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Cross-resistance of the chloroquine-derivative AQ-13 with amodiaquine in Cambodian *Plasmodium falciparum* isolates

Flore Nardella[1], Mélissa Mairet-Khedim[2], Camille Roesch[2], Steven P. Maher[3], Sopheakvatey Ke[2], Rithea Leang[4], Didier Leroy[5] and Benoit Witkowski[2]*

[1]Unité Biologie des Interactions Hôte-Parasite, Département de Parasites et Insectes Vecteurs, Institut Pasteur, CNRS ERL9195, INSERM Unit U1201, 25–28 Rue du Dr Roux, Paris, 75015, France; [2]Malaria Molecular Epidemiology Unit, Pasteur Institute in Cambodia, Phnom Penh, Cambodia; [3]Center for Tropical and Emerging Global Diseases, University of Georgia, 500 D.W. Brooks Dr., Athens, GA 30602, USA; [4]National Center for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia; [5]Medicine for Malaria Venture, Geneva, Switzerland

*Corresponding author. E-mail: bwitkowski@pasteur-kh.org

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Background: Expanding resistance to multiple antimalarials, including chloroquine, in South-East Asia (SEA) urges the development of new therapies. AQ-13, a chloroquine derivative, is a new drug candidate for treating malaria caused by *Plasmodium falciparum.*

Objectives: Possible cross-resistance between the 4-aminoquinolines amodiaquine, piperaquine and AQ-13 has not been assessed. *In vitro* parasite growth assays were used to characterize the susceptibility of multidrug-resistant and susceptible *P. falciparum* patient isolates to AQ-13.

Methods: A [^{3}H]hypoxanthine uptake assay and a 384-well high content imaging assay were used to assess efficacy of AQ-13 and desethyl-amodiaquine against 38 *P. falciparum* isolates.

Results: We observed a strong cross-resistance between the chloroquine derivative amodiaquine and AQ-13 in Cambodian *P. falciparum* isolates (Pearson correlation coefficient of 0.8621, $P < 0.0001$).

Conclusions: In light of the poor efficacy of amodiaquine that we described recently in Cambodia, and its cross resistance with AQ-13, there is a significant risk that similar clinical efficacy of AQ-13-based combinations should be anticipated in areas of amodiaquine resistance.

Introduction

Chloroquine is an easily-synthesized, affordable 4-aminoquinoline antimalarial that was highly efficacious until the emergence of resistance in the late 1950s.[1] Worldwide spread of resistance has restricted its use to areas of low resistance in Central America for the treatment of uncomplicated *Plasmodium falciparum* malaria.[2] Resistance to chloroquine is mediated by point mutations in *pfcrt* (notably K76T), a transporter that induces an efflux of chloroquine outside of the digestive vacuole, where it exerts its action.[3] To overcome and prevent the spread of resistance to single antimalarials, the current recommendation for the treatment of uncomplicated malaria is the use of artemisinin-based combination therapies (ACTs) that combine a fast-acting artemisinin derivative with a long-lasting partner drug. Six combinations are marketed, two of which are in combination with a chloroquine derivative: dihydroartemisinin/piperaquine (DHA-PIP) and artesunate/amodiaquine (AS-AQ). However, resistance to ACTs is occurring as several mutations in *pfcrt* participate in the resistance to DHA-PIP[3–5] while a mutation in *pfmdr1* seems to be associated with AS-AQ treatment failures.[6] Drug-failure to ACTs in the Greater Mekong Subregion (GMS), notably with DHA-PIP, urges the development of new combinations to control and eradicate malaria. AQ-13 is a short chain chloroquine derivative that has been developed to circumvent chloroquine resistance. AQ-13 properties have recently been reviewed by Mengue *et al.*[7] It showed non-inferiority to artemether/lumefantrine (AL) in a Phase II clinical trial in Mali, and successfully cured 100% of patients regardless of the status of chloroquine resistance.[8] However, we have recently shown that the clinical efficacy of AS-AQ precludes its implementation in Cambodia.[6]

In order to evaluate the activity of this 4-aminoquinoline in development in a context of high drug resistance, we tested the activity of AQ-13 against Cambodian multidrug-resistant isolates that were adapted to culture. We show here for the first time that

AQ-13 is cross-resistant with desethyl-amodiaquine (dAQ), the major metabolite of amodiaquine, in South-East Asian strains.

