International Journal for Parasitology: Drugs and Drug Resistance 17 (2021) 186–190

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Lumefantrine attenuates *Plasmodium falciparum* artemisinin resistance during the early ring stage

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ARTICLE INFO

Keywords:
Artemisinin
Drug resistance
Isobologram
Lumefantrine
Malaria

ABSTRACT

Emerging artemisinin resistance in *Plasmodium falciparum* malaria has the potential to become a global public health crisis. In Southeast Asia, this phenomenon clinically manifests in the form of delayed parasite clearance following artemisinin treatment. Reduced artemisinin susceptibility is limited to the early ring stage window, which is sufficient to allow parasites to survive the short half-life of artemisinin exposure. A screen of known clinically-implemented antimalarial drugs was performed to identify a drug capable of enhancing the killing activity of artemisinins during this critical resistance window. As a result, lumefantrine was found to increase the killing activity of artemisinin against an artemisinin-resistant clinical isolate harboring the C580Y *kelch13* mutation. Isobologram analysis revealed synergism during the early ring stage resistance window, when lumefantrine was combined with artemether, an artemisinin derivative clinically partnered with lumefantrine. These findings suggest that lumefantrine should be clinically explored as a partner drug in artemisinin-based combination therapies to control emerging artemisinin resistance.

1. Introduction

Malaria drug resistance is a major public health threat to people living in endemic regions worldwide (WHO 2019). One of the most recent pressing issues is the emergence of parasites with reduced susceptibility to artemisinins, the first-line drugs for *Plasmodium falciparum* malaria treatment (White 2016). This resistance phenotype manifests clinically in the form of delayed parasite clearance following artemisinin treatment and is associated with mutations at *kelch13* (PF3D7_1343700), most commonly at position C580Y (Dondorp et al., 2009; Ariey et al., 2014). Complete loss of efficacy for this antimalarial class would be disastrous to the global endeavor to control and eradicate *P. falciparum* malaria. Artemisinin and its derivatives are the only antimalarial drugs currently in clinical use capable of rapid clearance of *P. falciparum* in patients by acting against every asexual erythrocytic stage (White 2008). This emerging crisis, if left unchecked, could have devastating effects that will extend beyond Southeast Asia. Parasites harboring drug-resistant mutations against antifolates and chloroquine, which originated in Southeast Asia, have spread to the rest of the world, costing millions of lives and setting back malaria control efforts worldwide (Wellems and Plowe 2001). Molecular surveillance studies based on the C580Y *kelch13* mutation showed that these parasite populations

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https://doi.org/10.1016/j.ijpddr.2021.09.005
Received 28 February 2021; Received in revised form 25 September 2021; Accepted 28 September 2021
Available online 2 October 2021

have already spread in many countries in Southeast Asia (Amato et al., 2018).

The nature of emerging artemisinin resistance is unique in comparison to a typical full-blown resistance phenotype in that the parasites manifest reduced artemisinin susceptibility only during the early ring stage. During the rest of the erythrocytic cycle, there is no significant difference in artemisinin susceptibility between resistant and sensitive parasites (Klonis et al., 2013). Taking into consideration the short half-life of artemisinins, this resistance window provides ample time for parasites to survive the short artemisinin-exposure time (Woodrow et al., 2005). Due to poor pharmacokinetic profiles of artemisinins, it is strategically important to administer an additional antimalarial drug in the form of artemisinin-based combination therapies (ACTs) to eliminate residual surviving parasites (Eastman and Fidock 2009).

A high-throughput screen was developed and implemented to identify compounds that could boost activity of artemisinins against parasites with reduced susceptibility during the early ring stage resistance window. A panel of known anti-malarial drugs was included as a control. Interestingly, lumefantrine fitted the selection profile of compounds that could attenuate artemisinin resistance. Furthermore, artemether and lumefantrine were found to be synergistic during the early ring stage resistance window as shown by isobologram analyses. These findings highlight the benefit of lumefantrine as a partner drug for combating emerging artemisinin resistance.

