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3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors Lovastatin and Simvastatin Inhibit In Vitro Development of *Plasmodium falciparum* and *Babesia divergens* in Human Erythrocytes

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The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors lovastatin and simvastatin inhibit the in vitro intraerythrocytic development of *Plasmodium falciparum* and *Babesia divergens*, with concentrations inhibiting parasite growth by 50% in the ranges of 10 to 20 and 5 to 10 $\mu g \cdot ml^{-1}$, respectively. For *P. falciparum*, the 50% inhibitory concentrations were in the same range whatever the chloroquine susceptibility of the strains tested (strain F32/Tanzania [chloroquine susceptible] or FcB.1/Columbia [resistant]). The stage-dependent susceptibility of *P. falciparum* to simvastatin was studied by subjecting synchronized cultures to 6-h pulses of drug throughout the 48-h erythrocytic life cycle. The most important inhibitory effects were observed between the 12th and 30th hours of the cycle, corresponding to the trophozoite stage. This period precedes the S phase and the nuclear divisions. Parasites in the newly formed ring stage (time zero to the 6th hour of the cycle) and the schizont stage (30th to 48th hour of the cycle) were weakly or not susceptible to simvastatin pulses.

The Apicomplexa hemoparasites *Plasmodium falciparum* and *Babesia divergens*, the causative agents of human malaria and bovine babesiosis, respectively, still constitute major health and economic problems in most developing countries (12, 24). The increasing resistance of *P. falciparum* to antimalarial drugs as well as the often fatal diseases due to *B. divergens* in cattle and in some cases in humans indicate a need for new chemotherapeutic approaches.

During their erythrocytic life cycle, the hemoparasites multiply at a rapid rate, completing their development in about 8 h for *B. divergens* from the merozoite (infective cell) into 2 or 4 new merozoites (21) and in about 48 h for *P. falciparum*, with about 16 new merozoites. Such a rapid growth of parasites requires lipids for the biogenesis of membranes. However, both types of parasites are unable to synthesize fatty acids and cholesterol de novo (8, 21, 22) and depend on their import from the host's plasma. With the use of a serum-free medium, we have recently found a unidirectional transfer of phospholipids from human high-density lipoproteins to the intraerythrocytic *P. falciparum* by ducts and vesicles moving from the erythrocyte to the parasitophorous vacuole membranes (6). A similar phospholipid transfer was observed in *B. divergens*-infected erythrocytes (RBC), but no ducts or vesicles were observed (21). High-density lipoproteins support the in vitro growth of *B. divergens* and *P. falciparum* in the absence of other major serum components and appeared to be a lipid source for the parasites (6, 7, 21). Although cholesterol is not synthesized by *P. falciparum*-infected RBC, we have shown evidence for an isoprenoid metabolism until, at least, the farnesyl PP_i step (16). This pathway, with numerous end products, is essential for various cellular functions such as mitochondrial electron transport, tRNA synthesis, control of cell growth, protein glycosylation, and intracellular targeting (4). In both prokaryotic and eukaryotic cells, the isoprenoid pathway is highly regulated through feedback regulations at the level of two sequential enzymes involved in the synthesis of mevalonate: 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase (4). HMG-CoA reductase inhibitors are available as hypocholesterolemic agents in humans. The goal of our study was to establish whether the HMG-CoA reductase inhibitors lovastatin and simvastatin are able to inhibit the in vitro development of *P. falciparum* and *B. divergens*.

MATERIALS AND METHODS

Lovastatin and simvastatin, both in the lactone form, were kindly provided by J. C. Mazière (Hôpital St-Antoine, Paris, France) and by C. Foussard (Merck Sharp Dohm Production, Riom, France). They were converted to the active form by dissolving 7 mg of the lactone form in 100 μl of 100% ethanol, adding 200 μl of 0.2 M KOH, and then adding 0.2 M HCl for neutralization to pH 7.2. Then the desired volume was adjusted with 0.1 M sodium phosphate buffer, pH 7.2. Aliquots of the stock solutions were stored frozen at −20°C.

