ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2009, p. 5069–5073 Vol. 53, No. 12
0066-4804/09/$12.00 doi:10.1128/AAC.00638-09

In Vitro Activities of Piperaquine, Lumefantrine, and Dihydroartemisinin in Kenyan *Plasmodium falciparum* Isolates and Polymorphisms in *pfcrt* and *pfmdr1*[▽]

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Received 12 May 2009/Returned for modification 9 August 2009/Accepted 9 September 2009

We have analyzed the in vitro chemosensitivity profiles of 115 Kenyan isolates for chloroquine (CQ), piperaquine, lumefantrine (LM), and dihydroartemisinin in association with polymorphisms in *pfcrt* at codon 76 and *pfmdr1* at codon 86, as well as with variations of the copy number of *pfmdr1*. The median drug concentrations that inhibit 50% of parasite growth (IC_{50}s) were 41 nM (interquartile range [IQR], 18 to 73 nM), 50 nM (IQR, 29 to 96 nM), 32 nM (IQR, 17 to 46 nM), and 2 nM (IQR, 1 to 3 nM) for CQ, LM, piperaquine, and dihydroartemisinin, respectively. The activity of CQ correlated inversely with that of LM ($r^2 = -0.26$; $P = 0.02$). Interestingly, parasites for which LM IC_{50}s were higher were wild type for *pfcrt-76* and *pfmdr1-86*. All isolates had one *pfmdr1* copy. Thus, the decrease in LM activity is associated with the selection of wild-type *pfcrt-76* and *pfmdr1-86* parasites, a feature that accounts for the inverse relationship between CQ and LM. Therefore, the use of LM-artemether is likely to lead to the selection of more CQ-susceptible parasites.

Chemotherapy is still the main approach for the control of malaria, and current strategies for malaria treatment rely on the use of combinations of drugs that include artemisinin compounds. Although this strategy is designed to reduce the chance of resistance emerging, there is considerable concern that this will inevitably occur.

For instance, the combination of lumefantrine (LM) and artemether (ATM), known as Coartem, has become the first-line treatment for malaria in many African countries, including Kenya (19). ATM is converted in vivo to dihydroartemisinin (DHA). Emerging reports indicate that the use of LM (in Coartem) selects for parasites that show increased tolerance to Coartem, and these parasites select for a wild-type *pfmdr1* genotype or show increased copy numbers of *pfmdr1*, a gene associated with chloroquine (CQ) and mefloquine (MFQ) resistance (7, 13, 15, 20, 36, 38). Thus, there is concern that resistance to LM could emerge rapidly. On the other hand, recent reports from Southeast Asia indicate that resistance to artemisinin derivatives is increasing, threatening the concept of artemisinin-based combinations (8).

Another combination, piperaquine (PQ) and DHA, known as Artekin, is undergoing clinical evaluation (17, 39, 42). This drug is efficacious, safe, and affordable and thus is likely to become an alternative to Coartem. PQ is a bisquinoline derivative consisting of two linked CQ molecules. Although reports indicate that PQ retains potency against CQ-resistant parasites (3), there is concern that PQ could become less susceptible against a backdrop of high CQ resistance (17, 22).

In this paper, we sought to analyze the in vitro activities of the antimalarials LM, DHA, and PQ in relation to polymorphisms in *pfcrt* at codon 76 (*pfcrt-76*) and in *pfmdr1* at codon 86 (*pfmdr1-86*) and in relation to *pfmdr1* copy number variations in Kenyan isolates. We used CQ as a reference drug.

MATERIALS AND METHODS

CQ was purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). LM, PQ, and DHA were gifts from Steve Ward, Liverpool School of Tropical Medicine, Liverpool, United Kingdom.

Parasite adaptation. *Plasmodium falciparum* parasites were collected from malaria patients as part of several clinical studies that took place between 2005 and 2008 in the Kilifi district of Kenya. From the field, blood samples were collected, placed in "transport medium" (containing RPMI medium and albumin), and taken to the laboratory for long-term culture adaption, and other isolates were cryopreserved in liquid nitrogen before adaption. The detailed description of this long-term parasite adaptation procedure is given elsewhere (32). Provision of informed consent for the original studies was ensured by the Kenya Medical Research Institute, Nairobi.

