

SHORT REPORT: LACK OF PREDICTION OF AMODIAQUINE EFFICACY IN TREATING *PLASMODIUM FALCIPARUM* MALARIA BY *IN VITRO* TESTS

AGNÉS AUBOUY, JUSTICE MAYOMBO, ANNICK KEUNDJIAN, MOHAMED BAKARY, JACQUES LE BRAS, AND PHILIPPE DELORON

Centre International de Recherches Médicales de Franceville, Unité de Parasitologie Médicale, Franceville, Gabon;
Unité de Parasitologie, Institut de Médecine Tropicale du Service de Santé des Armées, le Pharo, Marseille Armées, France;
Hôpital de Bakoumba, Bakoumba, Gabon; Hôpital Bichat-Claude Bernard, Laboratoire de Parasitologie, Paris, France;
Institut de Recherche pour le Développement, Unité de Recherche 010 Mother and Child Health in the Tropics, Paris, France

Abstract. Amodiaquine (AQ) is currently a major candidate for new antimalarial combinations, although *in vivo* and *in vitro* tests have been rarely simultaneously investigated. The efficacy of AQ was assessed at the dose of 30 mg/kg in treating *Plasmodium falciparum* malaria attacks in 74 children from southeast Gabon, and the *in vitro* activity of monodesethylamodiaquine (MdAQ), the main metabolite of AQ, was measured against *P. falciparum* parasites isolated from these children. Treatment failures were observed in 40.5% of the children, while 5.4% of the isolates showed *in vitro* resistance to MdAQ. No relationship was observed between *in vivo* and *in vitro* susceptibility. The *in vitro* activities of MdAQ and chloroquine were correlated. The reasons for such disparities between *in vivo* and *in vitro* AQ activities are discussed and the issue of the validity of *in vitro* tests to measure AQ efficacy is raised.

In vivo and *in vitro* tests have been in use for more than 30 years for assessing the activity of drugs against *Plasmodium falciparum*.¹ Although it has been clearly shown that the *in vitro* activity of chloroquine (CQ) was highly correlated with CQ treatment efficacy,² this is not the case with numerous other antimalarials. Amodiaquine (AQ) is one of the cost-efficient alternatives to resistant parasites in Africa. However, very few studies investigated AQ activity, simultaneously using both *in vivo* and *in vitro* tests, and the relationship between both methods remains poorly documented. Therefore, we compared results of both methods for AQ and analyzed the reasons for the disparities.

The study was part of a larger treatment efficacy assay in children (<10 years old) comparing sulfadoxine-pyrimethamine versus AQ described elsewhere.³ Briefly, AQ treatment efficacy (30 mg/kg over a three-day period given under supervision, Camoquin®; Parke Davis, Dakar, Senegal) was assessed by the revised World Health Organization (WHO) *in vivo* protocol for areas of intense transmission,⁴ extended to 28 days of follow-up. A total of 125 children were enrolled in this study, of whom 118 completed the 28-day follow-up. Merozoite surface protein 1 (*msp* 1) and *m*sp 2 genotyping was used to distinguish recurrence of malaria due to recrudescence from those due to reinfection. *In vivo* responses were classified according to the WHO scheme⁴ for an adequate clinical and parasitologic response, early treatment failure, late clinical failure, or parasitologic failure. Plasma samples were collected at day 3 for measurement by high-performance liquid chromatography of the levels of AQ and monodesethylamodiaquine (MdAQ), the main metabolite of AQ. The study was reviewed and approved by the Center International de Recherches Médicales de Franceville ethical committee, and informed consent was obtained from all parents or guardians.

The *in vitro* drug sensitivity assay was assessed by the isotopic microtest with MdAQ and CQ, as described.⁵ The 50% inhibitory concentration (IC₅₀) value was defined as the drug concentration corresponding to 50% of the uptake of ³H-hypoxanthine in the drug-free control wells. The calculation was based on linear regression analysis of the logarithm of concentrations plotted against the percentage of growth

inhibition. According to previously published data,^{2,6} the threshold IC₅₀ values for CQ and MdAQ *in vitro* resistance were fixed at 100 nM (according to a direct comparison of *in vivo* and *in vitro* data) and 60 nM (more arbitrarily), respectively. Data were analyzed with the Statview software (SAS Institute Inc, Cary, NC).

