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RESEARCH

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Atorvastatin prevents *Plasmodium falciparum* cytoadherence and endothelial damage

Zacharie Taoufiq^{1,4*}, Paco Pino^{1,5}, Nadine N'dilimabaka¹, Issam Arrouss¹, Serge Assi^{1,6}, Florent Soubrier², Angelita Rebollo^{1†}, Dominique Mazier^{1,3*†}

Abstract

Background: The adhesion of *Plasmodium falciparum* parasitized red blood cell (PRBC) to human endothelial cells (EC) induces inflammatory processes, coagulation cascades, oxidative stress and apoptosis. These pathological processes are suspected to be responsible for the blood-brain-barrier and other organs' endothelial dysfunctions observed in fatal cases of malaria. Atorvastatin, a drug that belongs to the lowering cholesterol molecule family of statins, has been shown to ameliorate endothelial functions and is widely used in patients with cardiovascular disorders.

Methods: The effect of this compound on PRBC induced endothelial impairments was assessed using endothelial co-culture models.

Results: Atorvastatin pre-treatment of EC was found to reduce the expression of adhesion molecules and *P. falciparum* cytoadherence, to protect cells against PRBC-induced apoptosis and to enhance endothelial monolayer integrity during co-incubation with parasites.

Conclusions: These results might suggest a potential interest use of atorvastatin as a protective treatment to interfere with the pathophysiological cascades leading to severe malaria.

Background

Malaria remains a major threat to public health with 40% of the world population currently at risk. Every year, an estimated 500 million cases of clinical malaria and at least one million deaths are reported [1,2]. Fatal cases of malaria occur mainly in young children in Africa and consist of an acute neurological syndrome (cerebral malaria), either in isolation or concomitantly with multi-organ failure (pulmonary distress, acute renal failure) [3]. Anti-malarial drugs, such as quinine and artemisinin derivatives are administered intravenously as an emergency treatment. However, although these drugs effectively and rapidly clear parasites from the blood, 15%-20% of patients still die, probably as the result of impaired host responses [4,5]. Such a situation together with the difficulty in developing vaccines highlights the

urgent need of novel strategies for complement therapeutics.

The severity of *Plasmodium falciparum* infection depends largely on the ability of parasitized red blood cells (PRBC) to adhere on endothelial cells (EC) and sequester in the capillary network of vital organs (e.g. brain, lungs, kidneys, liver.) Moreover, activation of endothelial cells resulting from the adhesion of infected erythrocytes leads to an overexpression of different mediators, such as adhesion molecules ("hyperadhesion" phenomenon), pro-inflammatory cytokines, coagulation factors and contributes by itself to the pathology [6]. Among endothelial receptors, some are known to interact with PRBC, mainly via PfEMP1 (*P. falciparum* erythrocyte membrane protein 1), a highly polymorphic parasite ligand exported on the infected erythrocyte surface. A number of the cytoadherence receptors have been identified, such as ICAM-1, CD36, VCAM-1 or P-selectin [7]. It was previously shown that *P. falciparum* adhesion to human endothelial cells can specifically trigger proinflammatory gene expression [8-10], oxidative stress [11] and caspases activation [11], further leading

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to perturbation of the endothelial barrier integrity [12,13]. In addition PRBC adhesion to EC induces redox and rho-kinase dependent EC activation and apoptosis, which can be reversed respectively by the addition of anti-oxidants or fasudil rho-kinase inhibitor [12,14].

Atorvastatin is an oral drug that lowers the level of cholesterol in the blood. All statins, including atorvastatin, prevent the production of cholesterol by blocking the enzyme HMGCoA reductase. However, there is increasing evidence that statins may also exert effects beyond cholesterol lowering. Many of these cholesterol-independent or "pleiotropic" vascular effects of statins appear to involve restoring or improving the endothelial function through increasing the bioavailability of nitric oxide, promoting re-endothelialization, reducing oxidative stress, and inhibiting inflammatory responses [15]. Thus, the endothelium-dependent effects of statins are thought to contribute to many of the beneficial effects of statin therapy in cardiovascular disease [16]. In addition to its lipids lowering effects, atorvastatin has been shown to promote nitric oxide (NO) production by decreasing caveolin-1 expression in EC, regardless of the level of extracellular LDL-cholesterol [17]. Statins retard the initiation of atherosclerosis formation through the improvement of NO bioavailability by both up-regulation of endothelial-nitric oxide synthase (eNOS) mRNA and decrease of superoxide anion O_2^- production in EC [18]. Statins modulate the adhesion cascade at multiple points by targeting both the endothelium and leukocytes and affect cell adhesion by inhibiting chemokine expression (MCP-1) in activated leukocytes and endothelial cells. There is also evidence that statins decrease ICAM-1 expression in stimulated EC and monocytes [19]. The effects of atorvastatin on mature EC are correlated with the activation of the anti-apoptotic Akt pathway, as determined by the phosphorylation of Akt and eNOS [20]. Therefore, activation of Akt represents a mechanism that can account for some of the beneficial side effects of statins.

