In Vitro Activity of Antifolate and Polymorphism in Dihydrofolate Reductase of *Plasmodium falciparum* Isolates from the Kenyan Coast: Emergence of Parasites with Ile-164-Leu Mutation[▽]

Steven M. Kiara,^{1†} John Okombo,^{1†} Victor Masseno,¹ Leah Mwai,¹ Isabella Ochola,¹
Steffen Borrmann,^{1,2} and Alexis Nzila^{1,3*}

Kenya Medical Research Institute (KEMRI)/Wellcome Trust Collaborative Research Program, P.O. Box 230, 80108, Kilifi, Kenya¹;
Institute of Hygiene, University of Heidelberg School of Medicine, Heidelberg, Germany²; and University of Oxford,
Nuffield Department of Medicine, John Radcliffe Hospital, Oxford, United Kingdom³

Received 6 March 2009/Returned for modification 17 May 2009/Accepted 8 June 2009

We have analyzed the activities of the antifolates pyrimethamine (PM), chlorcycloguanil (CCG), WR99210, trimethoprim (TMP), methotrexate (MTX), and trimetrexate (TMX) against Kenyan *Plasmodium falciparum* isolates adapted in vitro for long-term culture. We have also assessed the relationship between these drug activities and mutations in dihydrofolate reductase (*dhfr*), a domain of the gene associated with antifolate resistance. As expected, WR99210 was the most potent drug, with a median 50% inhibitory concentration (IC₅₀) of <0.075 nM, followed by TMX, with a median IC₅₀ of 30 nM. The median IC₅₀ of CCG was 37.80 nM, and that of MTX was 83.60 nM. PM and TMP were the least active drugs, with median IC₅₀s of 733.26 nM and 29,656.04 nM, respectively. We analyzed parasite *dhfr* genotypes by the PCR-enzyme restriction technique. No wild-type *dhfr* parasite was found. Twenty-four of 33 parasites were triple mutants (mutations at codons 108, 51, and 59), and only 8/33 were double mutants (mutations at codons 108 and 51 or at codons 108 and 59). IC₅₀s were 2.1-fold (PM) and 3.6-fold (TMP) higher in triple than in double mutants, though these differences were not statistically significant. Interestingly, we have identified a parasite harboring a mutation at codon 164 (Ile-164-Leu) in addition to mutations at codons 108, 51, and 59. This quadruple mutant parasite had the highest TMP IC₅₀ and was in the upper 10th percentile against PM and CCG. We confirmed the presence of this mutation by sequencing. Thus, TMX and MTX are potent against *P. falciparum*, and quadruple mutants are now emerging in Africa.

The inhibition of dihydrofolate reductase (DHFR) remains an attractive drug target against *Plasmodium falciparum*. The combination of pyrimethamine (PM), an inhibitor of DHFR, and sulfadoxine (SD), an inhibitor of dihydropteroate synthase (DHPS), has been widely used for the treatment of malaria but is now abandoned for mass treatment due to the spread of drug resistance (39). However, this drug is still used for intermittent preventive treatment of pregnant women and children (55). Another combination, chlorproguanil-dapsone, known as Lapdap, has been developed (7). Chlorproguanil is converted to the active metabolite chlorcycloguanil (CCG), an inhibitor of DHFR; dapsone is an inhibitor of DHPS. An artemisinin-based combination, Lapdap with artesunate (known as CDA), has also been evaluated (11), but both drugs have recently been phased out because of the toxicity of dapsone.

Another antifolate, cotrimoxazole, is a combination of trimethoprim (TMP), a potent inhibitor of bacterial DHFR, and sulfamethoxazole (SMZ), an inhibitor of DHPS. TMP-SMZ is used as an antibacterial agent (30) and has been recommended by the World Health Organization (WHO) for the treatment of childhood febrile diseases and for prophylaxis against op-

portunistic infections in human immunodeficiency virus-infected patients in Africa (59). This use as a prophylactic agent has been associated with a reduction in malaria incidence in many parts of Africa (32, 53). This drug has also been shown to treat malaria infection (6, 10), and in some studies, it was reported to be as efficacious as PM-SD (20, 44). A recent study indicated that the combination of cotrimoxazole-artesunate was as effective as amodiaquine-artesunate in Nigeria (13).

Mutations in the *dhfr* gene lead to antifolate resistance. For instance, PM resistance is associated with the mutation of the amino acid Ser to Asn at codon 108 of DHFR (Ser-108-Asn). Ancillary mutations of Asn-51-Ile and Cys-59-Arg are associated with an increase in resistance, and the presence of the Ile-164-Leu mutation results in a higher level of PM resistance and decreased CCG activity (39). In vivo, PM-SD resistance is associated with the presence of triple mutant DHFR (Ser-108-Asn Asn-51-Ile Cys-59-Arg) with or without a mutation in DHPS (39). The presence of Ile-164-Leu (quadruple mutant parasites) is associated with a substantial increase in PM-SD resistance and a decrease in Lapdap efficacy in vivo (54, 57).

As part of our previous work, we provided evidence that the anticancer drugs methotrexate (MTX) and trimetrexate (TMX) are potent antimalarials, and these two drugs have a potential to be used as antimalarials (37). In this paper, we sought to (i) investigate the activities of PM, CCG, MTX, TMX, and TMP against recent *P. falciparum* field isolates from Kenya adapted in vitro for long-term culture and (ii) establish

* Corresponding author. Mailing address: Kenya Medical Research Institute (KEMRI)/Wellcome Trust Collaborative Research Program, P.O. Box 230, 80108 Kilifi, Kenya. Phone: 254-41-522535. Fax: 254-41-522390. E-mail: anzila@kilifi.kemri-wellcome.org.