The median (range) IC₅₀ values were 146.5 (3–629) nM and 20 (2–257) nM for CQ and MdAQ, respectively. The IC₅₀ values for 75.6% and 5.4% of the isolates were greater than the threshold of *in vitro* resistance to CQ and MdAQ, respectively. The IC₅₀ values for both drugs showed a correlation with each other (Figure 1) ($r = 0.45$, $P < 0.0001$, by linear regression analysis). Discrepancies between *in vivo* and *in vitro* results were observed in 31 of the 74 cases successfully studied by both methods: 29 isolates were susceptible *in vitro* (IC₅₀ ≤ 60 nM) to MdAQ, but led to treatment failures, while 2 of the 4 isolates with *in vitro* resistance to MdAQ led to treatment successes. The role of the factors that may influence treatment outcome was investigated (Table 1). Subjects with *in vivo* failures were younger ($P = 0.04$, by Mann-Whitney U test). Day 3 plasma concentrations of MdAQ, or MdAQ plus AQ did not differ in children in which AQ treatment outcome was a success or a failure. Similarly, these concentrations did not correlate with IC₅₀ values for CQ or MdAQ.

In this study, 5.4% of the isolates showed *in vitro* resistance to MdAQ, while a treatment failure to AQ occurred in 40.5% of the children. No clear relationship between treatment response and the *in vitro* IC₅₀ of AQ was detected. Similarly, Brasseur and others⁷ reported a two-fold higher rate of resistance *in vivo* than *in vitro*. However, the *in vitro* tests were carried out with AQ instead of MdAQ. Conversely, Trenholme and others,⁸ Ringwald and others,⁹ and Basco and others¹⁰ reported lower treatment failure rates compared with the rate of *in vitro* resistance. In endemic areas, the higher prevalence rate of *in vitro* resistance may be related to the immune response, adding its effect to that of the drug in clearing the parasite infection. Although differences in the methodology of *in vivo* and *in vitro* tests do not allow an accurate comparison of these studies to our results, there was no correlation between resistance *in vivo* and *in vitro* in most of these studies.

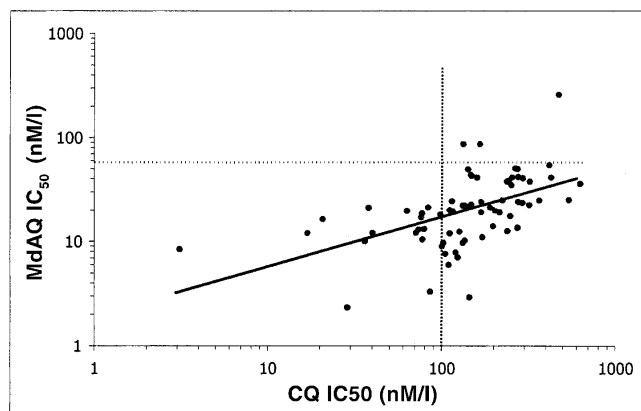


FIGURE 1. Correlation between *in vitro* responses to monodesethylamodiaquine (MdAQ) and chloroquine (CQ) among 72 *Plasmodium falciparum* Gabonese isolates from Bakoumba, Gabon, 2000. Resistance thresholds are shown at 60 nM for MdAQ and 100 nM for CQ. Two samples, one amodiaquine (AQ) resistant and one AQ sensitive, were not studied with CQ. IC₅₀ = 50% inhibitory concentration.

The accuracy of the threshold for MdAQ *in vitro* resistance may be questioned. The threshold for CQ was established 25 years ago^{2,6} by direct comparison of data from treatment efficacy of the standard therapeutic regimen of CQ and *in vitro* tests results. In the case of MdAQ, as well as most other antimalarial drugs, this threshold was calculated from the mean IC₅₀ of a number of isolates plus two standard errors.⁶ These determinations were done in areas where the level and the prevalence rate of AQ resistance were then low. Nevertheless, in our study, MdAQ IC₅₀ values were similarly low in isolates from both adequate responses and therapeutic failures.

