Published in final edited form as:

Exp Parasitol. 2010 October ; 126(2): 273–277. doi:10.1016/j.exppara.2010.05.012.

Inhibition of *Plasmodium* sporozoites infection by targeting the host cell

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Abstract

There is a great need of new drugs against malaria because of the increasing spread of parasite resistance against the most commonly used drugs in the field. We found that monensin, a common veterinary antibiotic, has a strong inhibitory effect in *Plasmodium berghei* and *P. yoelii* sporozoites hepatocyte infection *in vitro*. Infection of host cells by another apicomplexan parasite with a similar mechanism of host cell invasion, *Toxoplasma* tachyzoites, was also inhibited. Treatment of mice with monensin abrogates liver infection with *P. berghei* sporozoites *in vivo*. We also found that at low concentrations monensin inhibits the infection of *Plasmodium* sporozoites by rendering host cells resistant to infection, rather than having a direct effect on sporozoites. Monensin effect is targeted to the initial stages of parasite invasion of the host cell with little or no effect on development, suggesting that this antibiotic affects an essential host cell component that is required for *Plasmodium* sporozoite invasion.

Malaria, one of the most important parasitic diseases in humans, remains one of the major infectious diseases worldwide (Fauci, 2008). Considering the increasing problem of drug resistance in the parasite, there is an urgent need to identify new molecules with antimalarial activity. Since malaria affects mainly populations in developing countries who cannot afford most available treatments, the cost effectiveness analysis should be one prevalent paradigm taken into consideration when developing novel antimalarial therapies.

The liver stage is the initial step of natural malaria infection in mammals, which is initiated when a mosquito inoculates *Plasmodium* sporozoites into the skin of the host. The sporozoites migrate in the dermis, reach blood vessels and are carried rapidly via the bloodstream to the liver, where they invade hepatic parenchymal cells and begin a period of asexual reproduction, producing exo-erythrocytic forms (EEFs) (Ejigiri and Sinnis, 2009). Upon maturation, infected hepatocytes release merozoites that invade erythrocytes, initiating the blood stage of the disease. Inhibitors of *Plasmodium* growth in the liver assume a particular relevance as prophylactic agents, but the list of applied drugs is very limited (Fauci, 2008).

Monensin is a Na⁺ ionophore that exchanges specifically Na⁺ for H⁺, resulting in elevated cytosolic Na⁺ concentration and pH (Mollenhauer et al., 1990). Monensin, isolated from

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Spreptomyces cinnamomensis, is a well-known polyether antibiotic (Haney and Hoehn, 1967). Introduced in 1975 and massively produced, this inexpensive drug is extensively used as a feed additive for cattle, working as a growth enhancer (Meinert et al., 1992; Zinn et al., 1994). It also controls pasture or grain induced bloat (Goodrich et al., 1984). Additionally, it is also used as a coccidiostatic in pigs and poultry (Haberkorn, 1996).

In the present study, we characterize the monensin inhibitory effect in the liver stage of the parasite. *Plasmodium berghei* and *P. yoelii* sporozoites development within hepatocytes is inhibited by monensin *in vitro*, as well as murine hepatic infection *in vivo*. Most importantly, we show that monensin inhibition is achieved by targeting both the parasite and the host cell. We have determined that monensin treatment specifically inhibits sporozoite invasion of host cells, rendering hepatocytes resistant to sporozoite infection.

Monensin inhibits *in vitro* hepatocyte infection by *P. berghei* and *P. yoelii* sporozoites and *Toxoplasma gondii* tachyzoites

In order to characterize the effect of monensin on the pre-erythrocytic stage of malaria, we infected cultures of Hepa 1-6, a mouse hepatoma cell line, with *P. berghei* NK65 sporozoites expressing RedStar fluorescent protein (Frevert et al., 2005) in the presence of different doses of monensin. Monensin was added to host Hepa1-6 cells 2 h prior to addition of sporozoites and kept during 24 h of incubation before quantification of infection. We found that incubation of the cells with monensin at the concentration of 1 nM partially inhibits infection, with 10 nM and higher concentrations resulting in complete inhibition (Fig. 1a). After 24 h of incubation with monensin at concentrations ranging from 0.1 nM to 1 μ M no detectable effects in the total number of Hepa1-6 cells, adhesion to plastic, vacuolization or viability (determined by trypan blue) could be observed.

