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Ex vivo anti-malarial drugs sensitivity profile of Plasmodium falciparum field isolates from Burkina Faso five years after the national policy change

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Abstract

Background: The recent reports on the decreasing susceptibility of Plasmodium falciparum to artemisinin derivatives along the Thailand and Myanmar border are worrying. Indeed it may spread to India and then Africa, repeating the same pattern observed for chloroquine resistance. Therefore, it is essential to start monitoring P. falciparum sensitivity to artemisinin derivatives and its partner drugs in Africa. Efficacy of AL and ASAQ were tested by carrying out an in vivo drug efficacy test, with an ex vivo study against six anti-malarial drugs nested into it. Results of the latter are reported here.

Methods: Plasmodium falciparum ex-vivo susceptibility to chloroquine (CQ), quinine (Q), lumefantrine (Lum), monodesethylamodiaquine (MDA), piperaquine (PPQ) and dihydroartemisinin (DHA) was investigated in children (6 months – 15 years) with a parasitaemia of at least ≥4,000/μl. The modified isotopic microtest technique was used. The results of cellular proliferation were analysed using ICEstimator software to determine the 50% inhibitory concentration (IC50) values.

Results: DHA was the most potent among the 6 drugs tested, with IC50 values ranging from 0.8 nM to 0.9 nM (Geometric mean IC50 = 0.8 nM; 95% CI [0.8 - 0.9]). High IC50 values ranged between 0.8 nM to 166.1 nM were reported for lumefantrine (Geometric mean IC50 = 25.1 nM; 95% CI [22.4 - 28.2]). MDA and Q IC50s were significantly higher in CQ-resistant than in CQ-sensitive isolates (P = 0.0001). However, the opposite occurred for Lum and DHA (P < 0.001). No difference was observed for PPQ.

Conclusion: Artemisinin derivatives are still very efficacious in Burkina Faso and DHA-PPQ seems a valuable alternative ACT. The high lumefantrine IC50 found in this study is worrying as it may indicate a decreasing efficacy of one of the first-line treatments. This should be further investigated and monitored over time with large in vivo and ex vivo studies that will include also plasma drug measurements.

Background

Artemisinin-based combination therapy (ACT) has been deployed worldwide and is currently the only available effective treatment for falciparum malaria [1-4]. Nevertheless, the recent reports on the decreasing susceptibility of Plasmodium falciparum to artemisinin derivatives along the Thailand and Myanmar border [5-8] and more

recently in Kenya [9] are worrying. Indeed, artemisinin-resistant malaria parasites at the border of Thailand and Myanmar may spread to India and then Africa, repeating the same pattern observed for chloroquine resistance [10]. It is, therefore, important to document the efficacy of currently used anti-malarials and provide early warnings that would allow an adequate response and the containment of resistance [11]. It is essential to start monitoring P. falciparum sensitivity to artemisinin derivatives and its partner drugs in Africa. This could be done by carrying out standard in vivo drug efficacy tests

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recommended by the World Health Organization (WHO). In addition, considering that there are no validated molecular markers related to artemisinins [6,8], ex vivo tests may have a role as several drugs, including those in a specific ACT, can be tested at the same time [12-15].

In Burkina Faso, a new malaria treatment policy was adopted in 2005 and was fully implemented in 2006; for uncomplicated falciparum malaria, artesunate-amodiaquine (ASAQ) or alternatively artemether-lumefantrine (AL) are recommended as first-line treatments, whereas quinine is recommended for severe malaria [16]. Efficacy of AL and ASAQ were tested by carrying out an in vivo drug efficacy test, with an ex vivo study against six anti-malarial drugs nested into it. Results of the latter are reported here.

Methods
Plasmodium falciparum field isolates
The study was carried out from December 2008 to December 2010 in Bobo-Dioulasso situated at 365 km from Ouagadougou. The rainy season occurs from June to October (average rainfall: 1,000 mm/year; mean temperature >25°C) and is followed by a cold dry season from November to February (minimum temperature 15°C) and a hot dry season from March to May. Malaria transmission is seasonal, from June to December. The commonest vectors are Anopheles gambiae s.s., Anopheles funestus and Anopheles arabiensis, and P. falciparum is the predominant malaria parasite [17]. All children (6 months-15 years) with fever (axillary temperature ≥ 37.5°C) or history of fever in the last 24 hours were screened for malaria infection. Children weighing 5 kg or more with a P. falciparum mono-infection at a density between 4,000 and 200,000/μl and haemoglobin > 7 g/dL were included in the study if their parent/guardian provided the informed consent. Venous blood (5 – 10 ml) was collected in a tube coated with EDTA (Turumo, Escap, Belgium) for ex vivo tests. This study was reviewed and approved by the Institutional Ethics Committee of Center Muraz, and was registered at ClinicalTrials.gov (ID: NCT00808951).