Chemosensitivity testing. Routine cultures were carried out in RPMI 1640 medium (Gibco BRL, United Kingdom) supplemented with 15% (vol/vol) normal human serum, 25 mM bicarbonate, 2 mM glutamine, 25 mM HEPES buffer, and 3.6 nM *para*-aminobenzoic acid. The human blood used to culture the parasites was obtained from healthy subjects and was washed three times with RPMI culture medium that was not supplemented with serum. CQ was dissolved in distilled water, and LM and PQ were dissolved in 90% methanol plus 10% HCl; DHA was dissolved in 70% ethanol. The ranges tested (in a threefold dilution) were 7,180 to 0.12 nM for CQ and PQ, 17,505 to 0.3 nM for LM, and 163 to 0.22 nM for DHA. Assays were carried out in 200 μl of culture containing 0.5% parasitemia and 1.5% hematocrit, in 96-well microtiter plates. Cultures were incubated at 37°C in a gas mixture of 90% N_2, 5% CO_2, and 5% O_2 for 66 h. Thereafter, [^{3}H]hypoxanthine was added to the culture for another 18 h. Cells were then lysed by freeze-thawing; radiolabeled DNA was harvested on fiberglass paper (TomTec, Inc., and Perkin-Elmer); and the amount of ionizing radiation was determined using a Wallac 1450 MicroBeta counter.

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▽ Published ahead of print on 21 September 2009.

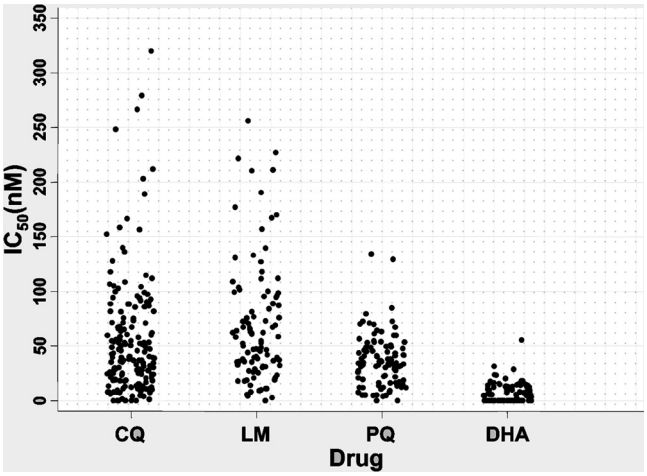

FIG. 1. In vitro activities of CQ, LM, PQ, and DHA against *Plasmodium falciparum* isolates.

Results were expressed as the drug concentration required for 50% inhibition of [^{3}H]hypoxanthine incorporation into parasite nucleic acid (IC_{50}), obtained by nonlinear regression analysis of the dose-response curve. We employed two reference *P. falciparum* laboratory strains: V1S, a multidrug-resistant strain, and 3D7, a drug-sensitive strain. IC_{50}s were measured two and four times for field isolates and reference strains, respectively.

Genotyping of *pfcrt* at codon 76 and of *pfmdr1* at codon 86. Blood samples (50 μl) of in vitro-adapted isolates were spotted onto filter paper, air dried, and stored in plastic bags with silica gel at ambient temperature. DNA was extracted by boiling, and single-base changes at *pfcrt-76* and *pfmdr1-86* were detected as reported previously (24).

Assessment of the copy number of *pfmdr1*. The *P. falciparum* reference isolates used in the analysis of *pfmdr1* copy numbers were DD2 (2 to 3 copies), D10 (1 copy), and 3D7 (1 copy). To estimate the copy numbers of *pfmdr1*, we used a relative-quantification TaqMan real-time PCR assay published elsewhere (29). In summary, the relative expression of *pfmdr1* in each isolate was compared with that of a housekeeping gene (β-tubulin) using specific fluorescently labeled probes (6-carboxyfluorescein and 2,7-dimethoxy-4,5-dichloro-6-caboxy-fluorescein) in a real time PCR assay. Every TaqMan run contained three calibrator DNA reference strains with known copy numbers. All assays were run in triplicate on an Applied Biosystems real-time PCR system, model 7500.

