Use of a Rhesus *Plasmodium cynomolgi* Model to Screen for Anti-Hypnozoite Activity of Pharmaceutical Substances

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Abstract. There remains a need for new drugs to prevent relapse of *Plasmodium vivax* or *P. ovale* infection. The relapsing primate malaria *P. cynomolgi* has been used for decades to assess drugs for anti-hypnozoite activity. After sporozoite inoculation and blood-stage cure of initial parasitemia with chloroquine, rhesus macaques were treated on subsequent relapses with chloroquine in conjunction with test regimens of approved drugs. Tested drugs were selected for known liver or blood-stage activity and were tested alone or in conjunction with low-dose primaquine. Tinidazole and pyrazinamide prevented relapse when used in conjunction with chloroquine and low-dose primaquine. Triamterene and tinidazole administered without primaquine achieved radical cure in some animals. All other tested drugs or combinations failed to prevent relapse. The rhesus macaque–*P. cynomolgi* model remains a useful tool for screening drugs with anti-hypnozoite activity. Tinidazole and pyrazinamide require further investigation as agents to enable dose reduction of primaquine.

INTRODUCTION

Although primaquine (PQ), an 8-aminoquinolone antimalarial remains the only therapeutic option for prevention of hypnozoite-induced relapse in *Plasmodium vivax* and *P. ovale* infection, its use is limited by significant hematologic toxicity in persons with glucose 6-phosphate dehydrogenase (G-6PD) deficiency. The necessity of screening for G-6PD enzyme activity before administration, along with the requirement for a 14-day dosing regimen, are impediments to wide-spread use of PQ in malaria-endemic countries.^{1,2} Identification of new classes of drugs with anti-hypnozoite activity but lacking the potential of G-6PD-related hematologic toxicity or agents that might enable simplified dosing regimens would be a significant advancement. Similarly, because the G-6PD related hemolytic effects of PQ are dose dependent,¹ identification of drugs that might enable a reduction in PQ dose or duration when co-administered might remove barriers to wider use of this drug.

A model in which rhesus monkeys (*Macaca mulatta*) are infected with the relapsing primate malaria parasite *P. cynomolgi* has been used to test drugs for efficacy in preventing relapse since 1948.³ This species has been demonstrated to have observable hypnozoite forms.⁴ No other model using a non-human malaria has been demonstrated to have the potential for relapse, implying a stage analogous to the hypnozoites seen in *P. vivax*. Although models using *P. vivax* infection in *Aotus* and *Saimiri* monkeys have been developed, inconsistent timing of infection, lack of demonstrated relapse, and the requirement of splenectomy limit their usefulness for drug screening programs.⁵ Radical curative activity in *P. cynomolgi* infection of rhesus macaques has been found to correlate with relapse prevention in human *P. vivax* infection.⁶

Existing approved drugs have not been adequately screened for anti-hypnozoite activity. If an existing, approved drug were found to be efficacious either as a treatment to

prevent relapse, or as a means to enable dose reduction of PQ, it would have immediate clinical utility if safety in G-6PD deficiency were increased. For this reason, we have an ongoing project screening selected drugs for anti-hypnozoite activity. As part of a pre-clinical research program testing prophylactic antimalarial drugs, we have an active program of experiments in which rhesus monkeys undergo sporozoite challenge. Because animals not protected from challenge are expected to have multiple relapses, rather than terminating the infections in these animals with PQ, they are used to screen candidate drugs for anti-hypnozoite activity. In conjunction with administration of chloroquine to cure blood stage parasitemia, animals receive candidate drugs either alone, or in combination with subtherapeutic doses of PQ. We present updated results of these screening experiments.

MATERIALS AND METHODS

All of the experiments presented herein were conducted under a protocol with approval of the United States Army Medical Component, Armed Forces Research Institute of Medical Sciences Institutional Animal Care and Use Committee in accordance with Code of Federal Regulations Title 9, Chapter 1, Subchapter A, Parts 1–3. Animals were maintained in accordance with established principles under the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996).⁷ The United States Army Medical Component, Armed Forces Research Institute of Medical Sciences animal care and use program has been accredited by The Association for the Assessment and Accreditation for Laboratory Animal Care International since 1999.