Materials and methods

P. falciparum clinical isolates

Isolates were collected from Cambodian patients with uncomplicated *P. falciparum* malaria enrolled in WHO therapeutic efficacy studies (2011–16). Venous blood was collected into acid-citrate-dextrose tubes (Becton-Dickinson, Franklin Lakes, NJ, USA). Parasites were adapted to *in vitro* culture at 2% haematocrit (O+ human blood, Centre de Transfusion Sanguine, Phnom Penh, Cambodia) in RPMI-1640 medium supplemented with 0.5% (w/v) albumax II, 2.5% (v/v) decomplemented human plasma (mixed serogroups) under an atmosphere of 5% CO_2 and 5% O_2 and kept at 37°C.

In vitro susceptibility determination

In vitro susceptibility of the parasites to AQ-13 and dAQ was determined using the [^{3}H]hypoxanthine uptake inhibition assay[9] against 38 *P. falciparum* isolates. AQ-13 [Ro 47-0543: N-(7-chloroquinolin-4-yl)-N′,N′-diethylpropane-1,3-diamine] was obtained from Medicine for Malaria Venture, monodesethylamodiaquine (dAQ) and DMSO (used as vehicle) were obtained from WWARN & Sigma Aldrich, Singapore, respectively. Parasites were synchronized at ring stage using two 5% D-sorbitol treatments (0–6 h post-invasion) and exposed to a concentration range of AQ-13 or dAQ (0.7 to 500 nM) for 48 h in presence of 0.5 μCi of [^{3}H]hypoxanthine (Perkin-Elmer, Waltham, USA). Tritium incorporation was measured with a β-counter (Trilux microbeta; Perkin-Elmer Waltham, USA). Inhibitory concentrations values (IC_{50}) were determined using IVART online software (https://www.wwarn.org/ivart).[10] Four *P. falciparum* laboratory reference strains were used as controls: 3D7, 7G8, W2 and Dd2. We chose an IC_{50} value >60 nM to define resistance to amodiaquine, according to previous studies.[11]

Parasite survival using high content imaging

We used two different strains: the laboratory AQ-susceptible strain 3D7 and a Cambodian AQ-resistant patient isolate collected in 2016 (Cambodia) having a dAQ IC_{50} of 239 nM. Parasites synchronized at ring stage (0–3 h post invasion) were diluted to 3% parasitaemia and 0.01% haematocrit and exposed to a concentration range of AQ-13 and dAQ (0.7 to 500 nM) for 72 h, in a 384 well-plate. After 72 h incubation, cells were then fixed for 15 min with 0.44% glutaraldehyde, and red blood cells were permeabilized with 3% Triton for 10 min. Parasite DNA was then stained with 80 nM YOYO™-1 Iodide for 45 min at room temperature in the dark. Pictures were taken using the Lionheart™ FX Automated Microscope (BioTek), covering the surface of each well containing YOYO™-1 Iodide-stained parasites.

Ethics

All isolates were collected during therapeutic efficacy studies (TES) upon protocol acceptance from the Cambodian National Ethical Committee (NECH # 071, 073, 079, 0.136, 0168 and 0273).

Statistical analysis

All statistical analyses were performed using Graphpad Prism 8.0 software. The correlation between AQ-13 and dAQ IC_{50}s was assessed using a Pearson's test (Figure 1a) and a Mann–Whitney test was used for comparing the difference in IC_{50} between AQ-susceptible (AQ-S) and -resistant (AQ-R) groups (Figure 1b). Comparison of survival between AQ-S and AQ-R strains in Figure 1(c) was done using a two-way ANOVA with Bonferroni's multiple comparison test. A P value <0.05 was considered significant.