To determine whether monensin inhibition of infection is mediated by an effect on host cells and/or directly on the parasite, we first pre-incubated Hepa1-6 cells for 2 h before infection with different concentrations of the drug. After this time, cells were washed twice and *P. berghei* sporozoites were added. We found a similar inhibition of infection when monensin was used to pre-treat host Hepa1-6 cells (Fig. 1b) and when the drug was present throughout the infection process (Fig. 1a), suggesting that monensin inhibitory effect is mediated by the host cell. The drug was also added to Hepa 1-6 cultures for 2 h, washed from the culture and *P. berghei* sporozoites were added at different times after the wash. We found complete inhibition of infection when sporozoites were added immediately after the washes and a progressive loss of inhibitory effect when the sporozoites were added at later time points (Fig. 1c). To determine if the inhibitory effect could arise from monensin that had remained on the wells after washing, monensin was also added to Hepa1-6 cells and washed immediately before it had time to affect the cells. After addition of sporozoites, the number of infected cells 24 h later showed no statistically significant difference (data not shown).

To test the effect of monensin on the parasite, *P. berghei* sporozoites were pre-incubated with different concentrations of monensin for 30 min, washed twice, and added to Hepa 1-6 cells. Sporozoites cannot be incubated for times longer than 30 min, even in the absence of any drug, because it results in almost complete loss of infectivity. The number of infected cells after 24 h was only significantly decreased in sporozoites pre-treated with 1 μ M monensin compared with vehicle treated ones (Fig. 1d). Concentrations of monensin that result in complete inhibition of infection when added to host cells: 10 and 100 nM, do not affect sporozoite infectivity. These data suggest that the inhibition of host cell infection by *P. berghei* is mediated primarily by monensin activity on host cells, with a minimal effect on sporozoites.

The high infectivity observed in sporozoite treated with monensin at 10 and 100 nM, confirms the lack of toxicity of monensin on sporozoites. It is likely that the penetration of monensin in host cells is more effective than in apicomplexan parasites because of the triple membrane that surrounds these parasites (Kappe et al., 2004).

We also tested the effect of host cell pre-incubation with monensin on *P. yoelii* sporozoites infection (Fig. 1e). We found that monensin could also inhibit this parasite, however a higher concentration of monensin was required compared to *P. berghei*, may be reflecting the different pathways used for infection of hepatocytes in these two parasites (Silvie et al., 2007).

The effect of monensin infection on *Toxoplasma gondii*, another apicomplexan parasite that shares a similar mechanism of infection with *Plasmodium* sporozoites (Sibley, 2004), was tested. We observed again that pre-incubation of host cells with 1 μ M monensin drastically inhibits *T. gondii* tachyzoites infection of host cells (Fig. 1f).

Monensin impairs *Plasmodium* Sporozoites Invasion of Host Cells

We next determined which step of infection is targeted by monensin. The drug was added to Hepa 1-6 cultures during different time periods before or after the addition of *P. berghei* sporozoites. Monensin was washed from the cultures after the corresponding incubation period. We found complete inhibition of infection when monensin is added to the hepatocytes 2 h prior to addition of sporozoites or during the first 2 h after (Fig. 2a). Since parasite infectivity is progressively lost after incubation at 37°C (Vanderberg, 1974), this early time effect suggests that monensin inhibits infection by interfering with sporozoite invasion and/or early EEF development. The effect of monensin was progressively decreased when added at later time periods (Fig. 2a), suggesting that the drug does not interfere preferentially with late parasite development in the hepatocyte.

To determine whether monensin pre-treatment of host cells inhibits invasion by *Plasmodium* sporozoites, we performed an invasion assay that differentiates intracellular from extracellular sporozoites. For these experiments, cultures of Hepa 1-6 cells were pretreated with 1 μ M monensin for 2 h. Subsequently, monensin was washed several times and sporozoites were added to the cells. After 1 h incubation, quantification of the percentage of intracellular sporozoites performed by differential staining of intracellular and extracellular parasites (Coppi et al., 2005), indicates that monensin reduces sporozoites host cell invasion (Fig. 2b). However, in this assay, a fraction of the intracellular sporozoites correspond to parasites observed during the process of migration through cells (Mota et al., 2001) and cannot be differentiated from sporozoites that have truly infected Hepa 1-6 cells. Intracellular malaria parasites that have infected a host cell are surrounded by a parasitophorous vacuole membrane (PVM), as opposed to migrating sporozoites, which are found free in the cytosol of host cells (Mota et al., 2001). UIS4 is a protein expressed in the liver-stage PVM (Mueller et al., 2005) and therefore, staining of UIS4 can be used to differentiate sporozoites migrating through cells (UIS4 negative) from sporozoites that have infected the host cell (UIS4 positive). We did not detect any UIS4 staining in host cells pre-treated with monensin before addition of *P. berghei* sporozoites, indicating complete absence of PVM formation and inhibition of effective sporozoite invasion. In non-treated hepatocytes, approximately 15% of parasites show strong UIS4 staining of the PVM (Fig. 2c). The remaining 85% of parasites represent extracellular sporozoites and sporozoites that are migrating through host cells.

