

Methotrexate Is Highly Potent Against Pyrimethamine-Resistant *Plasmodium vivax*

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Resistance of *vivax* malaria to treatment with antifolates, such as pyrimethamine (Pyr), is spreading as mutations in the dihydrofolate reductase (*dhfr*) genes are selected and disseminated. We tested the antitumor drug methotrexate (MTX), a potent competitive inhibitor of *dhfr*, against 11 *Plasmodium vivax* isolates ex vivo, 10 of which had multiple *dhfr* mutations associated with Pyr resistance. Despite high-grade resistance to Pyr (median 50% inhibitory concentration [IC₅₀], 13,345 nM), these parasites were all highly susceptible to MTX (median IC₅₀, 2.6 nM). Given its potency against Pyr-resistant *P. vivax*, the antimalarial potential of MTX deserves further investigation.

In the early 1970s, 7 patients were successfully and safely treated for *Plasmodium vivax* with low doses of methotrexate (MTX) [1]. MTX, a potent antifolate, had then long been used to treat leukemia and severe psoriasis. However, these observations were not followed further. First, they coincided with reports of MTX

hepatotoxicity in persons ingesting low doses (2.5 mg/kg) over prolonged periods (3 months to 6 years) [2], making the use of MTX for malaria prophylaxis or for repeated treatment of endemic residents untenable. Second, MTX offered no obvious advantages over other antifolates such as pyrimethamine (Pyr), which were then used effectively against malaria. Forty years on, widespread use of Pyr to treat malaria (principally in combination with sulphadoxine) has led to the selection and global dissemination of antifolate resistance, not only for *P. falciparum* but also for *Plasmodium vivax* [3–4]. Resistance to Pyr in *P. falciparum* and *P. vivax* is conferred by one or more mutations in the dihydrofolate reductase gene (*dhfr*) [5]. Interestingly, it was reported that MTX remains active against laboratory lines of Pyr-resistant *P. falciparum* that carry multiple *dhfr* mutations [6]. We wished to determine whether Pyr-resistant *P. vivax* isolates were also susceptible to MTX. Secondly, we wished to investigate the effect of multiple mutations in another important antifolate resistance gene: *P. vivax* dihydropteroate synthase (*dhps*) on *P. vivax* MTX sensitivity.

METHODS

Isolate Collection and Culture

Twenty *P. vivax* isolates were collected from May to June 2009 from malaria patients attending the Shoklo Malaria Research Unit (SMRU) clinics, Mae Sot region of Tak Province in northwestern Thailand. These samples were collected from patients with no prior antimalarial therapy and with microscopically confirmed *P. vivax* (parasitemia range 3,000–10,000 parasites/μL). After written consent, blood samples were collected by venipuncture in 5-ml-volume lithium heparinized tubes, which were transported to the laboratory at SMRU within 5 hours of collection. In 14 isolates, the majority (>80%) of the parasites were at the mid-trophozoite stage (~20 h after invasion), and no schizonts were detected. These isolates were chosen for drug susceptibility testing. After platelets and leukocytes removal, the susceptibility of these *P. vivax* isolates to MXT (Methotrexate hydrate MW 454.4; Sigma-Aldrich) or Pyr (Pyrimethamine Vetranal MW248.7; Sigma-Aldrich) was assessed as described elsewhere [7], with one important change. Folate-reduced media based on McCoy's 5A (Biowest) were used: folic acid 0.01 mg/mL and *p*-amino benzoic acid 0.0005 mg/L, as opposed to the regular McCoy's 5A medium (Gibco) of 10 mg/L and 1 mg/L, respectively. To determine the effect of reduced-folate media on *P. vivax* growth and sensitivity to drugs, assays on the first 6 isolates were carried out in parallel using one or the other of McCoy's 5A media types (Figure 1).