For *P. falciparum*, the experiments were performed with the chloroquine-susceptible F32/Tanzania strain and the chloroquine-resistant FcB.1/Columbia strain, kindly provided by H. Jepsen (Statens Seruminstitut, Copenhagen, Denmark) and by H.-G. Heidrich (Max Planck Institut für Biochemie, Martinsried bei München, Germany), respectively. In vitro cultures

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were realized by maintaining the parasites on human type O^+ RBC in RPMI 1640 culture medium (Gibco Laboratories) supplemented with 27.5 mM $NaHCO_3$, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 11 mM glucose, and 10% (vol/vol) human O^+ serum in an atmosphere of 3% CO_2, 6% O_2, and 91% N_2 at 37°C (20). The strain susceptibilities to chloroquine and to HMG-CoA reductase inhibitors were determined for cultures in 96-well plates according to the semiautomated microdilution technique of Desjardins et al. (2). Different concentrations of drugs were added to asynchronous parasite cultures (0.5% parasitemia and 1% hematocrit) for 24 h, at 37°C, prior to the addition of 0.5 μCi of [^{3}H]hypoxanthine monochloride (1 to 5 Ci · mmol^{-1}; Amersham, Les Ulis, France) per well. After further incubation for 18 h, the cells were harvested from each well with a cell harvester (Skatron, Lier, Norway) onto filter papers. The dried disks were counted in a scintillation spectrometer.

The *B. divergens* Rouen 1987 isolate (21) and Weybridge 8843 isolate (kindly provided by Rhône-Mérieux S. A., Lyon, France) were maintained in vitro in the same manner as *P. falciparum* (5, 21). The inhibitory assays were performed with cultures in 24-well plates. The cultures (1% parasitemia and 1% hematocrit) were incubated in the presence of different concentrations of drugs for 24 h at 37°C. Parasite growth was estimated either by [^{3}H]hypoxanthine incorporation (addition of 1 μCi of [^{3}H]hypoxanthine per well 16 h after the beginning of the experiment and measurement of the incorporated radioactivity as described for *P. falciparum*) or by determining the parasitemia on Giemsa-stained smears made at the end of the experiment.

In both assays, all the drug concentrations were tested in triplicate for each experiment. The growth inhibition for each concentration was determined by comparison of the treated and the control cultures without inhibitor. The concentrations causing 50% inhibition (IC_{50}) were obtained from the drug concentration-response curves, and the results are expressed as the means ± the standard deviations determined in several independent experiments.

The synchronization of *P. falciparum* was performed by successive treatments with 5% (wt/vol) sorbitol (13) and by concentration of trophozoite- or schizont-infected RBC by flotation in gelatin solution (Plasmagel; Laboratoire Roger Bellon, Neuilly, France) (17). The cultures were first treated with sorbitol to destroy RBC infected with the oldest parasites (trophozoites and schizonts), and the young parasites were left to grow in culture until they reached the schizont stage. The infected cells were then concentrated to 80 to 95% parasitemia by the Plasmagel procedure and put back in culture, and the parasitemia was adjusted to 20% with fresh RBC. Two hours after the beginning of merozoite release, the cultures were treated with sorbitol in order to eliminate the residual schizont-infected RBC (13) and returned to culture conditions. This procedure yields stock cultures constituted of parasite populations synchronized to a 2-h window. In the experiments, the initial parasitemia was adjusted to 1 to 2%, and time zero (t_0) was defined as immediately after sorbitol treatment, the oldest parasites being 2 h of age. From t_0, four aliquots were taken every 6 h from the stock culture (t_0, t_{6h}, t_{12h}, t_{18h}, t_{24h}, t_{30h}, and t_{36h}). Three aliquots from each sampling time were maintained for 6 h in culture medium, at 37°C, in the presence of three concentrations of simvastatin in the vicinity of the previously determined IC_{50} (2, 10, and 20 μg · ml^{-1}). The fourth aliquot, which served as the control, was maintained in culture medium without drug and was processed in the same manner as the treated samples. After a 6-h incubation, the cells were carefully washed by three incubations in normal culture medium and put back in culture. At t_0 + 72 h, a period corresponding to the trophozoite stage of the second erythrocytic cycle in the control experiments, the parasitemias were determined by counting 3,000 cells on Giemsa-stained smears made from all samples. The inhibitory effects of the drug were estimated by comparison of treated cultures at t_0 + 72 h with the control cultures (without drug) and proceeded under the same conditions of incubation. Furthermore, before each incubation with simvastatin, the number of nuclei per parasite was estimated by using the bisbenzimide H33258 (9). This fluorescent specific DNA probe was added to the cultures, at 37°C, with a final concentration of 10 μg · ml^{-1}. After 10 min of incubation, the cells were washed twice with RPMI 1640 before observation.

