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Ex Vivo Responses of *Plasmodium falciparum* Clinical Isolates to Conventional and New Antimalarial Drugs in Niger

Mariama Issaka,[a] Adamou Salissou,[a] Ibrahim Arzika,[a] Julia Guillebaud,[a] Abani Maazou,[b] Sabine Specht,[c] Halima Zamanka,[a] Thierry Fandeur[a,d]

Unité de Parasitologie, Centre de Recherche Médicale et Sanitaire, Niamey, Niger[a]; Programme National de Lutte contre le Paludisme, Ministère de la Santé, Niamey, Niger[b]; Institute for Medical Microbiology, Immunology & Parasitology, University Hospital of Bonn, Bonn, Germany[c]; Unité de Parasitologie Médicale, Centre International de Recherches Médicales de Franceville, Franceville, Gabon[d]

Little is known about resistance of *Plasmodium falciparum* to antimalarials in Sahelian countries. Here we investigated the drug susceptibilities of fresh isolates collected in Niger post-deployment of artemisinin-based combination therapies (ACTs). We found that the parasites remained highly susceptible to new (dihydroartemisinin, lumefantrine, pyronaridine, and piperaquine) and conventional (amodiaquine and chloroquine) antimalarial drugs. The introduction of ACTs in 2005 and their further deployment nationwide have therefore not resulted in a decrease in *P. falciparum* susceptibilities to these antimalarials.

The WHO estimates that 50% of the world's population is exposed to malaria. In 2010, 216 million cases and more than 650,000 deaths from malaria were reported. Despite a decrease in the number of confirmed cases in some parts of the world, the situation remains heterogeneous and worrying in Africa (1). Together with respiratory infections and diarrheal diseases, malaria is one of the leading causes of death in Niger. Malaria transmission rates are high, with a mean incidence of 80 cases per 1,000 inhabitants. Despite the complementary nature of interventions in the field, which have strengthened in recent years, the number of cases has steadily risen over the last 20 years, reaching three million in 2010 (2, 3). There are several reasons for the deterioration of the public health situation with regard to malaria, and the increase in resistance to antimalarial drugs is considered a key factor. In Niger, infections are currently treated with combinations of drugs including an artemisinin (ART) derivative (artemisinin-based combination treatment, or ACT) (4). Since 2005, ACT has been proposed as the first-line treatment for the management of uncomplicated malaria. The use of such treatments throughout Niger was greatly expanded in 2010 by the implementation of a Global Fund Affordable Malaria Facility mechanism (5). Thereby, the drug pressure exerted on *Plasmodium falciparum* increases the risk of selection of parasites with altered susceptibility to antimalarials. This seems to be inevitable, as demonstrated by recent observations in Asia, which have revealed the presence of parasites less sensitive to artemisinins (6). The risk of emerging resistance to ACT makes it necessary to monitor the susceptibilities of parasites to antimalarial drugs, particularly those used in combination with artemisinin derivatives, on a regular basis and to search for new molecules with antimalarial activity.

