Influence of Amitriptyline on Eryptosis, Parasitemia and Survival of *Plasmodium Berghei*-Infected Mice

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Key Words

Malaria • Cell volume • Phosphatidylserine • Sphingomyelinase • Ceramide

Abstract

Plasmodia express a sphingomyelinase, which is apparently required for their development. On the other hand, the sphingomyelinase product ceramide has previously been shown to delay parasite development. Moreover, ceramide triggers suicidal erythrocyte death or eryptosis, characterized by exposure of phosphatidylserine at the erythrocyte surface and cell shrinkage. Accelerated eryptosis of infected erythrocytes is considered to clear infected erythrocytes from circulating blood and, thus, to favourably influence the clinical course of malaria. The present experiments explored whether the sphingomyelinase inhibitor amitriptyline or genetic knockout of host acid sphingomyelinase influence *in vitro* parasite growth, eryptosis of *Plasmodium falciparum*-infected human erythrocytes, *in vivo* parasitemia and survival of *P. berghei*-infected mice. Phosphatidylserine exposure was determined by annexin V-binding and cell volume by forward scatter in FACS analysis. *In vitro* infection of human erythrocytes increased annexin-

binding, an effect blunted in the presence of amitriptyline ($\geq 50 \mu\text{M}$). Amitriptyline did not significantly alter intraerythrocytic parasite development but significantly ($\geq 1 \mu\text{M}$) delayed the increase in parasitemia *in vitro*. Most importantly, amitriptyline treatment (1 mM in drinking water) resulted in a significant delay of parasitemia and death of infected mice. However, upon infection, ceramide formation was stimulated in both, acid sphingomyelinase knockout mice (*Smpd1*^{-/-}) and their wild type littermates (*Smpd1*^{+/+}). Parasitemia following *P. berghei* infection was significantly lower in *Smpd1*^{-/-} than in *Smpd1*^{+/+} mice but did not significantly extend the life span of infected animals. In conclusion, mammalian and parasite sphingomyelinase contribute to ceramide formation during malaria, whereby the parasite sphingomyelinase ultimately determines the course of the infection. Amitriptyline presumably blocks both sphingomyelinases and, thus, its use might be a novel strategy to treat malaria.

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Introduction

The malaria pathogen *Plasmodium* expresses a sphingomyelinase, which appears to be important for

parasite development [1, 2]. On the other hand, ceramide has been shown to delay growth of *P. falciparum* [3]. Moreover, ceramide released upon activation of a mammalian sphingomyelinase stimulates suicidal erythrocyte death or eryptosis [4], which in turn may confer some protection against a severe course of malaria [5]. Sick cell trait, beta-thalassemia-trait, homozygous Hb-C and G6PD-deficiency trigger premature senescence and/or eryptosis upon infection with *Plasmodium*, thus leading to accelerated clearance of ring stage-infected erythrocytes [6-11]. Moreover, accelerated eryptosis parallels a favourable course of malaria in animals suffering from iron deficiency [12] or treated with lead ions [13].

Eryptosis is characterized by exposure of phosphatidylserine at the cell surface and cell shrinkage [11, 14-17]. The phosphatidylserine exposure results from scrambling of cell membrane phospholipids, which is triggered by increased cytosolic Ca^{2+} [11, 14-17]. The increase of cytosolic Ca^{2+} activity may result from activation of a Ca^{2+} permeable cation conductance, which is activated by osmotic shock, oxidative stress and energy depletion [18-21]. Moreover, activation of cation channels, Ca^{2+} entry and cell membrane scrambling are typical features of erythrocytes infected with the malaria pathogen *Plasmodium* [15, 22-24].

Macrophages are equipped with receptors for phosphatidylserine [25, 26] and phosphatidylserine exposing erythrocytes are recognized, engulfed, degraded and thus eliminated from circulating blood [27]. Early clearance of infected erythrocytes may prevent tissue sequestration of the late stage-infected erythrocytes and lead to removal of erythrocytes prior to the development of trophozoites with the ability to intoxicate macrophages [28]. These results suggest critical, although partially counteracting functions of sphingomyelinase during infection of mammalian hosts by *Plasmodia*.