TABLE 1. Antiparasitic activities of HMG-CoA reductase inhibitors

Parasite strain	Mean IC_{50} (μg · ml^{-1}) ± SD	
	Lovastatin	Simvastatin
P. falciparum		
F32/Tanzania	15.7 ± 6.5[a]	16.2 ± 3.6[a]
FcB.1/Columbia	13.6 ± 3.7[b]	12.8 ± 2.5[b]
B. divergens		
Rouen 1987	8.4 ± 0.3[b]	5.0 ± 0.4[b]
Weybridge 8843	ND[c]	5.8[d]

[a] From four experiments.
[b] From three experiments.
[c] ND, not determined.
[d] From two experiments.

RESULTS

Table 1 presents the IC_{50} of lovastatin and simvastatin for *P. falciparum* and *B. divergens*. For *P. falciparum*, similar IC_{50} were obtained with the two drugs, i.e., in the range of 10 to 20 μg · ml^{-1}. The compounds were equally effective with the chloroquine-susceptible F32/Tanzania strain and the chloroquine-resistant FcB.1/Columbia strain (the IC_{50} of chloroquine were 13 ± 1 and 178 ± 10 ng · ml^{-1}, respectively). These results indicate that the susceptibility to the HMG-CoA reductase inhibitors is not dependent on the status of chloroquine resistance. Similar IC_{50} for lovastatin and simvastatin, in the range of 5 to 10 μg · ml^{-1}, were also observed for two *B. divergens* isolates which present differences in virulence: the Weybridge 8843 isolate is more virulent than the Rouen 1987 isolate, as shown by assays with gerbils (19). The IC_{50} were similar when *B. divergens* growth inhibition was estimated by [^{3}H]hypoxanthine incorporation or by determining the parasitemia on Giemsa-stained smears.

As the in vitro development of *P. falciparum* can be synchronized by different procedures, we investigated the susceptibilities of parasites in the different erythrocytic stages to simvastatin. During its erythrocytic phase, the merozoite undergoes in 48 h a sequential development through a ring (0 to 20 h postinfection), a trophozoite (20 to 35 h), and a schizont (35 to 48 h) stage. At the schizont stage, the parasite divides to produce about 16 new merozoites per infected RBC. The DNA synthesis phase (S phase) starts at the 30th to 35th hour of the cycle, a period corresponding to the entry of the parasite into schizogony (10).

The stage susceptibility of *P. falciparum* FcB.1/Columbia to simvastatin was studied with highly synchronized cultures. These were pulsed with drug for 6 h at fixed time intervals