Given the pleiotropic effects on endothelium of statins in general and atorvastatin in particular, atorvastatin was hypothesized to be useful as a protective drug against *P. falciparum* induced endothelial damages. Primary human lung endothelial cells (HLEC) were used here in co-culture with *P. falciparum*-infected erythrocytes. Previous studies have already shown that PRBC adhesion triggers inflammation, oxidative stress and apoptosis within lung endothelial cells [9,11,12,21,22]. Moreover, acute respiratory distress as a complication of malaria infection is rare, but with a very high rate of mortality [3,23]. The data presented in this manuscript show that, concomitantly with increased expression of Akt within HLEC, atorvastatin reduces adhesion of *P. falciparum*-infected erythrocytes, and as a

consequence, prevents the endothelial damages induced by PRBC.

Methods

Culture of human endothelial cell

Primary endothelial cells were isolated from human lung (HLEC) after enzymatic digestion and selected using a continuous gradient and immunomagnetic purification technique, as described elsewhere [24]. Endothelial cells of ninth to twelfth passages derived from one batch were used for the experiments. Before use, cells were verified for their expression of ICAM-1, CD36, Von Willebrand factor, VCAM-1, CD31, E/P-selectin and CSA. HLEC were raised in M199 medium (Gibco), supplemented with 10 μ g/ml of endothelial cell growth supplement (Upstate, NY) and 10% of fetal calf serum (Biowest), at 37°C with 5% CO₂, using 8 chamber-Labtek (Nunc International, Naperville), 35 mm glass bottom dishes (MatTek corp.), 96-well plates (Costar), or Transwell insert supports (Corning LifeSciences).

Plasmodium falciparum culture

The *P. falciparum* 3D7 clone was used for these experiments. Infected erythrocytes were maintained in culture according to Trager and Jensen's technique in a suspension of erythrocytes in RPMI (Gibco) supplemented with 8.3 g/l of HEPES, 2.1 g/l of NaHCO₃, 0.1 mg.ml⁻¹ of gentamycin, 2 g/l of dextrose, and 0.4% of Albumax II (Gibco, Invitrogen Corporation) [25]. The 3D7 clone was characterized for adhesion phenotype as previously described [25] and adheres to ICAM-1 and CD36. For each experiment, parasite cultures were enriched in mature forms by Plasmagel floating [26]. Briefly, erythrocytes were harvested from 5 to 10% parasitized cultures and centrifuged 5 minutes at 2000 rpm. Cells were resuspended in Plasmion[®] and incubated for 20 minutes at 37°C. The upper fraction containing mature trophozoites and schizonts was collected and washed three times in RPM, before adequate adjustment of the hematocrit and parasitemia.

Real time RT-PCR

HLECs were treated with atorvastatin (Pfizer) for 24 hours, and total RNA was prepared using RNeasy mini kit (Qiagen). RT and quantitative PCR were performed as previously described [27]. Primers used were as follow: ICAM-1: forward 5'-GCAATGTGCAA-GAAGA TAGCCA-3' reverse 5'-GGGCAAGACCT-CAGGTC ATGT-3' VCAM-1: forward 5'-GAGTACG CAAACA CTTTATGTCAATGT-3', reverse 5'-CTCG TC CTTTCGGGACCG-3', P-selectin forward 5'-AGA CTCCC CACCAATGTGTGA-3', reverse 5'-CCAC-GAGTGTCA GAACAATCCA-3' CD36 forward 5'-TA

ATGGCACA GATGCAGCCT-3', reverse 5'-ACAG CATAGATG GACCTGCAA-3' HPRT1 forward 5'-AA AGGACCC CACGAAGTGTT-3' reverse 5'-TCAAGG GCATATCCTACAACAA-3'.

