Susceptibility of Colombian *Plasmodium falciparum* isolates to 4-aminoquinolines and the definition of amodiaquine resistance in vitro

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There are wide variations in the threshold used to define in vitro resistance of Plasmodium falciparum *to amodiaquine (AQ), probably due to differences in methodology and interpretation. In vitro susceptibility data of Colombian* P. falciparum *strains to AQ and N-desethylamodiaquine is used to illustrate the need to standardized methodologies and compare inhibitory concentrations, instead of resistant/susceptible phenotypes, when studying the mechanisms of resistance to AQ and monitoring drug susceptibility trends in the field.*

Key words: chloroquine - amodiaquine - N-desethylamodiaquine - in vitro tests

Amodiaquine (AQ) is an antimalarial compound chemically and functionally related to chloroquine (CQ) (Hyde 2002). Currently, it is used (in combination with other antimalarials) as the first choice to treat uncomplicated *Plasmodium falciparum* malaria in some countries in Africa and South America (Brasseur et al. 1999, WHO 2001). In the body, AQ is rapidly metabolized by the hepatic cytochrome CYP2C8 to N-desethylamodiaquine (DAQ) (Li et al. 2002) which exerts the main antimalarial therapeutic effect (Churchill et al. 1985, Basco & Le Bras 1993). Unfortunately, as with most antimalarial drugs, in vivo therapeutic failures as well as in vitro resistance of *P. falciparum* to AQ have been reported (Olliaro & Mussano 2003). A methodology to assess the therapeutic efficacy of most antimalarials has been standardized. In contrast, there are wide variations in the methods used for in vitro testing and particularly for AQ.

The schizonts maturation and radioisotopic microtests are the two most frequently used methods for the in vitro evaluation of antimalarial drugs. The former is based on the microscopical assessment of the inhibition of schizonts maturation in the presence of different drug concentrations. The latter measures the incorporation of radio-marked hypoxanthine by live parasites exposed to different drug concentrations. Other two methods, based on ELISA (pLDH and HRP2), are in an advanced process of standardization (Noedl et al. 2003).

The in vitro susceptibility of malaria parasites to a pharmacological compound could be determined using the IC99, IC90, and the IC50 (IC: inhibitory concentration). The latter is defined as the concentration of the drug able to inhibit the growth of 50% of the parasites with respect to the control without drug, and is the most commonly used. Among others, factors such as percent of red blood cell parasited, hematocrit, the level of plasmatic proteins, and traces of antimalarial drugs in plasma affect the estimation of the ICs (Winstanley & Watkins 1992). The immune response with antibodies apparently do not play a significant role in the in vitro response to AQ, when the hematocrit levels are < 5% (Wensdorfer & Paine 1988).

When revising the literature, in spite of the differences found in the in vitro protocols, it seems that there is a consensus on the threshold to define resistance to DAQ (≥ 60 nM) (Ringwald et al. 1996, 2000, Brasseur et al. 1999, Aubouy et al. 2004). However, we found no standard criterion that defines resistance or susceptibility to AQ. In fact, the reported resistance thresholds for AQ vary from 4 to 400 nM (Table I).

The differences in the reported thresholds to define AQ resistance in vitro described in Table I could be partially explained by: (1) variations in the in vitro methodology such as incubation time of the parasite with the drug (from 24 to 50 h) (Childs et al. 1989, Reynes et al. 1997, Pradines et al. 1998, Chaparro & Wassermann 1999, Basco et al. 2002, Rason et al. 2002), the final hematocrit (from 1 to 1.5%) (Childs et al. 1989, Chaparro & Wassermann 1999), and the percent of red blood cell parasited (from 0.1 to 0.8%) (Pradines et al. 1998, Duraisingh et al. 1999, Basco et al. 2002); the most important parameter probably being the hematocrit, since the 4-aminoquinolines have the likeness to concentrate inside erythrocytes (Pussard et al. 1987, Winstanley et al. 1987); (2) the in vitro tests are performed with DAQ but the conclusions refer to AQ (Ringwald et al. 2000, Basco et al. 2002, Aubouy et al 2004); (3) the use of different commercial presentations of AQ without taking into account their different molecular weights (AQ base, AQ hydrochloride or AQ dihydrochloride); and (4) the use of parasites adapted to cultures in vitro and with incubation time > 24 h are likely to show different results from those obtained with fresh isolates (W Wernsdorfer, pers. commun.).