Inhibitors of sphingomyelinase include amitriptyline [29-31]. The present study has been performed to test, whether amitriptyline or genetic knockout of host acid sphingomyelinase modifies eryptosis of infected erythrocytes and the course of malaria.

Materials and Methods

Cells, animals and solutions

Human erythrocytes were drawn from healthy volunteers. The volunteers providing erythrocytes gave informed consent. The study was approved by the ethical commission of the University of Tübingen.

In addition, experiments were performed in SV129/J wild type mice (aged 4 months, both male and female), acid sphingomyelinase deficient mice (*Smpd1*^{-/-}) and their wild type littermates (*Smpd1*^{+/+}). Animal experiments were performed according to German animal protection law and approved by the local authorities (registration number PY 2/06). Mouse erythrocytes were drawn from animals by retroorbital venopuncture or by incision of the tail vein.

Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl₂, pH 7.4. Amitriptyline was added to the NaCl Ringer at final concentrations varying from 0.1 µM to 100 µM (Sigma, Taufkirchen, Germany). For *in vitro* amitriptyline treatment the final hematocrit was adjusted to 0.3 %. Similar results were obtained with erythrocytes washed in PBS prior to the experiments.

For treatment of animals with amitriptyline, 1 mM drug was added to the drinking water. Considering the bioavailability of amitriptyline [32, 33], and the fluid intake of the mice (4 ml/day), the estimated dosage was 40 mg/kg/day.

Determination of phosphatidylserine exposure

FACS analysis was performed as previously described [21]. After incubation in the presence or absence of amitriptyline, suspensions of uninfected erythrocytes were stained with annexin V-FLUOS (Roche, Mannheim, Germany) to determine phosphatidylserine exposure. Suspensions of *P. falciparum* infected erythrocytes were stained with annexin V-APC (BD Biosciences Pharmingen, Heidelberg, Germany) and/or with the DNA/RNA specific dye Syto16 (Molecular Probes, Göttingen, Germany) to identify phosphatidylserine exposing and infected erythrocytes, respectively. For annexin binding, erythrocytes were washed, resuspended in annexin-binding buffer (Ringer solution containing 5 mM CaCl₂, pH 7.4), stained with annexin V-APC (dilution 1:20) or annexin V-FLUOS (dilution 1:100), incubated for 20 min at room temperature, and diluted 1:5 with annexin-binding buffer. Syto16 (final concentration of 20 nM) was added directly to the diluted erythrocyte suspension or co-incubated in the annexin-binding buffer. Cells were analyzed by flow cytometry (FACS-Calibur, Becton Dickinson, Heidelberg, Germany) in FL-1 for Syto16 or annexin V-FLUOS fluorescence intensity (detected at 530 nm) and in FL-4 for annexin V-APC fluorescence intensity (detected at 660 nm).

Infection

For infection of human erythrocytes the human pathogen *P. falciparum* strain BinH [34] was grown *in vitro* [35] in banked human erythrocytes. Parasites were cultured as described earlier [36, 37] at a hematocrit of 2 % and a parasitemia of 2-10 % in RPMI 1640 medium supplemented with Albumax II (0.5%; Gibco, Karlsruhe, Germany) in an atmosphere of 90% N₂, 5% CO₂, 5% O₂. As described earlier [38], the *in vitro* intraerythrocytic parasite development is favoured by low O₂ tension.

For infection of mice *P. berghei* ANKA-parasitized mouse erythrocytes (1x10⁶) were injected intraperitoneally [39] into wildtype mice with free access to drug free or amitriptyline

(1 mM) containing drinking water from the day of infection. In another series of experiments, the *Smpd1*^{-/-} and *Smpd1*^{+/-} mice were infected with *P. berghei* ANKA-parasitized mouse erythrocytes (1x10⁶) intraperitoneally. Parasitemia was determined daily by Syto16-staining in FACS analysis.