In this context, a study was carried out in Niger in 2011, at Gaya in the Dosso region, 250 km south of Niamey (3.44°N, 11.9°E), to evaluate the response of *P. falciparum* isolates to lumefantrine (LUM) and amodiaquine diphosphate (AQ), both of which are widely used in the ACTs distributed in Niger. In addition, responses to alternative molecules, such as pyronaridine (PYD) and piperaquine (PIP), both currently not available in Niger, as well as dihydroartemisinin (DHA) and chloroquine (CQ), were investigated. *P. falciparum* isolates were obtained from 89 symptomatic children (<5 years old) with uncomplicated *P. falciparum* malaria (50.8% girls and 49.2% boys). The study protocol was approved by the National Ethics Committee for Health Research of the Nigerien Ministry of Health. The susceptibilities of the Nigerien isolates to drugs were assessed in a classical isotopic (48-h) test (7). Each isolate (5% hematocrit and 0.1 to 1% parasitemia) was tested in duplicate in microplates that were precoated with serial dilutions of drugs. Parasite growth was assessed by measuring the incorporation of [^{3}H]hypoxanthine (0.5 μCi/well), as previously described (8). The results of the *in vitro* assay are expressed as the 50% inhibitory concentration (IC_{50}) or geometric mean IC_{50} ($GMIC_{50}$). The predosed plates were prepared monthly and stored at −20°C until use. Their suitability for *in vitro* testing was regularly monitored using the reference strains 3D7 Africa and W2 Indochina, maintained in continuous culture and presenting known responses to the various drugs tested. Mean IC_{50}s were 1.8 to 2.4 nmol · liter^{-1} for DHA, 5.6 to 20 nmol · liter^{-1} for LUM, 17.4 to 43.1 nmol · liter^{-1} for AQ, 6.3 to 76.1 nmol · liter^{-1} for PYD, 10.5 to 33.0 nmol · liter^{-1} for PIP, and 11.4 to 230 nmol · liter^{-1} for CQ, respectively, for the 3D7 and W2 parasites. The cutoff IC_{50}s for *in vitro* resistance to CQ, DHA, LUM, and AQ were 100, 10.5, 150, and 60 nmol · liter^{-1}, respectively (9, 10). These thresholds allow classification of isolates as resistant or sensitive, and variations in antimalarial susceptibilities over time and between areas of endemicity can be followed. They were established using reference strains and clinical isolates. Since the cutoff IC_{50}s for PYR and PIP have not been determined, the threshold for reduced *in vitro* susceptibility to these drugs was defined statistically (>2 standard deviations [SD] above the mean).

The mean (95% confidence interval [CI]) age (in months) of the patients included in the *in vitro* study was 58.8(50.6 to 67). The geometric mean (95% CI) parasitemia (percent infected red blood

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Address correspondence to Thierry Fandeur, thierryfandeur@yahoo.fr.

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TABLE 1 *In vitro* susceptibilities of fresh Nigerien isolates of *Plasmodium falciparum* to new and conventional antimalarial drugs[a]

Antimalarial	No. of isolates tested	$GMIC_{50}$ (nM)	95% CI	IC_{50}				Cut-off (nM)	R/S	% R
				Min	Max	Q1 25%	Q3 75%			
DHA	37	2.9	2.2–3.5	0.7	7.3	1.8	4.6	10.5	0/37	0
LUM	33	7.1	3.9–10.3	0.2	44.3	3.9	14.7	150	2/33	0
PIP	34	24.2	0.3–48.0	2.5	311.5	12	59.6	150	2/34	6
CQ	29	12.7	1.9–23.4	2.1	144.7	8.6	18.9	100	2/29	7
AQ	26	17.3	11.9–22.6	6.9	64.2	11.3	24.6	60	1/26	4
PYD	34	9.8	7.7–11.9	4.7	32.9	6.5	14.3	20	1/34	3

[a] Dihydroartemisinin (DHA) (D7439), pyronaridine (PYD) (P0049), chloroquine (CQ) (C6628), and ammodiaquine diphosphate (AQ) (A2799) were obtained from Sigma Chemicals. Piperaquine (PIP) was obtained from Sigma Tau, and lumefantrine (LUM) was obtained from Novartis. The final concentrations ranged from 0.02 to 200 nM for DHA, from 1.95 to 2,000 nM for AQ and PIP, from 2.44 to 2,500 nM for CQ, and from 4.88 to 5,000 nM for PYD. $GMIC_{50}$, geometric mean IC_{50}; Min, minimum; Max, maximum; Q1 25%, 1st quartile; Q2 75%, 3rd quartile; R/S, no. resistant/no. susceptible (according to the *in vitro* cutoff); % R, percent resistant.

cells) at admission was 2.9 (0.5 to 5.3). Results were interpretable for at least one of the drugs tested for 37 (41.6%) of the 89 fresh isolates tested. The low yield for the *in vitro* assay observed for 52 isolates may have been due to suboptimal storage and transportation conditions of parasitized blood or to the presence of traces of the drug in the samples, rather than limitations of the technical approach.