Drugs
The drugs tested were from the following sources: chloroquine diphosphate (Sigma Aldrich, St. Louis, USA), quinine hydrochloride (Sigma Aldrich, St. Louis, USA), lumefantrine (Novartis Pharma, Basel, Switzerland) piperaquine phosphate (Sigma Tau., Rome, Italy), the active metabolite of artemisinin, dihydroartemisinin (Sigma Tau., Rome, Italy) and the active metabolite of amodiaquine, monodeshethylamodiaquine obtained from the World Health Organization (WHO/TDR, Geneva, Switzerland). The stock solutions of chloroquine and monodesethylamodiaquine were prepared in sterile distilled water, in methanol for quinine and dihydroartemisinin, in ethanol for lumefantrine and in lactic

acid for piperaquine. A three-fold serial dilution of stock solutions was made. The final concentrations ranged from 12.5 nM to 3200 nM for chloroquine, 7.5 nM to 1920 nM for monodesethylamodiaquine, 13.02 nM to 3333 nM for quinine, 1.25 nM to 320 nM for lumefantrine, 6.25 nM to 1600 nM for piperaquine and 0.25 nM to 64 nM for dihydroartemisinine. The distribution in the plates was done at 50 μl per well.

Ex vivo assay
Drug sensitivity tests were performed within 24 hours of bleeding, without culture adaptation. Blood samples were washed three times in RPMI 1640 medium (Sigma Aldrich, St. Louis, USA). The modified isotopic microtest technique [14] was used. The complete RPMI 1640 medium, i.e. supplemented with 10% of human serum type AB (obtained from the blood bank of Souro Sanon University hospital, Bobo-Dioulasso, Burkina Faso), gentamicin (10 μg/ml) and tritiated hypoxanthine (Amersham, little Chalfont, UK), was used for parasites cultivation. Infected erythrocytes were suspended in this medium at a haematocrit of 1.5% and an initial parasitaemia between 0.1% and 0.5%. This suspension was then added in quantities of 200 μl per well to the plates containing the drugs. These were incubated for 48 hours at 37°C in 5% CO_2. After the incubation period, plates were frozen and thawed to lyse the blood cells. Cultures were then harvested (Harvester Unifilter 96, Packard) on fibre glass paper (reader plates Unifilter 96 Perkin Elmer). The strips obtained were dried and transferred to a plastic bag containing 30 μl of scintillation fluid (Perkin Elmer Betaplate Scint, Wallac). The incorporation of tritiated hypoxanthine was measured using a scintillation Beta counter (Perkin Elmer Wallac MicroBeta Trilux, Turku, Finland).

Data analysis and statistical methods
For parasites growth assays, the results of cellular proliferation were expressed as counts per minute and analysed using ICEstimator software to determine the 50% inhibitory concentration (IC_{50}) values [18]. The IC_{50} is defined as the drug concentration able to inhibit 50% of the uptake of ^3H hypoxanthine as compared to that measured in drug-free control wells. The threshold IC_{50} for ex vivo resistance was defined at ≥ 100 nM for chloroquine, ≥ 60 nM for monodesethylamodiaquine and ≥ 800 nM for quinine [19,20]. The thresholds for lumefantrine, piperaquine and dihydroartemisinin are not well established yet. Data were entered in Excel version 97 and analysed using Stata version 8.0. Results were expressed as geometric mean IC_{50} values and the 95% confidence intervals were computed. Correlation of the IC_{50} values for different drugs (2 by 2) was calculated using Spearman rank-order correlation test. The activity of monodesethylamodiaquine, quinine piperaquine,

lumefantrine and dihydroartemisinin against chloroquine-resistant isolates and chloroquine-sensitive isolates were compared using the IC_{50} geometric means (GM). A p value <0.05 was considered statistically significant.

Results

Ex vivo susceptibility of *P. falciparum* isolates was tested for 440 samples. The culture success rate (interpretable tests) and the IC_{50} geometric means of the 6 drugs tested are summarized in Table 1. The average culture success rate was around 85% (range: 79.3% - 86.8%). Out of 382 samples successfully tested against chloroquine (Geometric mean IC_{50} = 69.2 nM; 95% CI [60.6 - 79.1]) and quinine (Geometric mean IC_{50} = 162.1 nM; 95% CI [148.3 - 177.3]), 161 (42.1%) were resistant to chloroquine while only 4 (1%) were resistant to quinine. Out of 377 samples successfully tested against monodeshethyla-modiaquine (Geometric mean IC_{50} = 19.3 nM; 95% CI [18.0 - 20.6]), 24 (6.4%) were resistant. The IC_{50} values for lumefantrine ranged between 0.8 nM to 166.1 nM (Geometric mean IC_{50} = 25.1 nM; 95% CI [22.4 - 28.2]) and that for piperaquine from 0.8 nM to 375.2 nM (Geometric mean IC_{50} = 6.3 nM; 95% CI [5.9 - 6.8]). Dihydroartemisinin was the most potent among the six drugs tested, with IC_{50} values ranging from 0.8 nM to 0.9 nM (Geometric mean IC_{50} = 0.8 nM; 95% CI [0.8 - 0.9]). However, three isolates had much higher IC_{50}, i.e. 19 nM, 21 nM and 38 nM (Figure 1).