2. Materials and methods

2.1. Parasite cultivation and preparation

P. falciparum 3D7 and ANL4 were cultivated in RPMI1640 complete medium containing 0.5% Albumax II (Gibco), 25 mM HEPES (culture grade), 1X GlutaMAX (Gibco) and 25 μg/mL gentamicin (Gibco). *P. falciparum* ANL4 was cultivated and provided by Mahidol Oxford Research Unit from a malaria case with delayed clearance of *Plasmodium falciparum* following artemisinin treatment (parasite clearance half-life of 8.5 h, above the 5-h cutoff point). It was chosen because of its stable and robust artemisinin-resistant phenotype during a screen. The 3D7 reference strain was used as a control during assay optimisation and isobologram analyses. The parasite cultures were maintained between 0.5% and 3% parasitaemia at 3% haematocrit in malaria gas chambers (1% O_2, 3% CO_2 and 96% N_2). Erythrocytic schizont-stage parasites were synchronised by using 70% Percoll (GE Healthcare) diluted in 10X RPMI containing 2.9% sorbitol in phosphate-buffered saline (PBS). After 4 h, parasites were treated with 5% sorbitol to obtain early ring stage parasites, confirmed by microscopic examination following Giemsa staining (VWR Chemicals).

2.2. Artesunate-booster compound screening

Combinatorial compound screening was performed in 96-well plates (Costar) in a total volume of 100 μL/well. *P. falciparum* culture containing early ring stage parasites (0-4 h post invasion) was prepared as described above and was adjusted to 1% parasitaemia at 2% haematocrit, to which 10 μM of each antimalarial together with 130 nM artesunate was added using HP D300 digital dispenser immediately before performing the assays. Artesunate at 130 nM was chosen for the screen because it provides the maximum difference in early ring stage anti-plasmodial activity between artemisinin-resistant (ANL4) and -sensitive (3D7) parasites and has an optimal signal-to-noise ratio for a two-drug combination readout with the lactate dehydrogenase-based inhibitory concentration assay (LICA) (Loesbanluechai et al., 2019). Parasite susceptibility was determined by LICA which was used for determining IC_{50} values of artemisinins by exposing early ring parasites to different drug concentrations (Loesbanluechai et al., 2019). In short, early ring stage parasites were incubated with different artesunate concentrations for 3 h. After a 3-h incubation, compounds were washed out thrice using malaria complete medium. The parasites were transferred to a new 96-well plate and cultured in drug-free complete medium for a further 48-h period before performing a colourimetric lactate dehydrogenase assay to determine parasite growth. LICA was chosen because it provides similar information to a survival assay and relies on the survival of early ring stage parasites after exposure to multiple drug concentrations including at 700 nM commonly used in a standard ring survival assay (Witkowski et al., 2013).

The total parasite inhibition control used in data normalisation was performed with 5 μM artesunate. Assays with antimalarial alone, artesunate alone and no drug were included in every assay as controls. Due to experimental fluctuations in non-specific background signal in the colourimetric LDH assay, absorbance readings in test wells were sometimes below that of the inhibition control. This was interpreted as 100% inhibition in each case, but for none of the combinations tested did this influence the outcome of the initial screening. Artesunate was chosen for the screen due to its high solubility, which makes it ideal to be mixed with concentrated screening compounds. The IC_{50} value of artesunate is within the same range as that of dihydroartemisinin, a common choice for an *in vitro* artemisinin susceptibility assay (de Vries and Dien, 1996). Artesunate was shown to be converted by human blood to the active metabolite, DHA, by blood esterase (Zhou et al., 1987), but parasite exposure to DHA in artesunate-treated cultures might not be directly comparable to *in vivo* exposure, as conversion to DHA in the mammalian liver is likely to be more effective.