In vitro growth assays of *P. falciparum*-infected human erythrocytes

The *P. falciparum* BinH strain was cultured and synchronized to the ring stage by sorbitol treatment as described previously [40]. For the *in vitro* growth assay, ring stage-synchronized parasitized erythrocytes were aliquoted in 96-well plates (200 µl aliquots, 1% hematocrit, 0.5-2% parasitemia) and grown for 48 h in the presence or absence of amitriptyline (0.1 µM-100 µM). The parasitemia was assessed at time 0 (ring stage) and after 48 h of culture (upon merozoite release and reinvasion) by flow cytometry. Parasitemia was defined by the percentage of erythrocytes stained with the DNA/RNA specific fluorescence dye Syto16 (Molecular Probes, Göttingen, Germany). To estimate the intraerythrocytic parasite development, the culture was ring stage-synchronized, and re-synchronized after 6 h of culture (to narrow the developmental parasite stage), aliquoted (200 µl aliquots, 2% hematocrit and 10% parasitemia) and cultured for further 16 h in the presence or absence of amitriptyline (0.1 µM-100 µM). At time 0 and 24 h, the DNA/RNA amount of the parasitized erythrocytes was determined by Syto16-fluorescence.

Measurement of *in vivo* clearance of fluorescence-labelled erythrocytes

Fluorescence-labelled mouse erythrocytes were obtained by staining erythrocytes with carboxyfluorescein diacetate, succinimidyl ester (CFSE) from Molecular Probes (Leiden, The Netherlands). The labelling solution was prepared by addition of adequate amounts of a CFSE stock solution (10 mM in DMSO) to phosphate-buffered saline (PBS) to yield a final concentration of 5 µM. Then, the cells were incubated with labelling solution for 30 min at 37°C. The cells were pelleted at 400 g for 5 min, washed and resuspended in fresh, prewarmed PBS. The fluorescence-labelled erythrocytes were injected into the tail veins of healthy SV129/J mice. At indicated time points, blood was taken from the mice and CFSE-dependent fluorescence intensity of the erythrocytes was measured in the fluorescence channel FL-1. The percentage of CFSE-positive erythrocytes was calculated in % of the whole cell population.

Measurement of ceramide

Ceramide was measured by a biochemical method using diacylglycerol kinase and [³²P] γ-ATP as described [41]. After incubation of erythrocytes of both *Smpd1*^{-/-} and *Smpd1*^{+/-} mice for 24 h in Ringer, the samples were extracted in chloroform:methanol:1 N HCl (100:100:1), the lower phase was collected and dried. Diacylglycerol was then degraded by alkaline hydrolysis of the samples in 100 µl of 0.1 N methanolic KOH at 37°C for 60 min. The samples were re-extracted, dried and resuspended in 20 µl of detergent solution (7.5% (w/v) n-octylglucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid (DETAPAC)). The samples

were then sonicated for 10 min in a bath sonicator and the kinase reaction was initiated by addition of 70 µl reaction mix consisting of 10 µl DAG-kinase (Merck, Darmstadt, Germany) in 5 mM potassium phosphate buffer (pH 7.0), 10% glycerol, 1 mM 2-mercaptoethanol, 0.005 M imidazole/HCl, 0.5 mM DETAPAC (pH 6.6), 50 µl assay buffer (0.1 M imidazole/HCl (pH 6.6), 0.1 M NaCl, 25 mM MgCl₂ and 2 mM EGTA), 2.8 mM DTT, 5 µM ATP and 10 µCi [³²P] γ-ATP (Amersham, Braunschweig or Hartmann Analytic GmbH, Braunschweig). The kinase reaction was performed for 30 min at room temperature. The reaction was terminated by addition of 1 ml of chloroform:methanol:1 N HCl (100:100:1). 170 µl of a buffered saline solution (135 mM NaCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, pH 7.2) and 30 µl of a 100 mM EDTA-solution were added. The samples were extracted, the lower phases collected, dried, dissolved in 20 µl of chloroform:methanol (1:1) and separated on a Silica G60 TLC plate with chloroform:methanol:acetic acid (65:15:5). The plate was exposed, ceramide identified by co-migration with an identical standard, scraped from the plate and quantified by liquid scintillation counting. The amount of ceramide was determined by comparison with a ceramide standard curve using C₁₆-ceramide (Alexis, Lausen, Switzerland) and extrapolated for 100 % parasitemia.