The *in vitro* responses obtained with samples from Niger are shown in Table 1. All isolates reacted in a satisfactory manner to DHA ($GMIC_{50}$ of 2.9; 95% CI, 2.2 to 3.5), with an IC_{50} of <10.5. Our data can be compared with those found earlier for clinical isolates collected in Cameroon and in other African countries in 1992 and 2002, before ACTs were deployed: the $GMIC_{50}$ values reported are 1.29 nM · $liter^{-1}$ for DHA and 3.46 to 5.66 nM · $liter^{-1}$ for various artemisinin derivatives (arteether, artemether, and artelinate) (11, 12). Thus, after several years of extensive use of artesunate plus amodiaquine and of artemether plus lumefantrine as first-line treatments for *P. falciparum* malaria in Niger, there is no evidence of a decline in sensitivity to artemisinin. Our data are similar to those reported in Senegal and Congo and are consistent with findings of studies indicating a lack of decrease in sensitivity to artesunate in Gabon, Senegal, and Djibouti despite the extensive and growing use of ACT in these regions (12–18). A decrease in parasite susceptibility to artemisinin derivatives has been reported to date only in Southeast Asia along the border between Thailand and Cambodia. In this region, resistance is characterized by a decrease in susceptibility to both artemisinins and ACTs and by delayed parasite clearance times (6, 19). However, since decreases in sensitivity to ACT *in vivo* are difficult to measure *in vitro*, caution is necessary when trying to determine the clinical significance of the *in vitro* responses of parasites to artemisinin derivatives (20). The *P. falciparum ATPase6* (*PfATPase6*) S769N mutation, which has been described as a potential molecular marker for *P. falciparum* resistance to artemisinin in South America, was not observed in 92 samples collected in Niger in 2003 and 2006 (21, 22).

The ACT concept is based on the use of a combination of an artemisinin derivative and another antimalarial drug with a different mode of action and a slower clearance rate. Despite the danger signs concerning the emergence of resistance along the Thai-Cambodian border, mutual drug protection seems to have been successfully achieved in areas of higher endemicity, particularly in Africa, where *in vivo* and *in vitro* monitoring over the last decade has revealed no evidence of a decrease in the susceptibility of parasites to ACT (4, 23). The success of ACT depends almost entirely on the efficacy of the partner molecule. Our data show that isolates from Niger have remained highly susceptible to current partner drugs, with IC_{50}s ranging from 0.2 to 44.3 nmol · $liter^{-1}$ ($GMIC_{50}$, 7.1 nmol · $liter^{-1}$; 95% CI, 3.9 to 10.3 nmol · $liter^{-1}$) and from 6.9 to 64.2 nmol · $liter^{-1}$ ($GMIC_{50}$, 17.3 nmol · $liter^{-1}$; 95% CI, 11.9 to 22.6 nmol · $liter^{-1}$) for LUM and AQ, respectively. These values are lower than those reported in Cameroon a decade ago, where the $GMIC_{50}$ for LUM was 11.9 nm · $liter^{-1}$ and that for AQ was 29.0 nm · $liter^{-1}$ (12). The IC_{50} of LUM was <150 nmol · $liter^{-1}$ for all isolates. The IC_{50} of AQ was <60 nmol · $liter^{-1}$in all but one isolate (64.2 nmol · $liter^{-1}$). This finding is of particular concern, because the use of AQ-containing combinations has been shown to select for infections that are less responsive to AQ in Uganda (24). However, no clear relationship was observed between the treatment response and the *in vitro* IC_{50} of AQ in Gabon, and selection of this type does not seem to be occurring in Niger, while resistances to AQ have already been reported in some other African countries (25, 26). Similarly, no sign of a decrease in susceptibility to LUM has been detected. This observation is consistent with earlier molecular data showing the prevalence of mutations of *P. falciparum mdr1* (*Pfmdr1*) sequences to be low in isolates from Niger, particularly for the Y86N mutation, which is thought to confer a selective advantage on parasites, rendering them tolerant to LUM (22, 27). PYD and PIP have been developed as alternative antimalarial drugs for inclusion in ART-containing combinations for the treatment of multidrug-resistant malaria. PIP is a 4-aminoquinoline that has been in clinical use in China for decades, but few data have been published addressing its activity against African isolates *in vitro*. PYD, which was first synthesized in China in the 1970s, is a Mannich base structurally related to AQ. It has potent *in vitro* activity against erythrocyte stages of *P. falciparum* and is effective against CQ-resistant isolates (28). The IC_{50}s for PYD ranged from 4.7 to 32.9 nmol · $liter^{-1}$ ($GMIC_{50}$, 9.8 nmol · $liter^{-1}$; 95% CI, 7.7 to 11.9 nmol · $liter^{-1}$). The range of IC_{50}s for PIP was broader, extending from 2.5 to 311.5 nmol · $liter^{-1}$ ($GMIC_{50}$, 24.2 nmol · $liter^{-1}$; 95% CI, 0.3 to 48.0 nmol · $liter^{-1}$). The IC_{50}s of PIP were <150 nmol · $liter^{-1}$ for all but two isolates (279.9 and 311.5 nmol · $liter^{-1}$), whereas those of PYD were <20 nmol · $liter^{-1}$ in all but one isolate (32.9 nmol · $liter^{-1}$). These data demonstrate that the ACT components PIP and PYD have excellent antimalarial activity against Nigerien isolates. These activities are broadly similar to those reported in Africa (12–14, 18). In Niger, the first cases of CQ resistance *in vitro* were reported at the end of the 1980s. These data are ancient and concern only a small number of isolates. The few

