Reduced in vitro susceptibility to artemisinin derivatives associated with multi-resistance in a traveller returning from South-East Asia.

Bruno Pradines, Lionel Bertaux, Christelle Pomares, Pascal Delaunay, Pierre Marty

To cite this version:
Bruno Pradines, Lionel Bertaux, Christelle Pomares, Pascal Delaunay, Pierre Marty. Reduced in vitro susceptibility to artemisinin derivatives associated with multi-resistance in a traveller returning from South-East Asia.. Malaria Journal, BioMed Central, 2011, 10 (1), pp.268. 10.1186/1475-2875-10-268 . inserm-00629634

HAL Id: inserm-00629634
https://www.hal.inserm.fr/inserm-00629634

Submitted on 6 Oct 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Reduced in vitro susceptibility to artemisinin derivatives associated with multi-resistance in a traveller returning from South-East Asia

Bruno Pradines¹,²*, Lionel Bertaux²,³, Christelle Pomares⁴,⁵, Pascal Delaunay⁴,⁵ and Pierre Marty⁴,⁵

Abstract
Decreased in vitro susceptibility to dihydroartemisinin (21.2 nM) and artesunate (16.3 nM) associated with decreased susceptibility or resistance to quinine (1131 nM), mefloquine (166 nM), lumefantrine (114 nM), pyronaridine (70.5 nM) and piperaquine (91.1 nM) is reported in a patient returning from South-East Asia after trekking along the Mekong from the south of Laos to the north of Thailand. Decreased in vitro susceptibility to artemisinin derivatives did not appear to be mediated by the number of copies of pfmdr1 or pfATPase6, pfcrt, pfmdr1 or pfmrp polymorphism. The high IC₅₀ to mefloquine of this Asian isolate was not associated with pfmdr1 copy number. Pfne-1 microsatellite ms4760 showed a profile 7 (ms4760-7) with three repeats of DNNND and one repeat of DDDHNDHNN, which is associated with high quinine reduced susceptibility. The patient recovered in three days without relapse after treatment with the association of quinine and doxycycline. Decreased in vitro susceptibility to quinine and the delayed effect of doxycycline may both have contributed to the delayed parasite clearance time, D₄ (0.5%) and D₇ (0.004%). The in vitro data, with IC₅₀ for dihydroartemisinin and artesunate were up to ten times those of the reference clone W₂, which suggests that this isolate may be resistant to artemisinin derivatives, associated with a decreased susceptibility to quinine.

Background
Artemisinin-based combination therapy (ACT) is now recommended by the World Health Organization as first-line treatment of uncomplicated falciparum malaria in all areas in which malaria is endemic. However, recent reports from Cambodia of delayed parasite clearance after treatment by ACT have now been confirmed [1]. The resistant phenotype is not yet reflected by the results of conventional in vitro drug susceptibility assays. Parasites with slow clearance rate after ACT did not show in vitro decreased susceptibility [1]. In vitro decreased susceptibility to artemisinin derivatives was never or very rarely reported in Cambodia [1-4]. No molecular marker has been identified, which impedes surveillance studies to monitor the spread of artemisinin resistant phenotype. Decrease of in vitro susceptibility to dihydroartemisinin and artesunate, associated with reduced susceptibility to standard anti-malarials, such as quinine, mefloquine and lumefantrine, and new drugs, such as pyronaridine and piperaquine, is reported here.

Case presentation
A 52-year old female visited rural areas in Laos (Nov 9 to 12, 2009), Cambodia (Nov 12 to 29) and Thailand (Nov 29 to Dec 1). She took part in trekking along the Mekong from the south of Laos to the north of Thailand. The patient presented with fever since November 29 and was hospitalized (Dec 2) in intensive care unit (Centre Hospitalier Universitaire l’Archet, Nice, France) for complicated malaria (15% parasitaemia and altered consciousness). The patient used irregularly doxycycline (100 mg/day) as chemoprophylaxis. The patient was treated by intra-venous quinine chlorhydrate (25 mg/kg/day) and doxycycline (200 mg/day) for seven days. The patient recovered in three days without relapse and was discharged.
*Plasmodium falciparum* parasites were identified at Day 0 (15%), D4 (0.5%) and D7 (0.004%) but were not detected at D43.

**Methods**

*In vitro* testing of drug susceptibility was performed by the standard 42-hour $^3$H-hypoxanthine uptake inhibition method [5]. Susceptibility to dihydroartemisinin, artesunate, and ten standard or new anti-malarial drugs, ie chloroquine, quinine, mefloquine, lumarfantrine, monodesethylamodiaquine (biologically active metabolite of amodiaquine), pyronaridine, piperaquine, atovaquone, doxycycline and pyrimethamine, was assessed. The laboratory-adapted clone W2, tested on the same day, was used as a reference. Isolates from imported malaria, tested on the same batch of plates, were used as comparators.