Results

AQ-13 IC_{50} values ranged from 18 to 133 nM while those of dAQ ranged from 20 to 190 nM. We observed a clear correlation between the IC_{50}s obtained for AQ-13 and dAQ (Pearson coefficient of 0.8621, $P < 0.0001$; Figure 1a). Also, IC_{50}s of AQ-13 were statistically different when we compared AQ-S and AQ-R isolates using the [^{3}H]hypoxanthine uptake inhibition assay [Mann–Whitney statistical test, median of 46.7 nM ($n = 14$) and 64.9 nM ($n = 24$) respectively, $P < 0.0001$; Figure 1b]. IC_{50}s obtained for AQ-13 with the reference strains AQ-S 3D7 and AQ-R 7G8 had the same trend: 20.9 nM and 44.3 nM, respectively. In general, AQ-13 IC_{50}s measured in Cambodian isolates exceeded by far the values obtained in laboratory strains (represented as coloured triangles in Figure 1a). This difference was confirmed by high content imaging using YOYO™-1 DNA staining (Figure 1c and d). At a concentration of 167 nM, up to 54% of AQ-R parasites survived to 72 h during AQ-13 treatment and up to 95% survived dAQ treatment while only 5% survived both drugs in the 3D7 AQ-S strain (Figure 1c).

Discussion

The pipeline of long-lasting antimalarials potentially suitable for developing new ACT is limited and these drug candidates are essential in the current context of drug resistance. Among those in Phase II, AQ-13 remains a promising option with both excellent tolerability and clinical efficacy.[7] Previous investigation conducted by Ridley *et al.*[12] showed a strong correlation of the susceptibility to both CQ and AQ-13 of isolates from Thailand and Tanzania, as well as reference strains. However, the IC_{50} of AQ-13 remained lower than 100 nM whereas chloroquine's reached up to 500 nM in the most-resistant isolates. While these data indicate a potential efficacy of AQ-13 in CQ-R strains, they also clearly show a shared tolerance (or resistance) mechanism and raise the question of AQ-13 cross resistance with other 4-aminoquinolines. Circulation of AQ-R parasites has been recently described in Cambodia.[6] In this context we have measured the susceptibility of Cambodian *P. falciparum* isolates to both AQ and AQ-13. Interestingly, we found a strong cross-resistance between AQ and AQ-13 and the IC_{50} values obtained with AQ-13 in some isolates were mainly above 100 nM. Confirming the conclusions of Ridley and colleagues,[12] our findings suggest a shared resistance mechanism to both AQ and AQ-13. Therefore, and despite AQ-13 never having been deployed at large scale, strains harbouring a relatively high resistance to this molecule are already circulating. This study was not designed to explain the mechanism of cross-resistance between AQ and AQ-13, but the structural relatedness between AQ-13 and AQ could be one explanation. Previous data suggests an association of *pfmdr1* polymorphism with AQ resistance observed *in vitro*, while *pfcrt* polymorphism is not implicated.[6] Unfortunately, *pfdmr1* and *pfcrt* genotypes are not available here to evaluate this association with AQ-13 resistance.

In summary, we report *in vitro* cross-resistance between dAQ (and hence AQ) and AQ-13. Further development of this molecule should consider this finding and carefully address the use of AQ-13 in the areas where AQ-R has been detected.

