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Interruption of the blood-stage cycle of the malaria parasite, *Plasmodium chabaudi*, by protein tyrosine kinase inhibitors

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Abstract

Malaria is a devastating disease caused by a unicellular protozoan, *Plasmodium*, which affects 3.7 million people every year. Resistance of the parasite to classical treatments such as chloroquine requires the development of new drugs. To gain insight into the mechanisms that control *Plasmodium* cell cycle, we have examined the effects of kinase inhibitors on the blood-stage cycle of the rodent malaria parasite, *Plasmodium chabaudi*. *In vitro* incubation of red blood cells for 17 h at 37ºC with the inhibitors led to a decrease in the percent of infected cells, compared to control treatment, as follows: genistein (200 μM - 75%), staurosporine (1 μM - 58%), R03 (1 μM - 75%), and tyrphostins B44 (100 μM - 66%) and B46 (100 μM - 68%). All these treatments were shown to retard or prevent maturation of the intraerythrocytic parasites. The diverse concentration ranges at which these inhibitors exert their effects give a clue as to the types of signals that initiate the transitions between the different developmental stages of the parasite. The present data support our hypothesis that the maturation of the intraerythrocytic cycle of malaria parasites requires phosphorylation. In this respect, we have recently reported a high Ca^{2+} microenvironment surrounding the parasite within red blood cells. Several kinase activities are modulated by Ca^{2+}. The molecular identification of the targets of these kinases could provide new strategies against malaria.

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During its intraerythrocytic cycle, the malaria parasite, *Plasmodium*, matures in distinct developmental stages, passing from the ring, through the trophozoite to the schizont form. The molecular events that control these transitions are largely unknown. We have recently reported that the hormone melatonin synchronizes malaria parasites (1) through calcium-mediated signaling. In addition, the parasite is surrounded by a high calcium environment and therefore is able to use the ion to signal its intracellular events (2). Kinase activity is largely modulated by calcium and signal transduction pathways. Growth regulation and differentiation in mammalian systems involve protein kinases at many points and in lower eukaryotes, such as *Plasmodium*, this type of regulatory activity is also thought to occur. The evidence is based on extensive molecular level data since a number of different protein kinases have indeed been identified in *Plasmodium* spp.

Kappes et al. (3) have surveyed the known kinase genes of *Plasmodium falciparum*. These comprise: a) the CMGC group (CLK, MAPK, GSK-3 and CDK families), which includes the family of i) cyclin-dependent protein kinases or CDK-like kinases (CLK), probably involved in cell cycle regulation in malaria. The CLK include PfPK5 (3), Pfmrk (3) and Pfcrk-1 (4); ii) the mitogen-activated protein kinase (MAPK) family, members of which are thought to be involved in regulation of cell proliferation and include PfMAP1 (5); iii) the glycogen-synthase kinase 3 (GSK-3) family protein PfPK1 (3). b) Another group of kinases includes the family of protein kinases regulated by Ca^{2+}-calmodulin kinase (CAMk), namely PfCDPK1 (6), PfCDPK2 (7), PfKIN (3), and PfPK2 (8). c) The AGC group (cyclic nucleotide-dependent enzymes) includes protein kinase A (PKA; 9), PKG and PKC.

The question then arises: to what extent does phosphorylation control the parasite's developmental cycle? Although the functional roles of the proteins phosphorylated by these kinases in *Plasmodium* are not yet known they may clearly be involved in intracellular signaling, since protein phosphorylation regulates many cellular processes. In addition to phosphorylation events reported in malaria parasites, it has been suggested that the selective phosphorylation of red blood cell (RBC) membrane proteins by *Plasmodium* kinases may modulate several changes in the host cell (10), which accompany the intraerythrocytic development of the parasite, for example diminished membrane deformability.