***Plasmodium falciparum* adhesion assay**

HLEC were raised in Labtek until confluence. Suspensions of mature pRBC were deposited onto cells and incubated for one hour at 37°C with gentle shaking every 10 minutes. After the incubation, the unbound pRBC were removed and the preparation was fixed for 30 minutes at room temperature with 2% glutaraldehyde, before staining with Giemsa. The number of parasites adhering to 700 HLEC was counted by direct observation with light microscope.

Nucleosome release assay

The proportion of apoptotic cells was assessed by measuring the intracytoplasmic release of mono- and oligo-nucleosomes as, using Cell Death Detection ELISA[®] (Roche) previously described [11]. Briefly, HLEC were raised in 96-well plate (Costar) until confluence and exposed 24 hours to PRBC (hematocrit 5% parasitemia 50%) or RBC (hematocrit 5%). After 5 PBS washing steps, endothelial cells were lysed and cytoplasm were analysed for their nucleosomes content.

Endothelial barrier integrity assay

Confluent endothelial monolayer were obtained by seeding 30,000 HLEC on Transwell permeable support (polyester, 3 µm pores, 6.5 mm diameter) and raised in M199 medium supplemented as above during 36 hours. Endothelial monolayers were then exposed during 12 hours to PRBC suspensions at 1% parasitemia and 0.5% of hematocrit. Transwell compartments were then washed three times and cell monolayers on Transwell inserts were transferred to a new plate containing PBS. Evans Blue (0.5 mg/mL) (ICN Biomedicals) was then added in the 'upper compartments'. After 5 min of incubation at 37°C and 5% CO₂, the 'under compartments' were collected for optic density analysis of diffused Evans Blue (630 nm) with a standard microplate reader (Bio-Tek™ EL311SX) [12].

Western blot

Cells (1×10^6) were lysed in Laemmli sample buffer and protein extracts were separated by SDS-PAGE, transferred to nitrocellulose, blocked (5% non-fat dry milk) in Tris-buffered saline (TBS: 20 mM Tris-HCl pH 7.5, 150 mM NaCl) plus 0.05% Tween-20 and incubated with the primary antibody over night at 4°C in TBS-0.5% non-fat dry milk. The membrane was washed and incubated with PO-conjugated secondary antibody for

two hours at room temperature. Secondary antibodies on western blot membranes were revealed using the ECL system.

Immunofluorescence and confocal microscopy

HLEC were raised in 35 mm glass bottom dishes, before fixation with 1% paraformaldehyde for 5 min, permeabilized and then incubated with polyclonal anti-Akt antibody (Cell Signaling) for 2 h in PBS 3% BSA at room temperature. FITC-secondary antibody was added and incubated for 1 h at room temperature. After several washing steps, samples were incubated with methanol at -20°C for 10 min, mounted with Vectashield medium and analysed by confocal microscopy.

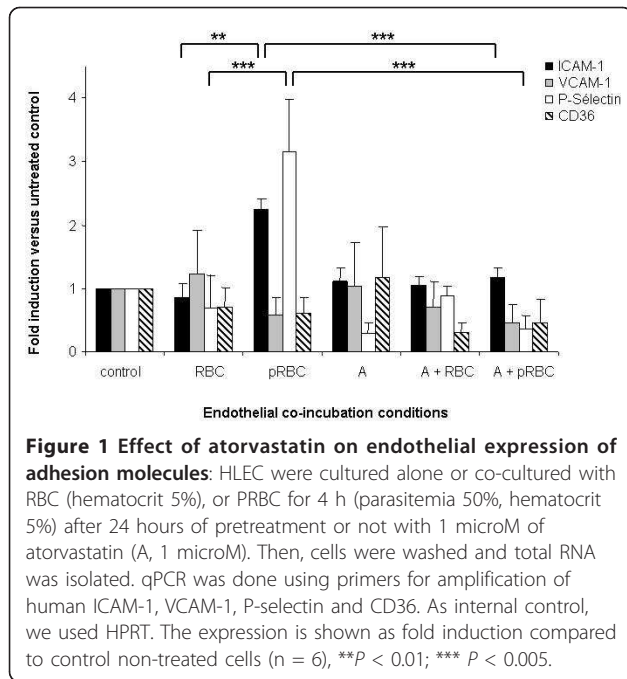
Statistical analysis

Differences between groups were analysed for statistical significance using the Games-Howell post-hoc (SPSS software). A *p* value at least less than 0.05 was considered significant.

