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Suppression of *Plasmodium berghei* parasitemia by LiCl in an animal infection model

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Abstract. Malaria, caused by the *Plasmodium* parasite is still a health problem worldwide due to resistance of the pathogen to current anti-malarials. The search for new anti-malarial agents has become more crucial with the emergence of chloroquine-resistant *Plasmodium falciparum* strains. Protein kinases such as mitogen-activated protein kinase (MAPK), MAPK kinase, cyclin-dependent kinase (CDK) and glycogen synthase kinase-3(GSK-3) of parasitic protozoa are potential drug targets. GSK-3 is an enzyme that plays a vital role in multiple cellular processes, and has been linked to pathogenesis of several diseases such as type II diabetes and Alzheimer's disease. In the present study, the anti-plasmodial property of LiCl, a known GSK-3 inhibitor, was evaluated *in vivo* for its anti-malarial effect against mice infected with *Plasmodium berghei*. Infected ICR mice were intraperitoneally administered with LiCl for four consecutive days before (prophylactic test) and after (suppressive test) inoculation of *P. berghei*-parasitised erythrocytes. Results from the suppressive test (post-infection LiCl treatment) showed inhibition of erythrocytic parasitemia development by 62.06%, 85.67% and 85.18% as compared to non-treated controls for the 100 mg/kg, 300 mg/kg and 600 mg/kg dosages respectively. Both 300 mg/kg and 600 mg/kg LiCl showed similar significant ($P<0.05$) suppressive values to that obtained with chloroquine-treated mice (86% suppression). The prophylactic test indicated a significantly ($P<0.05$) high protective effect on mice pre-treated with LiCl with suppression levels relatively comparable to chloroquine (84.07% and 86.26% suppression for the 300 mg/kg and 600 mg/kg LiCl dosages respectively versus 92.86% suppression by chloroquine). In both the suppressive and prophylactic tests, LiCl-treated animals survived longer than their non-treated counterparts. Mortality of the non-treated mice was 100% within 6 to 7 days of parasite inoculation whereas mice administered with LiCl survived beyond 9 days. Healthy non-infected mice administered with 600 mg/kg LiCl for four consecutive days also showed decreased mortality compared to animals receiving lower doses of LiCl; three of the seven mice intraperitoneally injected with the former dose of LiCl did not survive more than 24 h after administration of LiCl whereas animals given the lower LiCl doses survived beyond four days of LiCl administration. To date, no direct evidence of anti-malarial activity *in vivo* or *in vitro* has been reported for LiCl. Evidence of anti-plasmodial activity of lithium in a mouse infection model is presented in this study.

INTRODUCTION

The quest for new biochemical targets for the plasmodial parasite has become a vital undertaking for drug discovery in malaria. This is because malaria is still responsible for significant morbidity and mortality globally (Chung *et al.*, 2009). Additionally, the control and treatment of the disease has been complicated by the emergence of parasite resistance to commonly used anti-malarial drugs, such as chloroquine over the last two decades (Kalra *et al.*, 2006). Protein kinases represent attractive targets for anti-malarial chemotherapy in lieu of the vast phylogenetic distance between

malarial parasites and their human host as well important divergences in the properties of their protein kinases. An example of a protein kinase potentially suitable for this purpose is glycogen synthase kinase (GSK-3).

GSK-3 is a serine/threonine protein kinase first identified 30 years ago as one of several protein kinases capable of phosphorylating and inactivating glycogen synthase (Embi *et al*., 1980), and represents a highly conserved family of protein kinases involved in many cellular processes. The enzyme has been shown to be involved in the regulation of inflammatory processes, and thus implicated in prevalent diseases which involve inflammation (Jope *et al*., 2007). Given the central role of GSK-3 in physiological processes and its implication in human diseases, active research pursuits are currently being undertaken for potent and selective GSK-3 inhibitors. In the context of the malarial disease, subtle structural differences between the GSK-3 of the protozoan malarial parasites and of their hosts which may result in differential affinities of the enzyme molecule for inhibitory molecules, therefore offer possible therapeutic use of GSK-3 inhibitors in controlling these unicellular parasites. In addition, partially divergent sensitivity for inhibitors of *Plasmodium falciparum* GSK-3 and human GSK-3, also suggests the suitability of the parasite GSK-3 as a potential anti-malarial drug target (Droucheau *et al*., 2004). *In vivo* evidence of this proposition is however, lacking.