† S. M. Kiara and J. Okombo contributed equally to this work.

▽ Published ahead of print on 15 June 2009.

the relationship between polymorphism in the isolates' *dhfr* genes and these drug activities.

MATERIALS AND METHODS

The antimalarials PM, MTX, and TMP were purchased from Sigma Chemical Co. (Poole, United Kingdom). CCG and WR99210 were a gift from Steve Ward, Liverpool School of Tropical Medicine, Liverpool, United Kingdom, and TMX was a gift from Andre Rosowsky, Dana-Farber Cancer Institute, Boston, MA.

Parasite adaptation and drug assays. Samples infected with *P. falciparum* were collected as part of the malaria studies in Kilifi, Kenya, between 2006 and 2008 and were in vitro adapted for long-term culture as described elsewhere (46). Antimalarial activity was measured in the presence of varying concentrations of each compound using radioisotopic incorporation (49). Results were expressed as the drug concentration required for 50% inhibition of [³H]hypoxanthine incorporation into parasite nucleic acid (50% inhibitory concentration [IC₅₀]), using nonlinear regression analysis of the dose-response curve. Two reference *P. falciparum* laboratory strains were tested: 3D7, a fully PM sensitive isolate with a wild-type *dhfr* genotype, and V1/S, a highly PM resistant strain with point mutations at codons 108, 51, 59, and 164 of *dhfr* (quadruple mutant strain). *P. falciparum* cultures were carried out in RPMI 1640 (Gibco BRL, United Kingdom) medium supplemented with 10% (vol/vol) normal human serum, 25 mM bicarbonate, 2 mM glutamine, 25 mM HEPES buffer, and physiological concentrations of *para*-aminobenzoic acid (5 nM) and folic acid (23 nM). Human blood used to culture the parasites in vitro was obtained from healthy subjects and was washed three times with RPMI culture medium not supplemented with serum.

Genotyping of *dhfr*. After in vitro adaptation of parasites, infected blood samples were spotted onto filter paper and stored. Parasite genomic material from these filter papers was prepared using the methanol procedure, and point mutations at codons 108, 51, 59, and 164 of *dhfr* were analyzed by PCR and restriction enzyme digestion (PCR-restriction fragment length polymorphism [RFLP]) as described elsewhere (41).

Sequencing of *dhfr*. To confirm the PCR-RFLP result, we sequenced a 651-bp *dhfr* coding region from selected parasites. We included 3D7 and V1/S as controls. Two overlapping templates, each containing codons 51, 59, 108, and 164, were amplified separately through 35 cycles by PCR using primers Dfa1 (5'-CT CTTTATATGATGG-AACAAGTCTGCGACGTTTTCG-3'), F2 (5'-AAATT CTTGATAAACACCGGAACCTTTTA-3'), P51a (5'-AATGAGGTTTGGAA TAACTACACATTTAGAGG-3'), and Dfa2 (5'-TCATATGACATGTAT-CTT TGTCATATTCTTTAAAGGC-3'). The resulting amplification products were purified using the QIAquick PCR purification kit (Qiagen, United Kingdom) and were sequenced using the amplification primers given above, BigDye Terminator (version 3.1; Applied Biosystems, United Kingdom), and an ABI 3130xl capillary sequencer (Applied Biosystems, United Kingdom). The raw sequence data from forward and reverse reactions on each sample were assembled into contigs, analyzed to ensure sequence fidelity, and aligned using the Seqman and MegAlign programs (Lasergene, version 7.2; DNASTar, Madison, WI).

Analysis of MSP1 and MSP2. We used the *dhfr* quadruple mutant strain V1/S as our reference, which we cultured routinely in vitro. To rule out the possibility of contamination of our isolates with this strain, we have determined the merozoite surface protein 1 (MSP1) and MSP2 polymorphisms of V1/S and a quadruple mutant isolate we have identified in Kilifi, Kenya. These genes are highly polymorphic, with differing numbers of tandem repeats, conferring size polymorphism and allelic differences with MAD20, K1, and RO33 (7G8) for MSP1 and FC27 and IC types for MSP2. The study was performed by nested PCR as reported elsewhere (12), and DNA was prepared from filter paper samples as described under "Genotyping of *dhfr*" above.

Statistical analyses were carried out with the Stata program, version 9 (Stata, Inc.), using the Kruskal-Wallis nonparametric test for comparison of medians. The level of significance was set at a *P* value of <0.05.

RESULTS

We used V1/S and 3D7 as controls. The median IC₅₀s of PM, TMP, CCG, MTX, TMX, and WR99210 against V1/S were 6,929.14, 101,754, 92.3, 40.0, 24.8, and 0.063 nM, respectively. As expected, use of the antifolate-sensitive strain 3D7 gave lower median IC₅₀s of 3.9, 406.7, 0.011, 49.9, 1.9, and 0.015 nM for PM, TMP, CCG, MTX, TMX, and WR99210, respectively.

We have analyzed 33 fresh isolates collected in Kilifi, Kenya,

TABLE 1. Summary of the in vitro activities of PM, TMP, TMX, MTX, CCG, and WR99210 against field isolates of *Plasmodium falciparum* from Kenya

Drug	No. of isolates	Median IC ₅₀ (nM) (95% CI)
WR99210	15 ^a	0.072 (0.04–0.23)
TMX	23	30 (11.82–52.63)
CCG	33	37.80 (12.11–49.19)
MTX	33	83.60 (61.75–127.80)
PM	33	733.26 (229–941)
TMP	33	29,656 (6,686–62,448)

^a WR99210 IC₅₀s for 18 of 33 isolates were <0.01 nM and thus could not be estimated with accuracy according to our protocol. The data reported pertain to the remaining 15 isolates.

in the period from 2006 to 2008. The data are summarized in Table 1. Among the drugs tested, WR99210, as expected, was the most potent. For 18 of the 33 isolates (55%), WR99210 IC₅₀s were <0.01 nM and thus could not be estimated with accuracy according to our protocol. The remaining isolates (15/33) had a median IC₅₀ of 0.072 nM (95% confidence interval [95% CI], 0.04 to 0.23 nM). Subsequent analyses were carried out using these 15 isolates.