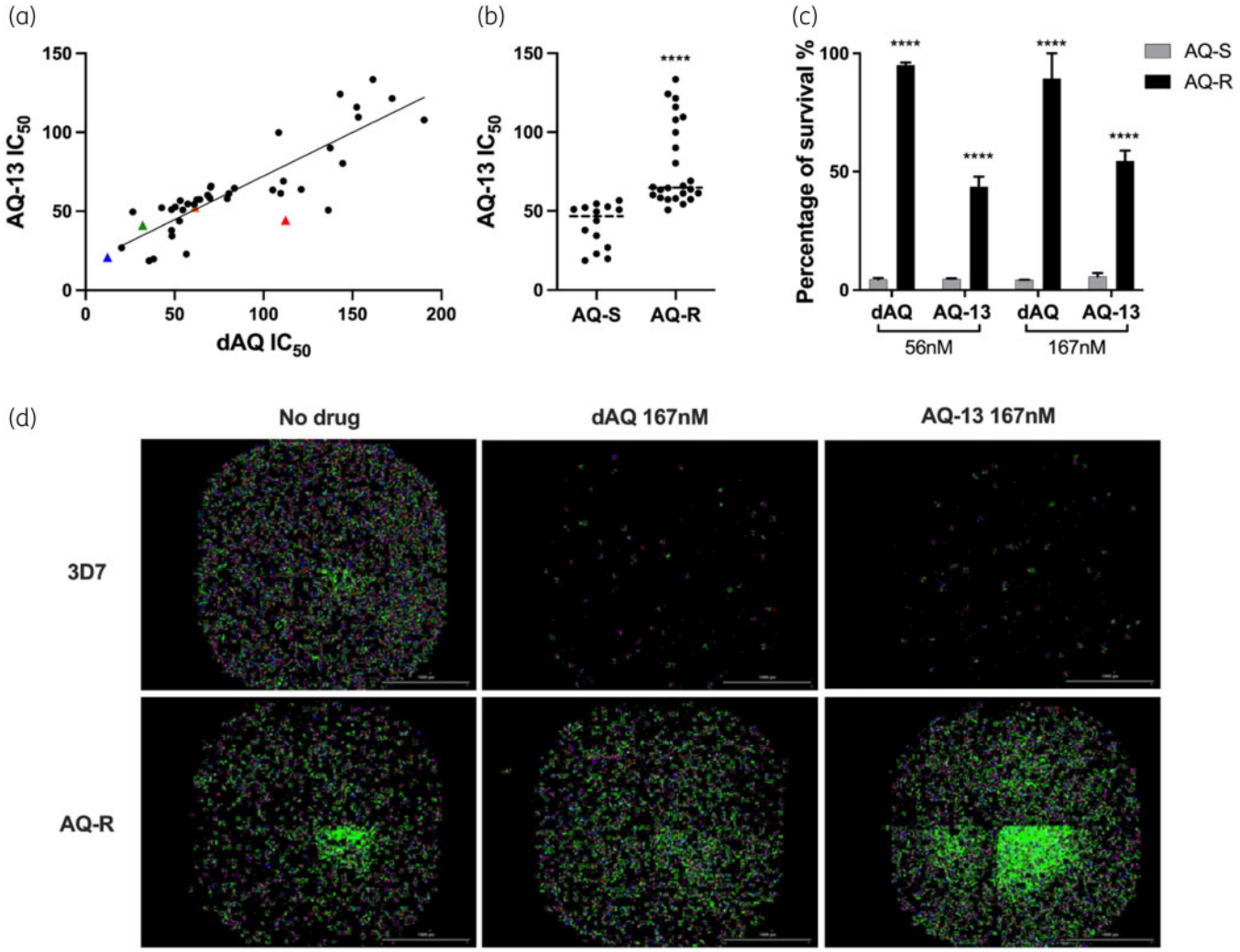

Figure 1. *In vitro* susceptibility of Cambodian *Plasmodium falciparum* isolates to AQ-13. (a) Correlation between AQ-13 and desethylamodiaquine (dAQ) IC_{50}s. Pearson test indicates a statistically significant correlation [$r = 0.8621$ (0.7487 to 0.9264), R squared = 0.7431, $P < 0.0001$]. Reference laboratory strains are represented as triangles: 3D7 (blue), Dd2 (green), W2 (orange), 7G8 (red). (b) Activity of AQ-13 in amodiaquine-susceptible (AQ-S) or resistant (AQ-R) *P. falciparum* isolates. Amodiaquine resistance was defined as an IC_{50}>60 nM, according to previous studies. Each dot represents individual IC_{50} values obtained with AQ-13 in the 38 isolates. Mann–Whitney statistical test indicates statistical difference between the two groups with a *P* value <0.0001. (c) Parasite multiplication after 72 h treatment with dAQ or AQ-13 in AQ-S (3D7) or AQ-R (isolate) parasites. Parasite numbers after drug exposure (56 and 167 nM) were quantified by high content imaging (YOYO™-1 DNA fluorescence quantification). Survival proportions were calculated in comparison to the untreated control. ****=$P < 0.0001$ (two-way ANOVA multiple comparisons test, using Bonferroni's correction). (d) Picture of the surface of a representative well (corresponding to Figure 1c data) using a high content imager, after 72 h treatment at 167 nM. Each dot represents a parasite stained with YOYO™-1.

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Transparency declarations

None to declare.

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