Commentary

In vitro tests for drug resistance in Plasmodium falciparum

The contribution on drug resistance in Plasmodium falciparum in five States of India by Anvikar et al¹ in this issue deserves particular attention since it underlines the importance of *in vitro* investigations for the clinical activity and its limitations for the efficacy of antimalarial drugs. On the limited sample from five States of India, it illustrates the lack of therapeutic activity of chloroquine, supported by restriction fragment length polymorphism analysis of *pfcrt* gene for the detection of K76T. Thus, chloroquine should be replaced by more effective drugs in the treatment of P. falciparum infections, wherever it is used in India. Also the record for monodesetylamodiaquine indicates reduced antimalarial activity in Orissa, Jharkand and Chhattisgarh, overlapping with resistance to chloroquine. On the contrary, the response to dihydroartesunate and to mefloquine seems to be fully preserved. By restricting the monitoring to 108 isolates of P. falciparum, the extent of the exercise seems to be well-below the critical mass, but it is a sound beginning, setting the signs for a major geographical expansion.

The creation of tests for the antimalarial activity against P. falciparum was preceded by the possibility of cultivating the parasite in vitro². Two years later, Rieckmann et al³ applied the in vitro culture technique to parasites in continuous culture. Based on these observations, it was apparent that natural isolates of P. falciparum could also be exposed, ex vivo, to mixtures of antimalarial drugs and plasma-free culture medium and to measure, hereby, the specific activity of the antimalarial drugs. These results were assessed under the auspices of the World Health Organization which issued in the 1980s the initial versions of the "in vitro micro-test for the assessment of the response of Plasmodium falciparum to chloroquine, mefloquine, quinine, sulfadoxine/pyrimethamine, and amodiaguine"⁴. Since then, a number of modifications

were introduced (Table). This Table also indicates additional compounds considered suitable for the application of the method to other chemical classes.

The test for *P. falciparum* is a schizont maturation inhibition test. It uses the natural ability of schizont formation in the intravascular environment to facilitate the reading of schizonts in the test environment and is, therefore, prone to over-breeding. Thus, particular attention has to be given to avoid over-breeding, an event leading to the cancellation of the results.

The test results are conveniently analysed by the method for the evaluation of dose-effect experiments¹³. The computerized programme provides an analysis of the inhibitory concentrations (ICs) and a series of regression parameters useful for comparisons with other log-probit regressions¹⁴, inter-alia the slope of the regression, the heterogeneity of results and the correlation coefficient.

The relative efficacy of antimalarial drugs is subject to wide variation, *e.g.* the value for the IC_{99} of monodesethylamodiaquine is well below 500 nM, whereas that of quinine lies above 10 µM. That of fosmidomycine is even higher. The evaluation of the micro-tests is to be adjusted to the blood concentrations of the compounds. This is also the basis for the calculation of the estimated IC₅₀, IC₉₀ and IC₉₉. A unique quotation of an IC₅₀ is wrong since it reflects only 50 per cent inhibition and omits the inhibition at higher ICs which may very well be situated outside the area of sensitivity. For the evaluation of grouped data, it may also be convenient to calculate the cutoff concentration of schizont maturation mean cut-off concentration (MCOCM) as it provides an overview of the general situation.

MCOC = Sum of first concentrations with complete interruption of Schizont maturation / n

Table. In vitro sensitivity micro-tests for P. falciparum		
Test compound	Remarks	Reference
Chloroquine	Better genomic pfcrt analysis for K76T	Wongsrichanalai et al, 20024
Mefloquine	Modifications of micro-test methodology require adjustment of dosing schedule	Gruber <i>et al</i> , 2009 ⁵
Quininine	The test for quinine is also applicable to other <i>Cinchona</i> alkaloids	Knauer et al, 2003 ⁶
Sulphadoxine/ Pyrimethamine	Microtests for sulphadoxine/pyrimethamine have been overcome by the detection of genomic mutations at codons 108, 51, 59 and 164 at the <i>dhfr</i> gene, and at codons 436, 437, 540, 581 and 623 of the <i>dhps</i> gene	Wongsrichanalai et al, 2002 ⁴
Amodiaquine	Replaced by tests for the metabolite, <i>i.e.</i> monodesethylamodiaquine. Procedure the same as for amodiaquine. Modifications of methodology require adjustment of dosing schedule	Gerstner <i>et al</i> , 2003 ⁷
Lumefantrine	A combination of lume fant rine and β -artemether has been introduced under the name of coartemether	Wernsdorfer, 2004 ⁸
Atovaquone/ Proguanil	Separate values for atovaquone and proguanil are far below the level of clinical activity. The joint testing reflects the clinical activity better, especially when tested with artemisinin	Wernsdorfer <i>et al</i> , 1995 ⁹ ; Luetgendorf <i>et al</i> , 2006 ¹⁰
Piperaquine	Suitable for <i>in vitro</i> testing. Delimitation of clinical activity still under discussion. Hydropiperaquine has been withdrawn	Basco & Ringwald, 2003 ¹¹
Artesunate derivative	Due to the strictly limited validity of dosed microtiter plates dosed with artesunate or similar drugs, plates with artemisinin are preferable (validity 3 months for cooled plates). The activity of artemisinin and other derivatives is strictly proportional	Wernsdorfer <i>et al</i> , 2000 ¹²