TMX was the second most active drug, with a median IC₅₀ of 30 nM (95% CI, 11.82 to 52.63 nM). The triazine CCG had a median IC₅₀ of 37.80 nM (95% CI, 12.11 to 49.19 nM). The IC₅₀ distribution of MTX showed a median IC₅₀ of 83.60 nM (95% CI, 61.75 to 127.80 nM). PM and TMP were the least active drugs, with median IC₅₀s of 733.26 nM (95% CI, 229 to 941 nM) and 29,656 nM (95% CI, 6,686 to 62,448 nM), respectively (Table 1). TMP was almost 40 times less active than PM.

We have analyzed the *dhfr* genotypes of the isolates. Of 33 isolates, none were wild type, and 8 were double mutants, of which 6 and 2 harbored the Ser-108-Asn and Cys-59-Arg mutations and the Ser-108-Asn and Asn-51-Ile mutations, respectively. The majority of isolates, 72.7% (24/33), were triple mutants (Ser-108-Asn Asn-51-Ile Cys-59-Arg). Interestingly, one isolate was a quadruple mutant, with an Ile-164-Leu mutation in addition to the mutations at the three codons (Ser-108-Asn Asn-51-Ile Cys-59-Arg Ile-164-Leu), and we named this isolate Kil-164. This is the first report of a parasite with Ile-164-Leu at our study site. Interestingly, this quadruple mutant parasite had the highest TMP IC₅₀ (154,736 nM); it had PM and CCG IC₅₀s of 3,690.79 and 87.68 nM, respectively, values that were within the upper 10th percentile (among the 10% of isolates that were most resistant) for the two drugs.

Since all of these drugs are known to target the parasite *dhfr*, we sought to investigate the relationship between mutations in *dhfr* and their chemosensitivity profiles. The data are summarized in Table 2. The median IC₅₀ for PM was 370 nM in the double mutant group, and this value increased almost twofold in the triple mutant group. Likewise, IC₅₀s for TMP increased from 9,308.6 nM in the double mutant group to 33,655 nM in the triple mutant group, a 3.6-fold increase; however, these differences were not significant. This may be due to the small number of double mutants compared to triple mutants or the small sample size. Interestingly, an increase in activity from double to triple mutants was noticed when TMX was used, with median IC₅₀s changing from 40.92 to 20.91 nM, but this

TABLE 2. Relationship between median IC₅₀s of drugs and parasite *dhfr* genotypes

DHFR genotype ^a	Median IC ₅₀ (nM) (no. of isolates)					
	PM	TMP	CCG	MTX	TMX	WR99210 ^b
Double mutant	371 (8)	9,308.6 (7)	38.06 (8)	84.89 (8)	40.92 (8)	0.069 (4)
Triple mutant	778.28 (24)	33,655 (15)	39.99 (24)	83.59 (24)	20.91 (24)	0.1 (10)
Quadruple mutant	3,690.79 (1)	154,736 (1)	87.6799 (1)	56.63 (1)	44.66 (1)	0.012 (1)

^a No wild-type *dhfr* isolate was identified. None of the tested differences between double and triple mutants were significant at a *P* value of 0.05 by the Kruskal-Wallis test.

^b WR99210 IC₅₀s for 18 out of 33 isolates were <0.01 nM and thus could not be estimated with accuracy according to our protocol. The data reported pertain to the remaining 15 isolates.

difference was not significant. No change was observed with CCG or MTX (Table 2).

We have also assessed the correlation between the activities of these antifolates. The activity of PM correlated strongly with those of TMP ($r^2 = 0.98$; $P < 0.0001$), CCG ($r^2 = 0.70$; $P < 0.0001$), and MTX ($r^2 = 0.58$; $P < 0.001$). The activity of CCG also correlated with those of TMP ($r^2 = 0.54$; $P < 0.01$) and MTX ($r^2 = 0.65$; $P < 0.01$) as well. None of the other antifolate combinations showed any correlation.

To confirm the presence of Ile-164-Leu in the Kil-164 isolate, we have sequenced its *dhfr* gene, along with the *dhfr* genes of the 10% of isolates most resistant to PM (a total of three isolates). We used V1S and 3D7 as controls. Kil-164, like V1S, had four mutations in the *dhfr* gene (at codons 108, 51, 59, and 164) (quadruple mutants). The other three isolates (with higher PM IC₅₀s) had mutations at codons 108, 51, and 59 (triple mutants), and the reference strain 3D7 was wild type. No new mutation was found. Thus, our PCR-RFLP data were confirmed by sequencing.

Both V1S and Kil-164 have Ile-164-Leu. To exclude the possibility of contamination of the Kil-164 isolate by the reference strain V1S, which we routinely cultured in vitro, we have analyzed the MSP1 and MSP2 polymorphisms of these two parasites. V1S and Kil-164 belong to the same MSP2 IC family; however, their length polymorphisms are different, around 550 and 425 bp, respectively, for V1S and Kil-164. Analysis of MSP1 has confirmed this difference. Indeed, V1S belongs to the RO33 family, with a size of 175 bp, while Kil-164 is member of the K1 family, with a size of 225 bp, a clear indication that these parasites are different. Thus, Kil-164 is a bona fide quadruple mutant parasite.