Many of the erythrocyte membrane proteins undergo reversible phosphorylation, including a tyrosine phosphorylation of the integral membrane protein, band 3. While there is as yet no evidence for a direct role of protein phosphorylation in modulating events in malaria parasites, a red-cell protein phosphorylation process appears to play a part in invasion of the cell by *P. falciparum* (11). On this basis, we have determined the effects of protein tyrosine kinase inhibitors on the intraerythrocytic development of *P. chabaudi*.

P. chabaudi (F IP-Pc1 clone) parasites were maintained in female mice (BALB/c strain) by weekly transfer. For inhibition assays, blood with parasites synchronized at the trophozoite stage and with 5-15% parasitemia was collected from the mice and the red cells were washed twice by centrifugation at 1500 *g* for 5 min in RPMI. The cells were resuspended at 10% hematocrit in RPMI 1640 (Gibco-BRL, Grand Island, NY, USA), supplemented with 10% calf serum (Gibco-BRL), 40 mg/l gentamicin, 50 mg/l hypoxanthine, 5.94 g/l HEPES and 4.2 ml of 5% (w/v) sodium bicarbonate for pH adjustment to 7.4 in 100 ml of medium. Aliquots of 200 µl were dispensed into 96-well plates and incubated with kinase inhibitors for 17 h at 37ºC. Inhibition of parasite development was assessed by counting the infected RBC per 1000 RBC on methanol-fixed, Giemsa-stained smears.

The kinase inhibitors, staurosporine, genistein, tyrphostin B44, and tyrphostin B46 were from Calbiochem-Novabiochem Ltd. (Nottingham, UK) and R03 was a gift from Hoffman LaRoche (Basel, Switzerland). Dimethylsulfoxide was used as the solvent for stock solutions of the kinase inhibitors and solvent blanks were performed as controls. The assays were used with 2% dimethylsulfoxide in the final volume. After 17 h in culture, the erythrocytes showed the same viability as the control and parasitemia was increased.

All experiments were carried out with at least three different cell preparations.

We attempted to define the importance of phosphorylation events in the intraerythrocytic growth cycle of *P. chabaudi* by examining the effects of several protein kinase inhibitors. Genistein (12), tyrphostin and R03 are generally considered to be selective inhibitors of protein tyrosine kinases, and stau-

rosporine is a serine/threonine kinase inhibitor (13).

Figure 1A shows the effect of genistein on the *P. chabaudi* parasite cell cycle. Addition of 20 μM genistein decreased parasitemia by 33% relative to control, while at 200 μM the parasitemia was reduced by 75%. Figure 1B shows that 1 μM staurosporine reduced the initial parasitemia by 58% of the control level. The kinase inhibitor R03 (Figure 1C) at a concentration of 1 μM reduced the parasitemia by 75% of the control level. Two inhibitors of another class, the tyrphostins, also showed activity, though within a higher concentration range. Figure 1D shows that tyrphostin B44 at 100 and 200 μM concentration reduced parasitemia by 66 and 79% of the control level, respectively. Similarly, tyrphostin B46 at 100 and 200 μM concentration reduced parasitemia by 68 and 83% of control, respectively (Figure 1E).

Studies on signal transduction pathways in malaria parasites have implicated several genes in the control of parasite growth and differentiation (3). Sequencing of these genes identified their products as adenylyl cyclase (14), calmodulin (15) and an E-F hand Ca^{2+}-binding protein (16). Recent studies on calcium dynamics of malaria parasites have indicated similarities to calcium regulation in mammalian cells (17). Briefly, these studies include the existence of a thapsigargin-sensitive pool in the human malaria parasite, *P. falciparum*, and in the rodent parasite, *P. chabaudi* (18). In addition to the endoplasmic reticulum calcium pool, the existence of acidic calcium pools in both parasites indicates that they possess multiple mechanisms of calcium storage during their intraerythrocytic developmental stage (18).

