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Desynchronizing *Plasmodium* Cell Cycle Increases Chloroquine Protection at Suboptimal Doses

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Abstract: We have previously shown that *in vivo* and *in vitro* the hormone melatonin is responsible for the synchronous development of *Plasmodia*. Melatonin can also mobilize calcium from internal stores in these parasites and this response is abolished by luzindole, a melatonin antagonist. We here demonstrate that *in vivo* alteration of parasite synchronous development, using luzindole, partially improves survival of infected mice and dramatically increases the antimalarial activity of chloroquine. The data presented may lead to a conceptually new paradigm for malaria infection therapy and provide novel evidence suggesting that the malaria parasite uses the cell cycle synchrony as one of the strategies to evade the host immune system.

Keywords: Malaria, Plasmodium, Rhythm, Chloroquine.

INTRODUCTION

Malaria is still the major cause of death in the world. Recent estimates [1] show that *Plasmodium falciparum* infection still affects 515 millions of human beings, and more than 2 billions people are at risk. This number is 50% bigger than the last WHO estimates. In Africa, continent that is most impaired by the burden, severe malaria causes 10% maternal mortality [2], and malaria is the main cause of children death in Angola [3] Though effective antimalarial therapies are available since a long time, not only the employed drugs have important toxic side effects, but over the last years Plasmodia strains resistant to classical treatments have evolved [4, 5].

The parasite has a complex life cycle, involving a vertebrate and a invertebrate host. In human malaria, the *Anopheles* mosquito bite delivers infective sporozoites forms that are taken by lymphatic and blood circulation [6], and reach the liver, where they invade the hepatocytes and mature into merozoites, which go back into circulation in a very particular fashion [7], avoiding the host immune system in the liver sinusoids. These forms are infective to the circulating red blood cells (RBC) (for review, see [8]). Once invaded, these RBCs are highly modified by the parasite, due to intense protein trafficking [9].

One of the most striking features of malaria in humans is its circadian rhythm, as revealed by the regularity of fever peaks, which occur with intervals multiple of 24 hours and are related to the synchronous development of the parasites within red blood cells, RBC, and the paroxystic release of pyrogens. This in turn depends on the fact that the processes of erythrocyte rupture and new cell invasion is highly synchronous [10].

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We have shown that the hormone melatonin, a known circadian marker [11] is able to synchronize the life cycle of *P. chabaudi* and *P. falciparum in vitro* and this effect is abolished by luzindole, a melatonin receptor antagonist [12]. The synchronism is also lost *in vivo* in pinealectomized mice (restored by melatonin administration) and upon injection in the animal of luzindole, a melatonin receptor antagonist [12].

As to the molecular mechanism of melatonin action in the parasites we have shown that melatonin can elicit an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in *Plasmodium* trophozoites [12, 13]. A great number of articles shows the importance of calcium signaling in these parasites [12-24]. In addition, in *Plasmodium falciparum*, we have demonstrated that the melatonin-signaling pathway involves a complex crosstalk between Ca^{2+} and cAMP [15], and further activation of PKA. Proteins kinases are key components in Plasmodium signaling pathways [25-27], as other components of transduction pathways, such as proteases [28], which could configure new targets to chemotherapy [29, 30].

In this report we addressed the problem of the evolutionary role of synchronicity by the following approach: using the murine strain *P. chabaudi* we have tested in the live mice whether Plasmodia cell cycle desynchronization has any beneficial effects on the development of the disease. The data demonstrate that *in vivo* desynchronization with luzindole has a small, but detectable protective effect against *Plasmodium* toxicity, and most important it dramatically synergizes with classical antimalarial drug in protecting the animal from the infection.

MATERIAL AND METHODS

Parasites

P. chabaudi (strain AJ, clone FIP-Pc1) was maintained in Balb/C mice by transfer of infection The procedure for collecting blood and removing platelets has been described previously by Hotta *et al.* 2000 [12].

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In Vivo Experiments with *P. chabaudi*

Balb/C mice were infected with 10^7 parasites in a photoperiodic regime (12hr light / 12hr dark) at day 0. Chloroquine (1 mg/Kg, 1,5mg/kg, 2mg/Kg and 3mg/Kg i.p) was administered at zeitgeber time 6 (ZT6) and luzindole at ZT12 (15mg/kg i.p.) (ZT0 corresponds to the beginning of the light phase of the daily cycle). The treatment with chloroquine and luzindole started simultaneously. Chloroquine was kept in PBS and Luzindole was diluted in 2% ETOH immediately before administration. On each day, at ZT11, blood samples were collected from tail blood to access parasitemia by counting no less than 1000 cells in Giemsa-stained blood smears. The survival rate was measured at ZT0 and ZT12.

