

PREVENTION OF SPOROGONY OF *PLASMODIUM VIVAX* IN *ANOPHELES DIRUS* MOSQUITOES BY TRANSMISSION-BLOCKING ANTIMALARIALS

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Abstract. The sporontocidal activity of four dihydroacridine-diones (WR-233602, WR-243251, WR-250547, and WR-250548) and three fluoroquinolones (WR-279135, WR-279298, and WR-279288) was determined against naturally circulating isolates of *Plasmodium vivax*. Laboratory-reared *Anopheles dirus* mosquitoes were infected with *P. vivax* by feeding them on gametocytemic volunteers reporting to local malaria clinics in Kanchanaburi and Tak provinces, Thailand. Four days after the infectious feed, mosquitoes were re-fed on uninfected mice treated 90 minutes previously with a given drug at a dose of 100 mg base drug/kg mouse body weight. Sporontocidal activity was determined by assessing both oocyst and sporozoite development. None of the fluoroquinolones exhibited sporontocidal activity against *P. vivax*, whereas all 4 dihydroacridine-diones affected sporogonic development to some degree. WR-233602 affected oocyst development, but had no impact on sporozoite production, WR-250548 affected oocyst development and had a limited effect on sporozoite production, and WR-243251 and WR-250547 had a marked impact on all phases of sporogony. These data demonstrate that experimental dihydroacridine-diones are capable of interrupting the sporogonic development of *P. vivax*. These compounds may be useful in preventing malaria transmission.

INTRODUCTION

The rapid emergence and spread of drug-resistant *Plasmodium falciparum* is a major factor affecting malaria control efforts.¹ More recently, chloroquine-resistance has been reported in *P. vivax* isolates from Southeast Asia and in South and Central America.^{2–5} The use of compounds that interrupt the transmission of malaria has been advocated as a means of preventing the development of drug-resistance or of limiting the spread of resistant parasites.^{6–9} Although it has been suggested that all new and existing antimalarials should be evaluated for gametocytocidal and/or sporontocidal action,¹⁰ little effort has been devoted towards establishing a practical experimental model for this purpose. In addition, relatively few studies have systematically characterized the gametocytocidal or sporontocidal properties of experimental antimalarials.

At present, primaquine and the various artemisinin derivatives are the only commonly used antimalarial agents capable of interrupting malaria transmission.^{8,11–13} Various other experimental compounds, including chlorguanide, pyrimethamine, atovaquone, tefenoquine (WR-238605), WR-250547, and others, can prevent malaria transmission by either gametocytocidal or sporontocidal activity.^{14–23} The current status of suspected gametocytocidal and/or sporontocidal compounds is summarized by Butcher.²⁴

In this study, we assessed the sporontocidal activity of four dihydroacridine-diones (WR-233602, WR-243251, WR-250547, and WR-250548) and three fluoroquinolones (WR-279135, WR-279298, and WR-279288) against naturally circulating isolates of *Plasmodium vivax*. Criteria used to assess the sporontocidal activity of the drugs included i) percent of mosquitoes with oocysts, ii) mean number of oocysts per infected mosquito, iii) average oocyst diameter and calculated oocyst volume, and iv) percent of mosquitoes with sporozoites in their salivary glands.

MATERIALS AND METHODS

Infection of mosquitoes with *Plasmodium vivax*. A colony of *Anopheles dirus* Peyton and Harrison mosquitoes has

been maintained for over 20 years at the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand. Procedures used for the infection of mosquitoes were modified from those described by Sattabongkot and others.²⁵ In brief, 200 laboratory-reared female *A. dirus* were fed on the arm of each gametocytemic patient volunteering to participate in the study. Volunteers were recruited from men aged 20 years or older reporting to government malaria clinics in Kanchanaburi or Tak Provinces in western Thailand between January and December 1999. Volunteers had at least one *P. vivax* gametocyte per 50 fields (700× oil immersion) in a Giemsa-stained thick film. Informed consent was obtained from all individuals participating in the study. The study protocol was reviewed and approved by the Ministry of Public Health, Thailand and the United States Army Human Subjects Research Review Board.