Drug intake before enrollment can modify the outcome of *in vivo* as well as *in vitro* tests results. An inadequate treatment regimen of AQ may also constitute a possible factor of discordance with *in vitro* results. The regimen of 25 mg/kg was first used and is still commonly used, but the higher efficiency of a 35 mg/kg regimen has been reported.^{11,12} No other comparative study was conducted to determine the best regimen,

but it was shown that increasing the doses of CQ from 25 to 35, 40, or 50 mg/kg only delayed the appearance of recrudescences.¹³

Our larger study reporting AQ versus SP efficacy for malaria treatment in Gabonese children³ showed that treatment failures were characterized by reduced MdAQ, and/or MdAQ plus AQ plasma concentrations. The metabolism of AQ via MdAQ depends on cytochrome P(450) 2C8 enzyme activity¹⁴ and large interindividual variation in metabolizing CYP2C8 substrates has been reported.¹⁵ Genetic variants of CYP2C8 have also been reported¹⁶ with various frequencies among different ethnic groups, and some variants being associated with altered activity.¹⁷ Therefore, the large interindividual variations in pharmacokinetics of AQ may contribute to *in vivo* and *in vitro* discrepancies.

In humans, the metabolism of AQ results in two metabolites other than MdAQ: bidesethyl-amodiaquine (BdAQ) and hydroxydesethylamodiaquine (HdAQ). The *in vitro* antimalarial activity of HdAQ is 1% that of AQ against CQ-sensitive strains.^{18,19} In addition, BdAQ was shown to have a very low *in vitro* activity and to achieve very low blood levels.²⁰ However, Mount and others showed that the *in vitro* activity of BdAQ was 10–30% that of AQ,¹⁹ suggesting that *in vitro* tests performed with MdAQ may not adequately reproduce the complex *in vivo* situation achieved following treatment with AQ. Amodiaquine was shown to be 1–3 times more effective *in vitro* than MdAQ,^{18,19} suggesting that remaining levels of AQ after metabolism should be considered when measuring blood concentrations of the drug. In our study, the addition of AQ concentrations to MdAQ did not alter the results or improve the *in vivo-in vitro* data agreement. This also suggests that it may be worthwhile to conduct *in vitro* testing not only with either AQ or MdAQ, but also with a mixture of these two drugs.

High-level transmission areas are characterized by large number of infective clones, as we previously showed at the site of this study.³ Such a factor may increase the complexity of *in vitro* tests, and raise questions about their significance. A resistant clone that is poorly represented in the blood may not have a sufficient effect to substantially increase the IC₅₀, but may later cause an *in vivo* failure. Furthermore, at the time of

TABLE 1

Malaria attack characteristics, *in vitro* activity of monodesethylamodiaquine (MdAQ) and chloroquine (CQ), and post-treatment plasma drug levels according to amodiaquine (AQ) treatment outcome Bakoumba, Gabon, 2000*

	Response to AQ treatment (%)		P
	Success (n = 44)	Failure (n = 30)	
<i>In vitro</i> MdAQ activity			
Resistance (%)	2 (4.5)	2 (6.7)	0.99†
IC ₅₀ MdAQ ± SE (nM/L)	29.0 ± 5.8	24.6 ± 3.5	0.80‡
<i>In vitro</i> CQ activity			
Resistance (%)	33 (75.0)	21 (75.0)§	0.99†
IC ₅₀ CQ ± SE (nM/L)	181.0 ± 18.4	172.9 ± 23.5	0.81‡
Day 3 plasma concentrations (± SE) MdAQ (ng/ml)	145.4 ± 17.3	110.6 ± 14.1	0.10‡
MdAQ plus AQ	149.1 ± 17.2	113.3 ± 14.2	0.33‡
Mean ± SE age (months)	52.5 ± 3.7	41.1 ± 4.4	0.04¶
Day 0 parasite density (/μL of blood)	55,336 (23,832–86,840)	59,053 (22,517–95,589)	0.73¶
Mean ± SE day 0 axillary temperature (°C)	38.1 ± 0.1	38.2 ± 0.1	0.94¶

* IC₅₀ = 50% inhibitory concentration.

† By chi-square test.

‡ By Kolmogorov-Smirnov test.

§ n = 28.

¶ By Mann-Whitney U test.

sampling, selected parasites may be sequestered and absent from the peripheral circulation.^{21,22}

New strategies are urgently needed to address the question of the waning lifetime of antimalarial treatments. Methods to evaluate treatment efficacy are of utmost importance because they help to develop treatment recommendations. Our data demonstrate that treatment failure may not necessarily be due to resistance, and that in the case of AQ and probably many other antimalarials, *in vitro* testing cannot replace *in vivo* testing and should instead used as an early warning tool. Our study raises the problem of the validity of *in vitro* tests to measure AQ efficacy, and stresses the necessity to validate an *in vitro* threshold of resistance and to validate its predictive value.

