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## In vitro evaluation of ganaplacide/lumefantrine combination against *Plasmodium falciparum* in a context of artemisinin resistance

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**Background:** Ganaplacide, also known as KAF156, is among the new antimalarial drug candidates that have successfully reached Phase III clinical trials, and is proposed in combination with lumefantrine. This combination could replace the current front-line artemisinin-based combination therapies (ACTs) in case of *Plasmodium falciparum* resistance to both artemisinins and partner drugs. Indeed, the African continent, where the malaria burden is the highest, is currently experiencing worrying multiple emergences and spread of artemisinin resistance, which urges for the exploration of the antiparasitic properties of KAF156 in this context.

**Objectives and methods:** The objectives of this work were firstly to evaluate the risk of cross-resistance between artemisinins and KAF156 alone, and in combination with lumefantrine, using a panel of artemisinin-resistant strains carrying different *pfk13* mutations and markers of other antiplasmodial drug resistances; secondly to explore *in vitro* the relevance of combining KAF156 and lumefantrine with artemisinins, based on the model of triple ACTs.

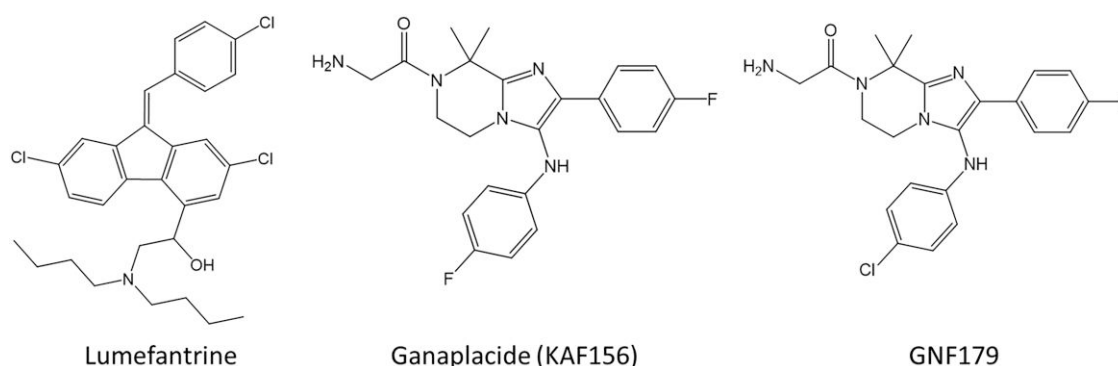
**Results:** Our results highlighted that KAF156 activity was not impaired by mutations in *pfk13*, *pfcr*, *pfmdr1*, *pfmdr2*, *pfdhps* and *pfdhfr* genes or by *pfmdr1* amplification. Moreover, we demonstrated that KAF156 alone and in combination with lumefantrine was active against artemisinin-resistant parasites, including when they are quiescent.

**Conclusions:** All these *in vitro* results evidence that multi-drug resistant parasites currently in circulation in the field might not affect KAF156 efficacy, and are encouraging signs for KAF156 use in a triple ACT to preserve the use of artemisinins for as long as possible.

### Introduction

Although efforts to develop new antimalarial drugs never stop, malaria continues to claim victims every year,<sup>1</sup> and few drug candidates succeed in reaching clinical trials. Among them is ganaplacide, proposed in combination with an improved solid dispersion formulation of lumefantrine (lumefantrine-SDF), resulting from a research programme conducted by Novartis and Medicines for Malaria Venture. Lumefantrine (Figure 1) is an antimalarial drug that inhibits the conversion by *Plasmodium* of toxic haem into non-toxic haemozoin,<sup>2</sup> but might have additional

targets. Lumefantrine is widely used with artemether in artemisinin (ART)-based combination therapy (ACT)<sup>3</sup> under the names of Coartem<sup>®</sup> or Riamet<sup>®</sup>. Ganaplacide, also known as KAF156 (Figure 1), belongs to the class of imidazolopiperazines. KAF156 is active in both the liver and blood stages of the parasite, and also has potent prophylactic and transmission-blocking properties. Its pharmacokinetic profile is compatible with once-daily dosing.<sup>4,5</sup> KAF156 acts in the endoplasmic reticulum (ER) by targeting protein folding and trafficking, and affects ER homeostasis and function.<sup>6</sup> Four months of *in vitro* drug pressure with GNF179 (Figure 1), a KAF156 analogue, were enough to obtain parasites