After signing consent forms, volunteers were interviewed and 100 mosquitoes fed on each arm of the volunteer for 30 mins. Volunteers were then provided with appropriate antimalarial treatment (200 mg chloroquine base/day for 3 days and 15 mg primaquine base/day for 14 days) by Thai Ministry of Public Health personnel. Unfed mosquitoes were discarded, and engorged mosquitoes were maintained on a 5% sugar solution at 25–27°C and 70–80% relative humidity.

Antimalarial drugs. The compounds examined in this study are listed below. All compounds were provided by the Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research.

Dihydroacridine-diones:

- Floxacrine (WR-233602): 7-chloro-3,4-dihydro-10-hydroxy-3-(4'-(trifluoromethyl)phenyl)-1,9(2H,1OH)acridinedione
- WR-243251: (7-Chloro-3-(2,4-dichlorophenyl)-1-[[3-(dimethylamino)propyl]imino]-1,2,3,4-tetrahydro-9(1OH)acridinone)
- WR-250548: (S)-7-Chloro-3-(2,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-[[3-(dimethylamino)propyl]imino]-9-acridinol
- WR-250547: (R)-7-Chloro-3-(2,4-dichlorophenyl)-1,2,3,4-

tetrahydro-1-[[3-(dimethylamino)propyl]imino]-9-acridinol

Fluoroquinolones:

- Norfloxacin (WR-279298): 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid
- Ofloxacin (WR-279288): (±)-9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid
- Ciprofloxacin (WR-279135): the monohydrochloride monohydrate salt of 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid

Assessment of sporontocidal activity of antimalarials.

Three days after being fed on gametocytemic malaria patients, mosquitoes were randomly separated into groups of 100 each and provided with water only. Twenty four hours later (4 days after the infectious feed), groups of 100 mosquitoes were allowed to feed for 30 minutes on 3 anesthetized mice that had received an intraperitoneal (ip) inoculation of a given drug 90 minutes earlier. Unengorged mosquitoes were removed from the cages, and mosquitoes were maintained as previously described until assessed for sporogonic development. Portions of this study requiring mice were approved by the Institutional Animal Care and Use Committee (IACUC) of the Armed Forces Research Institute of Medical Sciences and comply with all U.S. and Thai regulations pertaining to the humane care and use of laboratory animals.

Drug preparation. All drugs were diluted in a 1:3 suspension of 70% ethanol:phosphate buffered saline (PBS). Infected mosquitoes received the drugs by feeding on mice that had been inoculated with a given drug 90 min earlier. Each individually weighed mouse received a dose of 100 mg base drug/kg mouse body weight in a final volume of 0.25–0.30 ml diluent. Control animals received diluent only.

Assay for sporogonic development. Procedures used to determine oocyst and sporozoite production were modified from those previously described.^{14–16} In each experiment, mosquitoes were sampled for oocysts on day 10 post-infection and for sporozoites on day 21 post-infection. Midguts were stained with mercurochrome and oocysts counted using phase-contrast microscopy (200× and 400×). Oocyst development was quantified by measuring oocyst diameter using an ocular micrometer. Measurements were recorded for the 5 largest oocysts present in mosquitoes with at least 5 oocysts, and for all oocysts in mosquitoes with fewer than 5 oocysts. Oocyst volume was calculated using the formula: Volume = 4/3 (Radius³) * 3.14. Salivary glands were removed on day 21 post-infection and examined for the presence or absence of sporozoites using phase-contrast microscopy (400×).

Statistics. Due to the inherent variation found when mosquitoes are infected by feeding on a variety of naturally-infected patients, a total of 5–6 replicates were conducted using each drug evaluated. For each replicate, mosquitoes infected on a given patient were allowed to re-feed on mice treated with a given drug or with diluent only (controls).