The data reported above imply that the modulation of physiological events in *Plasmodium* must require changes in Ca^{2+} concentration as well as activation of kinases in an orchestrated manner. This correlation is indicated by the presence of Ca^{2+}-dependent protein kinases, such as PfCDPK1 (6), PfCDPK2

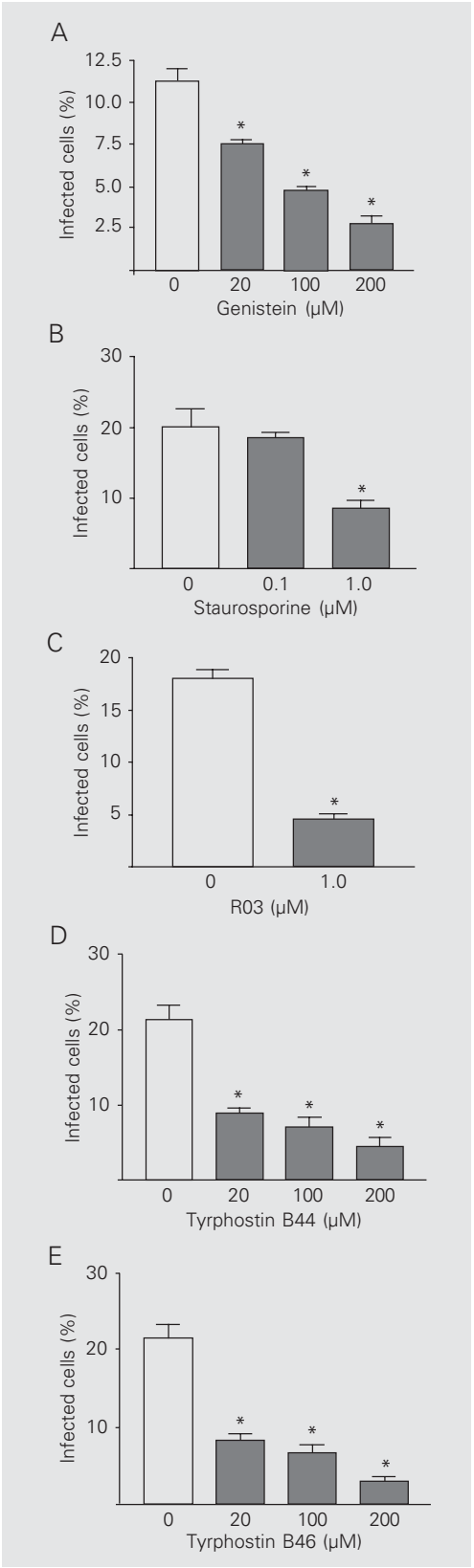

Figure 1. Effect of kinase inhibitors on *Plasmodium chabaudi* cell cycle development *in vitro*. *A*, Genistein; *B*, staurosporine; *C*, R03; *D*, tyrphostin B44, and *E*, tyrphostin B46. The bars indicate the percentage of infected red blood cells per 1000 cells after 17-h incubation. Results are reported as means ± SEM for three independent experiments. *P < 0.001 compared to control (one-way ANOVA and Newman-Keuls test).

(7) and PfKIN (3). This group of kinases also includes PfPK2, and the high expression levels of this kinase at the trophozoite stage in *P. falciparum* argues for a role of this enzyme in the growth of the parasites (8).

Unraveling the basis of signaling processes in *Plasmodium* development within host cells might open new avenues for therapies for the disease since the increase in the drug resistance phenomenon has limited the use of the best known antimalarial drug chloroquine. A clear picture of the problem of drug resistance is missing, since the mechanism involved in drug resistance is still a matter of debate.

In the present report we have shown that protein kinase inhibitors interrupt the blood-stage cycle of the rodent malaria parasite, *P. chabaudi*, thus clearly suggesting that phosphorylations are required for normal development of these parasites. Future work should establish whether these inhibitors block signaling pathways necessary for invasion, maturation, or both, during the asexual stage. Previous work by Ward et al. (19) on *P. knowlesi* and by Dluzewski and Garcia (20) on *P. falciparum* showed that staurosporine inhibits *Plasmodium* entry into RBC. In *P. falciparum* tyrphostin B46 was also shown to inhibit the invasion process (20).

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