PROPHYLACTIC EFFECT OF *IN SITU* SILVER SULFADIAZINE AND CHLOROQUINE GEL FORMULATIONS AGAINST RODENT MALARIA PARASITE *PLASMODIUM BERGHEI*

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Abstract. Despite technological advances made in the pharmaceutical antimalarial field, malaria remains one of the major causes of mortality worldwide. Resistance of malaria parasites to nearly all current antimalarials in clinical use is one of the primary challenges that face researchers. In this study, the prophylactic efficacy of a single-dose *in situ* gel formulation containing chloroquine or silver sulfadiazine compared to drug alone was examined in *Plasmodium berghei*-infected Balb/c mice. Intramuscular injection of *in situ* 30% polylactide-co-glycolide (1:1) in N-methyl-2-pyrrolidone gel containing silver sulfadiazine (5.25 mg/kg body weight) or chloroquine phosphate (40 mg/kg body weight) failed to protect a challenge of 10^6 parasitized erthrocytes intraperotoneal injected 4 days following treatment. This failure was due to rapid release and clearance of the antimalarial before appearance of parasitemia. The presence of *in situ* antimalarial-containg gels was not detrimental to murine kidney and liver funcrion based on measurements of standard biochemical blood markers. Additional studies are warranted to determine the optimal *in situ* antimalarial gel formulation to generate appropriate drug pharmacokinetics for a prophylaxis against malaria infection.

Keywords: *Plasmodium berghei*, chloroquine, *in situ* gel formulation, prophylaxis, silver sulfadiazine

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INTRODUCTION

Malaria is a life-threatening disease caused by *Plasmodium* parasites. According to the WHO (2021), there were an estimated 241 million cases of malaria worldwide in 2020. Children are more likely to have severe malaria symptoms (White, 2022). Public awareness regarding malaria increases the number of individuals seeking medical treatment when suffering from acute undifferentiated fever (Naing *et al*, 2017). Travel to sub-Saharan Africa, India, and Southeast Asia, has led to an increased number of imported malaria cases into non-endemic countries (Jelinek *et al*, 2002; Spira, 2003; UNWTO, 2004). For example, Denmark reported the number of imported cases to be as high as 2.5 and 714 per 100,000 travelers and 2.5 per 100,000 travelers to Thailand and Ghana respectively (Kofoed and Petersen, 2003). In addition, a recent review in 22 countries conclude that travel is a serious risk factor in the burden of malaria (Ahmed *et al*, 2020).

Currently, *in situ* gel implants are used for drug delivery. Polylactic-co-glycolic acid (PLGA) is a biodegradable, biocompatible polymer commonly used in drug formulations, and N-methyl pyrrolidone (NMP) is an organic physiologically compatible solvent that is miscible with water; together, they form an *in situ* gel that solidifies in an aqueous medium after introduction (Ayoub *et al*, 2020; Astaneh *et al*, 2006; Kamali *et al*, 2018). PLGA is degraded in vivo to lactic and glycolic acids and cleared in the urine (Davis *et al*, 1996).

One of the key steps in the prevention of malaria infection is the use of prophylactic drugs, such as atovaquone-proguanil, doxycycline, mefloquine, sulfadoxine-pyrimethamine, and chloroquine (Schwartz, 2012). The regimens of repeated doses need a personal commitment to take the doses at appropriate times, which cannot always be complied with. Thus, there is a need for development of a more convenient single-dose prophylactic drug strategy. *In situ* gel-containing drugs offer a more prolonged release compared to free drug formulations (Mahajan *et al*, 2011; Dawrea *et al*, 2018). In addition, our previous work showed that the cure rate of *P. berghei* infected Balb/c mice (males and females) is 73 and 100% post treatment with *in situ* gel formulation of silver sulfadiazine (Agsd) and chloroquine phosphate (CQ), compared to 0 and 67% cure rate associated with the free Agsd and CQ drug respectively (Zelai, 2017).

The study employed an *in situ* gel as a carrier for two antimalarial drugs, chloroquine phosphate (CQ) and Agsd, as prophylactic drugs against rodent malaria infection. The efficacy of the *in situ* gel formulations was compared to that of free drugs. In addition, the

effects of these formulations on liver and kidney enzyme levels, as well as their associated pharmacokinetics, were examined. Based on the previous results that showed that *in situ* gel formulation of the drugs, had a prolong release, it is expected that, this formulation may have a prophylactic role against malaria infection.