2.3. Stage-specific drug susceptibility assay

The synchronised early ring stage 3D7 and ANL4 parasites at 1% parasitaemia and 4% haematocrit were allocated to four experimental time points for subsequent drug exposure with three technical replicates; at 0–12 h post invasion (hpi), 12–24 hpi, 24–36 hpi and 36–48 hpi. Each parasite stage was exposed to lumefantrine (Sigma-Aldrich) for 12 h in ten concentrations ranging from 500 nM to 1 nM prepared by two-fold serial dilution in a 96-well plate with a total volume of 150 μL/well containing 1% parasitaemia at 2% haematocrit, followed by drug washout as described above. The growth of parasites and the half-maximal inhibitory concentration (IC_{50}) were determined after 48 h with SYBR green I (Molecular Probes) (Smilkstein et al., 2004). The microplate reader FLUOstar Omega (BMG Labtech) was used to read the SYBR green signal with the excitation and emission wavelength at $\lambda_{485\ nm}$ and $\lambda_{520\ nm}$, respectively. The IC_{50} plots and analyses were done using GraphPad Prism 8. Statistical significance was determined by Student's t-test. All assays were performed in three independent biological experiments.

2.4. Isobologram analysis

Isobologram was constructed for *P. falciparum* 3D7 and ANL4 using a chequerboard method (Martinez-Irujo et al., 1996). The drug plates containing a two-dimensional array of artemether and lumefantrine were prepared in a 96-well plate in a total volume of 100 μL/well parasite culture. The drugs were incubated with 1% early ring stage parasites (0-4 hpi) at 2% haematocrit for 3 h as performed in the screen. After treatment, the drugs were washed out thrice using malaria complete medium, and the parasites were transferred to a new culture plate and grown for 48 h. Parasite growth was determined by SYBR green I staining as previously described. The IC_{50} analyses were performed using GraphPad Prism 8. The degree of synergy was determined using a fractional inhibitory concentration (FIC) method, where ΣFIC of $\leq$0.5–1, >1–4 and >4 is considered synergistic, independent and antagonistic, respectively (Hall et al., 1983; Johnson et al., 2004).