The animals used were Indian-origin *Macaca mulatta* ranging in age from 2 to 10 years and in weight from 2.5 to 8.5 kg. Animals were either malaria naive or had not been infected with *P. cynomolgi* for more than one year before each experiment.

A splenectomized donor monkey was infected by intravenous inoculation with previously frozen *P. cynomolgi* *bastianellii* (B strain)-infected erythrocytes. When daily blood smears detected a parasitemia > 100,000 parasite/μL with gametocytemia > 100/μL, *Anopheles dirus* mosquitoes were allowed to feed on the donor animal. After 14–16 days,

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sporozoites were harvested from the mosquitoes by salivary gland dissection.

Sporozoites were counted in nine fields $\times$ two preparations of the 1:50 freshly harvested sporozoite suspension in phosphate-buffered saline by hemocytometer using phase-contrast illumination to calculate sporozoites/ μ L. Sporozoites in a concentration of 1×10^6 /mL were suspended in phosphate-buffered saline containing 5% bovine serum albumin and injected intravenously into each experimental animal. These procedures have been shown to produce reliable 100% infection rates with predictable relapse patterns in initial studies of more than 80 animals (Gettayacamin M., unpublished data).

Screening experiments were incorporated as follow-on studies in protocols designed to study causal prophylactic activity or radical curative activity of selected agents. In the event of relapse after primary infection, when parasitemia reached 5,000 parasites/ μ L, animals were eligible to be enrolled in anti-hypnozoite screening experiments for up to three relapses. After or concomitantly with chloroquine (CQ) treatment for clearance of blood stage parasites, study drugs were screened for anti-hypnozoite activity by serial microscopy blood smear evaluation after treatment with the protocol defined dosing regimen. Each drug was tested initially in two animals per group as a single arm of an experiment involving up to 12 animals. Each experiment included two control animals treated with CQ only for up to three relapses. Timing of subsequent relapse, measured as days from last dose of the prior drug regimen, was recorded in comparison with concurrent control animals at each relapse to assess for delays in relapse as an indicator of partial anti-hypnozoite activity. Animals were followed-up up to 100 days after drug treatment. Animals free of relapse at day 100 were considered to have radical cure. In the case of rifampin, because of concern that drug-drug interaction could lead to recrudescence,^{8,9} a recurrence of parasitemia was treated with an additional course of CQ. Recurrence of parasitemia a second time was interpreted as failure of anti-hypnozoite activity (relapse).

At the final relapse, animals were treated with a uniformly curative regimen of CQ, 10 mg/kg/day orally for 7 days, in combination with 1.78 mg/kg/day of PQ for 7 days without subsequent relapse in any animal.

Mean time to each relapse was determined for all CQ-treated controls. Because the timing of relapse varies as a function of relapse number (i.e., expected timing of first relapses is earlier than second or third relapses), time to relapse of each treatment arm was compared with the mean time to relapse for all controls for the same relapse number. That is, for a drug regimen tested in animals having a first relapse, their time to relapse was compared with controls undergoing CQ treatment of first relapses. For study drug regimens containing sub-therapeutic doses of PQ, comparison was made to control animals matched for relapse number and PQ dose. If no controls matched both PQ dose and relapse number, no statistical comparison was made. For animals without subsequent relapse during the study period, a duration of 100 days was used for the quantitative comparisons as this was the study observation period. The Student's *t*-test was used for all statistical comparisons, and calculations were performed by using SPSS version 16.0 (SPSS Inc., Chicago, IL). All *P* values were two-sided.

Drugs were selected for screening on the basis of factors such as known antiparasmodial blood or liver-stage activity and

mechanistic plausibility. Doses were selected on the basis of maximum tolerated doses in rhesus when known, or from allometric scaling from human doses.

RESULTS

In each of the reported experiments, control animals developed initial parasitemia uniformly eight days after sporozoite injection, confirming the infectivity of challenge procedures and suggesting a degree of uniformity in infectious dose. A typical course of infection among control animals is shown in Figure 1. Among control animals, timing of first and second relapses was earlier and more predictable than was the timing of later relapses (Table 1). Treatment with PQ at doses of 0.6 mg/kg/day prevented relapse in three of four animals receiving this dose and induced a pronounced delay in relapse in the fourth animal. Dosing of PQ at 0.3 mg/kg/day induced a significant delay in relapse in one experiment ($n = 2$) but not in a second experiment ($n = 2$). This dose did not protect any of the four tested animals from relapse establishing this dose to be sub-therapeutic (Table 2).