For each drug tested, Chi-square analysis was used to determine if the percentage of drug-treated mosquitoes with oocysts or sporozoites was different from the percentage of control mosquitoes. Multiple Analysis of Variance (MAN-

TABLE 1

Effect of seven experimental antimalarials on the production of *Plasmodium vivax* oocysts in *Anopheles dirus* mosquitoes*

Treatment	Controls		Treated		χ ²
	% of mosquitoes with oocysts (n)		% of mosquitoes with oocysts (n)		
Dihydroacridine-diones					
WR-233602	40.0% (62/155)		43.1% (56/130)		0.28
WR-250548	48.9% (87/178)		42.2% (70/166)		1.56
WR-250547	47.9% (81/169)		29.4% (42/143)		11.17†
WR-243251	40.0% (62/155)		22.2% (32/144)		10.95†
Fluoroquinolones					
WR-279288	38.7% (60/155)		34.0% (51/150)		0.73
WR-279298	52.9% (83/157)		54.2% (83/153)		0.06
WR-279135	52.9% (83/157)		54.1% (73/135)		0.04

* Data represents a total of 5–6 pooled replicates for each experimental compound.

† Treated mosquitoes significantly different from control mosquitoes ($P < 0.001$).

OVA) was used to determine if there were significant variations in the number of oocysts per experimental group or if the sizes of the oocysts in each group were significantly different.

RESULTS

Fluoroquinolones. A total of 5 replicates were conducted with WR-279298 and WR-279135, and 6 replicates with WR-279288. Results were pooled for each drug (Tables 1–4). At a dose of 100 mg/kg, none of the 3 fluoroquinolones significantly inhibited the percentage of mosquitoes with *P. vivax* oocysts (Table 1) or the number of oocysts per mosquito (Table 2). Neither WR-279298 or WR-279288 affected the size of oocysts; however, WR-279135 significantly reduced oocyst size (Table 3). When the replicates were considered separately, WR-279298, WR-279288, and WR-279135 significantly reduced the size of oocysts in 0/5, 2/6, and 2/5 replicates, respectively (Coleman RE and others, unpublished data). None of the 3 fluoroquinolones had any effect on the percentage of mosquitoes with salivary gland sporozoites (Table 4).

Dihydroacridine-diones. A total of 6 replicates were conducted with each of the 4 dihydroacridine-diones. Neither WR-233602 or WR-250548 had any effect on the percentage of mosquitoes with oocysts. WR-250547 and WR-243251 both significantly reduced the percentage of mosquitoes with oocysts (Table 1). Neither WR-250548 nor WR-250547 affected the number of oocysts produced per mosquito, whereas both WR-243251 and WR-233602 significantly reduced the numbers of oocysts per mosquito (Table 2). When the replicates were considered separately, WR-243251, WR-233602, WR-250548, and WR-250547 significantly reduced oocyst size in 6/6, 5/6, 5/6, and 6/6 replicates, respectively (Coleman RE and others, unpublished data). All four dihydroacridine-diones significantly reduced oocyst size when compared to controls (Table 3). All dihydroacridine-diones except WR-233602 significantly reduced the percentage of mosquitoes with salivary gland sporozoites (Table 4).

DISCUSSION

The emergence of resistance to multiple antimalarial drugs from diverse chemical classes is a major factor affecting the

TABLE 2
Effect of seven experimental antimalarials on the number of *Plasmodium vivax* oocysts in *Anopheles dirus* mosquitoes*

Treatment	Control mosquitoes		Treated mosquitoes		MANOVA	
	No. with oocysts	Mean no. of oocysts/ positive mosquito (\pm SEM)	No. with oocysts	Mean no. of oocysts/ positive mosquito (\pm SEM)	F value	DF
Dihydroacridine-diones						
WR-233602	62/155	8.1 (4.6)	56/130	39.3 (6.6)	7.31†	(1, 283)
WR-250548	87/178	9.3 (4.1)	70/166	12.5 (2.8)	1.85	(1, 342)
WR-250547	81/169	6.5 (4.1)	42/143	16.6 (3.1)	0.00	(1, 310)
WR-243251	62/155	8.1 (4.6)	32/144	5.6 (1.6)	6.39†	(1, 305)
Fluoroquinolones						
WR-279288	60/155	8.0 (4.6)	51/150	14.6 (4.4)	0.28	(1, 303)
WR-279298	83/157	3.4 (4.4)	83/153	20.9 (3.6)	0.19	(1, 307)
WR-279135	83/157	3.4 (4.4)	73/135	22.5 (4.5)	0.02	(1, 290)

* Data represents a total of 6 pooled replicates.