2.5. Drug pre-incubation assay

O^+ human red blood cells (human biological samples were ethically

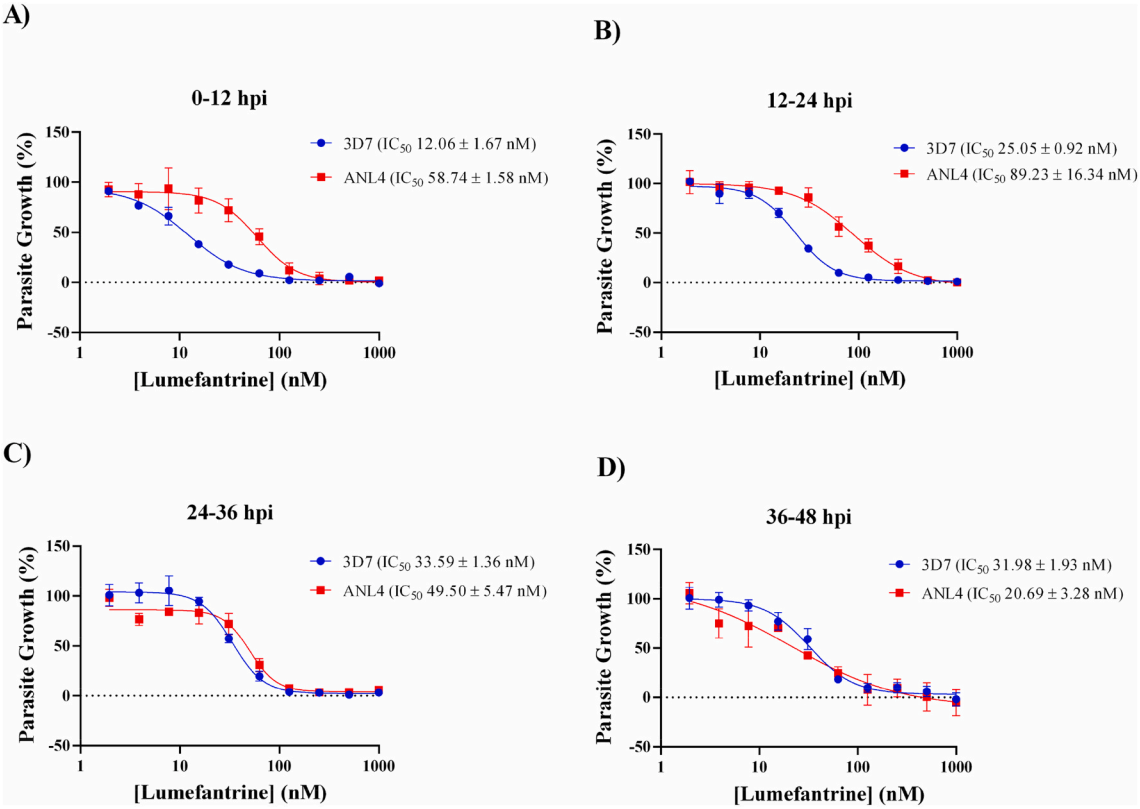

Fig. 1. Stage-specific drug susceptibility determination by the half-maximal inhibitory concentration analysis (IC_{50}). The IC_{50} assays were performed with tightly synchronised erythrocytic-stage 3D7 (blue) and ANL4 (red) parasites at 0-12 hpi (A), 12-24 hpi (B), 24-36 hpi (C) and 36-48 hpi (D). The IC_{50} and standard deviation values were calculated by GraphPad Prism 8. Three independent experiments were performed. Each experiment had three technical replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and anonymously obtained and provided by the Thai Red Cross, or the National Health Services Blood and Transplant, UK) at 5% haematocrit were incubated with 100 nM lumefantrine for 24 h in malaria complete medium. The drug-treated red blood cells were washed three times with malaria complete medium and then incubated with 1% synchronous schizonts obtained by the Percoll method mentioned above. Parasite growth was determined as mentioned above in comparison with the non-drug treated red blood cells infected with synchronous schizonts.

3. Results and discussion

We employed a colourimetric plate-format assay (Loesbanluechai et al., 2019) to perform a high-throughput screen for chemical compounds that improve the killing activity of artesunate during the critical early ring stage resistance window. During the screen, a control panel of twelve known antimalarial drugs (Supplementary Table S1) was included as a control. In short, the screen setup was performed using tightly-synchronised parasites in the early ring stage (0-4 hpi) which were exposed to a short 3-h pulse of 130 nM artesunate in combination with a series of clinically-implemented antimalarials at 10 μM. *P. falciparum* ANL4 shows higher survival than 3D7 when exposed to 130 nM artesunate and gives a higher signal-to-background ratio for the readout (Loesbanluechai et al., 2019), and the 3-h treatment was chosen to specifically target only the early ring stage interval. After 3 h, the drugs were thoroughly removed by washing three times. The treated parasites were transferred to a new plate and cultured for an additional 48-h period. *P. falciparum* ANL4 was cultivated by the Mahidol Oxford Research Unit from a malaria case with delayed clearance of *Plasmodium falciparum* following artemisinin treatment (parasite clearance half-life of 8.5 h compared to the 5-h cut-off value) (Ashley et al., 2014; Ponsuwanna et al., 2016). The genome of ANL4 (available at BioProject ID PRJNA742495) contains the C580Y *kelch13* mutation, the CVIET *crt* haplotype and the Y184F *mdr1* mutation without known *mdr1* amplification breakpoints and changes in *mdr1* sequencing coverage. Artesunate was chosen for the screen because it does not cause compound precipitation during the screening procedure. The hit criteria were set to focus on a drug that does not effectively kill early ring stage ANL4 by itself, but it boosts the parasite killing activity of artesunate during the early ring stage window. During the screen development phase, lumefantrine, one of the compounds in the control panel, emerged as being potent in the presence of artesunate compared to each drug alone (Supplementary Table S1).