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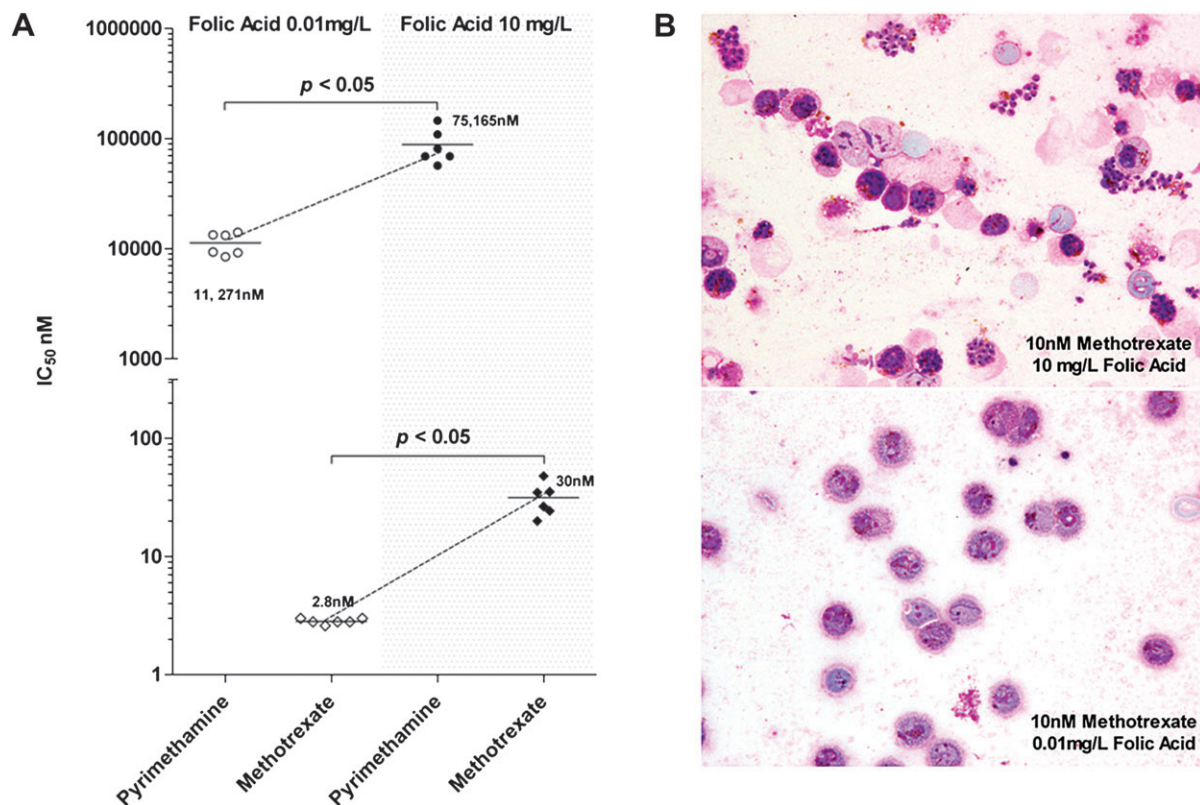


Figure 1. A, The effect of folic acid concentration in the growth media (McCoy's 5A), .01 mg/L (*open symbols*) vs. 10.0 mg/L (*solid symbols*), on the ex vivo susceptibility of 6 paired Thai *Plasmodium vivax* isolates to pyrimethamine and methotrexate (MTX). B, Photomicrographs of Giemsa-stained *P. vivax* thin films from a single isolate and grown in the presence of 10 nM MTX for 42h either in McCoy's medium with normal, 10.0 mg/L (*upper panel*), or reduced, .01 mg/L (*lower panel*), concentrations of folic acid. At the higher folic acid concentration, *P. vivax* was protected from an otherwise inhibitory effect of MTX (*lower panel*), as demonstrated by observation of numerous asexual stages that developed into mature schizonts (*upper panel*).

Antifolate susceptibility testing using the other 5 isolates was performed with the folic acid-reduced media.

Analysis

Dose response curves and 50% inhibitory concentration (IC₅₀) values were calculated by fitting the data to a sigmoidal inhibitory E-max pharmacodynamic model using Winnonlin version 4.1 (Pharsight). Eleven of the 14 drug response curves were successfully modeled according to criteria published earlier [7]. The median IC₅₀ values presented in Figure 1 were compared using a Wilcoxon matched-pairs test. Statistical analysis and graphics were carried out using GraphPad Prism 5 software (version 5).

Genotyping

In addition to confirmation of the parasite species by polymerase chain reaction [8], mutations present in the *P. vivax* *dhfr* and *dhps* genes (*Pvdhfr* and *Pvdhps*) for all the isolates were sought by amplification of the relevant fragments followed by DNA sequencing, as previously described [9].

Ethical Approval

The clinical samples examined in this study were collected under the following ethical guidelines in the approved protocols:

OXTREC 027-025 (University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, United Kingdom) and MUTM 2008-215 from the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok.

RESULTS AND DISCUSSION

Of the 11 Thai *P. vivax* isolates, 10 were successfully genotyped for mutations in residues of *Pvdhfr* and *Pvdhps* associated with antifolate resistance (Table 1). Nine had quintuple mutations in *Pvdhfr* (residues F57I, S58R, T61M, S117T, D156N), and one had quadruple mutations. All carried a triple mutation in *Pvdhps* (residues A383G, K512M, A553G), and in 4 isolates an additional mutation S382A was detected. Ex vivo drug assays showed that all 11 isolates were highly resistant to inhibition by Pyr, with median IC₅₀ = 13,345 nM (7036–15,287 nM [Table 1]), as predicted by the numerous mutations in *Pvdhfr*. By contrast, the 11 Pyr-resistant *P. vivax* isolates were highly susceptible to MTX, with median IC₅₀ = 2.6 nM (2.2–2.9 nM). Unsurprisingly, the presence of triple or quadruple mutations in *Pvdhps* did not affect the sensitivity of *P. vivax* to MTX.