MATERIALS AND METHODS

Drug source

Agsd, CQ, N-acetyl sulfadiazine (Agsd metabolite), and sulfamerazine (the internal standard to Agsd) were obtained from Riyadh Pharma, Riyadh, Saudi Arabia.

Parasite source

Plasmodium berghei ANKA strain was obtained from Addis Ababa University, Addis Ababa, Ethiopia. The parasite was maintained by serial intraperitoneal injection (200 μl) 10^6 parasitized erythrocytes in citrate saline into Balb/c male and female mice.

Mice source

Male and female Balb/c mice (20-30 g) were obtained from the Animal House of the College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. All mice were given food and water *ad libitum* for one week prior to conducting the experiments. Our previous study showed no differences between male and female mice in their ability to being infected and treated, and manifestations of all signs related to malaria infection (Zelai, 2017).

The animal experiments were approved by the Biomedical Ethics Research Committee, King Abdulaziz University, Saudi Arabia (reference no. 168-15; May 24, 2015). All methods were carried out in accordance with relevant guidelines and regulations, and results reported in accordance with ARRIVE guidelines (Percie du Sert *et al*, 2020).

Free drug preparation

Agsd suspension was prepared by mixing 50 mg of the compound in 50 ml of distilled water, followed by sonication for 5-10 minutes and incubation overnight at 25°C with shaking. CQ (50 mg) was first dissolved in 1 ml of distilled water and then distilled water was added to a final volume of 50 ml.

In situ gel drug preparation

A stock of 1.8 g of polylactide-co-glycolide (1:1) (PLGA) (CAS: 26780-50-7; 24-38 kDa; Sigma-Aldrich, Saint-Louis, MI) in 6 ml of N-methyl-2-pyrrolidone (NMP) (ChemSpider, Cambridge, United Kingdom) was prepared as reported previously (Tang and Singh, 2008). Individual precalculated drug doses were dispersed in 0.1 ml of PLGA-NMP, vortexed for 5 minutes, and incubated at 25°C with shaking for 24-72 hours

to obtain clear, viscous solutions. Each mouse received an intramuscular inoculum of 0.1 ml of PLGA-NMP preparation containing 5.25 and 40 mg/kg body weight (BW) Agsd and CQ respectively.

Mouse treatment groups

Mice were divided into five groups (n = 15 per group). Groups 1 and 2 received intramuscular prophylactic treatment of Agsd (1.05 mg/kg BW/day) for 5 days and CQ (10 mg/kg BW/day) for 4 days prior to parasite challenge (Wysor, 1975; Doolan, 2002), while Groups 3 and 4 received an inoculum of *in situ* drug-containing gel as described above. Control, Group 5, received *in situ* vehicle gel for 5 days. Four days after the (latest) treatment, all mice were injected intraperitoneally (within the peritoneal cavity) with 10^6 *P. berghei* parasitized erythrocytes and parasitemia assessed via collection of mouse tail blood on Days 3, 5, 7, and 9 post-infection. The experimental protocols are summarized in Table 1.

Liver and kidney enzyme analysis

Urea, creatinine, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALKP) levels in blood plasma were measured using a Vitros 350 instrument (Johnson & Johnson, Skokie, IL). Blood samples were collected by retro-orbital bleeding on Day 4 post-infection.

Pharmacokinetic analysis of the drugs and their primary active metabolites

Control murine plasma samples (250 µl) were spiked with serial dilutions of Agsd and its metabolite N-acetyl sulfadiazine and used as standards for in vivo pharmacokinetic analysis. Agsd was extracted from plasma of drug-treated mice samples spiked with sulfamerazine (62.5 µg/ml) as internal standard (USP, n.d.). Solutions of 1.95, 3.90, 7.81, 15.63, 31.25, and 62.50 µg/ml Agsd and N-acetyl sulfadiazine were prepared in mobile phase (1000:99:9, water:acetonitrile:phosphoric acid). In a separate tube, 62.5 µg/ml sulfamerazine was prepared in mobile phase. Blank plasma samples (250 µl) were spiked with different dilutions of 1:1 Agsd and N-acetyl sulfadiazine solutions.