Statistical analysis. Statistical analyses were carried out using the Stata program, version 9 (Stata, Inc.). We compared differences between groups using the Kruskal-Wallis nonparametric test. We measured correlation using pairwise correlation analysis. All pairwise correlations of drug activity were estimated using log-transformed IC_{50}s. The level of significance was set at a P value of <0.05.

RESULTS

Chemosensitivity test. We first established the IC_{50}s of drugs against reference strains. Against V1S, the IC_{50}s of CQ, PQ, LM, and DHA were 158 ± 75, 42 ± 10, 24 ± 14, and 2 ± 1 nM, respectively; against 3D7, these values were 6.5 ± 2.3, 27 ± 17, 96 ± 12, and 2.0 ± 0.1 nM, respectively.

We have adapted 115 isolates for long-term culture and analyzed their drug chemosensitivity profiles. The median IC_{50}s of CQ, PQ, LM, and DHA were 41 nM (interquartile range [IQR], 18 to 73 nM), 32 nM (IQR, 17 to 46 nM), 50 nM (IQR, 29 to 96 nM), and 2 nM (IQR, 1 to 3 nM), respectively (Fig. 1). As expected, DHA was the most active drug, followed by PQ, CQ, and LM. Interestingly, LM IC_{50}s were >100 nM for about 20% of isolates (Fig. 1).

We have assessed the correlation between the drugs tested. LM activity correlated with DHA activity ($r^2 = 0.4$; $P < 0.0001$) and with PQ activity ($r^2 = 0.19$; $P = 0.05$). Interestingly, we observed an inverse relationship between the activities of CQ and LM ($r^2 = -0.26$; $P = 0.02$), suggesting that selection of LM resistance would be associated with an increase in CQ activity (Table 1).

TABLE 1. Correlation of in vitro antimalarial activities[a]

Drug 1	Drug 2	Correlation coefficient (r^2)	P
LM	**CO**	**−0.26**	**0.02**
LM	**PQ**	**0.19**	**0.05**
LM	**DHA**	**0.40**	**<0.0001**
PQ	CQ	0.16	0.13
PQ	DHA	0.04	0.69
DHA	CQ	0.14	0.16

[a] All pairwise correlations of drug activity were estimated using log-transformed IC_{50}s. Significant associations are shown in boldface.

***pfcrt-76*.** We also analyzed the *pfcrt-76* genotype in association with drug activity (Fig. 2). Based on CQ IC_{50}s, isolates can be classified into three distinct groups. For the first group, which has wild-type *pfcrt-76* ($n = 31$), the median IC_{50} of CQ is 13 nM (IQR, 10 to 15 nM). The second group is composed of mixed-genotype (wild-type and mutant) isolates ($n = 11$), for which the median CQ IC_{50} is 21 nM (IQR, 15 to 35 nM). The third group, which forms the majority of isolates ($n = 70$), consists of *pfcrt-76* mutants for which the median CQ IC_{50} is 57 nM (IQR, 46 to 80 nM). These differences are significant ($P < 0.001$) (Fig. 2). Based on the *pfcrt* genotype, more than 60% of isolates are resistant to CQ.

Median PQ IC_{50}s were very similar for *pfcrt-76* mutant (38 nM [IQR, 20 to 58 nM]) ($n = 39$) and wild-type (35 nM [IQR, 18 to 56 nM]) ($n = 23$) parasites. However, the median LM IC_{50} was lowest (43 nM [IQR, 27 to 90 nM]) for the *pfcrt-76* mutant group ($n = 40$), intermediate (56 nM [IQR, 42 to 84 nM]) for the mixed-genotype group ($n = 9$), and highest (67 nM [IQR, 48 to 111 nM]) for wild-type parasites ($n = 23$); these differences were statistically significant ($P = 0.03$) (Fig. 2). Thus, wild-type *pfcrt-76* parasites were associated with decreased susceptibility to LM, in line with the inverse relation-