Lithium can either inhibit GSK-3 by binding directly to the enzyme or by inhibiting the phosphorylation of specific serine residues in the N-terminal of the enzyme structure (Jope, 2006). Other studies on GSK-3 have shown that lithium reduces GSK-3 activity by either directly or indirectly increasing the inhibitory phosphorylation of GSK-3 (Jope, 2003). In parasites, LiCl has been described to inhibit cell growth of the trypanosomatid protozoa, *Herpetomonas muscarum muscarum* and *Blastocrithidia culicis* in a concentration-dependent manner *in vitro*, and also to arrest the cell division process, stimulate cell differentiation, and affect metabolism of these parasites (Spiegel & Soares 1999).

The present study is designed to evaluate the therapeutic and prophylactic anti-malarial potentials of LiCl against *P. berghei* infection in a mouse infection model.

MATERIALS AND METHODS

Drug

Anhydrous lithium chloride was purchased from Fluka Analytical. For *in vivo* experiments, lithium chloride was freshly prepared and diluted to concentrations of 100 mg/kg, 300 mg/kg and 600 mg/kg in normal saline (0.9% saline solution).

Animals

Male ICR mice, 6-8 weeks old, weighing approximately 25±4 g, were obtained from the Animal House facilities at Universiti Kebangsaan Malaysia. Animals were maintained on standard food pellets and water *ad libitum*. Permission and approval for animal studies were obtained from the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC).

Parasite inoculation

The chloroquine-sensitive *P. berghei* NK65 strain was obtained from the Institute for Medical Research, Malaysia, and maintained in ICR mice. The inoculums consisted of 5×10^7 *P. berghei*-parasitised erythrocytes/mL, determined based on the percentage of parasitemia and the erythrocyte count of the stock mouse and diluting the blood with Alsever's buffer.

Evaluation of anti-plasmodial activity on early infection (4-day suppressive test)

Anti-plasmodial activity of lithium was performed in mice according to the 4-day suppressive test described by Peters *et al.* (1975). Mice divided into five groups of seven mice each were inoculated on day

0, intraperitoneally, with 0.2 mL of infected blood containing about 5 x 10^7 *P. berghei*-parasitised erythrocytes. The animals were then administered intraperitoneally with 100 mg/kg/day, 300 mg/kg/day or 600 mg/kg/day of lithium chloride (LiCl); 10 mg/kg/day of chloroquine (as anti-malarial reference drug controls); or an equivalent volume of normal saline (as non-treated controls), for four consecutive days (starting at 1 hour after parasite inoculation on day 0). On day 4, thin blood films were prepared from the tail blood of each mouse, and the parasitemia level was determined by counting the number of infected erythrocytes from more than 200 erythrocytes in random fields of the microscope. Average percentage of chemosuppression was calculated as 100 x [(A-B)/A], where A is the average percentage of parasitemia in the non-treated control group, and B is the average percentage of parasitemia in the test groups.

Evaluation of prophylactic activity

In this prophylactic experiment, groups of seven mice were injected intraperitoneally with three different doses of LiCl diluted in normal saline (100 mg/kg/day, 300 mg/kg/day or 600 mg/kg/day) for four consecutive days (starting day -3 till day 0). Mice administered 10 mg/kg/day of chloroquine were used as controls. On day 0, 0.2 mL of infected blood containing 5 x 10^7 *P. berghei*-parasitised erythrocytes were injected intraperitoneally, immediately after the last treatment of LiCl or chloroquine. Parasitemia levels were determined using thin blood smears starting on day 3 post-parasite inoculation onwards and compared with non-treated controls. Surviving mice were monitored every day until day 30.

***In vivo* toxicity test**

Toxicity of LiCl in mice was evaluated via the intraperitoneal route. Healthy 8-week-old male ICR mice were administered with 100 mg/kg/day, 300 mg/kg/day or 600 mg/kg/day LiCl once a day for four consecutive days. Mortality was recorded for 30 days.

Statistical analysis

Data obtained from the study were analysed statistically using Student's t-test, and values of $P>0.05$ were considered significant.