Plasma samples (n = 9) were collected from mice intramuscular injected with Agsd suspension (1.05 mg/kg BM) for 5 days or with *in situ* gel containing 5.25 mg/kg BW. Plasma samples were collected 6 hours after the first, second, third and fifth Agsd suspension doses, respectively, and then on the seventh, ninth, and fifteenth days after both Agsd suspension and *in situ* gel treatment.

All samples and standards were processed and analyzed in the same manner. Briefly, 20-µl aliquot of 65% perchloric acid was mixed with

250 µl of sample, centrifuged at 11,300 g for for 5 minutes, supernatant removed and added with 50 µl of 2M dipotassium hydrogen phosphate, centrifuged as described above for 1 minute, and resulting supernatant then spiked with 200 µl of sulfamerazine and injected into a high performance liquid chromatography (HPLC) system (Nexera XS lite; Shimadzu Corporation, Tokyo, Japan) using conditions previously described (Amini and Ahmadiani, 2007). Standard curve and standard equation were derived using a Microsoft Excel program.

Data analysis

Parasitemia, pharmacokinetics, liver and kidney enzyme data are expressed as mean ± standard error of the mean (SEM). All data were tested for Gaussian distribution using a D'Agostino-Pearson omnibus and Shapiro-Wilk normality tests. Experiments were designed with no matching or pairing. One-way analysis of variance and Tukey's multiple comparisons test were employed to analyze data for significance, with a p-value <0.05 considered statistically significant. Calculations were performed using GraphPad Prism 7.

RESULTS

In this study, Agsd, *in situ* Agsd-containing gel, CQ, and *in situ* CQ-containing gel were used as prophylactic formulations against *P. berghei* infection in Balb/c mice four days after (last) drug treatment. All drug formulations were delivered by intramuscular injection: Agsd (1.05 mg/kg BW/day) for 5 days, CQ (10 mg/kg BW/day) for 4 days, and Agsd (5.25 mg/kg BW)- and CQ (40 mg/kg BW)-containing gels one time (Table 1).

Infected mice showed signs of weakness, shivering, hepatomegaly, and splenomegaly. Parasitemia began to be apparent in all mice groups (except for the group treated with CQ) and rose steadily until termination of the study (Day 9 post-infection) (Fig 1). On Day 9, no significant differences in parasitemia were observed among all groups, except that treated with CQ, parasitemia of which became only noticeable on Day 7. Parasitemia of Agsd-treated murine group is significantly lower compared to control group on Day 7, but not by Day 9. It is worth noting that the efficacy of CQ was lost when adminstered as an *in situ* gel formulation.

When kidney and liver functions were evaluated, there are no significant differences among the parameters measured (on Day 4 post-(last) drug treatment) among all mice groups, including control and normal groups, except for a significantly higher blood plasma AST level in the *in situ* CQ-containing gel treated group (p = 0.025) (Table 2).

Table 1

Design of the experiments

Criterion	Mouse group					Number of Balb/c mice/group
	Group 1	Group 2	Group 3	Group 4	Group 5 (Control)	
Treatment	Agsd	CQ	*In situ* Agsd-containing gel	*In situ* CQ-containing gel	*In situ* vehicle-containing gel	15
Dose	1.05 mg/kg BW/day	10 mg/kg BW/day	5.25 mg/kg BW	40 mg/kg BW	NA	
Dosage	5 days	4 days	1 day	1 day	5 days	
Treatment route	Intramuscular	Intramuscular	Intramuscular	Intramuscular	Intramuscular	
Plamodium berghei infection on Day 4 post-(last) treatment	10^6 parasitized erythrocytes	10^6 parasitized erythrocytes	10^6 parasitized erythrocytes	10^6 parasitized erythrocytes	106 parasitized erythrocytes	
Parasitemia	Determined on Days 3, 5, 7, and 9 post-infection	Determined on Days 3, 5, 7, and 9 post-infection	Determined on Days 3, 5, 7, and 9 post-infection	Determined on Days 3, 5, 7, and 9 post-infection	Determined on Days 3, 5, 7, and 9 post-infection	
Murine viability	Monitored	Monitored	Monitored	Monitored	Monitored	
Liver and kidney enzymes	Tested	Tested*	Tested	Tested*	Tested	3
Pharmacokinetics	Tested	Tested*	Tested	Tested*	Tested	3