Results and discussion

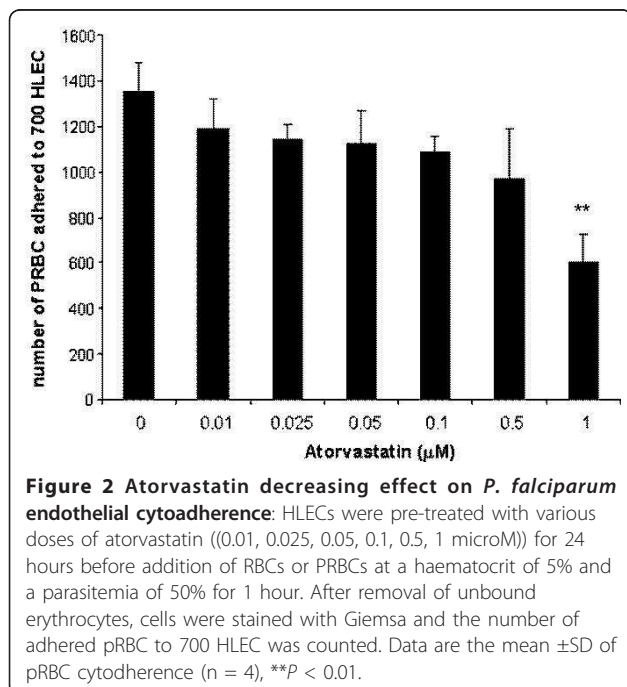
Atorvastatin prevents *P. falciparum*-induced endothelial adhesion molecules upregulation

In addition to their anti-cholesterol function, statins are also involved in expression regulation of some adhesion molecules in lymphocytes, monocytes and endothelial cells. The role of statin atorvastatin in the control of expression of CD36, ICAM-1, VCAM-1 and P-Selectin, four adhesion molecules that are involved in tethering, rolling and adhesion processes, was analysed in a co-culture of endothelial cells and *P. falciparum* parasitized red blood cells (PRBC). Atorvastatin doses, ranging from 0.01 to 1 microM, showed no toxic effect on human lung endothelial cells (HLEC) *in vitro*, doses ranging from 1.5 microM to 5 microM showed low toxic effects, whereas atorvastatin doses above 6 microM showed mitochondrial toxic effects on HLEC (MTT mitochondrial-based cell viability assay). HLEC were pre-treated 24 hours with 1 microM of atorvastatin before being exposed to PRBC or control RBC during four hours. Total RNA of EC was extracted and adhesion molecules expression was assessed by qPCR. On the one hand, our data show that PRBC increase the expression of endothelial cell (EC) adhesion molecule ICAM-1 and P-selectin. Indeed, PRBC cytoadherence was previously shown to increase cytoadherence itself through increase of EC adhesion molecules expression ('hyperadhesion') [28], contributing to the microcirculation perturbation in severe malaria pathology. More importantly, the data clearly showed that the *P. falciparum*-induced increase of ICAM-1 and P-Selectin expression is suppressed by atorvastatin pre-treatment (Figure 1).



Atorvastatin decreases *P. falciparum* cytoadherence and *P. falciparum*-induced endothelial apoptosis

Given the effect of atorvastatin endothelial treatment on the expression of adhesion molecules, its effect on PRBC ability to adhere on endothelial cells was analysed (Figure 2). HLEC were pre-incubated 24 hours with various doses of atorvastatin (0.01, 0.025, 0.05, 0.1, 0.5, 1 microM), before being exposed to PRBC ad