Polymorphisms of pfct, pfmdr1, pfmrp and pfhhe-1, involved in quinine resistance, and in pfATPase6, postulated to be involved in artemisinin resistance, and the copy number of pfmdr1 were assessed [6].

The French malaria consensus [7] and the WHO [8] recommend to clinically examine patient and control parasitaemia at D0, D3, D7 and D28 to evaluate anti-malarial efficacy. Blood controls were performed at D0, D4, D7 and D43. The genotyping of parasites was assessed at D0, D4 and D7 using six microsatellite loci (microsatellites 7A11, pf2689, pf2802, C4M79, TRAP, C4M69) [9], msp1 and msp2 [10].

**Consent**

Informed consent was not required as the sampling procedures and testing are part of the French national recommendations for the care and surveillance of malaria.

**Results**

This isolate showed decreased susceptibility to dihydroartemisinin (21.2 nM) and artesunate (16.3 nM) associated with decreased susceptibility or resistance to quinine (1131 nM), mefloquine (166 nM), lumarfantrine (114 nM), pyronaridine (70.5 nM) and piperaquine (91.1 nM) with high ratio in comparison with W2 (Table 1). These IC$_{50}$ and W2 ratios were higher than those of other imported isolates.

Mutations were not identified in pfmdr1, pfct and pfmrp genes. Only one copy of pfmdr1 was found. Two synonymous mutations were detected in pfATPase6 (N460N and I898I), which were previously described [11]. Pfhhe-1 microsatellite ms4760 showed a profile 7 (ms4760-7) with three repeats of DNNND and one repeat of DDDNDHNDHNN, which is associated with high quinine reduced susceptibility [12]. Two repeats of DNNND were seen to be associated with high IC$_{50}$ in quinine clinical failure in traveller from Senegal [14].

**Conclusion**

This isolate showed reduced susceptibility to artemisinin derivatives, but also to other ACT components commonly used in Asia or in clinical trials, such as mefloquine, lumarfantrine, pyronaridine or piperaquine. Surprising, this isolate was susceptible *in vitro* to chloroquine and monodesethylamodiaquine. It was also susceptible to doxycycline. *Plasmodium falciparum* parasite with high IC$_{50}$ to artesinin and artemether (20.1 nM and 21.4 nM, respectively), but with low IC$_{50}$ to dihydroartemisinin and artesunate (1.8 nM and 6.2 nM, respectively) was recently isolated in a traveller returning from Nigeria, who took artesunate prophylactically (two 50 mg tablets weekly for 4 weeks) [13]. Yet, recent clinical trials of oral artesunate monotherapy suggest that the loss of ACT efficacy might result from decreased efficacy of artemisinin derivatives [1,3]. The median parasite clearance time was 36 hours longer in patients from Western Cambodia, where the efficacy of ACT is decreasing [1]. Nevertheless, this phenomenon was not correlated with artemisinin derivatives IC$_{50}$.

Only one copy of pfmdr1 was found. The high IC$_{50}$ to mefloquine of this Asian isolate was not associated with pfmdr1 copy number. Pfhhe-1 microsatellite ms4760 showed a profile 7 (ms4760-7) with three repeats of DNNND and one repeat of DDDNDHNDHNN, which is associated with high quinine reduced susceptibility [12]. Two repeats of DNNND were seen to be associated with high IC$_{50}$ in quinine clinical failure in traveller from Senegal [14].

The persistence of parasites seven days after the start of treatment with quinine chloride and doxycycline, but without clinical signs, is consistent with the high IC$_{50}$ and with the profile ms4760 of pfhhe-1. The action of doxycycline is delayed. There is a relationship between the amount and duration of exposure and the effect of doxycycline on the erythrocytic stages with an increased activity during the second cycle. Doxycycline has been shown to exert its effect during the first 48 h, but is only detectable in second-generation parasites near the 96 hour time point [15-17]. The French malaria consensus recommends quinine associated with doxycycline for Asian and South-American *P. falciparum* (and not only quinine as for African parasites). Clinical failure with quinine has been shown in a patient returning from French Guiana treated by quinine only [18]. The patient recovered in three days without relapse and left intensive care due to the treatment she received, the association of quinine and doxycycline, as recommended by the French malaria consensus. *In vitro* quinine IC$_{50}$ and pfhhe-1 ms4760 analysis suggest a reduced susceptibility to quinine. This decreased
susceptibility to quinine and the delayed effect of doxycycline may both have contributed to the delayed parasite clearance time, D4 (0.5%) and D7 (0.004%).