RESULTS

To determine the influence of LiCl treatment on the course of malaria *in vivo*, mice were intraperitoneally administered with *P. berghei*-parasitised erythrocytes and then injected (i.p.) with LiCl for four consecutive days. Parasitemia levels were determined starting from day 2 post-parasite inoculation. LiCl produced a dose-dependent 4-day chemosuppressive effect at the various doses used in the study corresponding to suppression of 62.06%, 85.67% and 85.18% by 100 mg/kg, 300 mg/kg and 600 mg/kg LiCl respectively (Table 1) as compared to parasitemia detected in non-treated controls. Both 300 mg/kg and 600 mg/kg LiCl showed similar significant ($P<0.05$) suppressive values to that obtained with the reference drug (chloroquine)-treated mice (10 mg/kg/day) (86% suppression) (Fig. 1). Parasitemia in non-treated control mice reached 10.12%±2.0 on day 4 post-parasite inoculation and increased abruptly to 37.6%±4.5 within the next two days (day 6 post-infection) (Fig. 2). On day 6, the inhibition of parasite growth by both doses of LiCl also showed significantly ($P<0.05$) high percentage of parasite inhibition (72.13%±1.3 and 73.38%±1.0 for 300 mg/kg/day and 600 mg/kg/day dosage respectively), although relatively lower than the suppression seen on day 4. LiCl-treated animals survived longer than their non-treated counterparts; mice treated with 100 mg/kg/day LiCl were still alive at day 12, and for the higher doses at day 14; whereas all non-treated animals did not survive beyond day 7.

To evaluate the prophylactic effects of LiCl, mice were injected via the intraperitoneal route with LiCl (100 mg/kg/day, 300 mg/kg/day and 600 mg/kg/day) for four days before parasite inoculation.

The parasitemia levels measured at day 3 indicated a significantly ($P<0.05$) high protective effect on mice pre-treated with LiCl. This effect is particularly evident in mice treated with LiCl where suppression of 84.07% and 86.26% was obtained respectively for the 300 mg/kg/day and 600 mg/kg/day dosage. As comparison, the reference drug, chloroquine at 10 mg/kg/day resulted in 92.86%±0.5 suppression

Table 1. Inhibition of development of *P. berghei* blood stages by the 4-day suppressive test

Drug	Dose (mg/kg)	Average parasitemia on day 4 (%)	Average inhibition on day 4 (%)	Average inhibition on day 6 (%)	Mortality from Day 1-4 (n/N)
Lithium Chloride	100	3.84±0.92	62.06±0.92	60.64±5.0	0/7
	300	1.45±0.92*	85.67±0.92*	72.13±1.3*	0/7
	600	1.5±1.1*	85.18±1.1*	73.38±1.0*	3/7
Chloroquine	10	1.42±0.8*	86.0±0.8*	84.31±1.3*	0/7
Saline solution (non-treated control)	0.2 ml	10.12±2.0	–	–	0/7

Mice (7 mice per group) were inoculated intraperitoneally with *P. berghei* parasite and were administered 1 hour later with LiCl at the indicated doses. The LiCl injections were repeated daily for 3 days. For the toxicity test group (7 mice per group), mice were treated with the same doses of LiCl for four consecutive days. Mortality is defined as n/N, where n is the number of dead mice and N is the number of mice in each group. (* shows significant value, $P<0.05$)