DISCUSSION

We have confirmed that a high proportion of parasites circulating in Kilifi, Kenya, are triple mutants (>70% of isolates), and we report for the first time the presence of a parasite carrying the Ile-164-Leu mutation in Kilifi, our study site. Experience in the use of PM-SD in Southeast Asia and South America indicates that PM-SD can select for Ile-164-Leu, and this mutation is associated with high resistance to PM-SD and decreased efficacy of Lapdap (54, 57). In Africa, this mutation was identified for the first time in Tanzania, using the yeast complementation approach, which is based on the expression of *Plasmodium dhfr* genes in *Saccharomyces cerevisiae* followed by the selection of cells expressing highly resistant alleles (22). When this discovery was made, it was anticipated that the rate of parasites carrying this mutation would rise in this population

as a result of PM-SD pressure. Surprisingly, 5 years afterwards, we did not find this mutation by use of standard PCR-based methods (43). Recently, this mutation has been found in Malawi, in parasites from human immunodeficiency virus-positive pregnant women, by using a real-time PCR method and a heteroduplex tracking assay (1, 26); however, this has not been confirmed in a subsequent study in the same area (42). This Ile-164-Leu mutation has also been reported in Uganda and the Central African Republic (16, 35, 51), and a recent study showed prevalences of 4 to 14% at two sites in Uganda where *P. falciparum* is endemic (31). Interestingly, this mutation has been found in the western part of Kenya by using standard PCR methods and cloning; surprisingly, its presence was not associated with reduced PM-SD efficacy (21, 34), questioning its clinical relevance. There has been debate as to whether this mutation would be efficiently selected in Africa, and if so, whether it would be associated with reduced antifolate efficacy (23, 40). In this paper, we have provided evidence that the Ile-164-Leu mutation is present in Africa and that it is associated with a substantial increase in resistance to PM and TMP in vitro and with a decrease in CCG activity. Thus, in vivo, this mutation would be associated with increased PM-SD resistance and decreased Lapdap efficacy, though this mutation did not render PM-SD ineffective in the western part of Kenya (21).

Resistance to PM-SD started rising in Kilifi, Kenya, in the mid-1990s, even before the drug became the first line of treatment in the country (in 1998) (48). By the early 2000s, this drug was ineffective in most parts of Kenya, including Kilifi, and it was officially withdrawn in 2004 and replaced with the combination of lumefantrine and artemether (Coartem) (2). However, PM-SD is still available in the country for the management of malaria in pregnancy and for intermittent preventive treatment (8, 52). In addition, because it is cheap and affordable, this drug is likely to continue to be used for self-medication, thus maintaining drug pressure for the selection of resistance, explaining the emergence of the quadruple mutant. However, it remains puzzling that from the time PM-SD was widely used up to its official withdrawal, only limited numbers of quadruple mutant parasites were found in Africa, in spite of extensive investigations (43, 45, 56).

We focused our study on the *dhfr* gene only. The pressure for PM-SD is also exerted on the *dhps* gene. However, we and other groups have demonstrated that the *dhfr* gene is the critical marker associated with PM-SD (39); hence the focus on *dhfr*. Recently, a report has indicated that copy number variation in GTP-cyclohydrolase, the first enzyme in the folate

pathway, is likely to influence antifolate sensitivity in Southeast Asia, an area where Ile-164-Leu is dominant (36). Thus, it will be important to establish the role of this gene in the context of the emergence of Ile-164-Leu in Africa.

We have provided data on the in vitro activity of CCG against recent Kenyan isolates. Overall, this drug is potent, with IC_{50} s of <40 nM for more than 95% of parasites. However, the quadruple mutant isolate we have identified was among the 10% of isolates that were least susceptible, and experience with the use of Lapdap in Southeast Asia, an area with a high rate of the parasite with Ile-164-Leu, clearly indicates that this mutation would be associated with decreased Lapdap efficacy (57). Based on our in vitro data, this mutation would also compromise Lapdap efficacy in Africa. However, this drug has been phased out because of the toxicity of dapsone.

We have investigated the activity of the antibacterial agent TMP, a component of cotrimoxazole. Since cotrimoxazole is commonly used in Africa, there had been debate as to whether the use of this drug could have selected for mutations in *dhfr*, which would eventually have brought about resistance to PM. Jelinek et al. reported the selection of an unusual *dhfr* genotype (wild type at codon 108 but mutant at codons 51 and 59) following the use of TMP-SMZ (25), yet experience with PM-SD indicates that a mutation at codon 108 of *dhfr* is always a precondition for mutations at codons 51 and 59. Extensive work at many African sites has never confirmed this genotype, and it is now accepted that mutations in *dhfr* are driven by PM-SD and that these mutations are associated with decreased TMP activity, by virtue of cross-resistance with PM (24, 27). We found a high correlation between PM and TMP ($r^2 = 0.98$), which supports this observation.

Our data also indicate that PM is more active than TMP against field isolates, as reported previously (5, 24). However, in vivo, several reports have indicated comparative efficacies of PM-SD and TMP-SMZ (20, 44). These comparative in vivo efficacies could be explained by the better pharmacokinetic properties of TMP. Indeed, in vivo, doses of 1.25 and 40 mg/kg are used for PM and SD, respectively, while 8 mg/kg/day is used for TMP and 40 mg/kg/day for SMZ. At these doses, 2.5 μ M concentrations of PM and 5 to 20 μ M concentrations of TMP are achieved in vivo, yielding free and pharmacologically active TMP at >2.5 μ M (less than 40% of TMP is bound to protein), a value five times higher than the free concentration of PM (0.5 μ M) (28, 54). Thus, when normal doses of these drugs are used, the free concentration of TMP is at least five times higher than that of PM, which would compensate for the relatively low activity of TMP, explaining, at least partly, the comparative efficacies of TMP-SMZ and PM-SD. Thus, in areas of high PM-SD efficacy (low rates of *dhfr* mutants), PM-SD and TMP-SMZ could have comparative efficacies, as supported by in vivo investigations (14, 44, 50). However, based on our in vitro data, TMP-SMZ, like PM-SD, will be of marginal efficacy in the context of a high rate of *dhfr* triple mutant parasites. In support of this hypothesis, an increase in TMP-SMZ treatment failure has been reported in an area of PM-SD resistance (29). Thus, TMP-SMZ is a good antimalarial in the context of the high efficacy of PM-SD, so this antibacterial agent does not offer any advantage over PM-SD,

probably explaining why it was abandoned as an antimalarial (39).