Statistics

Data are expressed as arithmetic means ± SEM. Statistical analysis was made by two tailed unpaired t-test or ANOVA using Tukey's test as post hoc test, where appropriate. p<0.05 was considered statistically significant.

Results

To test the effect of amitriptyline on malaria, we first assessed eryptosis upon infection of murine erythrocytes with the rodent malaria parasite *P. berghei*. As a measure of eryptosis, cell membrane scrambling was determined by binding of fluorescent annexin V to erythrocytes and subsequent FACS analysis. In erythrocytes freshly drawn from uninfected mice the percentage of annexin V-binding was low (1.97 ± 0.41, n=6) and not significantly altered upon treatment with 1 mM amitriptyline (1.51 ± 0.12, n=6). Staining with Syto16 for identification of infected cells revealed that parasitized erythrocytes from *P. berghei*-infected mice (46 ± 6 % parasitemia; n=4) showed higher annexin V-binding (30 ± 4 %; n=4). The treatment with 1 mM amitriptyline significantly lowered the annexin V-binding of the *P. berghei*-infected mouse erythrocytes (15.5 ± 1.0 %, n=6).

Similar to uninfected mouse erythrocytes, human erythrocytes exhibited low annexin-binding even when cultured for 48 h at 37°C (7.5 ± 0.3 % annexin-binding

Fig. 1. Effects of amitriptyline on phosphatidylserine exposure of *P. falciparum*-infected and uninfected human erythrocytes and on intraerythrocytic parasite growth. A. Arithmetic means $\pm$ SEM (n=8) of annexin-binding of infected (closed symbols) and uninfected (open symbols) erythrocytes in the absence and presence of amitriptyline as a function of the amitriptyline concentration. * indicates significant difference (p<0.05) from absence of amitriptyline, # indicates significant difference from uninfected erythrocytes. B. Arithmetic means $\pm$ SEM (n=6-10) of forward scatter of late stage-infected (closed circles) and early stage-infected (closed squares) as well as uninfected (open circles) erythrocytes as a function of the amitriptyline concentration. * indicates significant difference (p<0.05) from absence of amitriptyline, # indicates significant difference from uninfected erythrocytes. C. Intraerythrocytic DNA amplification as a function of the amitriptyline concentration (arithmetic means $\pm$ SEM, n=8). D. *In vitro* increase in parasitemia of *P. falciparum*-infected human erythrocytes as a function of amitriptyline concentration (arithmetic means $\pm$ SEM, n=8). * indicates significant difference (p<0.05) from absence of amitriptyline.

cells; n=8). In sharp contrast, *P. falciparum*-infected human erythrocytes (when ring stage-synchronized and further incubated for 24 h) and - to a significantly lower extent - co-cultured uninfected bystander cells showed enhanced annexin V-binding (Fig. 1A). Treatment with amitriptyline during the 24 h-incubation period dose-dependently decreased the annexin V-binding of the infected erythrocytes (Fig. 1A). This suggests that membrane scrambling of the infected erythrocytes was at least in part due to formation of ceramide.

In addition to the decrease in annexin V-binding, amitriptyline increased the cell size of both the early and late stage-infected erythrocytes as estimated from forward scatter in FACS analysis (Fig. 1B). On the other hand, amitriptyline had only minor effects on the uninfected bystander cells (Fig. 1B). In contrast to the alterations of the host erythrocyte, amitriptyline did not affect the intraerythrocytic development of *P. falciparum* as deduced from the 24 h-increase in DNA/RNA content of the parasitized erythrocytes (Fig. 1C).