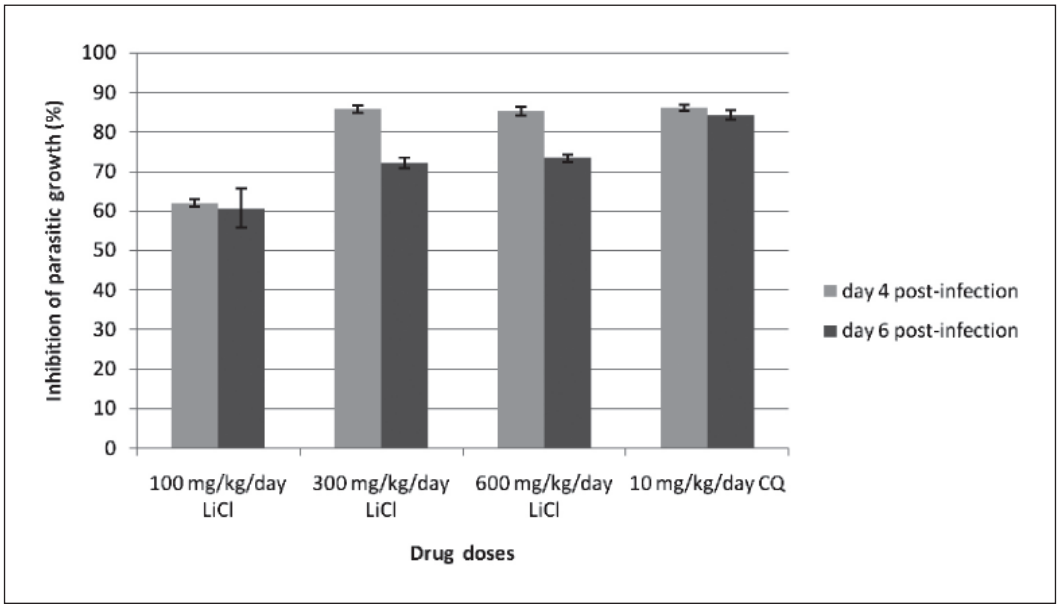

Figure 1. Percentage of inhibition of *P. berghei* growth
Percentages of inhibition of parasite growth *in vivo* and standard deviations at day 4 and 6 post-infection in all groups of mice infected with *P. berghei NK65* intraperitoneally and treated with indicated doses of LiCl compared with non-treated controls. Day 0 corresponds to the day of *P. berghei* infection

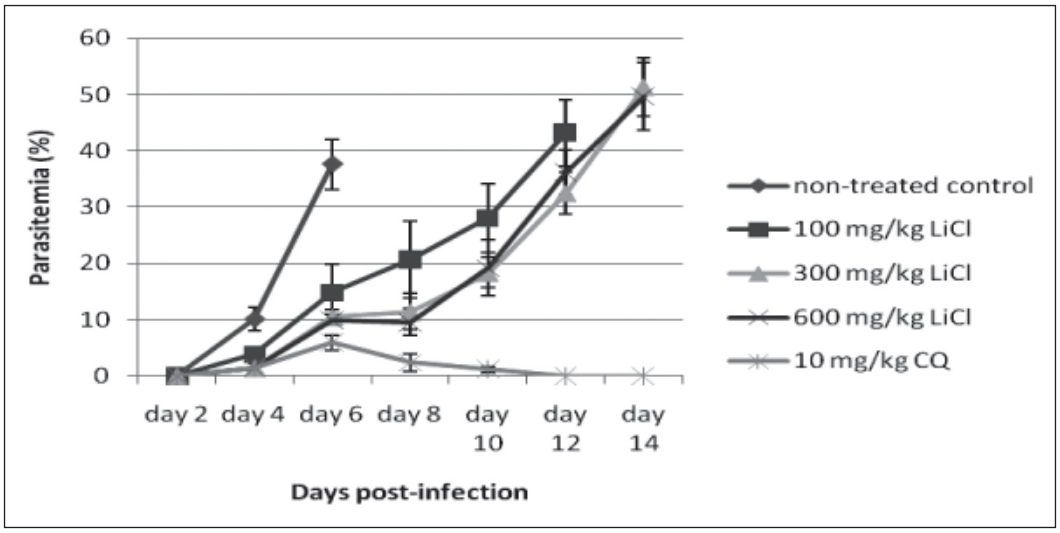

Figure 2. Percentage of inhibition of *P. berghei* growth
Effects of LiCl (100 mg/kg, 300 mg/kg and 600 mg/kg) on early infection of *P. berghei* compared with chloroquine, CQ (10 mg/kg) as the anti-malarial drug reference and control (0.9% saline solution)

(Fig. 3) (Table 2). The average suppression by LiCl as determined on day 5 were however lower than that observed on day 3 (i.e. with suppression levels of 57.36%±5.1, 70.32%±3.7 and 73.57%±3.2 for 100 mg/kg/day, 300 mg/kg/day and 600 mg/kg/day, respectively). All the non-treated mice died from a high level of parasitemia 6 days after parasite inoculation, whereas all LiCl-treated animals were still alive at day 9 (100 mg/kg/day LiCl group) and day 13 (300 mg/kg/day and 600 mg/kg/day) (Fig. 4).