In our lab, the susceptibility to AQ and DAQ of 22 *P. falciparum* Colombian isolates and two reference strains (W2 and D6, resistant and sensitive to CQ respectively) was determined using the radioisotopic method (Desjardins et al. 1979, Cerutti et al. 1997). The isolates that had been kept frozen in liquid Nitrogen were thawed

Financial support: OPS, CNT0400132.002
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Received 26 January 2006
Accepted 24 April 2006

following the methodology described by Cerutti et al. (1997) and maintained in continuous culture in supplemented RPMI 1640 (Trager & Jensen 1976, Desjardins et al. 1979). The samples were synchronized with Sorbitol 1% (Cerutti et al. 1997), evaluated with a percent of red blood cells parasited of 0.5% and a hematocrit of 1.5% and exposed to concentrations of AQ base (provided by WRA) and DAQ (provided by WHO) ranging from 5 to 320 nM. The final concentration of ^{3}[H] hypoxanthine was 0.5 µCi/well and of albumax was 0.5%. The samples were incubated at 37°C for 48 h (Desjardins et al. 1979, Cerutti et al 1997); each assay was done by duplicate. The counts per minute (cpm) were measured in a scintillation counter (Beckman LS7500) and the IC50 and IC99 were calculated using the PROBIT program in SPSS 7.5 for windows 98 (SPSS. Inc., Chicago 1996).

TABLE I

Thresholds used to define in vitro resistance of *Plasmodium falciparum* to amodiaquine

Threshold	Method	Reference
IC_{50} ≥ 0.004 mM (**4 nM)**	Isotopic	Chaparro & Wasserman 1999
IC_{50} ≥ **30 nM**	Schizont maturation	Childs et al. 1989
IC_{50} ≥ **59 nM**	Isotopic	Basco et al. 2002
IC_{50} ≥ **60 nM**	Isotopic	Reynes et al. 1997
IC_{50} ≥ **80 nM**	Isotopic	Pradines et al. 1998, Rason et al. 2002
	Schizont maturation	Ringwald et al. 1996
IC_{50} ≥ 0.4 mM/l (**400 nM)**	Schizont maturation	Segurado et al. 1997
MIC ≥ 0.4 mMol/l of blood (**400 nM)**	Schizont maturation	Draper et al. 1988
IC_{90} ≥ 400 nMol/l blood (**400 nM)** in non-immune populations	Schizont maturation	Wernsdorfer & Paine 1988
IC_{99} ≥ 400 nM/l blood (**400 nM)** in immune populations	Schizont maturation	Wernsdorfer & Paine 1988

TABLE II

Amodiaquine (AQ) and desethylamodiaquine (DAQ) inhibitory concentrations (IC) in *Plasmodium falciparum* Colombian and two reference strains
(samples with IC_{50} of AQ ≥ 30nM and DAQ ≥ 60nM are highlighted)

Reference Strains	AQ IC50	DAQ IC50	DAQ/ AQ ratio IC50	AQ IC99	DAQ IC99
W2	21.3	**145.6**	6.8	77.6	1120.9
D6	9.4	25.3	2.6	50.2	106.4
Field strains					
Q1114	9.6	47.5	4.9	45.8	142.4
Q1147	**30.0**	**125.1**	4.1	230.	501.5
Q1306	**34.4**	**122.7**	3.5	200.5	696.2
Q1266	14.2	51.8	3.6	255.0	2294.9
TA7519	25.7	**71.4**	2.7	150.6	707.2
TA7529	13.2	**62.7**	4.7	97.7	650.0
TA4609	26.7	**79.7**	2.9	217.2	346.9
TA6182	26.7	**177.5**	6.6	173.1	4308.0
TA4641	23.0	38.3	1.6	128.3	367.4
TA4640	20.1	**86.0**	4.2	216.8	1060.3
TA10254	**30.5**	**111.8**	3.6	53.8	230.4
TU384F	13.1	**62.4**	4.7	55.1	156.9
TU9288	27.2	**69.9**	2.5	52.5	547.8
TU741	**41.0**	**355.**	8.6	845.2	4783.9
TU545F	18.4	**159.9**	8.6	77.1	330.7
TU9255	22.2	**101.5**	4.5	189.7	530.9
TU8064	11.6	39.5	3.3	105.1	493.6
TU11365F	**39.6**	**222.3**	5.6	237.8	16281.9
CA2855	**64.6**	**198.0**	3.0	225.1	2831.4
BV5029	**37.1**	**122.4**	3.	161.9	286.8
BV5029F	**33.3**	**94.8**	2.8	316.1	968.6
BV5037	**30.7**	**94.2**	3.0	126.2	449.2
Mean	27.0	113.4	4.2	189.1	1771.2
SD	12.4	74.0	1.8	164.9	3492.1