However, in 48 h re-invasion assays amitriptyline decreased the *in vitro* parasitemia, an effect statistically

significant at 1 μM amitriptyline concentration (Fig. 1D). Thus, amitriptyline influenced the number of re-infected erythrocytes, suggesting that it impairs either the release of the merozoites or the invasion of the pathogens into hitherto uninfected erythrocytes.

Further experiments were performed to determine, whether amitriptyline influences the clearance of erythrocytes from circulating blood *in vivo*. To this end, erythrocytes from mice that were either left untreated or treated with amitriptyline (1 mM in drinking water) were labelled with the dye CFSE and injected into the tail vein of the donor mice. As shown in Fig. 2A, the clearance of the re-injected erythrocytes was significantly slower in the mice treated with amitriptyline (1 mM in drinking water) than the clearance of erythrocytes in untreated mice.

Additional experiments explored the effect of amitriptyline treatment on the course of malaria *in vivo*. Following infection with *P. berghei*, parasitemia gradually increased 8 days after infection. Amitriptyline treatment significantly delayed the increase of parasitemia (Fig. 2B,C) and significantly enhanced survival of

Fig. 2. Effects of amitriptyline on *in vivo* clearance of mouse erythrocytes and parasitemia of *P. berghei*-infected mice. A. Disappearance of CFSE labelled erythrocytes from circulating blood of untreated and amitriptyline treated mice. Mice were treated with amitriptyline (1 mM) in drinking water for two weeks before erythrocyte retrieval and re-injection into the donor mouse and post-treated until the end of the clearance experiments. Arithmetic means $\pm$ SEM (n=8) of the percentage CFSE labelled erythrocytes after injection into untreated (open symbols) or amitriptyline treated (closed symbols) mice. * indicates significant difference from erythrocytes of untreated mice. B. Arithmetic means $\pm$ SEM (n=12-14) of parasitemia in mice without treatment (open circles) or with 1 mM amitriptyline in drinking water (closed circles) as a function of days after infection with *P. berghei*. * indicates significant difference from untreated animals. C. Increase in parasitemia as calculated from (B) by linear regression between day 10 and 21 post infection in untreated (open bar, n=14) and amitriptyline (1 mM) treated mice (closed bar; n=12). * indicates significant difference from untreated animals. D. Survival of mice without treatment (open circles) or with 1 mM amitriptyline (closed squares) as a function of days after infection with *P. berghei*.

Fig. 3. Ceramide production in the erythrocytes of uninfected and *P. berghei*-infected (i) *Smpd1*^{-/-} and *Smpd1*^{+/+} mice. A. Arithmetic means $\pm$ SEM (n=5, parasitemia extrapolated to 100%) of ceramide formation in erythrocytes from uninfected (shaded bar) and infected (open bar) wild type mice (*Smpd1*^{+/+}). * indicates significant difference from uninfected *Smpd1*^{+/+} mice. B. Arithmetic means $\pm$ SEM (n=5, parasitemia extrapolated to 100%) of ceramide formation in erythrocytes from uninfected (shaded bar) and infected (i, closed bar) erythrocytes from acid sphingomyelinase knock out mice (*Smpd1*^{-/-}). * indicates significant difference from uninfected *Smpd1*^{-/-} mice.

amitriptyline treated mice (Fig. 2D).

In order to test, whether the effect of amitriptyline was due to inhibition of host sphingomyelinase, additional experiments were performed in animals lacking acid sphingomyelinase (*Smpd1*^{-/-}) and their wild type littermates (*Smpd1*^{+/+}). As illustrated in Fig. 3, ceramide formation was stimulated following infection with *P. berghei* in both, *Smpd1*^{-/-} (Fig. 3B) and *Smpd1*^{+/+} mice (Fig. 3A).

Thus, ceramide formation in infected mice was not fully dependent on host acid sphingomyelinase. Accordingly, the increase of parasitemia was slightly but signifi-

Fig. 4. Parasitemia and survival of *P. berghei*-infected mice. A. Arithmetic means $\pm$ SEM (n=19-21) of parasitemia in acid sphingomyelinase knockout mice (*Smpd1*^{-/-} closed circles) and their wild type littermates (*Smpd1*^{+/+}, open circles) as a function of days after infection with *P. berghei*. * indicates significant difference from *Smpd1*^{+/+} mice. B. Increase in parasitemia as calculated from (A) by linear regression between day 10 and 21 post infection in *Smpd1*^{-/-} (closed bar) and *Smpd1*^{+/+} (open bar) mice. * indicates significant difference from *Smpd1*^{+/+} mice. C. Survival of *Smpd1*^{-/-} (closed circles) and *Smpd1*^{+/+} (open circles) mice as a function of days after infection with *P. berghei*.

cantly delayed in *Smpd1*^{-/-} mice (Fig. 4A, B). Survival of infected mice was, however, not significantly prolonged in *Smpd1*^{-/-} mice (Fig. 4C).

Discussion

The present observations disclose that treatment with amitriptyline decreases *in vitro* parasitemia, delays the development of parasitemia *in vivo* and extends the survival of *P. berghei*-infected mice.

The effect of amitriptyline is presumably the result of a concerted action of the drug on the mammalian and parasite sphingomyelinase, which apparently impedes the infection of erythrocytes with the parasite. Intraerythrocytic growth and survival of the pathogen is apparently not significantly affected by the drug.

Infection with *P. falciparum* or *P. berghei* triggers eryptosis, which is characterized by annexin V-binding and cell shrinkage. Intraerythrocytic *Plasmodia* impose oxidative stress to the host cell [19, 35, 39], which in turn activates the Ca²⁺ permeable cation channels [18, 19]. The following Ca²⁺ entry leads to stimulation of cell membrane scrambling [15, 22-24]. The oxidative stress during *P. falciparum* infection does not only trigger eryptosis of infected erythrocytes, but to a smaller extent also of uninfected bystander cells. The eryptosis of those cells was not significantly modified by amitriptyline.

Amitriptyline attenuates infection-induced phosphatidylserine exposure and eryptotic cell shrinkage. The effect is, however, probably not relevant for the beneficial effect of amitriptyline on the course of malaria. Rather, amitriptyline seems to be predominantly effective by impairing the release of merozoites from infected erythrocytes or the invasion of the pathogens into hitherto uninfected erythrocytes. In the contrary, enhanced

eryptosis during iron deficiency [12] and following treatment with lead [13] is paralleled by decreased parasitemia and enhanced survival of *P. berghei*-infected mice.

Comparison of the course of malaria in amitriptyline treated animals and acid sphingomyelinase knockout animals suggests that the effect of amitriptyline is not exclusively caused by inhibition of host acid sphingomyelinase. Accordingly, formation of ceramide is observed in acid sphingomyelinase knockout animals. *Plasmodia* express an own sphingomyelinase [1, 2] and thus do not depend on the host sphingomyelinase. Therefore, the course of malaria is only slightly different in acid sphingomyelinase knockout animals and their wild type littermates. Hence, amitriptyline may, in addition to its known effect on acid sphingomyelinase in the host [29-31] similarly inhibit *Plasmodium* encoded sphingomyelinase, or it influences infection by some other, sphingomyelinase independent mechanism. Further studies are required to delineate the exact effects of amitriptyline on the parasite sphingomyelinase.

Eryptosis and/or ceramide formation are triggered by stimulation of CD95/Fas [42] and several substances including listeriolysin [43], paclitaxel [44], cyclosporine [45], methylglyoxal [46], amyloid peptides [47], anandamide [48], Bay-Y5884 [49], curcumin [50] and valinomycin [51]. Several of those substances have been shown to favourably influence the course of malaria, an effect presumably related to eryptosis.

In conclusion, amitriptyline decreases *in vitro* parasitemia and favourably influences the course of

malaria *in vivo*. The effect may be due to its inhibitory effect on both host and parasite sphingomyelinases.

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