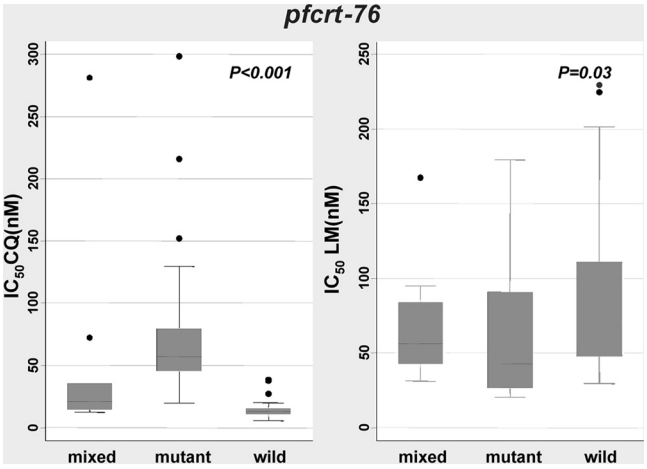

FIG. 2. Relationship between CQ and LM IC_{50}s and the *pfcrt* genotype at codon 76.

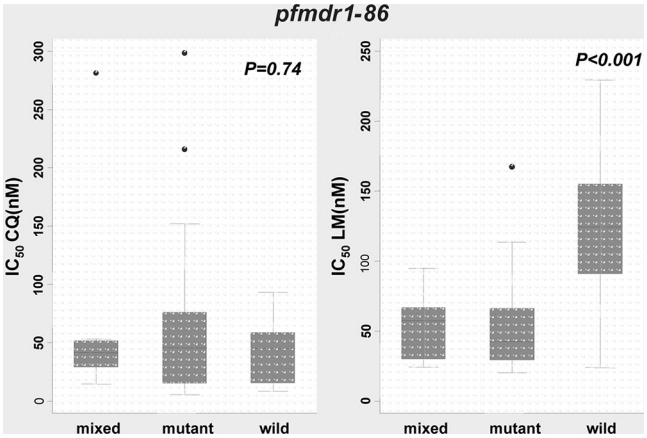

FIG. 3. Relationship between CQ and LM IC_{50}s and the *pfmdr1* genotype at codon 86.

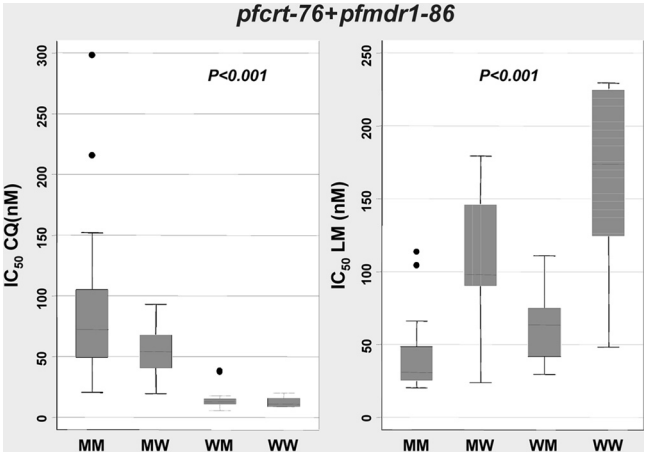

FIG. 4. Relationship between CQ and LM IC_{50}s and the *pfcrt-76* and *pfmdr1-86* genotypes at codons 76 and 86, respectively. MM, mutant *pfcrt-76* and *pfmdr1-86*; MW, mutant *pfcrt-76* and wild-type *pfmdr1-86*; WM, wild-type *pfcrt-76* and mutant *pfmdr1-86*; WW, wild-type *pfcrt-76* and *pfmdr1-86*.

ship with CQ resistance. DHA IC_{50}s did not differ as a function of the *pfcrt-76* genotype.

***pfmdr1-86*.** Median CQ IC_{50}s were similar for wild-type and mutant *pfmdr1-86* parasites (Fig. 3). However, the median DHA IC_{50} was higher (though marginally so) for *pfmdr1* wild-type isolates ($n = 23$) than for mutant isolates ($n = 47$) (3 nM [IQR, 3 to 4 nM] versus 2 nM [IQR, 1 to 3 nM]; $P = 0.04$). Interestingly, as in the case of *pfcrt-76*, the median LM IC_{50} was highest (124 nM [IQR, 90 to 155 nM]) for *pfmdr1-86* wild-type parasites ($n = 17$), intermediate (57 nM [IQR, 30 to 67 nM]) for mixed genotypes ($n = 8$), and lowest (43 nM [IQR, 29 to 66 nM]) for mutant isolates ($n = 47$); these differences were statistically significant ($P < 0.001$) (Fig. 3). Thus, *pfmdr1-86* contributes significantly to reduced susceptibility to LM.