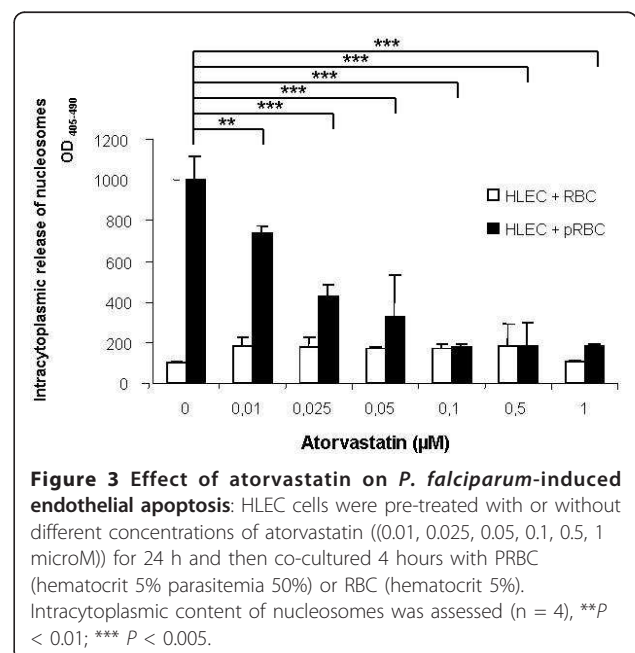


cytoadherence assay. The data showed that PRBC cytoadherence is significantly decreased by doses that are higher than 0.5 microM of atorvastatin. The cytoadherence decreased to 44% \pm 9, for EC pre-incubated with 1 microM of atorvastatin compared to untreated controls. Atorvastatin is thus capable of interfering efficiently with the adhesion of *P. falciparum* infected erythrocytes on endothelial cells.

PRBC adhesion was previously shown to specifically trigger endothelial apoptosis [9]. The effect of atorvastatin (0.01-1 microM) on endothelial apoptosis was tested in the co-culture model. HLEC were pre-treated 24 hours with atorvastatin before being exposed for four hours to PRBC or control uninfected RBC. Endothelial apoptosis was then quantitatively assessed using a method to determine HLEC intracytoplasmic content of nucleosomes, a well known late apoptosis marker (Figure 3). The data showed decreasing endothelial apoptosis correlated with increasing doses of atorvastatin pre-treatment. PRBC-induced apoptosis was found completely abolished with doses higher than 0.05 microM.

Atorvastatin protects endothelial barrier integrity from *P. falciparum*-induced impairments

PRBC were previously observed to trigger specific signaling pathway that leads to endothelial barrier permeabilization [12]. Given the effects of atorvastatin pre-treatments on endothelial apoptosis, atorvastatin was tested whether it could have also beneficial properties on endothelial barrier integrity. HLEC were pre-treated with 1 microM of atorvastatin for 24 h and then with PRBC for 4 hours.



After washing of endothelial monolayer, the cell barrier integrity was assessed by the standard Evans blue extrusion method. Figure 4 shows that co-culture of PRBC with endothelial cells induces endothelial barrier permeability, compared to unexposed control cells or RBC exposed cells. Pre-treatment of endothelial cells with atorvastatin 1 microM could strongly decrease PRBC-induced barrier permeabilization. This result suggests that atorvastatin is also very efficient to protect endothelial barrier integrity from *P. falciparum*-induced impairments. However, it should be noted that the relevance of these quantitative data must be restricted to the detection method of standard Evans blue extrusion.

Atorvastatin increases Akt expression in endothelial cells exposed to *P. falciparum*

Among the pleiotropic effects of statins, their role in the activation of cell survival protein kinase B or Akt signaling pathway is well established [29,30]. Given the protective effect of atorvastatin against cell death in our endothelial-PRBC co-culture model, the effect of atorvastatin treatment on endothelial Akt expression was analysed. HLEC were treated 24 hours with 0.05, 1 or 5 microM of atorvastatin. Figure 5A shows Western blot data and its respective quantitative analysis by densitometry imaging. It shows that pre-treatment of increasing doses of atorvastatin up-regulates Akt expression in HLEC. Similarly, Akt expression was up-regulated in co-cultures of HLEC exposed to RBC or PRBC. The effect of atorvastatin on the endothelial up-regulation of Akt expression was confirmed by confocal microscopy imaging analyses (Figure 5B). These data suggest that atorvastatin increases the Akt expression within endothelial cells.