Table 1. The ex vivo sensitivity of 11 Thai *Plasmodium vivax* isolates to methotrexate (MTX) and pyrimethamine (Pyr) relative to mutations in *dhps* and *dhfr*

Isolate	Species ^a	<i>Plasmodium vivax</i> gene locus										Antimalarial	
		<i>Pvdhps</i> ^b					<i>Pvdhfr</i> ^b					Average IC ₅₀ nM	
		S382A	A383G	K512M	A553G	V585	F57I	S58R	T61M	S117T	D156N	Pyr	MTX
WPP 5759	<i>Pv</i>	A	G	M	G	V	I	R	M	T	N	7,036	2.4
WPP 3839	<i>Pv</i>	A	G	K	G	V	I	R	M	T	N	15,287	2.8
WPP 3443	<i>Pv</i>	S+A	G	K	G	V	I	R	M	T	N	13,996	2.6
WPP 6225	<i>Pv</i> *	S+A	G	K	G	V	I	R	M	T	N	14,837	2.6
WPP 3597	<i>Pv</i>	S	G	K	G	V	I	R	T	T	N	10,691	2.2
WPP 4206	<i>Pv</i>	S	G	K	G	V	I	R	M	T	N	12,412	2.6
WPP 4767	<i>Pv</i>	S	G	K	G	V	I	R	M	T	N	12,259	2.9
WPP 3649	<i>Pv</i>	S	G	K	G	V	I	R	M	T	N	13,345	2.2
WPP 6100	<i>Pv</i>	S	G	K	G	V	I	R	M	T	N	14,085	2.6
WPP 6385	<i>Pv</i> *	S	G	K	G	V	I	R	M	T	N	13,220	2.2
WPP 3559	<i>Pv</i>	S	G	K	G	V	ND	ND	ND	ND	ND	15,287	2.6

NOTE. The mutant amino acid at each of the residues is boldface and italicized. ND = Not determined.

^a The presence of *P. vivax* (*Pv*) was confirmed by polymerase chain reaction; in 2 cases submicroscopic *P. falciparum* (*) was noted.

^b The position of the mutated residue is indicated, and the amino acid resulting from mutation is presented in boldface and italics.

The ex vivo assays used in this study were conducted using media with physiological folic acid levels (folic acid .01mg/ml). This contrasts with many studies where folate-enriched media (folic acid 10 mg/ml) were used, because it was thought that high levels of folic acid were needed to obtain the levels of *P. vivax* ex vivo maturation, which is necessary for meaningful dose response modeling [7]. In this study we used 6 of the isolates to establish that *P. vivax* maturation occurs efficiently in folate-depleted media and that under these conditions the IC₅₀'s were 5- to 10-fold lower (Figure 1) than those obtained in parallel using folate-enriched media (1,000-fold more folate). These observations underline the importance of using folic acid-reduced media to conduct antifolate sensitivity testing.

Whereas it is clear that high doses of MTX (130–300 mg kg⁻¹) taken over several weeks (with serum concentrations often >1,000 µM) to treat neoplastic disorders are associated with life-threatening adverse events [10], the lower doses of methotrexate (0.1–0.4 mg kg⁻¹ in children and 7.5–30 mg kg⁻¹ per adult) given once weekly for the management of rheumatoid arthritis, psoriasis, and juvenile idiopathic arthritis (including infants <one year old) have limited side effects [11–13]. Pharmacokinetic studies indicate that a daily dose of 5 mg in adults (0.05 mg kg⁻¹) results in serum MTX concentrations between 250 and 500 nM [13]. These levels are sufficient to fully inhibit *P. vivax* (this study) and *P. falciparum* [14]. Thus, we propose that at low doses MTX or novel drugs with similar structures may have utility for the short-term treatment of malaria in children and in adults. Studies to explore the potential of MTX as an anti-*P. falciparum* agent (clinicaltrials.gov, NCT00791531) are underway, and it is hoped

that similar investigations will eventually be conducted against *P. vivax*.

It should be noted that MTX-resistant *P. falciparum* could be relatively easily selected in culture [6], though the molecular basis of this resistance is yet to be determined. Therefore, it is important to ensure that any deployment of MTX for malaria treatment is done in the context of combination therapies. The *P. vivax* isolates that we collected in Thailand were more sensitive to MTX than to artesunate [15], a drug increasingly used in combination with piperazine to treat chloroquine-resistant vivax malaria as second-line treatment. Further studies are needed to establish whether the pharmacokinetic properties of MTX make it an adequate partner to artemisinins. It would also be of interest to examine the potential synergy in a combination of methotrexate with a *dhfr* inhibitor (such as WR99210), which might be expected to reduce even further the in vivo concentration of methotrexate required. We suggest that in vivo studies are warranted to explore the potential of low doses of MTX in the treatment of *P. vivax*.

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MI, BR, RS, AN, FN, and LR conceived and designed the experiments/study. MI, BR, RS, KS, and SK performed the experiments. MI, BR, RS, MLL, GS, FN, and LR analyzed the data. MI, BR, AN, MLL, APP, GS, FN, and LR contributed reagents/materials/analysis tools. AN, APP, and FN provided clinical support/information and ethical oversight. MI, BR, RS, AN, MLL, KS, SK, APP, GS, FN, and LR wrote the manuscript.

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