*From Zelai (2021)

Agsd: silver sulfadiazine; BW: body weight; CQ: chloroquine phosphate; mg/kg: milligram per kilogram; NA: not applicable

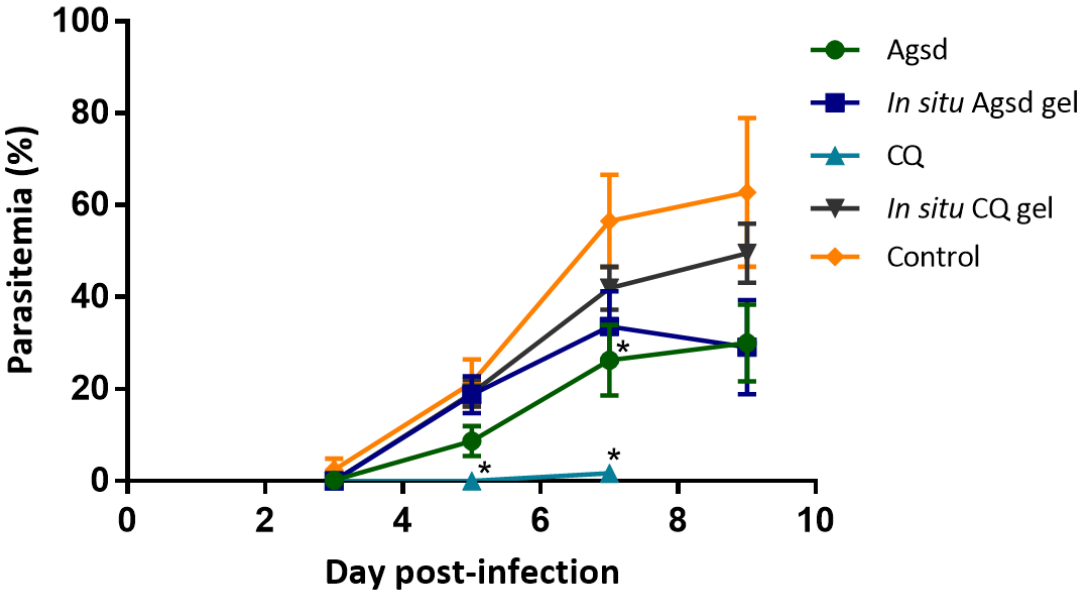

Fig 1 - Parasitemia in mice treated with silver sulfadiazine (Agsd), *in situ* gel containing Agsd, chloroquine phosphate (CQ), and *in situ* gel containing CQ

Mice (n = 15 per group) were treated as described in Table 1.
*p-value <0.05 compared to Control

Levels of Agsd and its main metabolite N-acetyl sulfadiazine were tracked in blood plasma of Agsd and *in situ* Agsd-containing gel treated mice as described above (but not *P. berghei* infected) over a period of 15 days post-(last) treatment. Agsd level was readily detected on Day 1 in plasma of the *in situ* gel treated mice, while a (3 folds) lower level was detected on Day 2 in Agsd-treated group, and Agsd levels were undetected in both groups by Day 3 (Fig 2a). N-acetyl sulfadiazine, as expected, was detected on Day 5 post-(last) treatment with Agsd and became undetectable on Day 7 onwards (Fig 2b). Surprisingly, there was no N-acetyl sulfadiazine in murine plasma in the *in situ* Agsd-containin gel group over the study period.

DISCUSSION

In this study, the efficacy of *in situ* Agsd- and CQ-cotaining gels formulations were evaluated compared to Agsd and CQ as prophylactic

Table 2

Changes in murine kidney function and liver enzymes following treatment with different malaria prophylaxis drug formulations