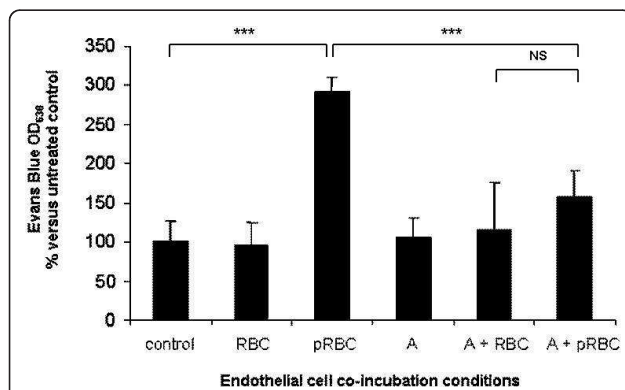


Figure 4 Protective role of atorvastatin against *P. falciparum*-induced endothelial barrier impairment. HLEC were pre-treated with 1 microM of atorvastatin for 24 h and then co-cultured with PRBC (hematocrit 0.5%/parasitemia 1%) or RBC (hematocrit 0.5%) for 4 h. The endothelial barrier permeability was estimated by Evan Blue diffusion through an endothelial monolayer. Results are represented related to the OD of the control HLEC cells. A, atorvastatin. (n = 6), *** P < 0.005, NS: non significant.

The data presented here demonstrate that atorvastatin can be used to reduce the cytoadherence of *P. falciparum* on endothelial cells, which is one of key events, along with the inflammatory burst, involved in the pathogenesis of human severe malaria cases. Atorvastatin can decrease the expression of adhesion molecules and also prevents the PRBC-induced overexpression of adhesion molecules. Moreover, atorvastatin shows an ability to strongly protect endothelial cell against *P. falciparum*-induced collateral damages, cell apoptosis and endothelial barrier permeabilization.

The observed cytoprotection effect on endothelial cells may mainly be due to the decrease of PRBC adhesion through the down-regulation of adhesion molecules, as PRBC contact was previously shown to specifically trigger pro-inflammatory and pro-apoptotic signaling cascades [9]. Moreover, atorvastatin stimulates Akt expression within HLEC, which is the major actor of anti-apoptotic and endothelial cell survival pathways [29]. Endothelial protection of atorvastatin appears then to be 'pleiotropic' via anti-adhesive, anti-inflammatory and anti-apoptotic synergistic effects. Indeed, PRBC cytoadherence is known to activate many deleterious cascades such as oxidative stress, via mitochondrial ROS (radical oxygen species) production, rho-kinase and NF-kappa B-dependent signaling to induce 'hyperadhesion' phenomenon, endothelial activation and apoptosis [8,11,12,14,28,31]. When phosphorylated, Akt inactivates pro-apoptotic elements such as Bad, preventing mitochondrial ROS release, and caspase 9 [32]. Statins can also increase the bioavailability and the physiological production of endothelial nitric oxide (NO) via Akt phosphorylation and downstream effector endothelial NO-synthase (eNOS), and via the stabilization of eNOS mRNA [30,33]. NO has many crucial functions in vascular endothelium particularly in maintaining permanently anti-inflammatory and anti-adhesive endothelium homeostasis. NO-donors have currently been under investigation in cerebral malaria adjunctive emergency treatments, the efficiency of which remains controversial [34]. Indeed, the deleterious scavenging of NO by ROS has not been considered with antioxidant co-treatment yet. As regard their molecular 'pleiotropic' effects, particularly with the antioxidant and the NO bioavailability promotion, statins may then be highly relevant to 'shield' the endothelium against both PRBC induced impairments and uncontrolled host responses in severe malaria cases.

There is increasing interest of using statins as complementary therapeutic to anti-plasmodial molecules. However, these studies with statins were performed on infected erythrocytes only and have shown interestingly to have a direct inhibitory effect on *P. falciparum* growth *in vitro*, using high doses ranging from 120 to 240 microM [35], from 5 to 40 microM [36] or from