We and other groups have shown the high potency of MTX against laboratory reference strains, with IC_{50} s for parasites ranging from 25 to 60 nM, and these values are not affected by polymorphism in the *dhfr* gene (9, 37). In the present work, we have provided data on the activity of MTX against field isolates. MTX is active, with a median IC_{50} of 83.5 nM and $IC_{90/99}$ around 250 to 350 nM (drug concentrations that kill 90 or 99% of parasitemia). MTX is used at high doses, as much as 5 to 12 g/m² per week (130 to 300 mg/kg) for several weeks for the treatment of cancer (4). This dose can yield concentrations of >1,000 μ M MTX, which can have life-threatening toxicity (4). On the other hand, a low dose of MTX (0.1 to 0.35 mg/kg [7.5 to 25 mg per adult]) is used for the treatment of rheumatoid arthritis and juvenile arthritis on a chronic basis, for as long as 5 years (18, 38). At this dose, MTX is safe and well tolerated and can yield MTX concentrations around 250 to 950 nM (15, 17). This information has led us to propose that a dose of <7.5 mg could be used to treat malaria. In support of this hypothesis, two small clinical trials in the 1970s demonstrated that doses as low as 2.5 mg/day (adult dose) for 3 to 5 days are safe and effective at treating malaria infection in humans (47, 58). We are currently reevaluating the antimalarial potential of this agent.

We have also studied the activity of TMX against field isolates. This drug has been used in the treatment of cancer (19, 33). It has been combined with the folate derivative folinic acid (FNA) in the treatment of *Pneumocystis jirovecii* infection (an opportunistic infection commonly associated with human immunodeficiency virus infection). TMX is potent against *P. jirovecii*, and this microorganism cannot transport folate derivatives. As a result, the combination of TMX with FNA retains potency against *P. jirovecii*, and the use of FNA protects the host against TMX toxicity. This combination is now the mainstay of *P. jirovecii* treatment (3, 33). As part of our previous work, we have shown that the most dominant form of folate, 5-methyl-tetrahydrofolate, is not utilized by *P. falciparum*; thus, we proposed that a low dose of TMX combined with 5-methyl-tetrahydrofolate be developed as an antimalarial (37), as it is used in the treatment of *P. jirovecii*. Our current data showing high activity of TMX against field isolates, even against triple and quadruple mutant parasites, lend support to the development of this drug as an antimalarial.

In conclusion, we have, for the first time, culture-adapted a *P. falciparum dhfr* Ile-164-Leu mutant from Africa. This isolate has high resistance to PM and CCG, and it has the highest resistance to TMP, as evidenced by the IC_{50} s of these drugs. We have confirmed that MTX and TMX are potent against *P. falciparum* and that their activities are independent of the parasite *dhfr* genotype. Thus, studies evaluating these two antifolates for the treatment of malaria are warranted.

ACKNOWLEDGMENTS

We thank the director of the Kenya Medical Research Institute for permission to publish these data.

This study was supported by a Pfizer-Royal Society (United Kingdom) award (to A.N.), by the EU Commission under Framework 6 as part of the AntiMal Integrated Project 018834, by the Wellcome Trust (grant WT077092 and 084538), and by the European and Developing Countries Clinical Trials Partnership (EDCPT). A.N. is an EDCPT

senior fellow, and L.M. is an EDCPT-funded Ph.D. student. S.B. is funded through a Junior Group grant (SFB544, A7) by the Deutsche Forschungsgemeinschaft (DFG).