In order to further explore this result, stage-specific susceptibility assays to lumefantrine were performed using synchronised ANL4 and 3D7 parasites at consecutive 12-h treatment periods covering one complete asexual life cycle (0–12, 12–24, 24–36, and 36–48 hpi). Overall, lumefantrine is broadly effective in every period of the parasite life cycle tested. ANL4 appears to be 4.9-fold and 3.6-fold less susceptible to lumefantrine in comparison to 3D7 during the 0-12 and 12-24 hpi intervals, respectively (Fig. 1A and B). The difference in lumefantrine susceptibility is less pronounced when parasites progressed to the trophozoite and schizont stages, corresponding to the 24-36 hpi and 36-48 hpi intervals (Fig. 1C and D). Nevertheless, this slight reduced susceptibility in ANL4 might not affect the treatment outcome since lumefantrine dosed orally for three consecutive days provides a high level of exposure with plasma concentration up to 7.5 μM (White et al., 1999). The increase in its IC_{50} value during the ring stage is much lower than the plasma concentration. Still, several pharmacokinetic factors would affect the amount of drug being exposed to the parasites. The difference between plasma drug concentrations and those used for *in vitro* drug assays needs to be fully explored in an *in vivo* model to determine whether the change in lumefantrine susceptibility is clinically

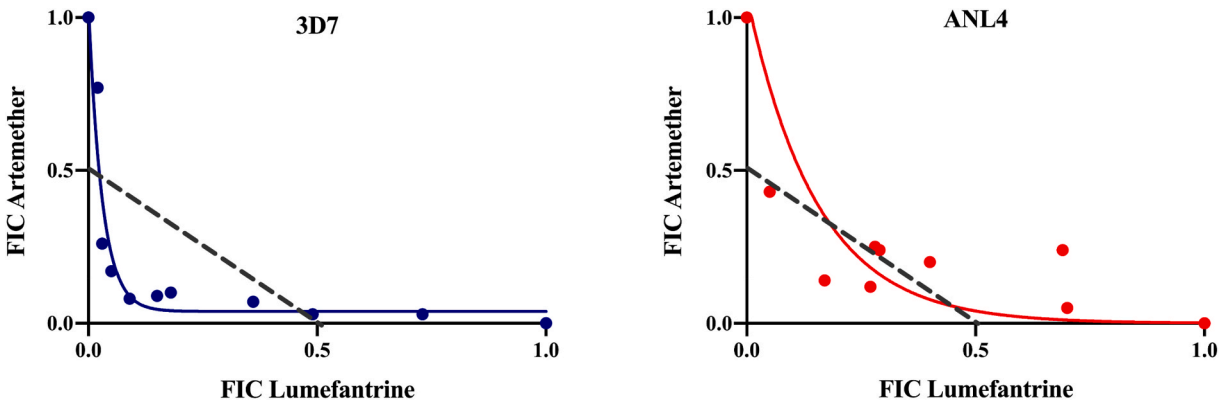

Fig. 2. Isobologram analysis between lumefantrine and artemether. The plots showing the fractional inhibitory concentration (FIC) values represented the drug interaction between lumefantrine and artemether in 3D7 and ANL4 during the early ring stages with two dashed lines marking the FIC values at 0.5 and 1. Two independent experiments were performed. Each experiment had three technical replicates for each parasite strain.

significant.

Since artemether, a closely-related compound to artesunate, and lumefantrine are clinically used together (known as Coartem®), their interactions on *P. falciparum* were evaluated using a chequerboard isobologram approach during the early ring stage (0-4 hpi). The interaction of two drugs in both 3D7 and ANL4 showed a concave isobologram curve (Fig. 2 and Supplementary Tables 2 and 3), indicating a synergistic effect between the drugs in both 3D7 and artemisinin-resistant backgrounds. This finding also supports the idea that lumefantrine could indeed boost the antimalarial activity of artemisinins during the critical resistance window. To rule out artefactual effects of residual lumefantrine lipophilicly bound to red blood cells even after extensive washing, an experiment was designed by pre-incubating lumefantrine for 24 h with red blood cells at 100 nM, a concentration that inhibits 100% growth of 3D7 strain (Fig. 1), followed by a thorough washing step (as performed in IC_{50} assays) prior to their use in parasite culture. This washing step used on lumefantrine-treated red blood cells was demonstrated to be effective in removing all the drug, allowing the treated red blood cells to support parasite growth without any significant difference from those of control red blood cells (Supplementary Fig. S1) indicating the washing step was adequate in removing any residual bioactive lumefantrine.