Decreased in vitro susceptibility to artemisinin derivatives did not appear to be mediated by the number of copies of pfmdr1 or pfATPase6 polymorphism. The in vitro data, with IC50 for dihydroartemisinin and artemesunate up to ten times those of the reference clone W2 or the geometric mean of the other isolates, suggest that this isolate could be resistant to artemisinin derivatives, even if there is no evidence that this isolate was clinically resistant to ACT, associated with decreased susceptibility to quinine. And its association with the other in vitro decreased susceptibilities is alarming, especially with the components of ACT used in Asia.

Acknowledgements and Funding
The authors thank R Amalvic, E Baret, N Benoit, H Bouchiba, S Charras, J Cren and D Traverse from IRBA and E Cua, H Hyvernat, C Dubois, S Melhem from CHU Nice for technical support. This study was supported by the Institut National de Veille Sanitaire.

Author details
1Unité de Recherche en Biologie et Epidémiologie Parasitaires - Unité de Recherche pour les Maladies Infectieuses et Tropicales Emergentes - UMR 6236, Institut de Médecine Tropicale du Service de Santé des Armées, Marseille, France. 2Centre National de Référence du Paludisme, Marseille, France. 3Unité de Recherche en Physiologie et Pharmaco Cinétique Parasitaires - UMR-MD3 Relations Hôte-Parasites - Pharmacologie et Thérapeutique, Institut de Médecine Tropicale du Service de Santé des Armées, Marseille, France. 4Laboratoire de Parasitologie-Mycologie, Centre Hospitalier Universitaire l'Arche, Nice, France. 5Inserm U 895, Équipe 6, Université de Nice Sophia Antipolis, Nice, France.

Authors’ contributions
LB carried out in vitro testing of drug susceptibility and drafted the manuscript. LB carried out the molecular genetic studies. CP, PD and PM carried out diagnostic, monitoring of the patient, collection of clinical and epidemiological data and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 20 July 2011 Accepted: 18 September 2011
Published: 18 September 2011

References
1. Dondorp AM, Nosten F, Yi P, Das D, Phyos AP, TAMING J, Lwin KM, Aney F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotvanshan K, Lim P, herdman T, An SS, Yeung S, Singhasivanon P, Day NPJ, Lindegardh N, Socheat D, White NJ: Artesinins resistance in Plasmodium falciparum malaria. N Engl J Med 2009, 361:455-467.
2. Jambou R, Legrand E, Niang M, Khim N, Lim P, Donney B, Eka LT, Bouchier C, Esterre P, Fandeur T, Merereau-Pujol D: Resistance of Plasmodium falciparum field isolates to in vivo artemether and point mutations of the Sera-type PfATPases. Lancet 2003, 366:1960-1963.
3. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM: Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 2008, 359:2619-2620.
4. Noedl H, Socheat D, Satimali W: Artemisinin-resistant malaria in Asia. N Engl J Med 2009, 361:540-541.
5. Briolant S, Baragatti M, Parola P, Simon F, Taal A, Solhna C, Maimoumbi MM, Koeck JL, Delmont J, Spiegel A, Castello J, Gardair JP, Trape JF, Kombila M, Minodier P, Fusai T, Roger C, Pradines B: A multi-normal distribution model suitable for the distribution of Plasmodium falciparum in vitro chemosusceptibility to doxycycline. Antimicrob Agents Chemother 2009, 53:688-695.
6. Pradines B, Dormio J, Briolant S, Bogreau H, Roger C: La résistance aux antipaludiques. Rev Fr Lab 2010, 422:51-62.
7. Management and prevention of imported Plasmodium falciparum malaria: Recommendations for clinical practice 2007 (revision 2007 of the 1999 consensus conference). Med Mal Infect 2008, 38:68-117.
8. World Health Organization: Methods for surveillance of antimalarial drug efficacy. [http://apps.who.int/malaria/docs/drugresistance/Protocol2009.pdf].
9. Bogreau H, Renaud F, Bouchiba H, Durand P, Assi SB, Henry MC, Garnotel E, Pradines B, Fusai T, Bade W, Adefosse E, Parola P, Kamil MA, Pujaljon O, Roger C: Genetic diversity and structure of African Plasmodium falciparum populations in urban and rural areas. Am J Trop Med Hyg 2006, 74:953-959.
10. Henry M, Dallol I, Bordes J, Ka S, Pradines B, Diatta B, Mbaye PS, Sane M, Thiam M, Gaye PM, Wade B, Toure JE, Debonne JM, Roger C, Fusai T.