The mean IC_{50} of the tested drugs were analysed by the parasites' susceptibility to chloroquine, i.e. chloroquine-resistant (IC50 ≥ 100 nM) against chloroquine-sensitive isolates (IC50 < 100 nM). Monodesethylamodiaquine and quinine IC_{50} mean values were significantly higher in chloroquine-resistant than in chloroquine-sensitive isolates (P = 0.0001) (Table 2). However, the opposite occurred for lumefantrine and dihydroartemisinin; their IC_{50} mean values were significantly higher in chloroquine-sensitive than in chloroquine-resistant isolates (P ≤ 0.001). No difference was observed for piperaquine (P = 0.382).

Cross-resistance between the six drugs is summarized in Table 3. A significant positive correlation (by ascending order) was found between monodeshetlyamodiaquine-piperaquine (r = 0.14; P = 0.008), dihydroartemisinin-quinine (r = 0.15; P = 0.002), dihydroartemisinin- piperaquine (r = 0.27; P < 0.0001), dihydroartemisinin-lumefantrine (r = 0.30; P < 0.0001), quinine - lumefantrine (r = 0.32; P < 0.0001), chloroquine-quinine (r = 0.51; P < 0.0001), monodeshetlyamodiaquine-quinine (r = 0.52; P < 0.0001) and chloroquine-monodeshetylamodiaquine (r = 0.86; P < 0.0001). For chloroquine-lumefantrine (r = − 0.10; P = 0.03) and monodeshetylamodiaquine-lumefantrine (r = − 0.11; P = 0.02) the correlation was significant but negative while for the other pair-wise comparisons no significant correlation was found.

Discussion

Since the policy change in 2005 in Burkina Faso, several studies on the therapeutic efficacy of both ASAQ and AL were carried out [21-25]. Nevertheless, the *ex vivo* susceptibility of *P. falciparum* to the different components of ACT had never been tested and this is the first study out of this kind. The prevalence of chloroquine resistant isolates (CQR) was higher than that against other drugs but lower than that reported in the same area in 2006 and estimated at 50% (Lea Bonkian, personal communication), suggesting that CQ resistance may be decreasing, possibly following the implementation of the new anti-malarial drug policy based on ACT. This is plausible when considering that a similar phenomenon has been observed in Malawi where, nine years after the withdrawn of CQ and its replacement with sulphadoxine-pyrimethamine, no CQR was found [26].

Despite withdrawal of chloroquine, CQR could persist due to the use of treatments with similar chemical structure, resulting in a strong selective pressure [27]. The positive correlation between *ex vivo* IC_{50} values of CQ and MDA or quinine and between quinine and AQ indicate cross-resistance and may explain the still high prevalence of CQR found in this study. Such cross-

Table 1 *Ex vivo* susceptibility of *Plasmodium falciparum* isolates against chloroquine, monodeshethylamodiaquine, quinine, lumefantrine and dihydroartemisinin

	Culture success rate % (n/N)	IC50 mean (nM) [95% CI]	Range (nM)		Resistant isolates (%)
			Minimum	Maximum	
Chloroquine	86.8 (382/440)	69.2 [60.6 – 79.1]	8.3	595.9	161 (42.1)
Quinine	86.8 (382/440)	162.1 [148.3 – 177.3]	10.2	950.3	4 (1.0)
Monodesethylamodiaquine	85.7 (377/440)	19.3 [18.0 – 20.6]	0.8	595.9	24 (6.4)
Lumefantrine	86.8 (382/440)	25.1 [22.4 – 28.2]	0.8	166.1	NA
Piperaquine	79.3 (349/440)	6.3 [5.9 – 6.8]	0.8	375.2	NA
Dihydroartemisinin	86.5 (381/440)	0.8 [0.8 – 0.9]	0.1	38.8	NA