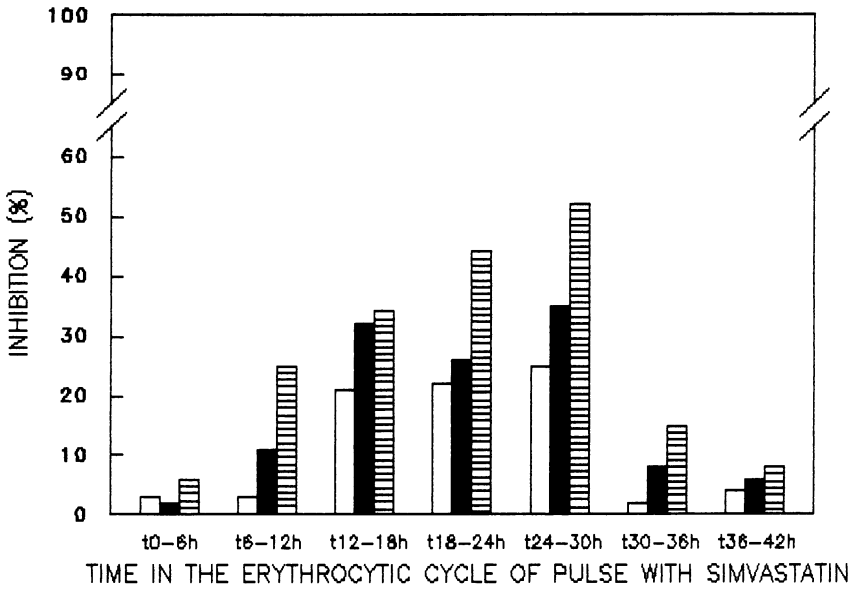

FIG. 1. Stage susceptibility of the *P. falciparum* erythrocytic cycle to simvastatin. A 2-h-synchronized culture (2% parasitemia) was subjected to 6-h pulses of different concentrations of simvastatin (2 $\mu g \cdot ml^{-1}$ [□], 10 $\mu g \cdot ml^{-1}$ [■], and 20 $\mu g \cdot ml^{-1}$ [▤]) every 6 h at all stages of the erythrocytic life cycle (t_{0h}, t_{6h}, t_{12h}, t_{18h}, t_{24h}, t_{30h}, and t_{36h}). After 6 h of incubation, the cells were washed and returned to normal culture conditions until t_0 + 72 h, corresponding to the second erythrocytic cycle. The inhibition of parasite development was then determined by comparison with the control cultures maintained without drug and processed in the same way. Each bar represents the mean of two values.

throughout the 48-h erythrocytic cycle, and the effect on parasite growth was evidenced at the second erythrocytic cycle. A typical pattern of parasite growth inhibition is depicted in Fig. 1. In these assays, the inhibition of parasite growth reflected parasite death following incubation with simvastatin. Inhibition of parasite growth was clearly concentration and stage dependent. The first significant inhibitory effects were observed only when the drug was applied at the sixth hour of the life cycle (Fig. 1, t_{6h}), indicating that parasites in the early ring stage (0 to 6 h of the life cycle) were not susceptible. The inhibition increased until the 30th hour (Fig. 1, t_{6h} to t_{24h}), the period corresponding to the transformation of the ring to the trophozoite stage. In fact, the more important inhibition was observed when 6-h pulses of simvastatin were applied between the 12th and the 30th hours of the life cycle. During this period, 20 to 25% and 35 to 55% inhibitions of parasite growth were observed with simvastatin at 2 and 20 $\mu g \cdot ml^{-1}$, respectively. In the experiment, at the 30th hour of the life cycle, more than 90% of the parasites started their schizogony, as evidenced by enlarged nuclei, typical of the S phase, or by the presence of two nuclei per parasite, indicating that the first nuclear division had been achieved. After the 30th hour, corresponding to the beginning of the schizont stage, the inhibition of growth was weak or totally absent.

Lovastatin and simvastatin are known to act as competitive inhibitors of HMG-CoA reductase and to block the proliferation of eukaryotic cells in the G_1 phase (11, 14, 15). For example, treated human bladder carcinoma T24 cells are viable for up to 72 h and are able to enter into the S phase after removal of the drug (11). To investigate if simvastatin has cytostatic effects on *P. falciparum* development as it does for for the eukaryotic cell lines, 6-h pulses of the drug were administered to 2-h-synchronized trophozoite cultures obtained 24 h after erythrocyte invasion, which was considered t_0 of the experiment. After removal of the drug (t_0 + 30 h), the parasite stage distribution was observed 18 h (t_0 + 48 h) and 28 h (t_0 + 58 h) later on Giemsa-stained smears, periods corresponding, in the control cultures without drug, to the mature schizont and the newly formed ring stages, respectively. The results of such an experiment are shown in Fig. 2. The parasite cultures (4.6% initial parasitemia) were incubated for 6 h with simvastatin at 15 $\mu g \cdot ml^{-1}$. At the time of drug removal (t_0 + 30 h), the parasites were all at the trophozoite stage, as in the control culture (Fig. 2, compare Con and Sim at t_0 + 30 h). Eighteen hours later, 55% inhibition of parasite growth was observed in the treated cultures and the dead parasites were easily detected by their altered morphology: typically rounded and reduced in size and intensely blue stained with Giemsa. Remaining parasites displayed the morphologies of the trophozoite and schizont stages (Fig. 2, Sim t_0 + 48 h) with, however, a delay in parasite growth and a loss of parasite synchronization compared with the control cultures, in which all the parasites were at the mature schizont stage (Fig. 2, Con t_0 + 48 h). Cytostatic effects of simvastatin on the development of *P. falciparum* were also confirmed at t_0 + 58 h, at which time almost all the treated parasites were at the schizont stage, whereas in the control cultures they reached the ring stage (Fig. 2, t_0 + 58 h). The schizonts of drug-treated cultures produced invasive merozoites (data not shown). Cytostatic effects were also observed with lower concentrations of simvastatin (2 and 5 $\mu g \cdot ml^{-1}$) but with reduced delays in parasite development.