REFERENCES

- Alker, A. P., V. Mwapasa, A. Purfield, S. J. Rogerson, M. E. Molyneux, D. D. Kamwendo, E. Tadesse, E. Chaluluka, and S. R. Meshnick. 2005. Mutations associated with sulfadoxine-pyrimethamine and chlorproguanil resistance in *Plasmodium falciparum* isolates from Blantyre, Malawi. *Antimicrob. Agents Chemother.* **49**:3919–3921.
- Amin, A. A., D. Zurovac, B. B. Kangwana, J. Greenfield, D. N. Otieno, W. S. Akhwale, and R. W. Snow. 2007. The challenges of changing national malaria drug policy to artemisinin-based combinations in Kenya. *Malar. J.* **6**:72.
- Amsden, G. W., S. F. Kowalsky, and G. D. Morse. 1992. Trimetrexate for *Pneumocystis carinii* pneumonia in patients with AIDS. *Ann. Pharmacother.* **26**:218–226.
- Barnhart, K., C. Coutifaris, and M. Esposito. 2001. The pharmacology of methotrexate. *Expert Opin. Pharmacother.* **2**:409–417.
- Basco, L. K., and J. Le Bras. 1997. In vitro sensitivity of *Plasmodium falciparum* to anti-folate agents (trimethoprim, pyrimethamine, cycloguanil): a study of 29 African strains. *Bull. Soc. Pathol. Exot.* **90**:90–93. (In French.)
- Bloland, P. B., S. C. Redd, P. Kazembe, R. Tembenu, J. J. Wirima, and C. C. Campbell. 1991. Co-trimoxazole for childhood febrile illness in malaria-endemic regions. *Lancet* **337**:518–520.
- Bukirwa, H., P. Garner, and J. Critchley. 2004. Chlorproguanil-dapsone for treating uncomplicated malaria. *Cochrane Database Syst. Rev.* **2004**:CD004387.
- Clarke, S. E., M. C. Jukes, J. K. Njagi, L. Khasakhala, B. Cundill, J. Otido, C. Crudder, B. B. Estambale, and S. B. Brooker. 2008. Effect of intermittent preventive treatment of malaria on health and education in schoolchildren: a cluster-randomised, double-blind, placebo-controlled trial. *Lancet* **372**:127–138.
- Dar, O., M. S. Khan, and I. Adagu. 2008. The potential use of methotrexate in the treatment of falciparum malaria: in vitro assays against sensitive and multidrug-resistant falciparum strains. *Jpn. J. Infect. Dis.* **61**:210–211.
- Daramola, O. O., P. L. Alonso, T. J. O'Dempsey, P. Twumasi, T. F. McArdle, and B. M. Greenwood. 1991. Sensitivity of *Plasmodium falciparum* in The Gambia to co-trimoxazole. *Trans. R. Soc. Trop. Med. Hyg.* **85**:345–348.
- Fanello, C. I., C. Karema, D. Ngamiye, A. Uwimana, V. Ndahindwa, C. Van Overmeir, W. Van Doren, J. Curtis, and U. D'Alessandro. 2008. A randomised trial to assess the efficacy and safety of chlorproguanil/dapsone + artesunate for the treatment of uncomplicated *Plasmodium falciparum* malaria. *Trans. R. Soc. Trop. Med. Hyg.* **102**:412–420.
- Färnert, A., M. Lebbad, L. Faraja, and I. Rooth. 2008. Extensive dynamics of *Plasmodium falciparum* densities, stages and genotyping profiles. *Malar. J.* **7**:241.
- Fehintola, F. A., A. A. Adedeji, G. O. Gbotosho, C. T. Happi, S. T. Balogun, O. A. Folarin, A. O. Sijuade, and A. Sowunmi. 2008. Effects of artesunate-cotrimoxazole and amodiaquine-artesunate against asexual and sexual stages of *Plasmodium falciparum* malaria in Nigerian children. *J. Infect. Chemother.* **14**:188–194.
- Fehintola, F. A., A. A. Adedeji, E. Tambo, B. B. Fateye, T. C. Happi, and A. Sowunmi. 2004. Cotrimoxazole in the treatment of acute uncomplicated falciparum malaria in Nigerian children: a controlled clinical trial. *Clin. Drug Investig.* **24**:149–155.
- Fox, R. I., S. L. Morgan, H. T. Smith, B. A. Robbins, M. G. Choc, and J. E. Baggott. 2003. Combined oral cyclosporin and methotrexate therapy in patients with rheumatoid arthritis elevates methotrexate levels and reduces 7-hydroxymethotrexate levels when compared with methotrexate alone. *Rheumatology (Oxford)* **42**:989–994.
- Francis, D., S. L. Nsohya, A. Talisuna, A. Yeka, M. R. Kamya, R. Mache-kano, C. Dokomajilar, P. J. Rosenthal, and G. Dorsey. 2006. Geographic differences in antimalarial drug efficacy in Uganda are explained by differences in endemicity and not by known molecular markers of drug resistance. *J. Infect. Dis.* **193**:978–986.
- Godfrey, C., K. Sweeney, K. Miller, R. Hamilton, and J. Kremer. 1998. The population pharmacokinetics of long-term methotrexate in rheumatoid arthritis. *Br. J. Clin. Pharmacol.* **46**:369–376.
- Gray, O. M., G. V. McDonnell, and R. B. Forbes. 2006. A systematic review of oral methotrexate for multiple sclerosis. *Mult. Scler.* **12**:507–510.
- Haller, D. G. 1997. Trimetrexate: experience with solid tumors. *Semin. Oncol.* **24**:S18–71–S18–76.
- Hamel, M. J., T. Holtz, C. Mkandala, N. Kaimila, N. Chizani, P. Bloland, J. Kublin, P. Kazembe, and R. Steketee. 2005. Efficacy of trimethoprim-sulfamethoxazole compared with sulfadoxine-pyrimethamine plus erythromycin for the treatment of uncomplicated malaria in children with integrated management of childhood illness dual classifications of malaria and pneumonia. *Am. J. Trop. Med. Hyg.* **73**:609–615.
- Hamel, M. J., A. Poe, P. Bloland, A. McCollum, Z. Zhou, Y. P. Shi, P. Ouma, K. Otieno, J. Vulule, A. Escalante, V. Udhayakumar, and L. Slutsker. 2008. Dihydrofolate reductase I164L mutations in *Plasmodium falciparum* isolates: clinical outcome of 14 Kenyan adults infected with parasites harbouring the I164L mutation. *Trans. R. Soc. Trop. Med. Hyg.* **102**:338–345.