The synergistic effect between artemether and lumefantrine during early ring stage is more prominent in 3D7 than ANL4 (Supplementary Tables 2 and 3). The lower level of synergism might be related to the fact that ANL4 is less susceptible to lumefantrine and artemisinins during the early ring stage. It is possible that the change in the degree of synergism could be related to the underlying genetic repertoires responsible for reduction in artemisinin susceptibility. Even though the combination can kill more ANL4 parasites than each individual drug by itself (Supplementary Table 3), this observation could be critical when the efficacy of artemether and lumefantrine is monitored in the field.

It is not possible to fully explain the molecular mechanism underlying the synergistic interaction between artemisinins and lumefantrine because the modes of action of both drugs are still being explored. However, artemisinin derivatives require haem-mediated activation, and the reduction in artemisinin susceptibility was shown to be related to changes in the amount of free haem and haemoglobin processing (Birnbaum et al., 2020; Bunditvorapoom et al., 2018). Lumefantrine was found to increase the amount of free haem in *P. falciparum* by an unknown mechanism (Combrinck et al., 2013), which might boost the activation of artemisinins.

The selection of an effective partner drug in ACTs is a vital step in the global malaria control effort since the full efficacy of ACTs for the treatment of *P. falciparum* plays a major role in driving down malaria morbidity and mortality. Here, we demonstrate that lumefantrine could improve the killing activity of artemisinin derivatives in clearing parasites during the critical resistance window. Furthermore, lumefantrine demonstrates an important property in acting synergistically with artemether during the early ring stage both in 3D7 and artemisinin-resistant backgrounds. The observation of synergism was made between the racemate form of benflumetol (lumefantrine) and artemether, especially at IC_{90} and IC_{99} in a 48-h exposure (Hassan Alin, Bjorkman et al., 1999). Lumefantrine has already been developed as one of the treatment combinations with artemether as Coartem®. This study shows that this synergistic interaction might assist in hampering emergence of artemisinin resistance in regions where Coartem® is prescribed, e.g. Laos (Mayxay et al., 2012). Lumefantrine was also chosen as a part of the newly developed triple drug regimen (van der Pluijm et al., 2020). In addition, it is of interest to test artemisinin compounds besides artemether to be tested for ring-stage synergy with lumefantrine or novel partner drug candidates in future studies. Our findings here provide an indication that lumefantrine could be explored as a partner drug particularly in areas where parasites are becoming less susceptible to artemisinins. Nevertheless, a recent report from Indonesia indicated that the use of artemether and lumefantrine in *P. falciparum* might still leave sub-microscopically detectable parasites (Lubis et al., 2020). Hence, it is necessary to determine the effect of evolving genetic repertoires in Southeast Asia on the response to each drug and their synergistic interaction. Alternative methods to preserve artemisinins in Southeast Asia should also be explored including two sequential regimens with two different partner drugs for an extended 6-day ACT course to prevent resistance (Schallig et al., 2017).

Declaration of competing interest

MGL, FJG and LMS are employees of GlaxoSmithKline and own shares of the company.

Acknowledgements

The work was supported by the Tres Cantos Open Lab Foundation, Wellcome (206194), the Bill and Melinda Gates Foundation (OPP1054480), Newton Advanced Fellowship under The Thailand Research Fund-Royal Society (DBG5980010) and the Faculty of Tropical Medicine, Mahidol University. DL was supported by Royal Golden Jubilee Ph.D. Program (Thailand Research Fund) and TRF-Newton Fund PhD Placement for Scholars (Thailand Research Fund and Newton Fund, United Kingdom).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.09.005.

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