RESULTS

Desynchronizing Plasmodium Cell Cycle Enhances the Protective Effect of Chloroquine *In Vivo*

Infection with *P. chabaudi* in mice is highly toxic. As shown in Fig. (**1**), when Balb/C mice were injected with 10^7 parasites, all animals were dead in 7 days. On the contrary, all animal survived the infection when they were treated with 3 mg/Kg of the classical antimalarial drug chloroquine. In Fig. (**1**), the protective effect of different doses of chloroquine on animal survival was also tested. The dose response is relatively sharp, with no protection at 1 mg/Kg and complete protection by 3 mg/Kg.

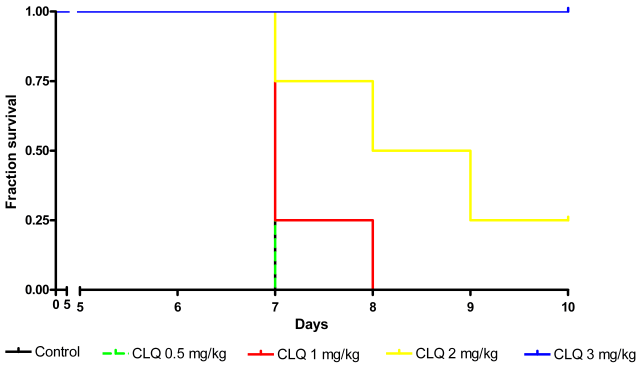

Fig. (1). Chloroquine effect on *P. chabaudi* infected animal survival. Balb/C mice were infected with at day 0 with 10^7 parasites in a photoperiodic regime (12hr light / 12 hr dark). Chloroquine (CLQ) was administered at zeitgeber time (ZT) 6. The survival rate was measured everyday, at ZT0 and ZT12. The curves are significally different by Logrank test (P = 0.0006).

In order to test whether *Plasmodia* synchronous development played a role in the development of the infection, in the experiment presented in Fig. (**2**), the animals were injected together with the parasites with the melatonin antagonist luzindole (15 mg/Kg). The treatment with luzindole continued throughout the duration of the experiments with one injection per day (see Methods). The luzindole treatment resulted in a modest, yet significant protection of the animals from the deadly effect of the parasites, in as much as 20 % of the animals survived at day 10, i.e. at a time where all controls treated with vector alone were dead. In order to further test whether this protective effect of luzindole could represent an additional therapeutic strategy to combat *Plasmodium* infection we tested whether luzindole treatment could act synergistically with suboptimal doses of chloroquine. As shown in Fig. (**2**), subobtimal dose of chloroquine (1.5mg/Kg) resulted in a small, yet significant, reduction in animal mortality, especially in the first 6 days of treatment. Strikingly, injection of the suboptimal chloroquine dose (1.5 mg/kg) and luzindole (15 mg/kg) strongly inhibited mice mortality, with over 70 % of the animals still alive at day 10.

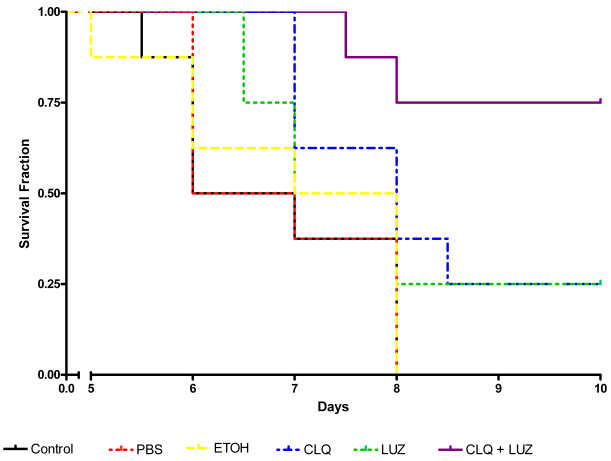

Fig. (2). Survival of Balc/C mice after infection with *P. chabaudi.* Balb/C mice were infected at day 0 with 10^7 parasites in a photoperiodic regime (12hr light / 12hr dark). Chloroquine was administered at zeitgeber time 6 (ZT6) and luzindole at ZT12 (ZT0 corresponds to the beginning of the light phase of the daily cycle). Every day of the experiment, at ZT11, blood samples were collected from tail blood, and parasitemia was counted on Giemsa-stained smears. Where indicated the animals were also injected with 1.5mg/kg Chloroquine (CLQ) and/or 15 mg/kg Luzindole (LUZ), solvent alone (PBS or ethanol) or no addition (control). 8 animals per group. Typical experiment of three independent trials. The survival rate was measured at ZT0 and ZT12. The curves are different controls with statistical significance by Logrank test (P = 0.002).