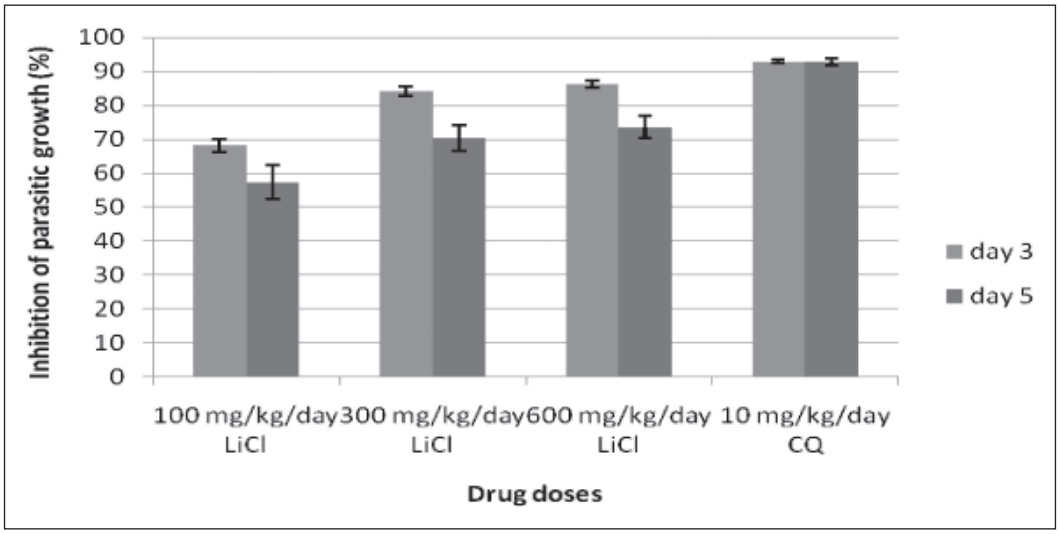

Figure 3. Percentages of inhibition of parasite growth in vivo and standard deviations at day 3 post-infection in all groups of mice infected with *P. berghei NK65* intraperitoneally and treated with indicated doses of LiCl compared with non-treated controls. Day 0 corresponds to the day of *P. berghei* infection

Table 2. Inhibition of development of *P. berghei* blood stages by the 4-day prophylactic test

Drug	Dose (mg/kg)	Average parasitemia on day 3 (%)	Average suppression on day 3 (%)	Average suppression on day 5 (%)
Lithium Chloride	100	5.8±1.9*	68.13±1.9*	57.36±5.1
	300	2.9±1.4*	84.07±1.4*	70.32±3.7
	600	2.5±0.9*	86.26±0.9*	73.57±3.2
Chloroquine	10	1.3±0.5*	92.86±0.5*	92.76±1
Saline water (control)	0.2 ml	18.2±3.1	–	–

Mice (7 mice per group) were treated with LiCl at the indicated doses for 4 consecutive days (day -3 to 0). On the last day of treatment (day 0), the mice were inoculated with *P. berghei* parasite immediately after the last LiCl injection. (* shows significant value, P<0.05)