Received January 23, 2004. Accepted for publication April 9, 2004.

Acknowledgments: We are grateful to the children who participated in the study, as well as to their mothers and guardians. We also thank J. Bourgeois (Le Site de la Société d'Exploitation du Parc de la Lékédi) for logistical support in Bakoumba.

Financial support: This work was supported by the French Ministry of Research (VIHPAL grant) and by the Fondation pour la Recherche Médicale. Agnes Aubouy was the recipient of a fellowship from the French Ministry of Research.

Authors' addresses: Agnes Aubouy and Philippe Deloron, Faculté de Pharmacie, Institut de Recherche pour le Développement, Unité de Recherche 010, 4 Avenue de l'Observatoire, 75006 Paris, France, Telephone: 33-1-53-73-96-22. Justice Mayombo, Center International de Recherches Médicales de Franceville, BP 769 Franceville, Gabon, Telephone: 241-677-096. Annick Keundjian, Unité de Parasitologie, Institut de Médecine Tropicale du Service de Santé des Armées, le Pharo, BP46, 13998 Marseille Armées, France, Telephone: 33-4-91-50116. Mohamed Bakary, Hôpital de Bakoumba, BP52, Bakoumba, Gabon, Telephone: 241-661-101. Jacques Le Bras, Hôpital Bichat-Claude Bernard, 75877 Paris, France, Telephone: 33-1-40-25-78-99.

Reprint requests: Philippe Deloron, Institut de Recherche pour le Développement, Unité de Recherche 010, Faculté de Pharmacie, 4 Avenue de l'Observatoire, 75006 Paris, France, Telephone: 33-1-53-73-96-22, Fax: 33-1-53-73-96-17, E-mail: Philippe.Deloron@ird.fr.