For drug regimens tested alone, in conjunction with CQ for blood-stage clearance, but without PQ co-administration, only two drug regimens, tinidazole and triamterene, induced radical cure in any animal (Table 3). A single animal treated with tinidazole, 150 mg/kg/day for 7 days, was protected from relapse. Other animals receiving the same ($n = 5$) or higher ($n = 2$) dose were not protected from relapse, although this regimen did induce delays in relapse compared with controls when used to treat primary parasitemia ($P < 0.01$) or to treat a first relapse ($P = 0.02$). Triamterene dosed at 12 mg/kg/day for 7 days protected one of two animals from relapse, although the second animal did not appear to have any delay in relapse. No other tested drug induced significant delays compared with control animals when tested without co-administration of PQ.

For drug regimens incorporating co-administration of sub-therapeutic PQ, radical cures were observed in two of two

FIGURE 1. Representative course of parasitemia for a rhesus monkey after infection with 1×10^6 sporozoites of *Plasmodium cynomolgi*. Parasitemia beginning with sporozoite inoculation on study day 0 and continuing until clearance of parasitemia after third relapse comes from a representative control animal (R532) from experiment number 3. Each episode of parasitemia was treated with chloroquine and for the third relapse with chloroquine-primaquine. Typical time course, decreasing parasitemia, and increasing time to relapse over the course of infection are demonstrated for illustrative purposes.

TABLE 1

Intervals, in days, between the primary infection and relapses for 14 control rhesus monkeys infected with 1×10^6 sporozoites of *Plasmodium cynomolgi**

Experiment	Animal	Primary infection	First relapse	Second relapse	Third relapse	Fourth relapse
2	R252	8	14	13	10	
	R338	8	11	11	18	
3	R532	8	11	11	19	
	R538	8	11	12	22	
7	R127	8	12	12		
	R205	8	11	14		
8	R420	8	14	16	27	34
	R421	8	8	12	12	14
9	R428	8	9	8	18	
	R444	8	9	10	19	
1	R752	8	7	14	28	
	R829	8	11	10	21	
5	R737	8	7	10	15	
	R739	8	7	6	8	
Mean		8.0	10.1	11.4	18.1	24.0
SD		0.0	2.4	2.6	6.2	14.1

* All animals were treated for each parasitemia with chloroquine (10 mg/kg/day for 7 days). Data are presented as days until onset of parasitemia of the listed relapse beginning with the last dose of chloroquine from prior treatment (or from time of sporozoite inoculation for the primary parasitemia). Infections in control animals from experiment 7 were radically cured with chloroquine/primaquine after the second relapse.

animals receiving tinidazole, 300 mg/kg/day for 7 days, in conjunction with PQ, 0.3 mg/kg/day for 7 days, followed by twice a week dosing of tinidazole for 4 weeks. Pyrazinamide, 90 mg/kg/day for 7 days, in conjunction with PQ, 0.3 mg/kg/day for 7 days, also prevented relapse in two of two animals. In each case, no statistical comparison was possible because of a lack of appropriate control animals because no animals had received PQ, 0.3 mg/kg/day for treatment of a second relapse.

Although animals were protected from relapse after treatment with tinidazole (2 of 2), doxycycline (1 of 1), and azithromycin (1 of 2) dosed in conjunction with PQ, 0.6 mg/kg/day for 7 days, these rates of protection could not be distinguished from the relatively high rates of radical cure observed with PQ, 0.6 mg/kg/day for 7 days, alone.

Early recrudescence was noted in one of two of the animals treated with rifampin. This parasitemia was cleared with a repeated course of CQ.