† Treated mosquitoes significantly different from control mosquitoes ($P < 0.01$).

treatment and control of malaria. The strategy of blocking malaria transmission has the potential to limit the emergence and spread of drug resistance and warrants further attention. However, few model systems have been developed that allow for rigorous evaluation of transmission-blocking activity.

Although transmission of *Plasmodium* parasites by mosquitoes can be prevented using either gametocytocidal or sporontocidal compounds, the particular developmental stage affected may depend on the drug used. As currently defined in our laboratory, gametocytocidal compounds interact directly with gametocytes. In contrast, sporontocidal compounds affect parasite development when administered to infected mosquitoes. The only method to accurately differentiate between gametocytocidal and sporontocidal modes of action is to expose the parasite to a particular drug during different stages of development. We differentiate between gametocytocidal and sporontocidal activity by administering a particular compound with the infectious bloodmeal (gametocytocidal activity) or by feeding previously infected mosquitoes on uninfected, drug-treated mice (sporontocidal activity). An antimalarial drug can possess both gametocytocidal and sporontocidal activity.

The experimental model that we present here for the assessment of the sporontocidal activity of experimental compounds against *P. vivax* provides advantages over previously used models. Although Golenda and others²⁶ reported an *in*

vitro system for the continuous culture of *P. vivax*, this system has not been established as a routine source of infectious gametocytes. In studies conducted over 40 years ago, mosquitoes were routinely fed on *P. vivax* infected patients (often prison volunteers) and then maintained on sucrose solutions spiked with an antimalarial agent.²⁷⁻²⁸ However, use of spiked solutions does not simulate the normal route of parasite exposure to the drug, as no metabolism of the drug occurs. In the model system that we describe here, naturally occurring strains of *P. vivax* are used to evaluate drug efficacy. Although the presumably heterogeneous parasite populations that occur using this system may result in greater variability in results, we believe that this is also a strength of this system. By using naturally occurring parasite populations we gain a more realistic understanding of how the drugs perform when compared to use of a single genotype, as typically occurs when using cultured *P. falciparum* isolates/clones. By allowing previously infected mosquitoes to feed on uninfected, drug-treated mice, gametocytocidal and sporontocidal activities can be differentiated. In addition, exposing mice to the drugs for 90 minutes prior to feeding mosquitoes allows for absorption and metabolism of the compound. This mode of drug delivery is presumably more realistic than feeding mosquitoes on sugar pads impregnated with the drugs, although drug metabolism by mice may differ from that by humans. Using oocyst numbers and size and sporozoite invasion of the salivary glands as criteria to as-

TABLE 3
Effect of seven experimental antimalarials on the size of *Plasmodium vivax* oocysts in *Anopheles dirus* mosquitoes*

Treatment	Control mosquitoes			Treated mosquitoes			MANOVA	
	No. with oocysts	No. oocysts measured	Mean oocyst volume (μm^3) (\pm SEM)	No. with oocysts	No. oocysts measured	Mean oocyst volume (μm^3) (\pm SEM)	F value	DF
Dihydroacridine-diones								
WR-233602	62	237	214.3 (64.2)	56	248	28.9 (7.0)	7.45†	(1, 116)
WR-250548	87	345	208.7 (45.7)	70	289	37.5 (5.9)	11.18†	(1, 155)
WR-250547	81	314	160.8 (11.1)	42	194	32.3 (10.0)	57.21†	(1, 121)
WR-243251	62	237	214.3 (64.2)	30	113	6.0 (1.4)	5.06†	(1, 90)
Fluoroquinolones								
WR-279288	60	222	217.6 (66.4)	51	193	136.9 (14.6)	1.21	(1, 109)
WR-279298	83	372	203.6 (11.4)	83	369	207.2 (10.4)	0.06	(1, 164)
WR-279135	83	372	203.6 (11.4)	73	313	164.5 (9.4)	6.73†	(1, 154)

* Data represents a total of 6 pooled replicates.

† Treated mosquitoes significantly different from control mosquitoes ($P < 0.01$).