Values near the limit of activity are a signal of impending resistance

Significant incidence of P. falciparum infections in India is marked with choloroquine as well as sulphadoxine/pyrimethamine. Chloroquine as well as sulphadoxine/pyrimethamine are not anymore useful, and the search has to be concentrated on the artemisinin based combination therapy (ACT) with mefloquine and a suitable artemisinin derivative, or on lumefantrine/βartemether. This exercise will be useful if it includes wide enough a case clientele for the selected area, *i.e.* one or two checking areas per State. For instance, none of the States at >90 ° East of Greenwich (Assam, etc.) was included in the analysis. In addition, these States are within easy reach of neighbouring Myanmar, a country already experiencing resistance to mefloquine and artemisinin. The situation calls for making the laboratory technicians familiar with the conduct and interpretation of the in vitro micro-test since in vivo tests are time consuming. This training should be carried out in an area with a significant incidence of P. falciparum.

Finally, it seems to be worth mentioning that also the *in vitro* assessment of antimalarial drugs against *P. vivax* has been developed to maturity¹⁵. It seems to be appropriate to India since *P. vivax* is the prevailing species in this country and chloroquine-resistance has reached Myanmar.

Walther H. Wernsdorfer

Institute of Specific Prophylaxis & Tropical Medicine Medical University of Vienna Kinderspitalgasse 15 A-1090 Vienna, Austria walter.wernsdorfer@meduniwien.ac.at

References

- Anvikar AR, Sharma B, Sharma SK, Ghosh SK, Bhatt RM, Kumar A, et al. In vitro assessment of drug resistance in *Plasmodium falciparum* in five States of India. Indian J Med Res 2012; 135: 494-9.
- 2. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976; *193* : 673-5.

- Rieckmann KH, Campbell GH, Sax LJ Mrema M. Drug sensitivity of *Plasmodium falciparum* - An *in vitro* microtechnique. *Lancet* 1978; 1: 22-3.
- Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR. Epidemiology of drug-resistant malaria. *Lancet Infect Dis* 2002; 2: 209-18.
- Gruber M, Wernsdorfer G, Satimai W, Wiedermann U, Congpuong K, Wernsdorfer WH. Pharmacodynamic interaction between mefloquine and retinol in *Plasmodium falciparum in vitro*. *Wien Klin Wochenschr* 2009; *121* (Suppl 3): 27-31.
- Knauer A, Sirichainsinthop J, Reinthaler FF, Wiedermann G, Wernsdorfer G, Wernsdorfer WH. *In-vitro* response of *Plasmodium falciparum* to the main alkaloids of *Cinchona* in northestern Thailand. *Wien Klin Wochenschr* 2003; *115* (Suppl 3): 39-44.
- Gerstner U, Prajakwong S, Wiedermann G, Sirichaisinthop J, Wernsdorfer G, Wernsdorfer WH. Comparison of the *in-vitro* activity of amodiaquine and its main metabolite monodesethyl-amodiaquine, in *Plasmodium falciparum*. *Wien Klin Wochenschr* 2003; *115* (Suppl 3): 33-8.
- 8. Wernsdorfer WH. Coartemether (artemether and lumefantrine): an oral antimalarial drug. *Expert Rev Antiinfect Ther* 2004; 2 : 181-96.
- 9. Wernsdorfer G, Landgraf B, Kilimali AEB, Wernsdorfer WH. *In vitro* sensitivity to atovaquone of *Plasmodium falciparum*

in northeastern Tanzania 1993. Mitt Oesterr Ges Trop Med Par 1995; 17: 229-34.

- Luetgendorf C, Rojanawatsirivet C, Wernsdorfer G, Sirichaisinthop J, Kollaritsch H, Wernsdorfer WH. Pharmacodynamic interaction between atovaquone and other antimalarial compounds against *Plasmodium falciparum in vitro. Wien Klin Wochenschr* 2006; *118* (Suppl 3): 70-6.
- 11. Basco LK, Ringwald P. *In vitro* activities of piperaquine and other 4-aminoquinolines against clinical isolates of *Plasmodium falciparum* in Cameroon. *Antimicrob Agents Chemoth* 2003; 47: 1391-4.
- Wernsdorfer WH, Wernsdorfer G, Prajakwong S, Woitsch B, Zatloukal C, Kollaritsch H. Activity correlation between artemisinin and dihydro-artemisinin in fresh isolates of *Plasmodium falciparum* from Thailand. *Mitt Oesterr Ges Trop Med Par* 2000; 22: 87-93.
- Litchfield JT, Wilcoxon F. A simplified method of evaluating dose effect experiments. *J Pharmacol Exp Med* 1949; 96: 99-113.
- 14. Wernsdorfer WH, Wernsdorfer MG. The evaluation of *in vitro* tests for the assessment of drug response in *Plasmodium falciparum*. *Mitt Oesterr Ges Trop Med Par* 1995; *17*: 221-8.
- Tasanor O, Noedl H, Na-Bangchang K, Congpuong K, Sirichaisinthop J, Wernsdorfer WH. An *in vitro* system for assessing the sensitivity of *Plasmodium vivax* to chloroquine. *Acta Trop* 2002; 83 : 49-61.