Group (3 mice in each group)	Kidney parameter (Mean ± SEM)[a]			Liver enzyme (Mean ± SEM)[a]		
	Urea (mM)	Creatinine (μM)	Uric acid (μM)	AST (U/l)	ALT (U/l)	ALKP (U/l)
Normal	6.1 ± 0.6	15 ± 0.6	104 ± 51	485 ± 215	132 ± 7	115 ± 85
Control	7.5 ± 0.3	19.3 ± 2.2	139 ± 65	237 ± 6	96 ± 14	88 ± 28
Agsd treatment	6.3 ± 0.4	17 ± 0.5	203 ± 44	340 ± 56	176 ± 92	174 ± 28
In situ Agsd-containing gel treatment	6.2 ± 0.5	16.7 ± 0.7	192 ± 47	203 ± 27	74 ± 9	102 ± 9
CQ treatment[b]	7.7 ± 1.2	14.7 ± 1.7	173 ± 92	233 ± 43	107 ± 5	150 ± 13
In situ CQ-containing gel treatment[b]	18.3 ± 3.1	12.0 ± 1.7	97 ± 60	617 ± 103*	404 ± 140	131 ± 8

Mice were treated with silver sulfadiazine (Agsd) or chloroquine phosphate (CQ) as described in Table 1.

[a]Measured on Day 4 post-(last) treatment using a Vitros 350 instrument (Johnson & Johnson, Skokie, IL); [b]From Zelai (2021)

*p-value = 0.025 compared to all other groups.

Agsd: silver sulfadiazine; ALKP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CQ: chloroquine; mM: millimeter; μM: micrometre; SEM: standard error of mean; U/l: Units per litre

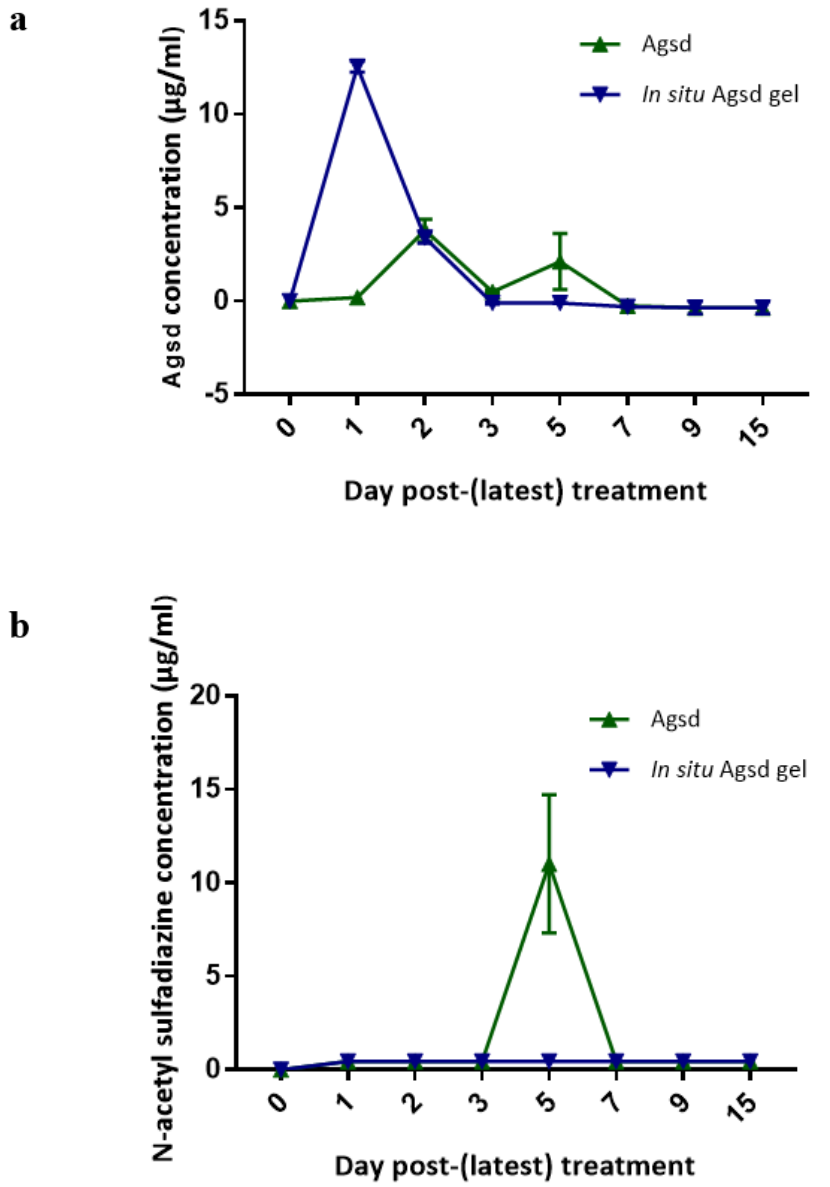

Fig 2 - Pharmacokinetics of silver sulfadiazine (Agsd) (a) and its metabolite N-acetyl sulfadiazine (b) in blood plasma of treated mice