SD: standard deviation

In the Colombian strains, the mean DAQ IC50 (113.45 nM) was 4.25 times higher than the one for AQ (27 nM). The IC50 results of W2 and D6 also followed this pattern (Table II). The mean DAQ and AQ IC99 of the field isolates (1771.27 nM and 189.17 nM, respectively) were well above the IC99s reported by Childs et al. (1989) in samples from Thailand (157 nM and 46,7 nM, respectively). The relatively high IC99s of Colombian isolates suggests the presence of AQ and DAQ resistance. However, our and Child's study used different methodologies (culture vs fresh isolates, and radioisotopic vs schizont maturation method) making them difficult to compare. Looking at Table I, the thresholds to define AQ resistance as IC50 > 60 nM (the proposed threshold for DAQ) contradict our findings and reports showing that AQ is three times more potent than DAQ (Pussard et al. 1987, Winstanley et al. 1987, 1990). Hence, the threshold to define AQ resistance should be lower than the one described for DAQ (Churchill et al. 1985, Childs et al. 1989).

When in vitro resistance to DAQ and AQ is defined as an IC50 ≥ 60 nM and ≥ 30 nM respectively, 82% (18/22) field isolates were resistant to DAQ and 41% (9/22) to AQ. All samples that were resistant to AQ were also resistant to DAQ but not all samples resistant to DAQ were resistant to AQ, which does not support the statement that resistance to DAQ predicts resistance to AQ (Basco et al. 2002, Aubouy et al. 2004). In fact, samples (including W2 strain) with DAQ IC50 > 120 nM (twice the defined threshold) showed AQ IC50 below 30 nM. The IC50 values of AQ and DAQ are highly correlated (Pearson r = 0,736 $P < 0.001$) suggesting that the inconsistencies in defining resistance could be explained by inaccurate thresholds. In fact, a linear regression analysis with log transformed data showed that, in this dataset, the DAQ IC50 60 nM was equivalent to AQ IC50 15.48 nM. This AQ IC50 will classify eight more of our isolates as AQ resistant. For AQ IC50 30 nM the equivalent DAQ IC50 was 119.28 nM, consistent with the threshold proposed by Childs et al. (1989) to define resistance to AQ (Childs et al. 1989). Based on this data, AQ IC50 values between 15 nM and 30 nM could be used to identify mutations or copy numbers associated with AQ resistance using a similar approach to that of Price et al. (2004) for mefloquine resistance. Nevertheless, this assumes that the 60 nM threshold used to define in vitro DAQ resistance is accurate, which is not necessarily true.

Pharmacokinetic and pharmacodynamic studies help to orientate the definition of a sensitive/resistance in vitro threshold to antimalarial drugs and should be expanded. In the meantime, it will be helpful to standardize methodologies and use continuous in vitro data (i.e. ICs rather than resistant/susceptible phenotypes) in studies dealing with the identification of the mechanisms of resistance to AQ and monitoring drug susceptibility trends in the field. In public health it is probably more relevant to monitor in vitro resistance to DAQ instead of AQ, since DAQ exerts the major antimalarial activity in vivo. On the other hand, understanding the mechanism of resistance to AQ would be useful in the design of new drugs, particularly 4-aminoquinoline derivatives.

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