- Hastings, M. D., S. J. Bates, E. A. Blackstone, S. M. Monks, T. K. Muta-bingwa, and C. H. Sibley. 2002. Highly pyrimethamine-resistant alleles of dihydrofolate reductase in isolates of *Plasmodium falciparum* from Tanzania. *Trans. R. Soc. Trop. Med. Hyg.* **96**:674–676.
- Hyde, J. E. 2008. Antifolate resistance in Africa and the 164-dollar question. *Trans. R. Soc. Trop. Med. Hyg.* **102**:301–303.
- Iyer, J. K., W. K. Milhous, J. F. Cortese, J. G. Kublin, and C. V. Plowe. 2001. *Plasmodium falciparum* cross-resistance between trimethoprim and pyrimethamine. *Lancet* **358**:1066–1067.
- Jelinek, T., A. H. Kilian, J. Curtis, M. T. Duraisingh, G. Kabagambe, F. von Sonnenburg, and D. C. Warhurst. 1999. *Plasmodium falciparum*: selection of serine 108 of dihydrofolate reductase during treatment of uncomplicated malaria with co-trimoxazole in Ugandan children. *Am. J. Trop. Med. Hyg.* **61**:125–130.
- Juliano, J. J., P. Trotman, V. Mwapasa, and S. R. Meshnick. 2008. Detection of the dihydrofolate reductase-164L mutation in *Plasmodium falciparum* infections from Malawi by heteroduplex tracking assay. *Am. J. Trop. Med. Hyg.* **78**:892–894.
- Khalil, I., A. M. Ronn, M. Alifrangis, H. A. Gabar, G. M. Satti, and I. C. Bygbjerg. 2003. Dihydrofolate reductase and dihydropteroate synthase genotypes associated with in vitro resistance of *Plasmodium falciparum* to pyrimethamine, trimethoprim, sulfadoxine, and sulfamethoxazole. *Am. J. Trop. Med. Hyg.* **68**:586–589.
- Kielhofner, M. A. 1990. Trimethoprim-sulfamethoxazole: pharmacokinetics, clinical uses, and adverse reactions. *Tex. Heart Inst. J.* **17**:86–93.
- Kilian, A. H., T. Jelinek, I. Prislis, G. Kabagambe, W. Byamukama, G. Mpigika, R. Korte, and F. von Sonnenburg. 1998. Resistance in vivo of *Plasmodium falciparum* to co-trimoxazole in western Uganda. *Trans. R. Soc. Trop. Med. Hyg.* **92**:197–200.
- Libecco, J. A., and K. R. Powell. 2004. Trimethoprim/sulfamethoxazole: clinical update. *Pediatr. Rev.* **25**:375–380.
- Lynch, C., R. Pearce, H. Pota, J. Cox, T. A. Abeku, J. Rwakimari, I. Naidoo, J. Tibenderana, and C. Roper. 2008. Emergence of a *dhfr* mutation conferring high-level drug resistance in *Plasmodium falciparum* populations from southwest Uganda. *J. Infect. Dis.* **197**:1598–1604.
- Malamba, S. S., J. Mermin, A. Reingold, J. R. Lule, R. Downing, R. Ransom, A. Kigozi, B. M. Hunt, A. Hubbard, P. J. Rosenthal, and G. Dorsey. 2006. Effect of cotrimoxazole prophylaxis taken by human immunodeficiency virus (HIV)-infected persons on the selection of sulfadoxine-pyrimethamine-resistant malaria parasites among HIV-uninfected household members. *Am. J. Trop. Med. Hyg.* **75**:375–380.
- Marshall, J. L., and R. J. DeLap. 1994. Clinical pharmacokinetics and pharmacology of trimetrexate. *Clin. Pharmacokinet.* **26**:190–200.
- McCollum, A. M., A. C. Poe, M. Hamel, C. Huber, Z. Zhou, Y. P. Shi, P. Ouma, J. Vulule, P. Bloland, L. Slutsker, J. W. Barnwell, V. Udhayakumar, and A. A. Escalante. 2006. Antifolate resistance in *Plasmodium falciparum*: multiple origins and identification of novel *dhfr* alleles. *J. Infect. Dis.* **194**:189–197.
- Menard, D., D. Djalle, F. Yapou, A. Manirakiza, and A. Talarmin. 2006. Frequency distribution of antimalarial drug-resistant alleles among isolates of *Plasmodium falciparum* in Bangui, Central African Republic. *Am. J. Trop. Med. Hyg.* **74**:205–210.
- Nair, S., B. Miller, M. Barends, A. Jaidee, J. Patel, M. Mayxay, P. Newton, F. Nosten, M. T. Ferdig, and T. J. Anderson. 2008. Adaptive copy number evolution in malaria parasites. *PLoS Genet.* **4**:e1000243.
- Nduati, E., A. Diriyee, S. Ommeh, L. Mwai, S. Kiara, V. Masseno, G. Kokwaro, and A. Nzila. 2008. Effect of folate derivatives on the activity of antifolate drugs used against malaria and cancer. *Parasitol. Res.* **102**:1227–1234.
- Niehues, T., and P. Lankisch. 2006. Recommendations for the use of methotrexate in juvenile idiopathic arthritis. *Paediatr. Drugs* **8**:347–356.
- Nzila, A. 2006. The past, present and future of antifolates in the treatment of *Plasmodium falciparum* infection. *J. Antimicrob. Chemother.* **57**:1043–1054.
- Nzila, A., E. Ochong, E. Nduati, K. Gilbert, P. Winstanley, S. Ward, and K. Marsh. 2005. Why has the dihydrofolate reductase 164 mutation not consistently been found in Africa yet? *Trans. R. Soc. Trop. Med. Hyg.* **99**:341–346.
- Nzila, A. M., E. K. Mberu, J. Sulo, H. Dayo, P. A. Winstanley, C. H. Sibley, and W. M. Watkins. 2000. Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. *Antimicrob. Agents Chemother.* **44**:991–996.
- Ochong, E., D. J. Bell, D. J. Johnson, U. D'Alessandro, M. Mulenga, S. Muangnoichareon, J. P. Van Geertruyden, P. A. Winstanley, P. G. Bray, S. A. Ward, and A. Owen. 2008. *Plasmodium falciparum* strains harboring dihydrofolate reductase with the I164L mutation are absent in Malawi and Zambia even under antifolate drug pressure. *Antimicrob. Agents Chemother.* **52**:3883–3888.
- Ochong, E., A. Nzila, S. Kimani, G. Kokwaro, T. Mutabingwa, W. Watkins, and K. Marsh. 2003. Molecular monitoring of the Leu-164 mutation of