Inhibition of liver infection in mice by a single injection of Monensin

To investigate the *in vivo* relevance of our findings, we treated mice with monensin by intraperitoneal (i.p.) administration, 1 h prior to infection of mice by the bite of 20 *P. berghei*-infected mosquitoes. As control, we used mice injected with the vehicle alone. There were no detectable toxic effects in the mice due to the injection of monensin. We found either complete inhibition or greatly reduced infection in monensin-treated mice compared to controls (Fig. 3). Only two of the five mice treated with monensin showed blood stage infection and in these mice the appearance of parasites in the blood was delayed four days compared to controls. Since each pre-patent day delay roughly corresponds to a ten fold reduction in the liver parasite load (Gantt et al., 1998), we conclude that monensin strongly reduced the liver parasite load. These results indicate that monensin is very effective in the inhibition of *P. berghei* liver stage infection in mice.

The malarial liver stage is not associated with any symptoms, but it is the gold standard for prophylaxis because it avoids the symptomatic blood stage (Fauci, 2008). The only currently existing prophylactic drugs for malaria are atovaquone and two related compounds, primaquine and tafenoquine. Of these, just primaquine is commonly available in a clinical setting in developing countries (Reidenberg, 2007). Atovaquone is very efficient, but its high cost renders its use limited. Primaquine and tafenoquine have significant toxicity and only partial efficacy (Shanks and Edstein, 2005). Our experiments show that monensin strongly inhibits rodent malaria liver stage infection, *in vitro* as well as *in vivo*.

Monensin activity against the blood stage of *P. falciparum* has been described *in vitro* with IC₅₀ of 1.1 nM (Adovelande and Schrevel, 1996), 1.4 nM (Gibot et al., 1999), 2.1 nM (Gumila et al., 1997) and 2.3 nM (Rochdi et al., 1996). A recent screening of drugs predicted by molecular topology has also found monensin inhibitory activity against the liver stage of *P. yoelii* and *P. falciparum* *in vitro* (Mahmoudi et al., 2008). The minimal dose of monensin required for inhibition of *P. yoelii* sporozoite infection was higher in this study compared to Mahmoudi et al. (Mahmoudi et al., 2008), where they describe an IC₅₀ lower than 10⁻³ nM, however, the different time of incubation of the cultures with the drug and the use of primary cultures versus the cell line used here could account for this. In this later study (Mahmoudi et al., 2008), it is assumed that monensin activity is targeted to the parasite, however, our studies demonstrate that monensin primarily acts on the host cell to inhibit *Plasmodium* infection of hepatocytes. Interestingly, the mechanism of action of monensin is directed to the inhibition of parasite entry in the host cell with little or no effect on parasite development.

The Na⁺ ionophore activity of monensin results in an influx of Na⁺ into the cell, with corresponding efflux of H⁺ and K⁺, leading to an increase in intracellular Ca²⁺ (Satoh and Satoh, 2007). The changes in the equilibrium of these essential cations affects a number of major cellular processes. Among the most prominent effects there are changes in intracellular pH, cell swelling, vacuolization of the Golgi cisternae and defects in intracellular protein transport (Tartakoff, 1983).

Since monensin affects central regulatory mechanisms of the host cell, it is difficult to identify the particular mechanism that inhibits *Plasmodium* sporozoite invasion. The effects of monensin on sporozoite invasion of host cells could be mediated by depletion of specific host membrane proteins that are required for *Plasmodium* invasion (Ejigiri and Sinnis, 2009) caused by defects on Golgi protein transport. It is also possible that changes in the intracellular Ca²⁺ concentrations would interfere with actin polymerization in the host cell that is required for sporozoite invasion (Gonzalez et al., 2009). However, it is also possible

that the change in membrane potential or in surface hepatocyte membrane induced by swelling could also interfere with sporozoite invasion.