DISCUSSION

Results of these experiments support the utility of this approach as a screen for drugs with anti-hypnozoite activity. Infections in control animals induced highly consistent infections with predictable relapses. Results observed with PQ treatment in doses of 0.3, 0.6, or 0.9 mg/kg/day are consistent with those reported by Schmidt in which he determined the 90% curative dose of PQ co-administered with CQ to be 0.62 mg/kg/day for 7 days for the B strain of *P. cynomolgi* inoculated at a dose of 2×10^5 to 2×10^6 sporozoites.¹⁰

When used as a single agent in conjunction with blood stage treatment with CQ, none of the agents tested effectively prevented relapse. Several agents caused a significant delay in relapse occurrence. Delay in relapse might be indicative of some degree of activity against hypnozoites, as was observed in animals treated with the sub-therapeutic 0.3 mg/kg/day regimen of PQ. Caution is warranted in interpreting delayed patency times, particularly in the setting of drugs with slow rates of clearance because the delay may indicate ongoing concentrations of drugs adequate to suppress blood-stage parasites. Although pharmacokinetic studies would help to exclude prolonged blood-stage suppression, none of the agents tested here would be expected to have clearance rates likely to delay relapse on the basis of suppression alone.

The results observed with tinidazole are particularly interesting in light of prior work with this compound in which it was found to prevent relapse of *P. vivax* in human volunteers in an open label clinical trial.¹¹ A subsequent clinical trial (NCT00811096) of the use of tinidazole with CQ to prevent relapse of *P. vivax* failed to show efficacy (Miller RS, unpublished data). Although results of the use of this drug as a single agent were inconsistent, there seems to be promise in the potential development of this agent to enable reduction of PQ doses.

TABLE 2

Timing of relapse parasitemia in rhesus monkeys treated for *Plasmodium cynomolgi* parasitemia with test drug regimens in combination with chloroquine*

Drug	No.	Relapse	Dose (mg/kg)	Schedule†	Days to relapse	P
Primaquine	2	First	0.3	Daily	9, 12	0.67
Primaquine	2	First	0.3	Daily	16, 49	< 0.01
Primaquine	2	First	0.6	Daily	NR, NR	< 0.01
Primaquine	2	Second	0.6	Daily	50, NR	< 0.01
Primaquine	2	First	0.9	Daily	NR, 42	< 0.01
Rifampin	2	First	30	Daily (8–21)	4,‡ 12	0.15
Clindamycin	2	First	40	BID	19, 15	0.19
Triamterene	2	Primary parasitemia	12	Daily	12, NR	< 0.01
Mebendazole	2	Primary parasitemia	20	Daily	11, 12	0.45
Tinidazole	2	Primary parasitemia	150	Daily	14, NR	< 0.01
Tinidazole§	2	Primary parasitemia	150	Daily	15, 20	0.19
Tinidazole	2	First	150	Daily	16, 17	0.02
Tinidazole	2	First	300	Daily	22, 28	0.12
Ciprofloxacin	2	First	100	BID	12, 7	0.59
Moxifloxacin	2	First	50	Daily	14, 21	0.32
Norfloxacin	1	First	100	BID	13	0.55
Norfloxacin	1	Second	100	BID	8	0.14
Ofloxacin	2	Second	100	BID	14, 17	0.58

* NR = no relapse (radical curative activity); BID = twice a day. Days to relapse are counted from the last day of chloroquine or the tested compound dosing. Statistical comparisons were made by using the Student's *t*-test. P values in **bold** are statistically significant.

† All dosing was delivered orally. All regimens were dosed on days 1–7 unless otherwise noted. All chloroquine doses were 10 mg/kg/d delivered orally for seven days.

‡ Early recrudescence was seen after rifampin dosing, with a subsequent re-treatment with chloroquine, which was followed by an additional relapse in 12 days.

§ Chloroquine in this arm was dosed from days 3 to 9.