Table 1 In vitro susceptibility to standard antimalarial drugs of the multidrug-resistant isolate in comparison with P. falciparum W2 clone and P. falciparum isolates tested with the same plate batches

| Drugs               | Isolate IC50 | Ratio IC50 Isolate/W2 | W2* IC50 | Isolates** Mean IC50 (C95%) | Ratio IC50 Isolates/W2 | Resistance cut-off |
|---------------------|--------------|-----------------------|---------|-----------------------------|------------------------|-------------------|
| Dihydroartemisinin  | 21.2 nM      | 11.8                  | 1.6 nM  | 2.2 nM (1.3-3.7)            | 1.2                    | > 10.5 nM         |
| Artesunate          | 16.3 nM      | 10.2                  | 1.6 nM  | 1.9 nM (1.0-3.2)            | 1.2                    | > 10.5 nM         |
| Quinine             | 1131 nM      | 1.5                   | 731 nM  | 201 nM (131-307)            | 0.3                    | > 800 nM          |
| Mefloquine          | 166 nM       | 4.5                   | 36.6 nM | 26.0 nM (15.9-42.5)         | 0.7                    | > 30 nM           |
| Lumeferantine       | 114 nM       | 4.0                   | 28.4 nM | 23.9 nM (14.1-40.8)         | 0.8                    | > 150 nM          |
| Pyronaridine        | 70.5 nM      | 7.8                   | 9.0 nM  | 26.2 nM (15.4-44.6)         | 2.9                    | ND                |
| Piperaquine         | 91.1 nM      | 3.3                   | 273 nM  | 66.2 nM (34.7-126.3)        | 2.4                    | ND                |
| Chloroquine         | 63.0 nM      | 0.14                  | 449 nM  | 63 nM (29-138)              | 0.1                    | > 100 nM          |
| Monodesethylamodiaquine | 34.4 nM    | 0.54                  | 63.2 nM | 32.4 nM (19.2-54.8)         | 0.5                    | > 80 nM           |
| Atovaquone          | 2.21 nM      | 0.74                  | 2.99 nM | 1.48 nM (1.06-2.07)         | 0.5                    | > 490 nM          |
| Doxycycline         | 18.5 μM      | 1.5                   | 12.1 μM | 10.0 μM (7.8-12.9)          | 0.8                    | > 35 μM           |
| Pyrimethamine       | 497 nM       | 0.04                  | 12621 nM| 107 nM (7-1681)            | 0.01                   | > 2000 nM         |

** Values are geometric mean and 95% confidence interval of 16 isolates from imported malaria, tested on the same batch of plates, and used as comparators.
ND: not determined.
Urban malaria in Dakar, Senegal: chemosusceptibility and genetic diversity of *Plasmodium falciparum* isolates. *Am J Trop Med Hyg* 2006, 75:146-151.

11. Bertaux L, Quang LH, Sinou V, Xuan Thanh N, Parzy D: New *pfATP6* mutations found in *Plasmodium falciparum* isolates from Vietnam. *Antimicrob Agents Chemother* 2009, 53:4570-4571.

12. Henry M, Briolant S, Zettor A, Pelleau S, Baragatti M, Baret E, Mosnier J, Arnalvict R, Fusai T, Rogier C, Pradines B: *Plasmodium falciparum Na⁺/H⁺ exchanger 1 transporter is involved in reduced susceptibility to quinine. Antimicrob Agents Chemother* 2009, 53:1926-1930.

13. Shahinas D, Lau R, Khairnar K, Handock D, Pillai DR: Artesunate misuse and *Plasmodium falciparum* malaria in traveller returning from Africa. *Emerg Infect Dis* 2010, 16:1608-1610.

14. Pradines B, Pistone T, Ezzedine K, Briolant S, Bertaux L, Receveur MC, Parzy D, Millet P, Rogier C, Malvy D: Quinine-resistant malaria in traveler returning from Senegal. 2007. *Emerg Infect Dis* 2010, 16:546-548.

15. Dahl E, Rosenthal P: Multiple antibiotics exert delayed effects against the *Plasmodium falciparum* apicoplast. *Antimicrob Agents Chemother* 2007, 51:3485-3490.

16. Pradines B, Spiegel A, Rogier C, Tall A, Mosnier J, Fusai T, Trape JF, Parzy D: Antibiotics for prophylaxis of *Plasmodium falciparum* infections: in vitro activity of doxycycline against Senegalese isolates. *Am J Trop Med Hyg* 2000, 62:82-85.

17. Pradines B, Rogier C, Fusai T, Mosnier J, Daries W, Baret E, Parzy D: In vitro activities of antibiotics against *Plasmodium falciparum* are inhibited by iron. *Antimicrob Agent Chemother* 2001, 45:1746-1750.

18. Bertaux L, Kraemer P, Taudon N, Grignon A, Mantelloni M, Saidi R, Parzy D, Pradines B, Simon F: Quinine-resistant malaria in traveler returning from French Guiana. 2010. *Emerg Infect Dis* 2011, 17:943-945.

doi:10.1186/1475-2875-10-268

Cite this article as: Pradines et al: Reduced in vitro susceptibility to artemisinin derivatives associated with multi-resistance in a traveller returning from South-East Asia. *Malaria Journal* 2011 10:268.

---

**Submit your next manuscript to BioMed Central and take full advantage of:**

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit