Beneficial effect of aurothiomalate on murine malaria

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Abstract

Background: Premature death of Plasmodium-infected erythrocytes is considered to favourably influence the clinical course of malaria. Aurothiomalate has previously been shown to trigger erythrocyte death or eryptosis, which is characterized by cell membrane scrambling leading to phosphatidylserine exposure at the cell surface. Phosphatidylserine-exposing cells are rapidly cleared from circulating blood. The present study thus tested whether sodium aurothiomalate influences the intraerythrocytic parasite development in vitro and the clinical course of murine malaria in vivo.

Methods: Human erythrocytes were infected with Plasmodium falciparum BinH in vitro and mice were infected (intraperitoneal injection of 1 × 10⁶ parasitized murine erythrocytes) with Plasmodium berghei ANKA in vivo.

Results: Exposure to aurothiomalate significantly decreased the in vitro parasitemia of P. falciparum-infected human erythrocytes without influencing the intraerythrocytic DNA/RNA content. Administration of sodium aurothiomalate in vivo (daily 10 mg/kg b.w. s.c. from the 8th day of infection) enhanced the percentage of phosphatidylserine-exposing infected and noninfected erythrocytes in blood. All nontreated mice died within 30 days of infection. Aurothiomalate-treatment delayed the lethal course of malaria leading to survival of more than 50% of the mice 30 days after infection.

Conclusions: Sodium aurothiomalate influences the survival of Plasmodium berghei-infected mice, an effect only partially explained by stimulation of eryptosis.
effect correlates with a favourable influence on parasitemia and host survival during murine malaria.

**Methods**

Human erythrocytes were drawn from healthy volunteers. The study was approved by the Ethical commission of the University of Tübingen.

Animal experiments were performed according to the German animal protection law and approved by the local authorities (registration number PY 4/09). Experiments were performed in healthy SV129/J wild type mice (aged 4 months, both male and female). The animals had free access to standard chow (C1310, Altromin, Lage, Germany) and drinking water. Blood was drawn by incision of the tail vein.

For infection of human erythrocytes the human pathogen *Plasmodium falciparum (P. falciparum)* strain BinH [58] was grown *in vitro* [37,59]. Parasites were cultured as described earlier [60-62] 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 and 5% O2 [62,63].

To estimate the *in vitro* growth of *Plasmodium falciparum* the BinH strain was cultured and synchronized to the ring stage by sorbitol treatment as described previously [14,63]. For the *in vitro* growth assay, 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 sodium aurothiomalate.

The parasitemia was assessed 0 h and 48 h after infection by flow cytometry of human erythrocytes and by counting of Giemsa-stained blood smears from infected mice. Parasitemia was defined as the percentage of erythrocytes stained with the DNA/RNA-specific fluorescence dye Syto16 or by identification of Giemsa-stained infected erythrocytes using light microscopy.

For Giemsa staining, the thick blood film was air-dried and fixed with methanol. 2% Giemsa solution (Sigma) was added for 30 min. The slide was rinsed with water and again dried. Then, the slides were analysed under a Leica CM E light microscope (100 ×, oil immersion).

To estimate DNA/RNA amplification of the intraerythrocytic parasite, 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 sodium aurothiomalate. Thereafter, the DNA/RNA amount of the parasitized erythrocytes was determined by Syto16 fluorescence as a measure of intraerythrocytic parasite copies.

For infection of mice *Plasmodium berghei* ANKA-parasitized murine erythrocytes (1 × 10⁶) were injected intra-peritoneally [64,65]. Where indicated, sodium aurothiomalate (10 mg/kg b.w. s.c) was administered from the 8th day of infection daily. Blood was collected from the mice daily starting 8 days after infection by incision of the tail. The hematocrit was determined by centrifugation in hematocrit capillaries. Parasitemia was determined by Syto16 staining in FACS analysis. *In vitro* 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 [66]. Aurothiomalate was added to the NaCl Ringer at final concentrations varying from 0.1 to 100 μM (Sigma, Schnelldorf, Germany). For *in vitro* treatment, the final hematocrit was adjusted to 0.3%.

For determination of phosphatidylserine exposure, FACS analysis was performed as described [10]. After incubation in the presence or absence of sodium aurothiomalate, suspensions of *Plasmodium falciparum*-infected erythrocytes were stained with annexin V-APC (BD Biosciences Pharmingen, Heidelberg, Germany) and/or with Syto16 (Molecular Probes, Göttingen, Germany) to identify phosphatidylserine-exposing and infected erythrocytes, respectively. For annexin V-binding, erythrocytes were washed, resuspended in annexin V-binding buffer (Ringer solution containing 5 mM CaCl₂, pH 7.4), stained with annexin V-APC (dilution 1:20), incubated for 20 min at room temperature, and diluted 1:5 with annexin V-binding buffer. Syto16 (final concentration of 20 nM) was added directly to the diluted erythrocyte suspension or co-incubated in the annexin V-containing buffer solution. Erythrocytes were analyzed by flow cytometry (FACS-Calibur, Becton Dickinson, Heidelberg, Germany) in fluorescence channel FL-1 for Syto16 (detected at 530 nm) and in FL-4 for annexin V-APC fluorescence intensity (detected at 660 nm).

Data are expressed as arithmetic means ± SEM, and statistical analysis was made by t-test or ANOVA using Tukey’s test as post hoc test, as appropriate. p < 0.05 was considered as statistically significant.

**Results and Discussion**

A first series of experiments explored the influence of aurothiomalate on the *in vitro* growth of *Plasmodium falciparum* in human erythrocytes. To this end, *P. falciparum*-infected erythrocytes were cultured in human erythrocytes and synchronized to the ring stage by sorbitol treatment. Within 48 hours the percentage of infected erythrocytes increased from 6.0% to 21.0% in the absence and to 9.8% in the presence of 100 μM aurothiomalate (Fig. 1A). Accordingly, aurothiomalate blunted the increase in the percentage of parasitized erythrocytes, an effect reaching statistical significance at ≥ 10 μM auro-
thiomalate (Fig. 1A). The halfmaximal inhibition (IC50) was achieved by 68 μM aurothiomalate. In contrast, at the concentrations tested, the presence of aurothiomalate did not influence the intraerythrocytic DNA amplification of the parasite (Fig. 1AB).

In order to determine the effect of infection and of aurothiomalate on eryptosis, phosphatidylserine-exposing erythrocytes were identified by measurement of annexin V-binding in FACS analysis. Within 24 hours, the infection with *P. falciparum* markedly increased the annexin V-binding of infected and noninfected erythrocytes (Fig. 2). The percentage of annexin V-binding was, however, significantly higher in infected than in noninfected erythrocytes (Fig. 2). The phosphatidylserine exposure of infected erythrocytes was significantly increased in the presence of aurothiomalate (Fig. 2), an effect reaching statistical significance at 50 μM aurothiomalate.

In a next series, mice were infected with *P. berghei* with or without sodium aurothiomalate treatment. Sodium aurothiomalate was administered daily from the 8th day of infection. Similar to the *in vitro* infection of human erythrocytes with *P. falciparum*, the infection of mice with *P. berghei* was followed by a marked increase in the percentage of phosphatidylserine-exposing erythrocytes.

![Figure 1](image.png)

**Figure 1 Effects of sodium aurothiomalate on intraerythrocytic amplification and in vitro parasitemia.** A. *In vitro* parasitemia with *P. falciparum* (left panel) in human erythrocytes as a function of the aurothiomalate concentration (arithmetic means ± SEM, n = 16). *, ** indicate significant difference (p < 0.05, p < 0.001) from the absence of aurothiomalate. Intraerythrocytic DNA amplification (right panel) as a function of the aurothiomalate concentration (arithmetic means ± SEM, n = 12). B. Intraerythrocytic DNA amplification (right panel) as in B for different time periods (arithmetic means ± SEM, n = 8).
The phosphatidylserine exposure of infected erythrocytes was significantly more pronounced following treatment with sodium aurothiomalate than the phosphatidylserine exposure of noninfected erythrocytes (Fig. 3).

The parasitemia was still low on the 8th day of infection (Fig. 4B). The percentage of infected erythrocytes gradually increased with or without sodium aurothiomalate treatment. However, the percentage of parasitized erythrocytes was significantly lower in sodium aurothiomalate-treated animals than in animals without sodium aurothiomalate treatment (Fig. 4A, B).

Since the FACS-dependent determination of parasitemia utilizes a DNA/RNA-specific dye, reticulocytes may also be counted as parasitized erythrocytes. Therefore, a second series of experiments was performed to compare the values for parasitemia determined by FACS analysis to those obtained from Giemsa staining. As shown in Fig. 4D, E, parasitemia was lower in the aurothiomalate-treated group of mice, irrespective of the methods applied.

The treatment with sodium aurothiomalate further resulted in enhanced survival of P. berghei-infected mice. As shown in Fig. 4C, all untreated animals died within 30 days after the infection. In contrast, 57% of the sodium aurothiomalate-treated animals were still alive 30 days after infection. All treated mice died, however, until day 44 after infection.

To investigate whether aurothiomalate treatment influences inflammation, the plasma levels of the inflammatory mediator TNFα, were determined on the 16th day of infection. As a result, the TNFα concentration in non-treated mice was 53.4 ± 29.7 pg/ml whereas the TNFα was below the detection limit in mice treated with sodium aurothiomalate (both n = 4).

As shown earlier, TNF-α may exert an antiparasitic effect in animal models [67-69], and high TNF production is associated with more rapid clinical and parasitologic recovery in humans [70]. Even though aurothiomalate does not seem to affect induction of TNF-alpha in phagocytic cell cultures [71], the present observations clearly demonstrate an effect of the drug on TNF production in vivo.

Malaria is paralleled by loss of erythrocytes leading to anemia. As shown in Fig. 5, the hematocrit of aurothiomalate-treated mice was significantly reduced. The effect could have been due to enhanced eryptosis or hemolysis. In noninfected erythrocytes aurothiomalate has previously been shown to trigger eryptosis rather than hemolysis [44].

The present study demonstrates that aurothiomalate had only mild effects on the parasite burden and moderately delayed the lethal course of malaria following infection of mice with P. berghei. Similar to what has been observed earlier [64], the infection of mice with P. berghei was followed by an invariably lethal course of malaria.
without aurothiomalate treatment. More than 50% of the sodium aurothiomalate-treated animals survived the infection for 30 days, even though they all died until day 44.

The effect of sodium aurothiomalate treatment may in part be due to a toxic effect on the pathogen, which compromises the intraerythrocytic growth of the parasite. As a matter of fact, gold-containing drugs have previously been shown to be toxic for Plasmodia [45-57]. Drugs could specifically enter infected erythrocytes, as the pathogen dramatically enhances the permeability of the erythrocyte membrane [1].

Alternatively, sodium aurothiomalate may exert a protective effect by accelerating the death of infected erythrocytes. Phosphatidylserine-exposing erythrocytes are engulfed by macrophages [25,26] and are thus rapidly cleared from circulating blood [27]. As eryptosis mainly affects infected erythrocytes, accelerated eryptosis should decrease the parasitemia and thus favourably influence the course of the disease [29]. On the other hand, eryptosis has been suggested to foster vascular derangements of metabolic syndrome [72].

The discrepancy between the moderate influence of aurothiomalate on parasitemia and the effect on survival of the infected host is suggestive for an additional effect of the drug on mouse survival. Possibly it is in part the anti-inflammatory effect of the drug, which accounts for at least part of the effect on host survival and the stimulation of eryptosis. As a matter of fact, aurothiomalate
treatment virtually abolished the increase in TNFα plasma concentration following infection.

**Conclusion**

In mice, sodium aurothiomalate delays the lethal course of malaria. Presumably, the effect is not only due to the toxicity for the pathogen and due to stimulation of eryptosis, but may involve the anti-inflammatory activity of the drug.

**List of abbreviations**

ANOVA: (analysis of variance); APC: (allophycocyanin); FACS: (fluorescence-activated cell sorter); FL: (fluorescence channel); Hb: (hemoglobin); TNF: (tumor necrosis factor).

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

IA carried out the flow cytometry analysis, DB participated in the in vivo experiments, SMQ analyzed the TNFα plasma levels, AE maintained the malaria parasite culture. MF and FL conceived the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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