Resistance threshold defined for chloroquine at 100 nM, quinine at 800 nM and monodeshethylamodiaquine at 60 nM; NA: Non applicable as the threshold is not defined yet.
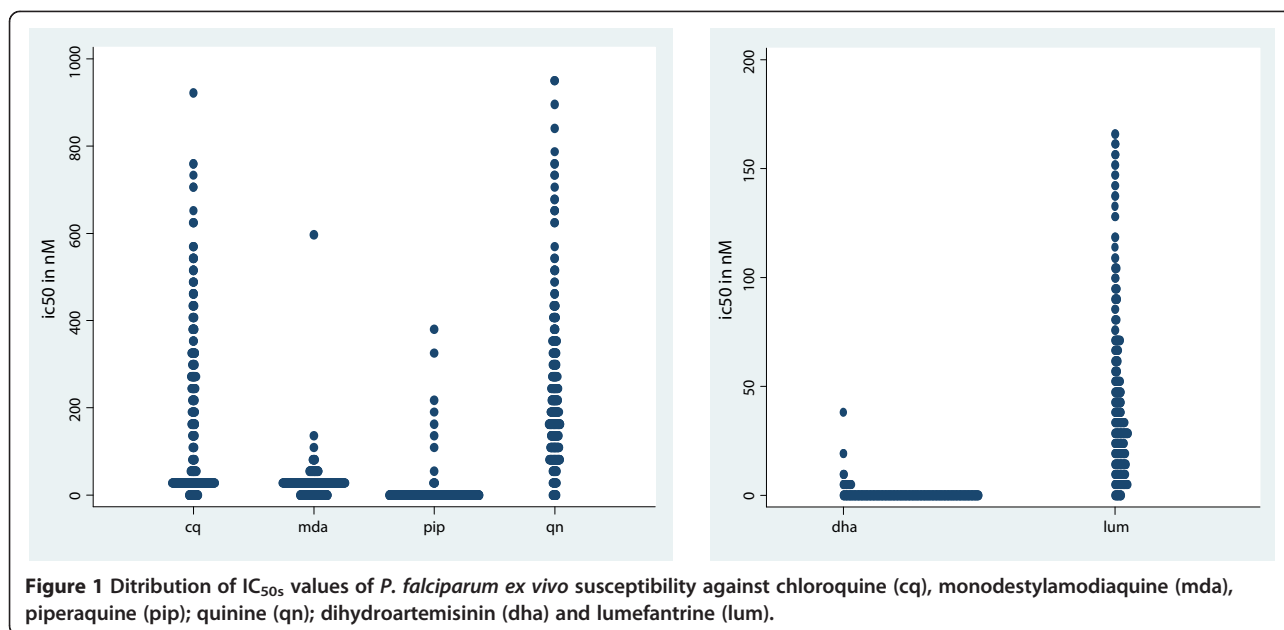
Figure 1 Ditribution of IC$_{50s}$ values of *P. falciparum ex vivo* susceptibility against chloroquine (cq), monodestylamodiaquine (mda), piperaquine (pip); quinine (qn); dihydroartemisinin (dha) and lumefantrine (lum).

resistance is not surprising as it has already been reported by several studies [20,28,29]. Nevertheless, the relationship between CQ and AQ efficacy is not a straightforward one as AQ may still be effective where CQ resistance is high [1,30-32]. This seems confirmed by the low prevalence of AQ resistant isolates as determined by our *ex vivo* test. Nevertheless, almost half of the isolates had AQ IC$_{50}$ values higher than 20 nM, a relatively high figure when considering that in Cameroun most isolates of recurrent infection after AQ treatment had IC$_{50}$ ranging between 25.6 and 115 nM, indicating that the threshold for AQ resistance might be lower than the standard value of ≥ 60 nM [19]. This raises the issue of defining appropriate drugs' *ex vivo* IC$_{50}$ thresholds able to predict *in vivo* outcomes [19,27,33,34].

In the new malaria treatment policy adopted in Burkina Faso, quinine is still the recommended treatment for severe malaria and for any treatment failure after administration of ASAQ or AL [16]. Only 4 isolates were found to be resistant to quinine, an extremely low prevalence when considering the potentially frequent use of this drug and cross resistance with CQ. Lowering the threshold for

resistance to 600 nM [28,35,36], increased the number of isolates classified as resistant but their prevalence remains low, i.e. 4% (17/382). Therefore, quinine can still be considered effective in Burkina Faso though recent reports of an increasing number of patients with recurrent infection after ACT treatment [21,24] may result in the frequent use of quinine as rescue treatment and higher drug pressure. There is the need of regularly monitoring the susceptibility of local isolates to quinine for the early detection of quinine resistance.

The resistance threshold for lumefantrine is not established yet but the mean IC$_{50}$ (25.1 nM) found in this study seems high compared to that (9.8 nM) reported by a study carried out in Senegal at approximately the same period [37]. Similarly, in Cameroon the mean IC$_{50}$ for lumefantrine was 11.9 nM in 1997 and 9.57 nM in 2003 [38]. Therefore, high lumefantrine IC$_{50}$ may indicate a decreasing susceptibility of local isolates to this drug, though baseline data are not available, possibly explained by the high AL use and hence drug pressure in this urban area. Indeed, though Burkina Faso has adopted two types of ACT as first-line treatments, ASAQ is

Table 2 *Ex vivo* IC$_{50}$ of *Plasmodium falciparum* isolates against monodeshethylamodiaquine, quinine, lumefantrine, piperaquine and dihydroartemisinin by chloroquine susceptibility