- dihydrofolate reductase in a highly sulfadoxine/pyrimethamine-resistant area in Africa. *Malar. J.* 2:46.
44. Omar, S. A., A. Bakari, A. Owiti, I. S. Adagu, and D. C. Warhurst. 2001. Co-trimoxazole compared with sulfadoxine-pyrimethamine in the treatment of uncomplicated malaria in Kenyan children. *Trans. R. Soc. Trop. Med. Hyg.* 95:657–660.
 45. Parola, P., B. Pradines, F. Simon, M. P. Carlotti, P. Minodier, M. P. Ranjeva, S. Badiaga, L. Bertaux, J. Delmont, M. Morillon, R. Silai, P. Brouqui, and D. Parzy. 2007. Antimalarial drug susceptibility and point mutations associated with drug resistance in 248 *Plasmodium falciparum* isolates imported from Comoros to Marseille, France in 2004–2006. *Am. J. Trop. Med. Hyg.* 77:431–437.
 46. Sasi, P., A. Abdulrahman, L. Mwai, S. Muriithi, J. Straimer, E. Schieck, A. Rippert, M. Bashraheil, A. Salim, J. Peshu, K. Awuondo, B. Lowe, M. Pirmohamed, P. Winstanley, S. Ward, A. Nzila, and S. Borrmann. 2009. In vivo and in vitro efficacy of amodiaquine against *Plasmodium falciparum* in an area of continued use of 4-aminoquinolines in East Africa. *J. Infect. Dis.* 199:1575–1582.
 47. Sheehy, T. W., and H. Dempsey. 1970. Methotrexate therapy for *Plasmodium vivax* malaria. *JAMA* 214:109–114.
 48. Shretta, R., J. Omumbo, B. Rapuoda, and R. W. Snow. 2000. Using evidence to change antimalarial drug policy in Kenya. *Trop. Med. Int. Health* 5:755–764.
 49. Sixsmith, D. G., W. M. Watkins, J. D. Chulay, and H. C. Spencer. 1984. In vitro antimalarial activity of tetrahydrofolate dehydrogenase inhibitors. *Am. J. Trop. Med. Hyg.* 33:772–776.
 50. Sowunmi, A., B. A. Fateye, A. A. Adedeji, F. A. Fehintola, A. E. Bamgboye, C. P. Babalola, T. C. Happi, and G. O. Gbotosho. 2005. Effects of antifolates—co-trimoxazole and pyrimethamine-sulfadoxine—on gametocytes in children with acute, symptomatic, uncomplicated *Plasmodium falciparum* malaria. *Mem. Inst. Oswaldo Cruz* 100:451–455.
 51. Staedke, S. G., H. Sendagire, S. Lamola, M. R. Kamya, G. Dorsey, and P. J. Rosenthal. 2004. Relationship between age, molecular markers, and response to sulphadoxine-pyrimethamine treatment in Kampala, Uganda. *Trop. Med. Int. Health* 9:624–629.
 52. Temperley, M., D. H. Mueller, J. K. Njagi, W. Akhwale, S. E. Clarke, M. C. Jukes, B. B. Estambale, and S. Brooker. 2008. Costs and cost-effectiveness of delivering intermittent preventive treatment through schools in western Kenya. *Malar. J.* 7:196.
 53. Thera, M. A., P. S. Sehdev, D. Coulibaly, K. Traore, M. N. Garba, Y. Cissoko, A. Kone, A. Guindo, A. Dicko, A. H. Beavogui, A. A. Djimde, K. E. Lyke, D. A. Diallo, O. K. Doumbo, and C. V. Plowe. 2005. Impact of trimethoprim-sulfamethoxazole prophylaxis on falciparum malaria infection and disease. *J. Infect. Dis.* 192:1823–1829.
 54. Watkins, W. M., E. K. Mberu, P. A. Winstanley, and C. V. Plowe. 1997. The efficacy of antifolate antimalarial combinations in Africa: a predictive model based on pharmacodynamic and pharmacokinetic analyses. *Parasitol. Today* 13:459–464.
 55. White, N. J. 2005. Intermittent presumptive treatment for malaria. *PLoS Med.* 2:e3.
 56. Wichmann, O., T. Jelinek, G. Peyerl-Hoffmann, N. Muhlberger, M. P. Grobusch, J. Gascon, A. Matteelli, C. Hatz, H. Laferl, M. Schulze, G. Burchard, S. da Cunha, J. Beran, P. McWhinney, H. Kollaritsch, P. Kern, J. Cuadros, M. Alifrangis, and I. Gyorup. 2003. Molecular surveillance of the antifolate-resistant mutation I164L in imported African isolates of *Plasmodium falciparum* in Europe: sentinel data from TropNetEurop. *Malar. J.* 2:17.
 57. Wilairatana, P., D. E. Kyle, S. Looareesuwan, K. Chinwongprom, S. Amradee, N. J. White, and W. M. Watkins. 1997. Poor efficacy of antimalarial biguanide-dapsone combinations in the treatment of acute, uncomplicated, falciparum malaria in Thailand. *Ann. Trop. Med. Parasitol.* 91:125–132.
 58. Wildbolz, A. 1973. Methotrexate in the therapy of malaria. *Ther. Umsch.* 30:218–222.
 59. Zachariah, R., and M. Massaquoi. 2006. Cotrimoxazole prophylaxis for HIV-positive TB patients in developing countries. *Trop. Doct.* 36:79–82.