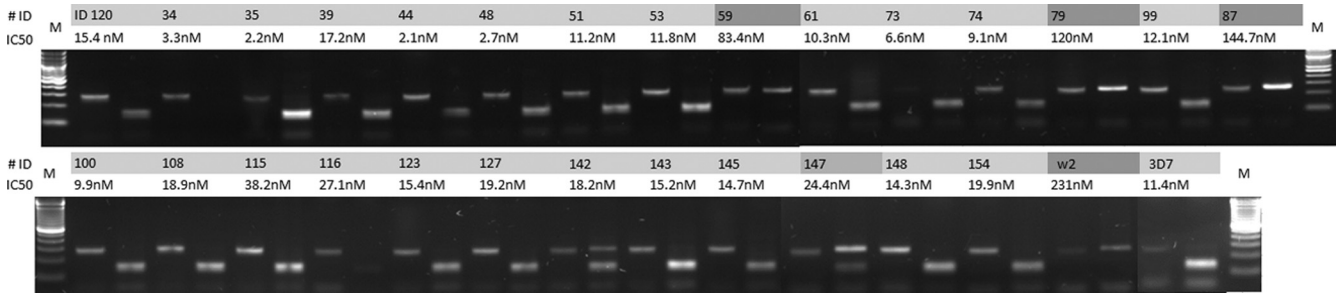

FIG 1 ApoI restriction digestion of *Pfcrt* fragments and genotype distribution among Nigerien isolates. Sample identification numbers (#ID) and the IC_{50} of CQ are indicated. For each sample, the first and second lanes correspond to undigested and ApoI-digested PCR products, respectively. M, SmartLadder SF (Eurogentec).

clinical trials carried out since then have confirmed the circulation of parasites resistant to this antimalarial drug but with an exceptionally low treatment failure rate, i.e., below 16% (29, 30). Consistent with these data, most of the Nigerien samples examined in the present study were found to respond adequately to CQ *in vitro* (CQ susceptible [CQS], 93%), with IC_{50}s ranging from 2.1 to 144.7 nmol · liter^{-1} ($GMIC_{50}$, 12.7 nmol · liter^{-1}; 95% CI, 1.9 to 23.4 nmol · liter^{-1}). Only two samples had IC_{50}s of >100 (120 and 144.7 nmol · liter^{-1}, respectively). There is a positive correlation between polymorphism at position 76 in the *P. falciparum crt* (*Pfcrt*) gene and the CQ-resistant (CQR) *in vitro* response. We therefore assessed the prevalence of the mutant 76T *Pfcrt* allele by PCR-restriction fragment length polymorphism (RFLP) (2 DNA samples were not available for typing) (31). Briefly, *crt* sequences (280-bp fragment) were amplified by conventional PCR with sense (5′CGACCTTAACAGATGGCTCA3′) and antisense (5′TTTGAATTTCCCTTTTTA TTTCCA3′) primers and digested with ApoI. Overall (Fig. 1), 23 samples harboring a wild-type K76 *Pfcrt* allele had IC_{50}s between 2.1 and 38.2 nmol · liter^{-1} ($GMIC_{50}$, 10.9 nmol · liter^{-1}; 95% CI, 7.5 to 14.3 nmol · liter^{-1}), whereas three others with a mutant 76T *Pfcrt* allele had significantly higher IC_{50}s (Mann-Whitney rank sum test, $P = 0.005$), ranging from 83.4 to 144.7 nmol · liter^{-1} ($GMIC_{50}$, 113.1 nmol · liter^{-1}; 95% CI, 78.3 to 147.9 nmol · liter^{-1}). One isolate (no. 147) with a mixed restriction pattern had an IC_{50} of 24.4 nmol · liter^{-1}. All CQS isolates other than no. 59 had wild-type *Pfcrt* K76; isolate no. 59 had an IC_{50} of 83 nmol · liter^{-1}. The two CQR isolates had a mutated *Pfcrt* 76T. Taken together, the *in vitro* and molecular data indicate a high level of susceptibility of Nigerien isolates to CQ. This observation must be viewed in light of the results of *in vivo* tests conducted in Niger a decade ago, for which a treatment success rate of about 85% was reported for CQ. The trend in 2011 (11% of isolates presenting a mutated codon 76) is therefore distinctly different from those reported for molecular studies carried out between 2003 and 2007 in Niger, in which the prevalence of *Pfcrt* 76T was reported to be between 32.4% and 50.8% (22, 32). However, given the limited number of isolates examined and the short time interval between these studies, it is too soon to draw any firm conclusions about a shift toward an improved susceptibility of parasites to CQ in Niger.

