PREVENTION OF SPOROGONY OF \textit{PLASMODIUM VIVAX} IN \textit{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 \textit{Plasmodium vivax}. Laboratory-reared \textit{Anopheles dirus} mosquitoes were infected with \textit{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 \textit{P. vivax}, whereas all 4 dihydroacridine-diones affected sporogonic development to some degree. WR-250548 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 \textit{P. vivax}. These compounds may be useful in preventing malaria transmission.

INTRODUCTION

The rapid emergence and spread of drug-resistant \textit{Plasmodium falciparum} is a major factor affecting malaria control efforts. More recently, chloroquine-resistance has been reported in \textit{P. vivax} isolates from Southeast Asia and in South and Central America.\textsuperscript{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.\textsuperscript{6–8} Although it has been suggested that all new and existing antimalarial agents should be evaluated for gametocytocidal and/or sporontocidal action,\textsuperscript{9} 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.\textsuperscript{8,11–13} Various other experimental compounds, including chloroguanide, pyrimethamine, atovaquone, tefenoquine (WR-238605), WR-250547, and others, can prevent malaria transmission by either gametocytocidal or sporontocidal activity.\textsuperscript{14–21} The current status of suspected gametocytocidal and/or sporontocidal compounds is summarized by Butcher.\textsuperscript{23}

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 \textit{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 \textit{Plasmodium vivax}. A colony of \textit{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.\textsuperscript{25} In brief, 200 laboratory-reared female \textit{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 \textit{P. vivax} gamocyte 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.

\textbf{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.

\textbf{Dihydroacridine-diones:}
- Floxacin (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]iminio]-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]iminio]-9-acridinol
- WR-250547: (R)-7-Chloro-3-(2,4-dichlorophenyl)-1,2,3,4-
tetrahydro-1-[[3-(dimethylamino)propyl]limino]-9-acyridinol.

**Fluoroquinolones:**
- Norfl Roxacin (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-4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid

**Assessment of sporontocidal activity of antimalarials.**
Three days after being fed on gametocytic 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 min 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.14–18 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 (Radius)³ • 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 (MANOVA) 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.

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**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).

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**DISCUSSION**

The emergence of resistance to multiple antimalarial drugs from diverse chemical classes is a major factor affecting the
used models. Although Golenda and others pounded against assessment of the sporontocidal activity of experimental com-
tivity. An antimalarial drug can possess both gametocy-
ted in our laboratory, gametocytocidal compounds in-
comparatives against sporontocidal activity by adminis-
teract directly with gametocytes. In contrast, sporontocidal
In the model system that we describe here, naturally
parasite exposure to the drug, as no metabolism of the drug
in greater variability in results, we believe that this is also a strength
this is not a routine source of infectious gametocytes. In studies conducted over 40 years ago, mos-
mosquitoes were routinely fed on P. vivax infected patients (of-
tain prison volunteers) and then maintained on sucrose so-
lutions spiked with an antimalarial agent. However, use of spiked solutions does not simulate the normal route of
parasite populations that occur using this system may result in greater
by using naturally occurring parasite populations we gain a more realistic understanding of how the
that will allow for vitals to occur. As naturally occurring parasite popu-
urally different from control mosquitoes (P < 0.01).
exhibit the potential to limit the emergence
in 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 activ-
ity.

Although transmission of Plasmodium parasites by mos-
quitoes 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 in-
controls we gain a more realistic understanding of how the
experimental model that we present here for the as-
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
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experimental model that we present here for the as-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control mosquitoes</th>
<th>Treated mosquitoes</th>
<th>MANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. with oocysts</td>
<td>Mean no. of oocysts/positive mosquito (± SEM)</td>
<td>No. with oocysts</td>
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<tr>
<td>Dihydroacridine-diones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WR-233602</td>
<td>62/155</td>
<td>8.1 (4.6)</td>
<td>56/130</td>
</tr>
<tr>
<td>WR-250548</td>
<td>87/178</td>
<td>9.3 (4.1)</td>
<td>70/166</td>
</tr>
<tr>
<td>WR-250547</td>
<td>81/169</td>
<td>6.5 (4.1)</td>
<td>42/143</td>
</tr>
<tr>
<td>WR-243251</td>
<td>62/155</td>
<td>8.1 (4.6)</td>
<td>32/144</td>
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<tr>
<td>Fluoroquinolones</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>WR-279288</td>
<td>60/155</td>
<td>8.0 (4.6)</td>
<td>51/150</td>
</tr>
<tr>
<td>WR-279298</td>
<td>83/157</td>
<td>3.4 (4.4)</td>
<td>83/153</td>
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<td>WR-279135</td>
<td>83/157</td>
<td>3.4 (4.4)</td>
<td>73/135</td>
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</tbody>
</table>

* Data represents a total of 6 pooled replicates.
† Treated mosquitoes significantly different from control mosquitoes (P < 0.01).

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control mosquitoes</th>
<th>Treated mosquitoes</th>
<th>MANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. with oocysts</td>
<td>Mean oocyst volume (μm³) (± SEM)</td>
<td>No. with oocysts</td>
</tr>
<tr>
<td>Dihydroacridine-diones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WR-233602</td>
<td>62/237</td>
<td>214 (64.2)</td>
<td>56/248</td>
</tr>
<tr>
<td>WR-250548</td>
<td>87/345</td>
<td>208.7 (45.7)</td>
<td>70/289</td>
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<tr>
<td>WR-250547</td>
<td>81/314</td>
<td>160.8 (11.1)</td>
<td>42/194</td>
</tr>
<tr>
<td>WR-243251</td>
<td>62/237</td>
<td>214.3 (64.2)</td>
<td>30/113</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WR-279288</td>
<td>60/222</td>
<td>217.6 (66.4)</td>
<td>51/193</td>
</tr>
<tr>
<td>WR-279298</td>
<td>83/372</td>
<td>203.6 (11.4)</td>
<td>83/369</td>
</tr>
<tr>
<td>WR-279135</td>
<td>83/372</td>
<td>203.6 (11.4)</td>
<td>73/313</td>
</tr>
</tbody>
</table>

* Data represents a total of 6 pooled replicates.
† Treated mosquitoes significantly different from control mosquitoes (P < 0.01).
Effect of seven experimental antimalarial on the production of *Plasmodium vivax* sporozoites in *Anopheles dirus* mosquitoes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of mosquitoes with sporozoites (n)</th>
<th>x²</th>
</tr>
</thead>
<tbody>
<tr>
<td>WR-233602</td>
<td>40.8% (31/76)</td>
<td>2.86</td>
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<tr>
<td>WR-250548</td>
<td>40.6% (43/106)</td>
<td>8.01</td>
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<tr>
<td>WR-250547</td>
<td>38.7% (36/93)</td>
<td>25.11</td>
</tr>
<tr>
<td>WR-243251</td>
<td>40.8% (31/76)</td>
<td>36.38</td>
</tr>
<tr>
<td>WR-279288</td>
<td>32.9% (28/85)</td>
<td>2.06</td>
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<tr>
<td>WR-279298</td>
<td>47.0% (39/83)</td>
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<tr>
<td>WR-279135</td>
<td>47.0% (39/83)</td>
<td>0.46</td>
</tr>
</tbody>
</table>

* Data represents a total of 5–6 pooled replicates for each experimental compound.
† Treated mosquitoes significantly different from control mosquitoes (P < 0.001).

**Acknowledgments**: The test compounds originated from the Chemical Synthesis Program of the Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, D.C.

**Disclaimer**: The views of the authors do not purport to represent the position of the Department of the Army or the Department of Defense.

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**REFERENCES**