DISCUSSION

In this study, we have shown that lovastatin and simvastatin, two HMG-CoA reductase inhibitors, possess antiparasitic activities in vitro against *P. falciparum* and *B. divergens*, with IC_{50} in the range of 10 to 20 and 5 to 10 $\mu g \cdot ml^{-1}$, respectively. For *P. falciparum*, the drug susceptibility appears independent of the chloroquine resistance status of the strains. The lower IC_{50} of lovastatin and simvastatin for *B. divergens* may result from

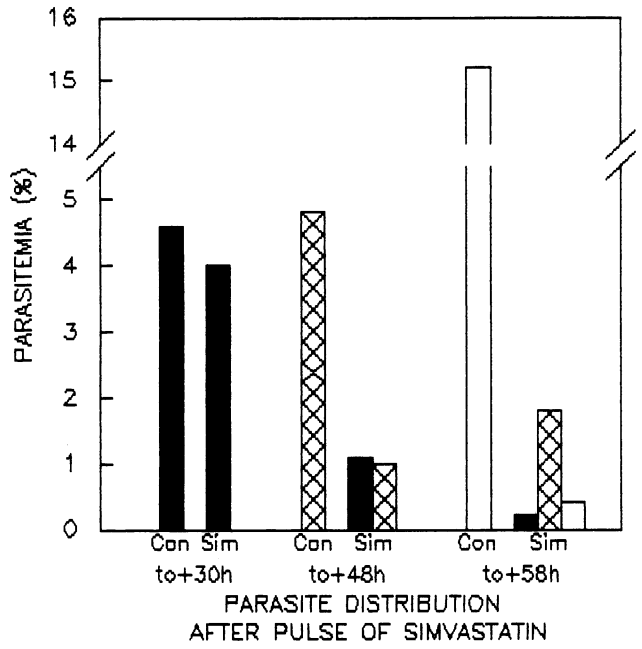

FIG. 2. Cytostatic effects of simvastatin on the development of *P. falciparum*. A 6-h incubation with simvastatin at 15 $\mu g \cdot ml^{-1}$ was performed with a 2-h-synchronized culture of *P. falciparum* at the 24th hour of the erythrocytic life cycle. After removal of the drug (t_0 + 30 h), the parasite stage distribution in the treated culture (Sim) was monitored at t_0 + 48 h and t_0 + 58 h and was compared with that in the control culture (Con) maintained without drug and processed in the same way. Each bar represents the mean of two values. Symbols: □, ring stage; ▩, trophozoite; ■, schizont.

cumulative effects of the drugs on several life cycles rather than from the higher susceptibility of the *B. divergens* isolates. It could be observed that in terms of the cell cycle, the in vitro *B. divergens* assays were performed over a 24-h period which corresponds to 3 erythrocytic cycles (8-h erythrocytic cycle) whereas the assays on *P. falciparum* were performed for 72 h, corresponding to 1.5 erythrocytic cycles (48-h erythrocytic cycle).

Inhibition assays with 6-h-pulse incubations of simvastatin with *P. falciparum* synchronized cultures have shown that the trophozoite stage (12th to 30th hour of the erythrocytic life cycle) is the stage at which the parasite is most susceptible to this drug. The inhibition of growth could be related to a direct effect of simvastatin on the parasite HMG-CoA reductase. This is in agreement with our previous studies (16) showing that the products of the *P. falciparum* isoprenoid metabolism were mainly detected at the same stage of parasite development: the isoprenoid metabolism increases progressively during the trophozoite stage to reach a maximum at the time the parasite enters into schizogony.

