45

The mode of action of atorvastatin is as yet unknown. The absence of cross-resistance with chloroquine, quinine, monodesethylamodiaquine, mefloquine, lumefantrine, dihydroartemisinin, atovaquone or doxycycline suggested different features in drug uptake and/or mode of action of atorvastatin and the other compounds.6 The hypothesis of inhibition of PfCRT is compelling. Atorvastatin is an HMG-CoA reductase inhibitor. Nevertheless, the presence of an HMG-CoA homologue was not revealed by BLASTX comparison of the P. falciparum genome with other protozoal HMG-CoA protein sequences. Parasites treated with mevastatin show depressed biosynthesis of dolichol and isoprenoid pyrophosphate. In addition, mevastatin decreases the viability of cells by inhibiting proteasome activity.

### Table 3. Association of the in vitro atorvastatin (AVA) response (IC50), mefloquine (MQ), AVA + MQ and polymorphism in the pfmdr1, pfmdr2 and pfmrp genes and the copy number of the pfmdr1 gene in 21 strains of Plasmodium falciparum

<table>
<thead>
<tr>
<th>Genotype</th>
<th>AVA</th>
<th>MQ</th>
<th>AVA + MQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation in codon 86 of pfmdr1 gene</td>
<td>0.4792</td>
<td>0.2166</td>
<td>0.0059</td>
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<td>Mutation in codon 1034 of pfmdr1 gene</td>
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<td>0.0593</td>
<td>0.4193</td>
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<tr>
<td>Mutation in codon 1246 of pfmdr1 gene</td>
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<td>0.7624</td>
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<tr>
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<td>0.0155</td>
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<td>Mutation in codon 437 of pfmrp gene</td>
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<td>0.0126</td>
<td>0.0155</td>
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*P*-value significance: NS, not significant.

### Notes

- **Table 3.** Association of the in vitro atorvastatin (AVA) response (IC50), mefloquine (MQ), AVA + MQ and polymorphism in the pfmdr1, pfmdr2 and pfmrp genes and the copy number of the pfmdr1 gene in 21 strains of Plasmodium falciparum

- **Notes:**
  - Mann–Whitney U-test or Kruskal–Wallis test; significance cut-off 0.005 (0.05/10, 10 tests, Bonferroni correction). S, significant; NS, not significant.

- Twenty-one strains may not be sufficient to reach definite conclusions. The validity of the conclusion should be further investigated by analysing more strains or isolates, particularly from Asia.

- Atorvastatin did not increase the in vitro activity of chloroquine and monodesethylamodiaquine. The data on combination with chloroquine were in concordance with those of Wrong and Davis. As predicted by the inhibition of Pgh in human cells by atorvastatin, the association between pfmdr1 copy numbers and synergy of mefloquine effects by atorvastatin was a valid hypothesis. The transporter PfCRT was not involved in chloroquine or monodesethylamodiaquine resistance.44,45 PFCT (P. falciparum chloroquine resistance transporter), which belongs to the drug and metabolite transporter (DMT) superfamily, was the transport protein involved in chloroquine and monodesethylamodiaquine resistance.44,45

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malaria treatment. All of these observations support calls for both an in vivo evaluation with a pharmacokinetic component and clinical trials of atorvastatin as an antimalarial therapy.

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**Transparency declarations**

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**References**