Drug	Chloroquine-resistant isolates (n = 161)	Chloroquine-sensitive isolates (n = 221)	P value
Chloroquine	289.8 [269.2 – 312.0)]	24.4 [22.8 – 26.1]	0.0001
Monodesethylamodiaquine	33.8 [31.4 – 36.4]	12.8 [12.0 – 13.6]	0.0001
Quinine	249.7 [223.3 – 279.1]	120.4 [107.2 – 135.2]	0.0001
Lumefantrine	21.9 [18.8 – 25.5]	27.8 [23.5 – 32.8]	0.0006
Piperaquine	6.5 [5.7 – 7.3]	6.1 [5.6 – 6.7]	0.382
Dihydroartemisinin	0.7 [0.6 – 0.8]	0.9 [0.8 – 1.0]	0.001

Table 3 Pairwise comparison of *ex vivo* IC$_{50}$ values

Drug pairs	r*	P - value
Chloroquine - Dihydroartemisinin	−0.08	0.09
Chloroquine - Monodestylamodiaquine	0.86	< 0.0001
Chloroquine – quinine	0.51	< 0.0001
Chloroquine - Piperaquine	0.02	0.63
Chloroquine - Lumefantrine	−0.10	0.03
Dihydroartemisinin- Monodestylamodiaquine	0.01	0.77
Dihydroartemisinin – Quinine	0.15	0.002
Dihydroartemisinin- Piperaquine	0.27	< 0.0001
Dihydroartemisinin - Lumefantrine	0.30	< 0.0001
Monodestylamodiaquine – Quinine	0.52	< 0.0001
Monodestylamodiaquine - Piperaquine	0.14	0.008
Monodestylamodiaquine - Lumefantrine	−0.11	0.025
Quinine - Piperaquine	0.07	0.16
Quinine – Lumefantrine	0.32	< 0.0001
Piperaquine - Lumefantrine	0.04	0.35

*Spearman's rank-order correlation coefficient (r).

mainly used in rural areas while AL in towns, including Bobo-Dioulasso where this study was carried out. This is also confirmed by informal discussions with local health practitioners who stated that they mostly prescribe AL for uncomplicated malaria. However, such hypotheses need to be confirmed by a well-planned survey. The high lumefantrine IC$_{50}$ could also be due to technical problems related to the execution of the *ex vivo* test. Indeed, lumefantrine is an amino alcohol and the drugs in this class are not easily soluble, a characteristic that may compromise the reproducibility of *ex vivo* test results [39]. Nevertheless, the use of the ethanol as solvent in this study should have addressed this problem so that a major bias related to the lumefantrine solubility is unlikely.

Dihydroartemisinin-piperaquine is one of the most recent ACT submitted for prequalification to the WHO [40] and represents an additional ACT for endemic countries, including Burkina Faso. Mean piperaquine IC$_{50}$ was extremely low, only two isolates had values above 200 nM, and similar to that observed in Uganda [41]. These results contrast with those found in Cameroon and Kenya, where the mean piperaquine IC$_{50}$ was 39 nM and 50 nM, respectively [42,43]. In addition, there was no correlation between piperaquine and chloroquine, lumefantrine and quinine IC$_{50}$s, a weak correlation with MDA, and piperaquine was equally active on both CQ sensitive and resistant isolates. All these elements support the use of dihydroartemisinin-piperaquine as an alternative ACT in Burkina Faso.

The mean dihydroartemisinin IC$_{50}$ was extremely low, indicating high susceptibility of local isolates, and in accordance with other studies carried out in sub-Saharan Africa [36,38,44]. In addition, dihydroartemisinin IC$_{50}$ was not correlated to that of MDA while the correlation with CQ IC$_{50}$ was a negative one, also shown by its higher activity against CQ resistant isolates as compared to sensitive ones. This is reassuring when considering the threat represented by the emergence of artemisinin resistance in South-East Asia where, besides a delay in parasite clearance, the *ex vivo* sensitivity of *P. falciparum* to artemisinin derivatives has declined substantially over the last few years [2,4-6,8,45,46], reaching in some regions mean IC$_{50}$ values as high 21.2 nM for dihydroartemisinin and 16.3 nM for artesunate [47]. Nevertheless, the interpretation of this results should also consider the weaknesses of the standard *ex vivo* tests, e.g. the 3H hypoxantine technique, in detecting artemisinin derivatives resistance [48]. Though in sub-Saharan Africa the *ex vivo* efficacy of artemisinin derivatives seems high, declining *in vivo* responsiveness of *P. falciparum* infections to ACT has been observed in Kenya [9].

Conclusion

In conclusion, this study confirms that artemisinin derivatives are still very efficacious and dihydroartemisinin-piperaquine seems a valuable alternative ACT in Burkina Faso. Similarly, both quinine and amodiaquine had a good sensitivity profile. The high lumefantrine IC$_{50}$ found in this study is worrying as it may indicate a decreasing efficacy of one the first line treatments. This should be further investigated and monitored over time with large *in vivo* and *ex vivo* studies that will include also plasma drug measurements.