REFERENCES

1. World Health Organization, 1996. *Assessment of Therapeutic Efficacy of Antimalarial Drugs for Uncomplicated falciparum Malaria in Areas with Intense Transmission*. Geneva: World Health Organization. WHO/MAL/96.1077.
2. Le Bras J, Ringwald P, 1990. Situation de la chimiorésistance de *Plasmodium falciparum* en Afrique en 1989. *Med Trop (Mars)* 50: 11–16.
3. Aubouy A, Bakary M, Mbomat B, Makita JR, Keundjian A, Migot-Nabias F, Le Bras J, Deloron P, 2003. Combination of drug dosage and parasite genotyping data for a better assessment of amodiaquine and sulfadoxine-pyrimethamine efficacy to treat *Plasmodium falciparum* malaria in Gabonese children. *Antimicrob Agents Chemother* 47: 231–237.
4. World Health Organization, 2002. *Monitoring Antimalarial Drug Resistance*. Geneva: World Health Organization. WHO/CDS/CSR/EPH/2002.17.
5. Le Bras J, Deloron P, 1983. *In vitro* study of drug sensitivity of *Plasmodium falciparum*: evaluation of a new semi-micro test. *Am J Trop Med Hyg* 32: 447–451.
6. Le Bras J, Simon F, Ramanamirija JA, Calmel MB, Hatin I, Deloron P, Porte J, Marchais H, Clausse JL, Biaud JM, Sarrouy J, Guiguemde TR, Carme B, Charmot G, Coulaud JP, Coulanges P, 1987. Sensibilité de *Plasmodium falciparum* aux quinolones et stratégies thérapeutiques: comparaison de la situation en Afrique et à Madagascar entre 1983 et 1986. *Bull Soc Pathol Exot Filiales* 80: 477–489.
7. Brasseur P, Agnamey P, Same Ekobo A, Samba G, Favenne L, Kouamouo J, 1995. Sensitivity of *Plasmodium falciparum* to amodiaquine and chloroquine in central Africa: a comparative study *in vivo* and *in vitro*. *Trans R Soc Trop Med Hyg* 89: 528–530.
8. Trenholme KR, Kum DE, Raiko AK, Gibson N, Narara A, Alpers MP, 1993. Resistance of *Plasmodium falciparum* to amodiaquine in Papua New Guinea. *Trans R Soc Trop Med Hyg* 87: 464–466.
9. Ringwald P, Keundjian A, Same Ekobo A, Basco LK, 2000. Chemoresistance of *Plasmodium falciparum* in the urban region of Yaounde, Cameroon. Part 2: Evaluation of the efficacy of amodiaquine and sulfadoxine-pyrimethamine combination in the treatment of uncomplicated *Plasmodium falciparum* malaria in Yaounde, Cameroon. *Trop Med Int Health* 5: 620–627.
10. Basco LK, Ndounga M, Keundjian A, Ringwald P, 2002. Molecular epidemiology of malaria in Cameroon. IX. Characteristics of recrudescence and persistent *Plasmodium falciparum* infections after chloroquine or amodiaquine treatment in children. *Am J Trop Med Hyg* 66: 117–123.
11. Hengy C, Garrigue G, Abissege B, Ghogomu NA, Gazin P, Gelas H, Kouka-Bemba D, Le Bras J, Jambou R, 1989. Surveillance de la chimiosensibilité de *Plasmodium falciparum* à Yaoundé et ses environs (Cameroun). Etude *in vivo* et *in vitro*. *Bull Soc Pathol Exot Filiales* 82: 217–223.
12. Carme B, Moudzeo H, Mbisi A, Ndounga M, Samba G, 1991. Stabilization of drug resistance (chloroquine and amodiaquine) of *Plasmodium falciparum* in semi-immune populations in the Congo. *J Infect Dis* 164: 437–438.
13. Sexton JD, Deloron P, Bugilimfura L, Ntilivamunda A, Neill M, 1988. Parasitologic and clinical efficacy of 25 and 50 mg/kg of chloroquine for treatment of *Plasmodium falciparum* malaria in Rwandan children. *Am J Trop Med Hyg* 38: 237–243.
14. Li XQ, Bjorkman A, Andersson TB, Ridderstrom M, Msimirembwa CM, 2002. Amodiaquine clearance and its metabolism to N-desethylamodiaquine is mediated by CYP2C8: a new high affinity and turnover enzyme-specific probe substrate. *J Pharmacol Exp Ther* 300: 399–407.
15. Ong CE, Coulter S, Birkett DJ, Bhasker B, Miners JO, 2000. The xenobiotic inhibitor profile of cytochrome P450 2C8. *Br J Clin Pharmacol* 50: 573–580.
16. Goldstein JA, 2001. Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol* 52: 349–355.
17. Dai D, 2001. Allelic frequencies of human CYP2C8 and genetic linkage among different ethnic populations. *FASEB J* 15: A575.
18. Churchill FC, Mount DL, Patchen LC, Bjorkman A, 1986. Isolation, characterization and standardization of a major metabolite of amodiaquine by chromatographic and spectroscopic methods. *J Chromatogr* 377: 307–318.
19. Mount DL, Patchen LC, Nguyen-Dinh P, Barber AM, Schwartz IK, Churchill FC, 1986. Sensitive analysis of blood for amodiaquine and three metabolites by high-performance liquid chromatography with electrochemical detection. *J Chromatogr* 383: 375–386.
20. Pussard E, Verdier F, Faurisson F, Scherrmann JM, Le Bras J, Blayo MC, 1987. Disposition of monodesethylamodiaquine after a single oral dose of amodiaquine and three regimens for prophylaxis against *Plasmodium falciparum* malaria. *Eur J Clin Pharmacol* 33: 409–414.
21. Daubersies P, Sallenave-Sales S, Magne S, Trape JF, Contamin H, Fandeur T, Rogier C, Mercereau-Puijalon O, Druilhe P, 1996. Rapid turnover of *Plasmodium falciparum* populations in asymptomatic individuals living in a high transmission area. *Am J Trop Med Hyg* 54: 18–26.
22. Farnert A, Snounou G, Rooth I, Bjorkman A, 1997. Daily dynamics of *Plasmodium falciparum* subpopulations in asymptomatic children in a holoendemic area. *Am J Trop Med Hyg* 56: 538–547.

Aubouy Agnès, Mayombo J., Keundjian A., Bakary M., Le Bras J., Deloron Philippe (2004)

Short report : lack of prediction of amodiaquine efficacy in treating Plasmodium falciparum malaria by in vitro tests

American Journal of Tropical Medicine and Hygiene, 71 (3), 294-296

ISSN 0002-9637