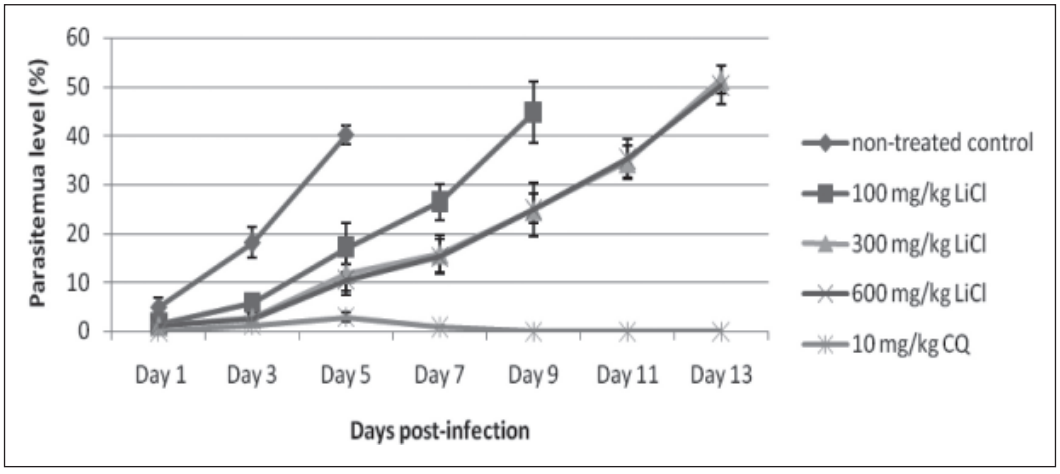

Figure 4. Effects of LiCl on parasitemia development
Effects of LiCl (100 mg/kg/day, 300 mg/kg/day and 600 mg/kg/day) when given prophylactically, where *P. berghei* infection was given 1 hour after the last treament of LiCl, compared with chloroquine, CQ (10 mg/kg/day) as the anti-malarial drug reference and non-treated control (given 0.9% saline solution)

Because of the promising anti-plasmodial activity of LiCl *in vivo*, toxicity tests utilising the same doses of LiCl (100 mg/kg/day, 300 mg/kg/day and 600 mg/kg/day) and the intraperitoneal route of infection were carried out with non-infected mice. The mortality of animals administered LiCl for four consecutive days were monitored until day 30 (Table 1). All mice treated with LiCl (100 or 300 mg/kg body weight) survived beyond 4 days after administration of LiCl. However, at 600 mg/kg/day, three of the seven mice in the group did not survive prior to day 4 (i.e. 24 hours after the last administration of LiCl) suggesting that intraperitoneal administration of LiCl at 600 mg/kg body weight was causing toxicity to the animals.

DISCUSSION

Lithium, a drug most commonly used to treat bipolar disorder, and an excellent inhibitor of mammalian GSK-3 (Jope, 2003), has been reported to inhibit recombinant *P. falciparum* GSK-3 (*Pf*GSK-3) *in vitro* (Droucheau *et al.*, 2004). To date, no direct evidence of anti-malarial activity *in vivo* or *in vitro* has been reported for lithium. Here, we present evidence of anti-plasmodial activity of lithium in a mouse infection model.

In the present study, three doses of LiCl; 100 mg/kg, 300 mg/kg and 600 mg/kg, were chosen based on the IC_{50} value of ~2 mM obtained for LiCl in a GSK-3β activity assay (Klein & Melton, 1996). At this concentration, lithium was shown to be a direct, reversible inhibitor of GSK-3β. We therefore postulate that *Plasmodium* infection in mice can be suppressed at the doses chosen, possibly through the inhibition of parasite GSK-3.

Lithium (300 mg/kg) demonstrated high suppression toward development of *Plasmodium* parasitemia in a mouse model without causing any apparent toxicity to the host. This conclusion is based on the results from both the 4-day suppressive test (an early infection test) and the prophylactic experiment; as well as the toxicity test *in vivo* carried out using LiCl administered by the intraperitoneal route. At the higher dose of 600 mg/kg tested, LiCl also showed high levels of suppressive activity but with concomitant toxicity. Similar toxicity findings were reported by Ogilvie & Lobb (1981), with an LD_{50} (24 h treatment) of 600 mg/kg attained from intraperitoneal injection of LiCl into albino mice. Poorer suppressive activity was observed in mice injected with 100 mg/kg/day LiCl in both the 4-day suppressive test (62.06%) and in the prophylactic test (68.13%).

However the anti-plasmodial activity of the LiCl observed in the present study admittedly is not comparable to that of the reference anti-malarial drug, chloroquine. One explanation is that this may be associated with the short term treatment with lithium (i.e. four consecutive day treatment) relative to its half life. A study using rats indicated only little serum lithium remained at 24 h after injection of 2.0 mequiv kg-1 lithium carbonate (Kling *et al.*, 1978). The half life of lithium in mice was reported to be 3.5 h (Wood *et al.*, 1986). In contrast, the half life of chloroquine in mice is approximately 7 h (Cambie *et al.*, 1994).

In conclusion, this represents the first report that lithium chloride exhibited suppressive activity against malarial parasites in both early infection and prophylactic tests. Since lithium chloride is a well-known inhibitor of GSK-3, it will be interesting to see whether GSK-3 has a role in the inhibitory effects observed on the parasitemia in this study. Ongoing work is in progress to evaluate whether similar results will be observed with other GSK-3 inhibitors.

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