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References

1. Zwang J, Olliaro P, Barennes H, Bonnet M, Brasseur P, Bukirwa H, Cohuet S, D'Alessandro U, Djimdé A, Karema C, Guthmann JP, Hamour S, Ndiaye JL, Mårtensson A, Rwagacondo C, Sagara I, Same-Ekobo A, Sirima SB, Van Den Broek I, Yeka A, Taylor WR, Dorsey G, Randrianarivelojosia M: **Efficacy of artesunate-amodiaquine for treating uncomplicated falciparum malaria in sub-Saharan Africa: a multi-centre analysis.** *Malar J* 2009, **8**:203.
2. World Health Organization: *Containment of Malaria Multi-Drug Resistance on the Cambodia-Thailand Border.* Switzerland, Geneva: Report of an Informal Consultation Phnom Penh; 2007:23.
3. World Health Organization: *Guidelines for the Treatment of Malaria.* 2nd edition. Switzerland, Geneva: WHO; 2010:166.
4. Maude RJ, Pontavornpinyo W, Saralamba S, Aguas R, Yeung S, Dondorp AM, Day NPJ, White NJ, White LJ: **The last man standing is the most resistant: eliminating artemisinin-resistant malaria in Cambodia.** *Malar J* 2009, **8**:31.
5. Rogers WO, Sem R, Tero T, Chim P, Lim P, Muth S, Socheat D, Ariey F, Wongsrichanalai C: **Failure of artesunate-mefloquine combination therapy for uncomplicated Plasmodium falciparum malaria in southern Cambodia.** *Malar J* 2009, **8**:10.
6. Noedl H, Se Y, Sriwichai S, Schaecher K, Teja-Isavadharm P, Smith B, Rutvisuttinunt W, Bethell D, Surasri S, Fukuda MM, Socheat D, Chan Thap L: **Artemisinin resistance in Cambodia: a clinical trial designed to address an emerging problem in Southeast Asia.** *Clin Infect Dis* 2010, **51**:82–89.
7. Lim P, Alker AP, Khim N, Shah NK, Incardona S, Doung S, Yi P, Bouth DM, Bouchier C, Puijalon OM, Meshnick SR, Wongsrichanalai C, Fandeur T, Le Bras J, Ringwald P, Ariey F: **Pfmdr1 copy number and artemisinin derivatives combination therapy failure in falciparum malaria in Cambodia.** *Malar J* 2009, **8**:11.
8. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ: **Artemisinin resistance in Plasmodium falciparum malaria.** *N Engl J Med* 2009, **361**:455–467.
9. Borrmann S, Sasi P, Mwai L, Bashraheil M, Abdallah A, Muriithi S, Frühauf H, Schaub B, Pfeil J, Peshu J, Hanpithakpong W, Rippert A, Juma E, Tsofa B, Mosobo M, Lowe B, Osier F, Fegan G, Lindegårdh N, Nzila A, Peshu N, Mackinnon M, Marsh K: **Declining responsiveness of Plasmodium falciparum infections to artemisinin-based combination treatments on the Kenyan coast.** *PLoS ONE* 2011, **6**:e26005.
10. Gharbi M, Flegg JA, Hubert V, Kendjo E, Metcalf JE, Bertaux L, Guérin PJ, Le Bras J, Aboubaca A, Agnamey P, Angoulvant A, Barbut P, Basset D, Belkadi G, Bellanger AP, Bemba D, Benoit-Vica F, Berry A, Bigel ML, Bonhomme J, Botterel F, Bouchaud O, Bougnoux ME, Bourée P, Bourgeois N, Branger C, Bret L, Buret B, Casalino E, Chevrier S, *et al*: **Longitudinal study assessing the return of chloroquine susceptibility of Plasmodium falciparum in isolates from travellers returning from West and Central Africa, 2000–2011.** *Malar J* 2013, **12**:35.
11. Sibley CH, Guerin PJ, Ringwald P: **Monitoring antimalarial resistance: launching a cooperative effort.** *Trends Parasitol* 2010, **26**:221–224.
12. Anderson T: **Mapping the spread of malaria drug resistance.** *PLoS Med* 2009, **6**:2.
13. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR: **Epidemiology of drug-resistant malaria.** *Lancet Infect Dis* 2002, **2**:209–218.
14. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD: **Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique.** *Antimicrob Agents Chemother* 1979, **16**:710–718.
15. Shretta R, Omumbo J, Rapuoda B, Snow RW: **Using evidence to change antimalarial drug policy in Kenya.** *Trop Med Int Health* 2000, **5**:755–764.
16. Gansané A, Nébié I, Soulama I, Tiono A, Diarra A, Konaté AT, Ouédraogo A, Sirima BS: **Change of antimalarial first-line treatment in Burkina Faso in 2005.** *Bull Soc Pathol Exot* 2009, **102**:31–35.
17. Tinto H, Zoungrana EB, Coulibaly SO, Ouedraogo JB, Traoré M, Guiguemde TR, Van Marck E, D'Alessandro U: **Chloroquine and sulphadoxine-pyrimethamine efficacy for uncomplicated malaria treatment and haematological recovery in children in Bobo-Dioulasso, Burkina Faso during a 3-year period 1998–2000.** *Trop Med Int Health* 2002, **7**:925–930.
18. *ICEstimator software.* http://www.antimalarial-icestimator.net, Version 1.