Mice (n = 3 per group) were intramuscularly injected with Agsd (1.05 mg/kg body weight) for 5 days or with *in situ* Agsd-containing gel (5.25 mg/kg body weight) once and blood plasma levels of Agsd and N-acetyl sulfadiazine determined by high performance liquid chromatography.

Vertical line denotes standard error of mean.

antimalarial drugs against *P. berghei* challenge in a murine malaria model. Disappointingly, only CQ provided protection. This could be attributed to a burst of plasma Agsd on Day 1 post-(last) treatment, which subsided by Day 3; whereas mice were infected with *P. berghei* on Day 4 post-(last) treatment and parasitemia could only be diagnosed on Day 9 post-(last) treatment (*ie*, on Day 5 post-infection).

Furthermore, in the free drug groups, lower levels of blood parasitemia was observed compared to those of *in-situ* gel groups. This may be attributed to the high burst of drugs released from the *in-situ* gel formulation shortly after administration, which would have occurred within the four-day period prior to infection. This hypothesis is supported by the pharmacokinetics of Agsd, which revealed a high burst of *in-situ* gel on Day 1 (Zelai, 2017). However, Zelai (2021) found that the single dose of CQ *in situ* gel, had similar release (for 15 days) as the free CQ that was given for four days, although they had a high burst in the first day.

Currently, there are limited studies on *in situ* gel-formulated drugs against malaria. Mahajan *et al* (2011) demonstrated that a biodegradable *in situ* gel polymer can be injected safely via intramuscular administration and that it offers an extended bioavailability for up to three days. They found that pluronic and hydroxy propyl methyl cellulose *in situ* gel is stable for over 90 days under different temperatures and humidity conditions. Although Mahajan *et al* (2011) and Dawrea *et al* (2018) reported that *in situ* gel prolongs drug release, this was not the case in our study. These differences could be due to the gel composition. Dawrea *et al* (2018) reported that, treatment with arteether-lumefantrine-polymeric lyotropic liquid crystalline phase *in situ* gel formulation at 1/40th of the therapeutic dose (3.2 mg/kg) as a single dose, cures mice from *P. berghei* infection, while mice treated with 3.2 mg/kg marketed arteether for three days, died within 20 days.

Thus, optimization of *in situ* gel formulations should be carried out to obtain a formulation allowing an initial burst drug release followed by an extended release required for prophylaxis. For example, Ahmed (2015) demonstrated partially water-miscible solvent (such as triacetin) with PLGA instead of water-miscible NMP reduces the initial burst of *in situ* gel formulations, and an increase in lactic acid:glycolic acid ratio (from 75:25 to 85:15) raises polymer hydrophobicity and extends the period release of drug release; moreover, high molecular weight polymers and high polymer concentrations in the solvent decrease the initial burst of the drug release.

CQ *in situ* gel had adverse effects in elevation of liver enzymes, although some of these effects are not significant, compared with normal mice group. The elevated level of AST in *in situ* gel treated group, may be attributed to the high burst of CQ that directed to the liver. However, treatment with Agsd *in situ* gel did not affect AST level comparing to normal mice. This may attributed to the low dose of Agsd in *in situ* gel formulation (5.25 mg/kg BW) compared to the CQ dose (40 mg/kg BW).

In conclusion, the formulation of *in situ* 30% polylactide-co-glycolide (1:1) in N-methyl-2-pyrrolidone gel containing silver sulfadiazine or chloroquine phosphate failed to prevent infection in a rodent malaria model. Given the lack of adverse side effects of this *in situ* gel formulation, future studies should focus on optimizing the gel composition to obtain appropriate pharmacokinetics of antimalarials released from *in situ* gel to provide prophylaxis in an animal malaria model, with the ultimate goal of applying this approach in humans.

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CONFLICT OF INTEREST DISCLOSURE

The author declares no conflict of interest.

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