The degree of *in vitro* cross-reactivity in drug responses was assessed by pairwise correlation analyses of IC_{50}s. A significant, positive association between CQ IC_{50}s *in vitro* and those of PYD and DHA and between the responses to AQ and PYD was detected. No significant correlation was found between the IC_{50}s of the other antimalarial drugs (Table 2). However, the positive correlations observed are very weak (r^2 value between 0.36 and 0.5) and may suggest a shared mode of "resistance" to antimalarials rather than a common mechanism of action of the drugs. Consistent with this interpretation, studies with mammalian cells have shown that changes in *mdr* gene expression in response to toxic stress may have pleiotropic consequences, affecting the sensitivity to several drugs simultaneously.

An inverse relationship between the levels of malaria transmission and resistance was found, and the highly diverse epidemiological contexts of the various collection sites undoubtedly influence the results of resistance studies (33). The Gaya site, on the border with Benin, has one of the highest levels of malaria endemicity in Niger (34). This region belongs to the Sudanese zone, which is the most highly populated, and may not be representative of northern regions of Niger in terms of malaria endemicity. Nevertheless, our results indicate that the parasites of this part of Africa have retained a high degree of susceptibility to the new therapeutic combinations and, unexpectedly, to CQ. These observations are reassuring, because Niger has adopted ACTs as the first-line treatment for malaria, and AQ and LUM are the leading partner drugs used in ACTs in this region. These data support the continuing collaboration of the health authorities of Niger with the Global Fund and the strengthening of the AMFm

TABLE 2 Correlation coefficients for *in vitro* antimalarial drug susceptibilities of Nigerien isolates

Drugs compared	No. of isolates	Correlation of responses	
		r value	*P* value
LUM and AQ	20	0.006	0.97
CQ and PIP	27	0.051	0.8
LUM and CQ	22	0.08	0.72
DHA and LUM	24	0.081	0.7
DHA and PIP	27	0.133	0.57
PIP and PYD	25	0.166	0.43
LUM and PYD	25	0.202	0.33
DHA and PYD	27	0.246	0.22
LUM and PIP	20	0.254	0.28
AQ and PIP	24	0.285	0.18
DHA and AQ	22	0.304	0.17
AQ and CQ	23	0.358	0.09
DHA and CQ	24	0.415	0.04
CQ and PYD	25	0.429	0.03
AQ and PYD	23	0.496	0.02

(Affordable Medicines Facility—malaria) system established in this country in 2010 with the aim of making ACTs more accessible to the population (5), yet no official decision has been announced about whether and how to continue the program (35). Further investigations are required to confirm these preliminary observations, particularly *in vivo* using the WHO therapeutic efficacy test; indeed, it is necessary to check that the global trend observed by *in vitro* testing applies to *in vivo* conditions and to extend monitoring to northern sentinel sites. This will make it possible to take into account the possible effects of the malaria transmission level on both the nature and prevalence distribution of resistance to antimalarial drugs in Niger.

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