19. Basco LK, Ndounga M, Keundjian A, Ringwald P: **Molecular epidemiology of malaria in cameroon. IX. Characteristics of recrudescent and persistent Plasmodium falciparum infections after chloroquine or amodiaquine treatment in children.** *Am J Trop Med Hyg* 2002, **66**:117–123.
20. Ringwald P, Meche FS, Bickii J, Basco LK: **In Vitro culture and drug sensitivity assay of Plasmodium falciparum with nonserum substitute and acute-phase sera.** *J Clin Microbiol* 1999, **37**:700–705.
21. Four T: **A head-to-head comparison of four artemisinin-based combinations for treating uncomplicated malaria in African children: a randomized trial.** *PLoS Med* 2011, **8**:e1001119.
22. Siribié M, Diarra A, Tiono AB, Soulama I, Sirima SB: **Efficacité de l'artéméther-luméfantrine dans le traitement du paludisme simple de l'enfant en milieu rural au Burkina Faso en 2009.** *Bull Soc Path Exot* 2012, **105**:202–207.
23. Zongo I, Dorsey G, Rouamba N, Dokomajilar C, Séré Y, Rosenthal PJ, Ouédraogo JB: **Randomized comparison of amodiaquine plus sulfadoxine-pyrimethamine, artemether-lumefantrine, and dihydroartemisinin-piperaquine for the treatment of uncomplicated Plasmodium falciparum malaria in Burkina Faso.** *Clin Infect Dis* 2007, **45**:1453–1461.
24. Zongo I, Dorsey G, Rouamba N, Tinto H, Dokomajilar C, Guiguemde RT, Rosenthal PJ, Ouedraogo JB: **Artemether-lumefantrine versus amodiaquine plus sulfadoxine-pyrimethamine for uncomplicated falciparum malaria in Burkina Faso: a randomised non-inferiority trial.** *Lancet* 2007, **369**:491–498.
25. Barennes H, Nagot N, Valea I, Koussoubé-Balima T, Ouedraogo A, Sanou T, Yé S: **A randomized trial of amodiaquine and artesunate alone and in combination for the treatment of uncomplicated falciparum malaria in children from Burkina Faso.** *Trop Med Int Health* 2004, **9**:438–444.
26. Kublin JG, Cortese JF, Njunju EM, Mukadam RAG, Wirima JJ, Kazembe PN, Djimdé AA, Kouriba B, Taylor TE, Plowe CV: **Reemergence of chloroquine-sensitive Plasmodium falciparum malaria after cessation of chloroquine use in Malawi.** *J Infect Dis* 2003, **187**:1870–1875.
27. Tinto H, Rwagacondo C, Karema C, Mupfasoni D, Vandoren W, Rusanganwa E, Erhart A, Van Overmeir C, Van Marck E, D'Alessandro U: **In-vitro susceptibility of Plasmodium falciparum to monodesethylamodiaquine, dihydroartemisinin and quinine in an area of high chloroquine resistance in Rwanda.** *Trans R Soc Trop Med Hyg* 2006, **100**:509–514.
28. Ouédraogo JB, Dutheil Y, Tinto H, Traoré B, Zampa H, Tall F, Coulibaly SO, Guiguemdé TR: **In vitro sensitivity of Plasmodium falciparum to halofantrine compared with chloroquine, quinine and mefloquine in the region of Bobo-Dioulasso, Burkina Faso (West Africa).** *Trop Med Int Health* 2003, **3**:159–164.
29. Pradines B, Tall A, Parzy D, Spiegel A, Fusai T, Hienne R, Trape JF, Doury JC: **In-vitro activity of pyronaridine and amodiaquine against African isolates (Senegal) of Plasmodium falciparum in comparison with standard antimalarial agents.** *J Antimicrob Chemother* 1998, **42**:333–339.
30. Gorissen E, Ashruf G, Lamboo M, Bennebroek J, Gikunda S, Mbaruku G, Kager PA: **In vivo efficacy study of amodiaquine and sulfadoxine/pyrimethamine in Kibwezi, Kenya and Kigoma, Tanzania.** *Trop Med Int Health* 2000, **5**:459–463.
31. Brasseur P, Guiguemde R, Diallo S, Guiyedi V, Kombila M, Ringwald P, Olliaro P: **Amodiaquine remains effective for treating uncomplicated malaria in west and central Africa.** *Trans R Soc Trop Med Hyg* 1999, **93**:645–650.
32. Staedke SG, Kamya MR, Dorsey G, Gasasira A, Ndeezi G, Charlebois ED, Rosenthal PJ: **Amodiaquine, sulfadoxine/pyrimethamine, and combination therapy for treatment of uncomplicated falciparum malaria in Kampala, Uganda: a randomised trial.** *Lancet* 2001, **358**:368–374.
33. White NJ: **Why is it that antimalarial drug treatments do not always work?** *Ann Trop Med Parasitol* 1998, **92**:449–458.
34. Aubouy A, Mayombo J, Keundjian A, Bakary M, Le Bras J, Deloron P: **Short report: lack of prediction of amodiaquine efficacy in treating Plasmodium falciparum malaria by in vitro tests.** *Am J Trop Med Hyg* 2004, **71**:294–296.
35. Pettinelli F, Pettinelli M-E, Eldin De Pécoulas P, Millet J, Michel D, Brasseur P, Druilhe P: **Short report: high prevalence of multidrug-resistant Plasmodium falciparum malaria in the French territory of Mayotte.** *Am J Trop Med Hyg* 2004, **70**:635–637.