Cytotoxic effects giving a complete inhibition of growth were observed for all parasite stages only with drug concentrations above 50 $\mu g \cdot ml^{-1}$ (data not shown). Lovastatin and simvastatin are known to reversibly arrest the cell cycle progression of mammalian cells in the G_1 phase with drug concentrations ranging from 1 to 10 $\mu g \cdot ml^{-1}$ for human bladder carcinoma T24 cells (11) and human fibroblasts (15) to 60 $\mu g \cdot ml^{-1}$ for rat brain glial cells (14). Blocked cells are viable, and cytostatic effects can be reversed by the addition of exogenous mevalonate, the product of the HMG-CoA reductase reaction. Such cytostatic effects on the development of *P. falciparum* are also observed and support the idea that the inhibition of parasite growth results from the inhibition of parasite HMG-CoA reductase. However, reversal of parasite growth inhibition by competition with an excess of exogenous mevalonate was unsuccessful. This observation could result from the impossibility for mevalonate to cross the three membranes surrounding the parasite: the parasitophorous and parasite membrane complex, whose composition is poorly known, and the highly charged erythrocyte membrane. We have previously observed that *P. falciparum*-infected RBC incubated in vitro with [^{14}C]mevalonate were not able to metabolize the radiolabelled precursor, whereas the same substrate is incorporated until the farnesyl PP_i step by cell extracts of these infected RBC (16).

In terms of the cell cycle, the intraerythrocytic development of *P. falciparum* is not clearly defined; however, the S phase is known to take place at the 30th to 35th hour of the life cycle (10). By analogy to the mammalian cell cycle, the trophozoite stage, on the basis of the susceptibility of parasites in this stage to the HMG-CoA reductase inhibitors and the cytostatic effects observed with these drugs, could be related to a G_1 phase. The early ring stage would correspond to the G_0 phase. As a matter of fact, we have previously shown that the parasite can develop in vitro to the ring form in the absence of serum components (7). Normal parasite development can be reinitiated by the addition of serum or of human high-density lipoproteins (7). This reinitiation of parasite development shows strong similarities to the proliferation of mammalian cells in G_0 phase after stimulation with lipoproteins (1).

As *P. falciparum* is unable to synthesize cholesterol de novo (8, 22), the effects of lovastatin and simvastatin could concern other aspects of the isoprenoid pathway, such as the production of ubiquinones which are involved in the mitochondrial electron transport chain or such as the dolichol pathway and the prenylation of proteins involved in the control of the cell cycle.

The effects of HMG-CoA reductase inhibitors on *P. falciparum* development show a selective inhibition during the trophozoite stage. In vivo drug assays must take into account this stage specificity, and drug concentrations in the plasma must be optimal when parasites are at the trophozoite stage. In vivo, in terms of bioavailability, these HMG-CoA reductase inhibitors have short half-lives in plasma (18, 23). In human beings, the maximum mean levels of simvastatin in plasma are 400 ± 200 $ng \cdot ml^{-1}$ 4 h after patients receive 100 mg by oral administration, and less than 10% of the peak remains after 12 h (23). These low concentrations and short half-lives in plasma are explained in part by the rapid uptake of the drugs by the liver, the major site of cholesterol synthesis in humans. At present, such concentrations of simvastatin in plasma are unsuitable for a blood eradication of malaria by the current usage of this cholesterol-lowering agent. However, derivatives with higher concentrations in plasma and lesser selectivity for the liver could be designed. One factor of possible importance in such a design could be the capacity of the drug to bind to plasma proteins. For example, lovastatin, simvastatin, and their active species are highly bound (95%) to human plasma proteins, whereas pravastatin, a less hydrophobic derivative, is approximately 50% bound (18). Presumably, only the unbound drug can penetrate the tissues (18). Tissue distribution studies with rats demonstrated that concentrations of simvastatin and lovastatin were 50% higher than that of pravastatin in liver during the 24 h after drug administration and were three to six times lower than pravastatin concentrations in peripheral tissues (3). The antiparasitic activities of pravastatin on the intraerythrocytic development of *P. falciparum* are under in-

vestigation. In the same way, the effects of HMG-CoA reductase inhibitors on the hepatocytic development of *P. falciparum* should also be tested. Because of their tendencies to concentrate in the liver, these drugs could block the transformation of sporozoites to hepatocytic schizonts. This step is of importance since one sporozoite is able to produce about 10,000 hepatocytic merozoites.

In this report, we give the first demonstration that HMG-CoA reductase inhibitors selectively block the development of *P. falciparum* at the trophozoite stage and also block the development of *B. divergens*. Such inhibitors could start new trends in antiparasitic chemotherapy. For the design of inhibitors for the parasite isoprenoid pathway, it is important to consider not only differences in specificity for the host and parasite enzymes but also their differential uptake by the infected and normal RBC and, obviously, host cell targeting, such as hepatocyte versus RBC.

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