***pfcrt-76* and *pfmdr1-86*.** We also investigated the effects of both *pfcrt-76* and *pfmdr1-86* mutations in relation to drug activity. To do so, we excluded parasites with mixed genotypes (at *pfcrt-76* or *pfmdr1-86*). Parasites were classified as *pfcrt-76* mutant and *pfmdr1-86* mutant (M-M), *pfcrt-76* mutant and *pfmdr1-86* wild type (M-W), *pfcrt-76* wild type and *pfmdr1-86* mutant (W-M), or wild type at both genes (W-W). The median CQ IC_{50} for parasites with the M-M genotype ($n = 45$) was significantly higher (72 nM [IQR, 49 to 105 nM]) than those for all others; the next highest median CQ IC_{50} (54 nM [IQR, 41 to 68 nM]) was that for M-W parasites ($n = 15$), and, as expected, the lowest CQ IC_{50}s (13 nM [IQR, 11 to 15 nM] and 11 nM [IQR, 9 to 15 nM]) were observed for W-M ($n = 21$) and W-W ($n = 6$) parasites, respectively (Fig. 4). Likewise, the median LM IC_{50} for M-M parasites ($n = 25$) was significantly lower (31 nM [IQR, 25 to 48 nM]) than those for M-W (99 nM [IQR, 90 to 146 nM]) ($n = 11$) and W-M (63 nM [IQR, 41 to 75 nM]) ($n = 17$) parasites. The highest median LM IC_{50}, 173 nM (IQR, 124 to 224 nM), was observed for W-W parasites ($n = 6$). These observations were statistically significant ($P < 0.001$) (Fig. 4). Thus, as expected, the presence of mutant genotypes in *pfcrt76* and *pfmdr186* is strongly associated with CQ resistance but also with increased susceptibility to LM.

Analysis of *pfmdr1* copy number. To gain more insight into the mechanism of decreased susceptibility to LM, we analyzed *pfmdr1* copy number variations in association with drug chemosensitivity profiles. We used the reference strain Dd2, which has 3 *pfmdr1* copies, though 2 copies have also been reported (5, 41), and the single-copy-number strains D10 and 3D7. Since we did not know the exact copy number for our Dd2 clone, we classified our data as isolates with 1 copy versus those with more than 1 copy. We eliminated data where threshold cycle (C_T) values were >35 and $\Delta\Delta C_T$ values were >1.5.

All 78 isolates analyzed for *pfmdr1* amplification were estimated to have one single copy of *pfmdr1*. Thus, no association could be established between *pfmdr1* amplification and drug activities.

DISCUSSION

Coartem has been adopted in many parts of Africa, including Kenya, as the first-line treatment against malaria. We have generated baseline information on the in vitro susceptibilities of *P. falciparum* isolates in Kilifi, an area of endemicity on the Kenyan coast, to LM and DHA. LM IC_{50}s are around 50 nM for most of the isolates; however, it is noteworthy that IC_{50}s for more than 20% of the isolates are >100 nM. The in vitro activity of LM against field isolates from several areas where malaria is endemic has been investigated using the WHO microtest. In all these studies, LM activity was high, with IC_{50}s of <30 nM for more than 95% of isolates (1, 4, 16, 21, 25, 26). In our study, we observed a higher LM IC_{50} range, and IC_{50}s of >200 nM for some isolates, findings similar to those of a report from Senegal (27).

Previous reports have indicated that the use of Coartem is associated with the selection of parasites with wild-type *pfmdr1-86*, which are tolerant of low LM concentrations (7, 15, 36–38). Our data clearly indicate that the presence of wild-type *pfmdr1-86* is associated with an increase in the LM IC_{50} (reduced susceptibility) and that this increase is more pronounced for parasites harboring a wild-type genotype at *pfcrt-76*, in line with a recent report (37). Since this genotype is associated with CQ susceptibility, it is not surprising that an inverse relationship

exists between CQ and LM activity, a feature that has been reported in other areas where malaria is endemic (27, 30).