36. Fall B, Diawara S, Sow K, Baret E, Diatta B, Fall KB, Mbaye PS, Fall F, Diémé Y, Rogier C, Wade B, Bercion R, Pradines B: **Ex vivo susceptibility of** *Plasmodium falciparum* **isolates from Dakar, Senegal, to seven standard anti-malarial drugs.** *Malar J* 2011, **10**:310.

37. Fall B, Pascual A, Sarr FD, Wurtz N, Richard V, Baret E, Diémé Y, Briolant S, Bercion R, Wade B, Tall A, Pradines B: *Plasmodium falciparum* **susceptibility to anti-malarial drugs in Dakar, Senegal, in 2010: an ex vivo and drug resistance molecular markers study.** *Malar J* 2013, **12**:107.

38. Basco LK, Ringwald P: **Molecular epidemiology of malaria in Cameroon. XXIV. Trends of in vitro antimalarial drug responses in Yaounde, Cameroon.** *Am J Trop Med Hyg* 2007, **76**:20–26.

39. Fule R, Meer T, Ajay Sav AP: **Solubility and dissolution rate enhancement of lumefantrine using hot melt extrusion technology with physicochemical characterisation.** *J Pharm Investig* 2013, **43**:305–321.

40. World Health Organization: *WHO Technical Report Series (UNEDITED REPORT).* Switzerland, Geneva: WHO; 2011:193.

41. Nsobya SL, Kiggundu M, Nanyunja S, Joloba M, Greenhouse B, Rosenthal PJ: **In vitro sensitivities of** *Plasmodium falciparum* **to different antimalarial drugs in Uganda.** *Antimicrob Agents Chemother* 2010, **54**:1200–1206.

42. Basco LKRP: **In vitro activity of piperaquine and other 4- aminoquinolines against clinical isolates of** *Plasmodium falciparum* **in Cameroon.** *Antimicrob Agents Chemother* 2003, **47**:1391–1394.

43. Mwai L, Kiara SM, Abdirahman A, Pole L, Rippert A, Diriye A, Bull P, Marsh K, Borrmann S, Nzila A: **In vitro activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan** *Plasmodium falciparum* **isolates and polymorphisms in pfcrt and pfmdr1.** *Antimicrob Agents Chemother* 2009, **53**:5069–5073.

44. Pascual A, Parola P, Benoit-Vical F, Simon F, Malvy D, Picot S, Delaunay P, Basset D, Maubon D, Faugère B, Ménard G, Bourgeois N, Oeuvray C, Didillon E, Rogier CPB: **Ex vivo activity of the ACT new components pyronaridine and piperaquine in comparison with conventional ACT drugs against isolates of** *Plasmodium falciparum.* *Malar J* 2012, **11**:9.

45. Na-Bangchang K, Ruengweerayut R, Mahamad P, Ruengweerayut K, Chaijaroenkul W: **Declining in efficacy of a three-day combination regimen of mefloquine-artesunate in a multi-drug resistance area along the Thai-Myanmar border.** *Malar J* 2010, **9**:273.

46. Woitsch B, Wernsdorfer G, Congpuong K, Rojanawatsirivet C, Sirichaisinthop J, Wernsdorfer WH: **Sensitivity to artemisinin, mefloquine and quinine of** *Plasmodium falciparum* **in northwestern Thailand.** *Wien Klin Wochenschr* 2007, **122 Suppl**(19–20 Suppl 3):76–82.

47. Pradines B, Bertaux L, Pomares C, Delaunay P, Marty P: **Reduced in vitro susceptibility to artemisinin derivatives associated with multi-resistance in a traveller returning from South-East Asia.** *Malar J* 2011, **10**:268.

48. Witkowski B, Amaratunga C, Khim N, Sreng S, Chim P, Kim S, Lim P, Mao S, Sopha C, Sam B, Anderson JM, Duong S, Chuor CM, Taylor WRJ, Suon S, Mercereau-Puijalon O, Fairhurst RM, Menard D: **Novel phenotypic assays for the detection of artemisinin-resistant** *Plasmodium falciparum* **malaria in Cambodia: in-vitro and ex-vivo drug-response studies.** *Lancet Infect Dis* 2013, **13**:1043–1049.