TABLE 4
Effect of seven experimental antimalarial on the production of *Plasmodium vivax* sporozoites in *Anopheles dirus* mosquitoes*

Treatment	% of mosquitoes with sporozoites (n)		χ^2
	Controls	Treated	
Dihydroacridine-diones			
WR-233602	40.8% (31/76)	27.3% (18/66)	2.86
WR-250548	40.6% (43/106)	23.5% (31/132)	8.01†
WR-250547	38.7% (36/93)	8.2% (8/98)	25.11†
WR-243251	40.8% (31/76)	2.4% (2/85)	36.38†
Fluoroquinolones			
WR-279288	32.9% (28/85)	23.6% (25/106)	2.06
WR-279298	47.0% (39/83)	51.9% (42/81)	0.39
WR-279135	47.0% (39/83)	52.0% (51/98)	0.46

* Data represents a total of 5–6 pooled replicates for each experimental compound.

† Treated mosquitoes significantly different from control mosquitoes ($P < 0.001$).

sess sporontocidal activity, active and inactive compounds can be differentiated.

We have previously assessed the gametocytocidal/sporontocidal activity of several of the compounds tested in this study (floxacrine, WR-250547 and WR-250548) against *P. berghei*^{15–16} and *P. falciparum*.¹⁶ All 3 of these dihydroacridine-diones were effective against *P. vivax* as well as *P. berghei* and *P. falciparum*. The relative efficacy of each compound was similar in all 3 model systems, with the compound WR-250547 appearing to be more effective than either floxacrine or WR-250548.

In general, the dihydroacridine-diones appear to be effective sporontocidal agents. All 4 agents tested against *P. vivax* in this study exhibited some degree of sporontocidal activity. WR-250547 and WR-243251 were particularly effective, producing significant reductions in the percentage of mosquitoes with sporozoites in the salivary glands. Although WR-250547 had no effect on the number of oocysts produced, both it and the other 3 dihydroacridine-diones significantly inhibited oocyst size and development. The failure of WR-250547 and WR-243251 to completely prevent the development of sporozoites may have been due to some of the mosquitoes only taking a partial blood-meal when feeding on the drug-treated mice, perhaps resulting in a lower drug dose. The results of this and prior studies^{15–16} have confirmed that all 4 dihydroacridine-diones tested so far (floxacrine, WR-250547, WR-250548, and WR-243251) have significant transmission-blocking activity against a variety of species of *Plasmodium*.

In contrast to the dihydroacridine-diones, the fluoroquinolones tested here do not appear to have sporontocidal activity. Neither oocyst numbers nor size nor sporozoite production were affected by either WR-279288, WR-279298, or WR-279135.

The model system presented here provides certain advantages over previously used models; however, it is clearly not perfect. Metabolism of drugs by mice is clearly different from that of humans, and results obtained in this study will need to be confirmed in humans if and when any of these compounds are approved for experimental human testing. The use of direct dosing of mosquitoes using sugar solutions spiked with a given drug has provided a great deal of information on the sporontocidal activity of a variety of antimalarial drugs for over 50 years.^{27–28} Although the present study

used mice to metabolize the drugs prior to exposing mosquitoes to the candidate compounds, it is not clear that metabolism of the drug by mice provides more relevant information than does direct dosing using spiked sugar solutions. Ideally, the two methods should be compared with one another and results compared with those obtained when humans are treated. Unfortunately, there are very few drugs with confirmed sporontocidal activity that can be safely tested in humans.

Although problems with toxicity, cost of development, or other factors may preclude further development of dihydroacridine-diones as anti-malarial drugs, we believe that further studies on the use of these compounds to control the spread of drug-resistant *Plasmodium* parasites is warranted. Although *Plasmodium*-infected mosquitoes would normally be exposed to a sporontocidal compound when feeding on a vertebrate (human) that had previously been treated with the drug, alternative routes of exposure may offer opportunities for interrupting the transmission of malaria. For example, anopheline mosquitoes are readily collected from resting boxes. It may be possible to develop a “bait station” consisting of a resting box that contains a sugar-bait spiked with a transmission-blocking antimalarial. Mosquitoes that enter the bait-station would ingest the drug while feeding on the sugar-bait, thereby exposing sporogonic-stage parasites to the drug. An advantage of such a system would be that asexual parasites would not be exposed to the blood, thereby minimizing the chance that development of resistance could occur.²⁹

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