We also investigated variation in the copy number of *pfmdr1*. There was no evidence of *pfmdr1* amplification in our isolates. This is consistent with previous data showing that *pfmdr1* amplification is a relatively rare event in African isolates compared with those in Southeast Asia (14, 36, 37, 40, 41); the high rate of parasites with more than 1 *pfmdr1* copy in Southeast Asia is likely due to the use of MFQ (23, 28, 29, 33). In Africa, MFQ is not used routinely for malaria treatment. Thus, under these conditions, one would expect parasites with multiple *pfmdr1* copies to be rare in Africa. In Southeast Asia, reduced LM susceptibility has been associated with an increase in the *pfmdr1* copy number (30); thus, it is possible that the continuous use of Coartem would select for parasites with increased *pfmdr1* copy numbers.

The significant decrease in LM susceptibility in vitro for parasites with wild-type *pfmdr1* and *pfcrt* genotypes that we report here is in line with previous work showing the emergence of LM-tolerant parasites after a single use of Coartem (7, 13, 15, 20, 36–38). However, it should be borne in mind that Coartem is effective at treating malaria, with high success rates (>90 to 95%), in many areas where malaria is endemic, including Kenya (11, 12, 18, 42). Thus, the mechanisms of Coartem resistance in vivo will likely involve other genes. Nevertheless, we propose that an increase in the frequency of parasites that are wild type for both *pfcrt-76* and *pfmdr1-86* (and probably in the *pfmdr1* copy number) in the population would be the first step in the selection of resistance to LM and that these parasites will form the backdrop for LM resistance and thus for Coartem resistance.

Artekin is now undergoing clinical evaluation and is likely to become an alternative to Coartem (17, 39, 42). PQ is active against Kilifi isolates, with IC_{50}s falling between 2 and 132 nM, and IC_{50}s of >100 nM were found for only two of the isolates tested. Similar results were obtained for isolates from other areas of malaria endemicity (2, 3, 6). We also demonstrate that polymorphism in *pfcrt-76* and *pfmdr1-86* is not associated with decreased susceptibility to PQ. However, a recent study using transgenic parasite lines has indicated that the presence of the *pfcrt* mutant decreases susceptibility to PQ in vitro (22), and in vivo, Artekin efficacy may be reduced in an area of high CQ resistance in Southeast Asia (17). In Africa, however, this drug remains effective, probably due to the lower level of CQ resistance in Africa than in Southeast Asia (17, 39, 42).

We have also generated data on the activity of DHA. As expected, this drug is very active against *P. falciparum*; most IC_{50}s are <25 nM. Our data indicate that wild-type *pfmdr1-86* parasites are less susceptible to DHA than mutant parasites (although this difference is marginal), in line with previous reports (9, 10, 31, 34). Likewise, previous investigations have indicated that an increase in the *pfmdr1* copy number and the presence of wild-type *pfcrt* genotypes are associated with reduced artemisinin susceptibility (20, 29, 33, 35). If this feature is translated in vivo, the emergence of LM resistance could be associated with a decrease in DHA efficacy. However, it remains to be seen whether the same would happen in vivo.

In conclusion, using laboratory-adapted African isolates, we have provided data showing that parasites that are less susceptible to LM in vitro are predominantly *pfmdr1* and *pfcrt* wild type. We propose that such parasites would form a backdrop on which LM resistance may thrive. Since Coartem has been rolled out in Africa, it is therefore critical to monitor LM and DHA activity in vitro and polymorphism in *pfcrt* and *pfmdr1*.

ACKNOWLEDGMENTS

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

This study was supported by the European Developing Countries Clinical Trials Partnership (EDCTP), The Wellcome Trust (WT077092 and WT084538), and The Global Health Research Award (GHRA-06-03) from the Health Research Board (Ireland) to Angus Bell and A.N. L.M. is an EDCPT Ph.D.-funded student. S.B. is funded through a Junior Group grant (SFB544, A7) by the Deutsche Forschungsgemeinschaft (DFG).

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