In order to verify whether luzindole treatment, alone or in combination with chloroquine, exerted its protective effect by reducing the amount of infected cells, parasitemia was measured by counting Giemsa-stained smears 4 days after

Table 1. ***In vivo*** **parasitemia measured at day 4. Mean of 8 animals. Typical experiment of three independent trials. The parasitemias are statistically different from each other by 1-way ANOVA variance test and Newman-Keuls post test (P < 0.0001), except for the pair CLQ 3 mg/Kg vs LUZ 15 mg/Kg + CLQ 1.5 mg/Kg**

Treatment	Control	CLQ 1.5 mg/kg	LUZ 15 mg/kg	CLQ 3 mg/kg	LUZ 15 mg/kg CLQ 1.5 mg/kg
Parasitemia	45.75 ± 2.04	32.63 ± 0.89	38.61 ± 1.6	3.20 ± 0.08	3.62 ± 0.13

injection of the parasites (Table **1**). Mice treated with optimal doses of chloroquine or luzindole + chloroquine (at suboptimal doses) had a drastic reduction in the number of intraerythrocyte parasites. Luzindole and suboptimal doses of chloroquine, when given separately, were not able to significantly inhibit the number of infected cells.

DISCUSSION

The rhythmicity of *Plasmodia* infection, its most distinctive trait, has been studied since the beginning of the XX century (for review, see Garcia *et al.* 2001). Attempts to take advantage of the periodicity of malaria infection have been made in the past, with little success [31, 32]. However, this chronotherapeutic approach has been investigated in a vast number of diseases e.g. cancer, arthritis, heart ischemia [33-36], often with good therapeuthical outcomes. The understanding of the parasite's rhythm, and its modulation, could serve malaria treatment by, for instance, enabling the use of lower dosage of antimalarial to clear out the disease.

The question then arises as to the evolutionary advantage for the parasites provided by synchronization of their cell cycle by host produced melatonin. One possible hypothesis is that the synchronous maturation of the Plasmodia is a strategy to evade the immune system [12, 16, 19, 37]. Indeed when the parasites synchronously burst the RBCs, they flood the circulation with a huge number of merozoites, overcoming the capacity of the immune system to efficiently deal with the infection. The immune system does kill some parasites, but a sufficient number of merozoites can infect other RBCs, leading the infection to another intraerythocytic cycle, i.e. away from the host cellular and humoral defenses. Additional roles for synchronicity has been suggested, in particular concerning the efficiency of vector infection. Here we have readdressed the problem, by taking advantage of the demonstration that the rhythmic cycle of Plasmodia *in vitro* and *in vivo* depends on the hormone melatonin. The rationale of the approach is that alteration in the Plasmodia synchronicity may favor the capacity of the host defense system that could more efficiently deal with parasites asynchronously bursting the RBC than billions of parasites coming out of the red blood cells all at the same time. The *in vivo* experiments with mice infected with P. chabaudi clearly showed that the disruption of the rhythmicity of the Plasmodium cell cycle, using luzindol, a melatonin antagonist, has a small beneficial effect on animal survival. It has also been reported that luzindol act as antioxidant [38]. The more striking results, however, has been the discovery that luzindole drastically improves the therapeutic effect of a suboptimal dose of chloroquine. Indeed, the associated treatment of the animals with the melatonin antagonist and 1,5 mg/Kg of chloroquine, a dose that hardly affects animal survival on its own, has a clear synergistic effect on the survival of Balb/C mice. In particular, at day 10, 25 % of the animals treated with either luzindole or cloroquine alone survived, while 75 % were still alive if treated with both drugs. The treatment with luzindole and cloroquine also decreases the parasitemia, measured on the fourth day of infection. It should be stressed that luzindole has no toxic effect on *Plasmodia in vitro* and thus the simplest explanation for its efficacy is that the host defense mechanisms become more effective when the burst of erythrocytes becomes asynchronous.

The current antimalarial drugs possess a large number of adverse effects, which are predominantly dose-dependent. By using a lower dose, combined with a desynchronizing agent, it is expected that these effects would be milder, making the treatment less toxic for the patient. The adverse effects include nausea, headache, pruritus. Toxic effects includes retinal, cardiovascular- hypotension, vasodilatation, arrhythmias, cardiac arrest - and neurological - convulsions and confusion – disorders. The chloroquine cardiovascular toxicity comes from its membrane stabilization properties, direct negative ionotrophic effects, arterial vasodilatation promotion and manifests as disturbances in cardiac rhythm and conductance, myocardiopathy or vasoplegic shocks [39]. Even in normal cases, when the dosage used in current chloroquine therapy is well tolerated by the patient, the concern with these adverse and toxic effects is always taken into account, whether for treatment or chemoprophylaxis.

In conclusion, we here demonstrate that a antagonist of melatonin, luzindole, while having some effects on its own on infected animal survival, is strongly synergistic with a classical antimalarial drug such as chloroquine. We suggest that the desynchronization of Plasmodia cell cycle by luzindole is beneficial because it allows the host defense mechanisms to more effectively combat the infection. The present data may be of practical significance. In particular, considering the toxicity of chloroquine (and of other antimalarial drugs) [40], the possibility to reduce the effective dose of these compounds by combining them with a drug such as a melatonin antagonist may represent a novel paradigm in malaria therapy and may turn out to be of advantage in the treatment of parasites that are becoming resistant to current therapies.

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