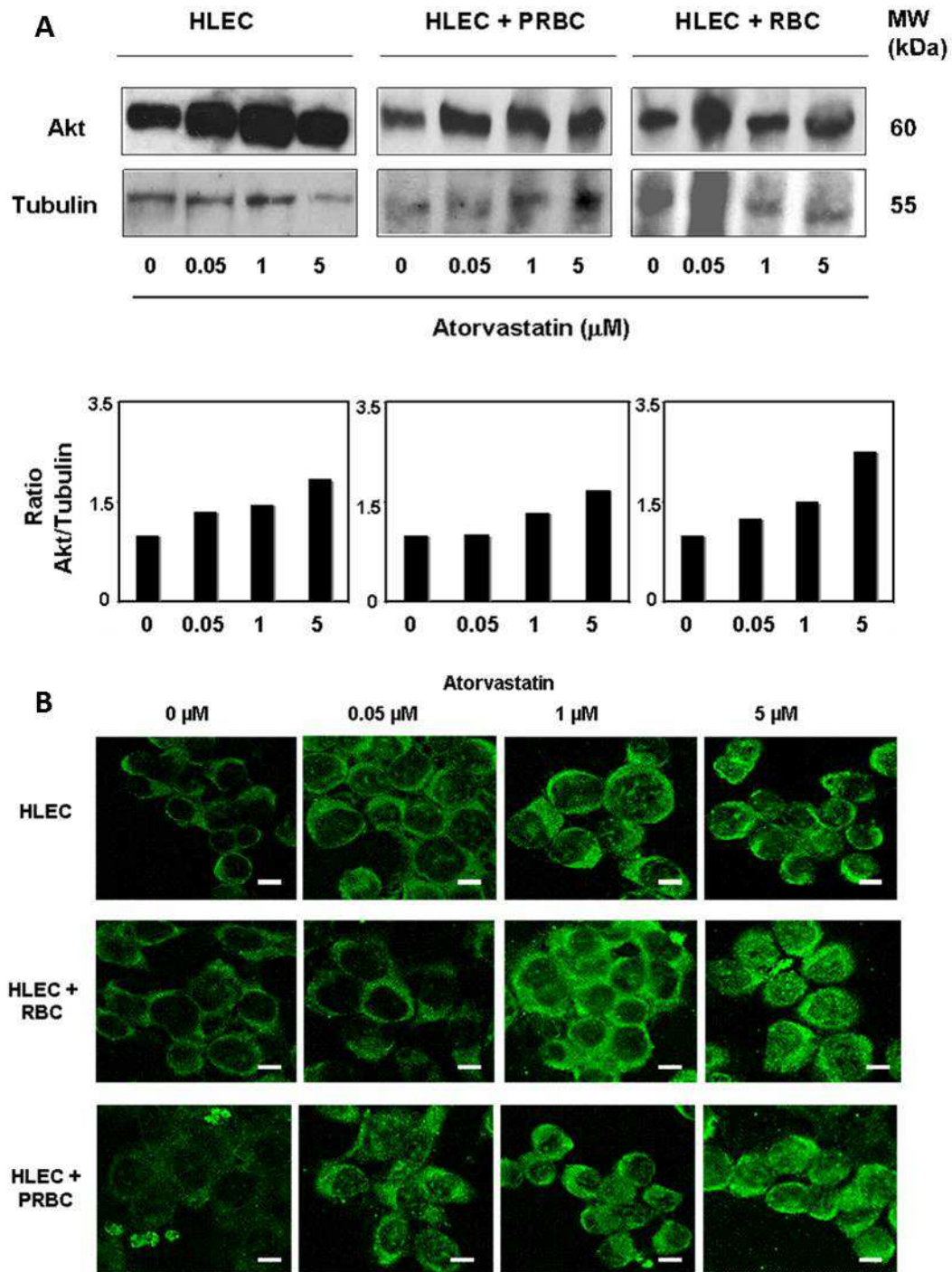


Figure 5 Atorvastatin promotes endothelial expression of Akt: A. Western blot. HLEC were pre-treated with different concentrations of atorvastatin (0.05, 1 or 5 microM) for 24 h and then cultured or co-cultured with PRBC (at 5% of hematocrit and 50% of parasitemia) or RBC (at 5% of hematocrit) for 4 h. Cells were harvested, washed, protein extracts separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Akt and anti-tubulin antibodies, the latter as internal control. Molecular mass of the corresponding proteins is shown. The Western blot data were then quantitatively analysed by densitometry imaging (ratio Akt/Tubulin). Similar results were obtained in three independent experiments. **B. Confocal microscopy.** HLEC were pre-treated with different concentrations of atorvastatin (0.05, 1 or 5 microM) for 24 h and then exposed to PRBC (at 5% of hematocrit and 50% of parasitemia) or RBC (at 5% of hematocrit) for 4 h. Fixed cells were stained with anti-Akt antibody and analysed by confocal microscopy. Similar results were obtained in 3 independent experiments. (Bar: 20 μm).