TABLE 3

Timing of relapse parasitemia in rhesus monkeys treated for *Plasmodium cynomolgi* parasitemia with test drug regimens in combination with chloroquine and sub-therapeutic doses of primaquine*

Drugs	No.	Experiment	Relapse	Dose (mg/kg)‡	Schedule†	Days to relapse	P
Trimethoprim/primaquine	2	3	Primary parasitemia	50 (0.3)	BID/daily	29, 27	NC
Promethazine/primaquine	2	3	First	40 (0.3)	BID/daily	38, 42	0.26
Clindamycin/primaquine	2	2	First	40 (0.3)	BID/daily	26, 33	0.60
Tinidazole/primaquine	2	2	Second	300 (0.3)	Daily then twice a week for 4 weeks/daily	NR, NR	NC
Minocycline/primaquine	2	2	Second	25 (0.3)	BID/daily	28, 26	NC
Pyrazinamide/primaquine	2	2	Second	90 (0.3)	Daily	NR, NR	NC
Triamterene/primaquine	2	8	Second	24 (0.3)	Daily	19, 45	NC
Tinidazole/primaquine	2	8	First	300 (0.3)	Daily	30, 68	0.36
Tinidazole/primaquine	2	8	First	300 (0.6)	Daily	NR, NR	NC
Doxycycline/primaquine	1	8	Second	50 (0.3)	Daily	17	NC
Doxycycline/primaquine	1	8	Second	50 (0.6)	Daily	NR	0.67
Azithromycin/primaquine	2	8	Second	50 (0.6)	Daily	31, NR	0.84
Clindamycin/primaquine	2	8	Third	100 (0.3)	Daily	23, NR	NC
Ciprofloxacin/primaquine	2	9	First	200 (0.3)	BID/daily (days 1–3)	11, 15	0.58

* BID = twice a day; NC = no comparator; NR = no relapse (radical curative activity).

† All dosing was delivered orally. All regimens were dosed on days 1–7 unless otherwise noted. All chloroquine doses were 10 mg/kg/d delivered orally for seven days.

‡ Doses are presented in mg/kg of study drug with the dose of primaquine in parentheses.

The observation of early treatment failure in one of two animals treated with rifampin in conjunction with CQ is likely caused by a drug-drug interaction, perhaps by induction of cytochrome P450 enzymes by rifampin. Chloroquine is a substrate of CYP 2C8 and 3A4/5, which facilitate metabolism to desethylchloroquine and bisdesethylchloroquine.¹² This finding of treatment failure is consistent with results from mice treated for *P. berghei* infection,⁸ and the finding of recrudescence after combination with quinine in humans.⁹ Although not mentioned as a potential drug interaction in the package insert for CQ,¹³ this scenario may arise clinically in cases in which malaria is treated concurrently with tuberculosis therapy. Given the results from animal experiments, clinicians should consider avoidance of co-administration of these medications.

The results of these screening experiments have several important limitations that are important to recognize. First, because these experiments were performed on animals after relapse from a prior infection, they are confounded by variable development of host immunity, variability in relapse number and variability in prior drug exposure. For this reason, positive findings of anti-hypnozoite activity observed in a screening experiment require confirmation in additional malaria-naïve animals during a primary infection without prior complicating drug exposure. Each of these complicating factors is unlikely to lead to false-negative results. Drugs failing to prevent relapse in screening experiments are unlikely to possess potent anti-hypnozoite activity in the regimens tested. Although we acknowledge this limitation, we believe it is outweighed by the benefit inherent in this approach that animals with predictable relapses from other unrelated experiments can be used to screen drugs that otherwise may never have been tested if doing so would have required generation of a *de novo* experiment. This approach enables a more efficient and ethical means of maximizing the useful information gathered from each experiment.

Use of non-human primates in a screening program also causes sample size limitations because of logistical and feasibility constraints of using larger numbers of animals. Although this factor limits the sensitivity of this model for detecting small degrees of anti-hypnozoite activity, the effect is somewhat mitigated by the highly predictable nature of the infections and the goal of identifying only agents with potent activity.

This limitation can be further mitigated by aggregating results from multiple experiments to increase sample size and discriminatory power. Standardization and exact duplication of study procedures makes this approach possible. The high degree of consistency of results of control animals across experiments supports the validity of this approach.

Although we acknowledge the need for duplication and additional study of drugs, such as pyrazinamide, tinidazole, and triamterene, which appeared to induce radical cure in this screening, we believe that there is value in the reporting of the negative results from the other drugs tested. Identification of useful new antimalarial drugs will be advanced by transparent and open sharing of results within the research community to prevent duplication of efforts. We anticipate periodic updates to these results as additional drugs are screened in this model.

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