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► **To cite this version:**

Flore Nardella, Mélissa Mairet-Khedim, Camille Roesch, Steven Maher, Sopheakvatey Ke, et al.. Cross-resistance of the chloroquine-derivative AQ-13 with amodiaquine in Cambodian Plasmodium falciparum isolates. *Journal of Antimicrobial Chemotherapy*, Oxford University Press (OUP), 2021, 76 (10), pp.2565-2568. 10.1093/jac/dkab219 . pasteur-03480931

HAL Id: pasteur-03480931

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Submitted on 15 Dec 2021

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

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Received 25 February 2021; accepted 3 June 2021

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 [³H]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 *pfcr* (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 *pfcr* 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) decomplexed human plasma (mixed serogroups) under an atmosphere of 5% CO₂ and 5% O₂ and kept at 37°C.

In vitro susceptibility determination

In vitro susceptibility of the parasites to AQ-13 and dAQ was determined using the [³H]hypoxanthine uptake inhibition assay⁹ 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 [³H]hypoxanthine (Perkin-Elmer, Waltham, USA). Tritium incorporation was measured with a β-counter (Trilux microbeta; Perkin-Elmer Waltham, USA). Inhibitory concentrations values (IC₅₀) 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 IC₅₀ 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 IC₅₀ 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 YOYOTM-1 Iodide for 45 min at room temperature in the dark. Pictures were taken using the LionheartTM FX Automated Microscope (BioTek), covering the surface of each well containing YOYOTM-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 IC₅₀s was assessed using a Pearson's test (Figure 1a) and a Mann-Whitney test was used for comparing the difference in IC₅₀ 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 IC₅₀ values ranged from 18 to 133 nM while those of dAQ ranged from 20 to 190 nM. We observed a clear correlation between the IC₅₀s obtained for AQ-13 and dAQ (Pearson coefficient of 0.8621, *P* < 0.0001; Figure 1a). Also, IC₅₀s of AQ-13 were statistically different when we compared AQ-S and AQ-R isolates using the [³H]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]. IC₅₀s 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 IC₅₀s 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 YOYOTM-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 IC₅₀ 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 IC₅₀ 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 *pfcr1* polymorphism is not implicated.⁶ Unfortunately, *pfmdr1* and *pfcr1* 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.

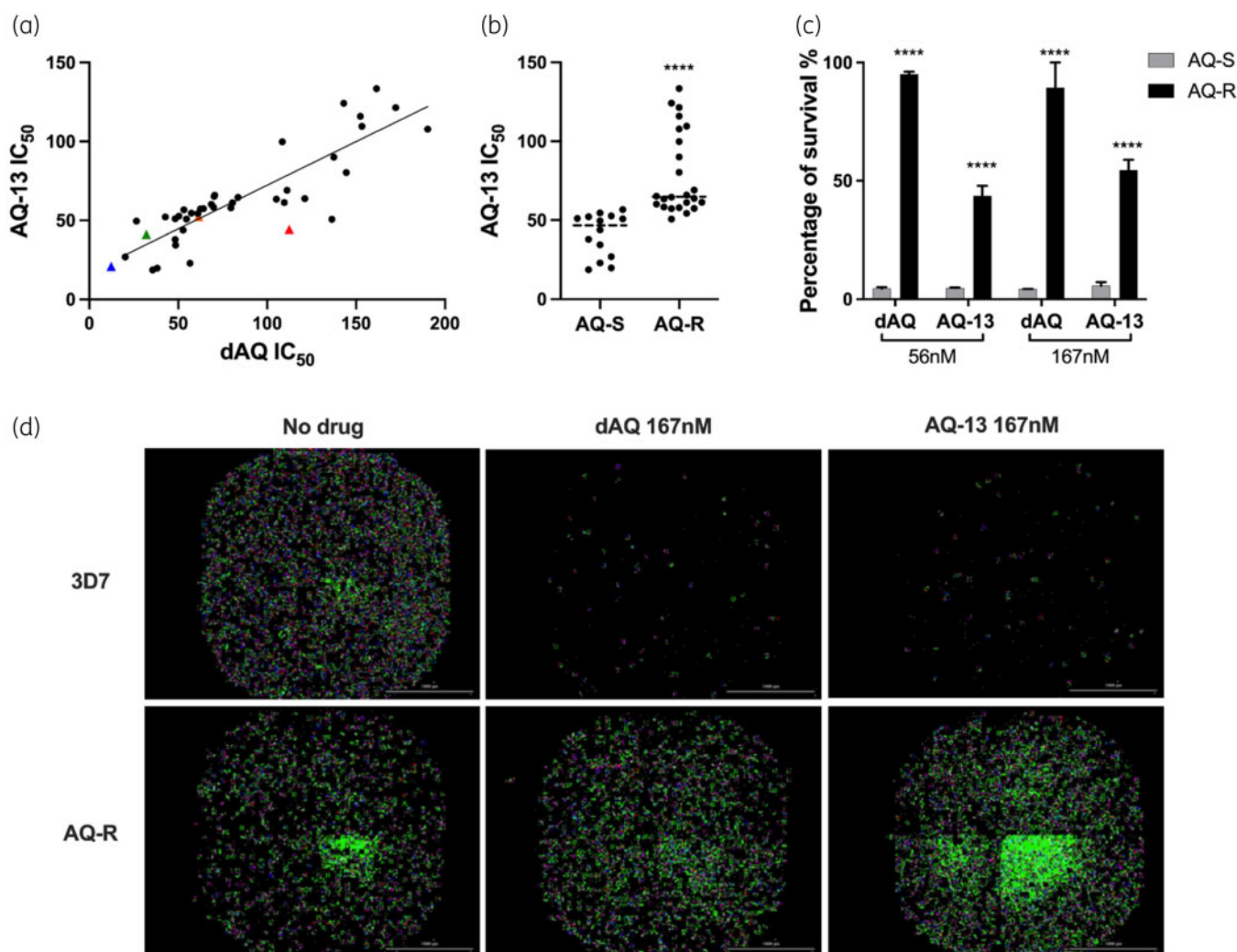


Figure 1. *In vitro* susceptibility of Cambodian *Plasmodium falciparum* isolates to AQ-13. (a) Correlation between AQ-13 and desethylamodiaquine (dAQ) IC_{50} s. Pearson test indicates a statistically significant correlation [$r=0.8621$ (0.7487 to 0.9264), $R^2=0.7431$, $P<0.0001$]. Reference laboratory 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 (YOYOTM-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 YOYOTM-1.

Acknowledgements

We would like to thank the patients who agreed to participate in TES studies and the Cambodian Health Centres staff for their contribution.

Funding

This study was supported by Medicine for Malaria Venture. Flore Nardella was supported by a fellowship from the *Fondation Pierre Ledoux Jeunesse Internationale* and the Pasteur Institute International Network. Melissa Mairet-Khedim was supported by Initiative 5% Grant (MIVS-ACT Grant #15SANIN211).

Transparency declarations

None to declare.

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