2.5 to 10.8 microM [37]. Atorvastatin was shown to be 10 times more active against *P. falciparum* compared to mevastatin, simvastatin, lovastatin, fluvastatin or pravastatin [36], suggesting that atorvastatin is the best candidate among statins for therapy prospects in malaria treatment. Simvastatin was also used in mouse model of cerebral malaria (C57BL/6 mice infected with *Plasmodium berghei*), but failed to decrease significantly parasitemia or to prevent death [38,39]. Atorvastatin may have a beneficial effect on mice survival, only when administered in combination with artesunate [40]. Indeed, atorvastatin was recently shown to reduce the IC50 of the anti-plasmodial activity of quinine, mefloquine and dihydroartemisinin [41-43]. However, the pathogenesis of cerebral malaria in murine model relies solely on the inflammatory response unlike the pathogenesis of human cerebral malaria. Indeed, the cytoadherence phenomenon does not exist in *P. berghei* mice infections [44,45]. Atorvastatin was used in pre-treatment to specifically evaluate the potential effects on endothelial cells. Endothelial damage is almost universal in severe malaria pathogenesis and statins have proved their efficiency in clinical use for 20 years against cardiovascular diseases [29]. Interestingly, significant endothelial protection effects were obtained with doses as low as 100 nanoM, 500 nanoM or 1 microM.

Although the 'pleiotropic' effects of statins are well documented, the precise molecular mechanism by which the PI3K/Akt/eNOS pathway is activated remains unknown [29,46]. Since the cholesterol lowering property of statins by itself ameliorates the endothelial function, statins beneficial effects were for long attributed to the inhibition of HMG-CoA reductase and the cholesterol synthesis pathway. However, the endothelial function improvement was shown to occur earlier than cholesterol lowering and independently from the cholesterol rate in blood serum [47,48]. Statins are inhibiting the synthesis of mevalonate, the second biochemical reaction in the cholesterol synthesis pathway. Mevalonate is also an important precursor of isoprenoid intermediates such as farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP). Isoprenoids serve as cell surface inner membrane-anchoring molecules to functionalize Rho-GTPases, such as Rho (GGPP) and Ras (FPP) [49,50], key signaling molecules, after geranylation and farnesylation post-translational modifications. Rho-GTPases act as molecular switches (active and inactive when bound to GTP and GDP, respectively), and are the first intermediates of the intracellular signaling engagement of these receptors with the downstream effector Rho-kinase. We have previously demonstrated that *P. falciparum*-induced endothelial collateral damages are precisely dependent on Rho-kinase signaling [12]. Rho-kinase pathway by many aspects is antagonist to PI3K/Akt/eNOS endothelial cell

survival pathway [46]. In fact, strong endothelial protective effects, obtained with atorvastatin against *P. falciparum*, may likely be related to both rho-kinase pathway inhibition and Akt cell survival pathway promotion. However, it was previously reported that the rho-kinase inhibition by fasudil could not decrease significantly the number of parasites and *P. falciparum* cytoadherence [12,51].

Conclusions

In conclusion, data from this report demonstrate that HMG-CoA reductase inhibitor atorvastatin prevents *P. falciparum* cytoadherence and confers the ability to the endothelium to resist against consequent cellular damages. In conclusion, this study suggests that atorvastatin may be a good candidate for further studies or clinical trials on the use of statins in malaria treatment. Moreover, the safety of statins on malaria treatment needs to be addressed, since these molecules were not originally designed for these therapeutic approaches.

List of abbreviations

EC: endothelial cell; RBC: red blood cells; PRBC: parasitized red blood cells; HLEC: human lung endothelial cells.

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Authors' contributions

ZT contributed to write the manuscript, to design and to conduct the experiments. PP contributed to design the experiments. NN, IA and SA contributed to perform some experiments. FS made substantial constructive advice in the initial design of the project. AR and DM contributed to write the manuscript and to design the experiments. All authors have read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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