The rapid appearance of resistant parasites after introduction of a new anti-malarial drug in the field has prompted the search for drugs that would limit the generation of resistance, such as compounds targeted to host components. Since monensin anti-*Plasmodium* activity is mediated mainly by its effects on the host cell, it would be of great interest to investigate the mechanism of action of this drug to discover new host cell pathways that are required for *Plasmodium* infection and could lead to new therapeutic targets.

Acknowledgments

We would like to thank Stefan Kappe (Univ. of Washington) and Isabelle Coppens (Johns Hopkins University) for providing anti-UIS4 and anti-SAG1 antibodies. Sandra Gonzalez and Jean Nonon for producing *P. yoelii* and *P. berghei* sporozoites. R.L. is the recipient of fellowship from Fundacao para a Ciencia e Tecnologia, Portugal. A.R. was supported by NIH grant R01AI053698.

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Figure 1. Monensin inhibits Hepa 1-6 infection by *Plasmodium* sporozoites and *Toxoplasma* tachyzoites in vitro

(a–c) Cultured hepatoma Hepa1-6 cells in 24-well plates were incubated with different concentrations of monensin 2 h before addition of 5×10^4 /well *P. berghei* RedStar sporozoites. Cultures were incubated for 24 h. Monensin was either kept throughout the 24 h incubation (a), washed after 2 h incubation immediately before addition of sporozoites (b) or washed after 2 h incubation at 1 μ M and sporozoites were added at the indicated times after the wash (c). (d) *P. berghei* Red Star sporozoites were incubated or not with 1 nM monensin for 30 min. After 2 washes, sporozoites were added to Hepa 1-6 cells. Infection was determined 24 h later. (e,f) Hepa1-6 cells in 24-well plates were incubated with monensin at the indicated concentrations (e) or at 1 μ M (f) 2 h before addition of 5×10^4 /well *P. yoelii* sporozoites (e) or 1×10^4 /well *T. gondii* tachyzoites (f). In (f), after 2 h of incubation with *T. gondii* tachyzoites, cells were again washed to remove non-adherent parasites and then incubated for 24 h. *P. yoelii* and *T. gondii* infected cells were detected by staining with anti-Hsp70 (Tsuiji et al., 1994) and anti-SAG1 antibodies, respectively. The number of infected cells was determined in each coverslip after fixation by counting in 50 microscopic fields. Each condition was assayed in triplicate. Results are expressed as mean of triplicates $\pm$ SD. *, $p < 0.05$; **, $p < 0.01$ when compared to control by t-test. Each graph shows one representative of two independent experiments.

Figure 2. Monensin inhibits *Plasmodium* sporozoite invasion of Hepa 1-6 *in vitro*

(a) Monensin (1 μ M) was added to Hepa 1-6 cultures for 2 h at the indicated times before or after the addition of *P. berghei* Red Star sporozoites ($t=0$). After the incubation period, cells were washed three times and incubated with fresh medium. Infection was determined after 24 h incubation. (b,c) Cultures of semiconfluent Hepa 1-6 cells (5×10^4 /well) were pretreated with 1 μ M monensin for 2 h. Cells were washed twice before the addition of sporozoites. (b) After 1 h, cells were fixed and differentially stained to determine the numbers of intracellular and extracellular sporozoites. Results are expressed as percentage of intracellular sporozoites from total. (c) After 4 h, cells were fixed and stained with anti-UIS4 anti-serum for PVM labeling. Sporozoites were quantified by counting 50 fields per well in three independent wells. Results are expressed as mean of triplicates $\pm$ SD. *, $p < 0.05$;

**, $p < 0.01$ when compared to control by t-test. Each graph shows one representative of two independent experiments.

Figure 3. Monensin inhibits *Plasmodium berghei* infection in mice

Female Swiss Webster mice were injected i.p. with 10 mg/Kg of monensin or vehicle (control) 1 h before allowing 20 *P. berghei*-infected mosquitoes to feed on the mice. Procedures were approved by IACUC. Mice were housed in our animal facility and anesthetized before mosquito bites. Mice parasitemia was determined daily for the initial ten days and weekly until 45 days. Pre-patent period is the number of days before blood-stage infection is detected (black circles). In three out of five mice treated with monensin blood stage infection was never detected (n.i., not infected, white circles). Comparison of groups by t-test, $p < 0.01$.