**Figure 1.** Chemical structures of lumefantrine and of two imidazolo-piperazines, ganaplacide (KAF156) and GNF179.

resistant to KAF156 due to mutations in *pfcarl* (cyclic amine resistance locus, PF3D7\_0321900), *pfugt* (UDP-galactose transporter, PF3D7\_1113300) or *pfact* (acetyl-CoA transporter, PF3D7\_1036800) genes.<sup>7–10</sup> This resistance is probably associated with modifications in fatty acid transport and membrane protein trafficking.<sup>11</sup> To reduce the risk of resistance emerging in the field, KAF156 was combined with lumefantrine. Results of a Phase II clinical trial highlighted an adequate parasitological and clinical response in patients on Day 29 with ganaplacide 400 mg plus lumefantrine-SDF 960 mg once daily for 3 days (NCT03167242),<sup>12</sup> enabling ganaplacide/lumefantrine to move forward to a Phase III study in 2023.<sup>13</sup> This drug combination could replace ACTs in case of parasite resistance to both ARTs and partner drugs.<sup>1</sup> *P. falciparum* ART resistance<sup>14</sup> is mainly associated with mutations in the *pfkelch13* (*pfk13*) gene,<sup>1,15,16</sup> and leads to the survival of a subset of the parasite population (even clonal) due to cell cycle arrest, called quiescence, upon ART treatment. This mechanism makes ART resistance a partial resistance. Once ARTs have been eliminated, parasites are able to multiply again.<sup>17,18</sup> ART resistance, first reported in South-East Asia,<sup>19,20</sup> recently emerged in South America<sup>21</sup> and Africa.<sup>22,23</sup> The major malaria-endemic area, i.e. sub-Saharan countries, is currently experiencing a worrying increase in the prevalence and regional spread of ART-resistant parasites.<sup>24,25</sup> Therefore, as the ganaplacide/lumefantrine combination will sooner or later have to face ART resistance, it is essential to explore its antiparasitic potential in this context. Some data are already available regarding KAF156 antiplasmodial efficacy against ART-resistant parasites with nanomolar *in vitro* activity.<sup>26</sup> In a clinical trial conducted in Thailand and Vietnam, parasite clearance times and clearance rates were similar in infections with or without K13 mutations.<sup>27</sup> Here, we aimed to complete these data by assessing the risk of cross-resistance between ART and KAF156 alone and in combination with lumefantrine, at pharmacologically relevant concentrations, with a panel of ART-resistant strains carrying different *pfk13* mutations. Moreover, because ARTs enable a drastic and very rapid reduction in parasite load, even in infections with ART-resistant parasites, these drugs remain valuable medicines as long as the partner drug in ACT is effective.<sup>28,29</sup> This is why, based on the model of the triple ACT (TACT) artemether/lumefantrine/amodiaquine<sup>30</sup> moving to Phase III trial in 2023,<sup>13</sup> we also explored the possibility to use KAF156+lumefantrine (LUM) in combination with ART.

## Materials and methods

### Drugs

Atovaquone (ATQ) and dihydroartemisinin (DHA) were purchased from TCI Chemicals (Belgium), and chloroquine diphosphate salt (CQ) and lumefantrine (LUM) from Sigma Aldrich/Merck (Germany). KAF156, with a purity >99% (HPLC), was synthesized in our lab according to Nagle et al.<sup>31</sup>

### Parasite strains

F32-ART is a laboratory strain, obtained after ART pressures and carrying the M476I mutation on the *pfk13* gene responsible for its ART resistance.<sup>15</sup> F32-TEM is its twin ART-susceptible strain.<sup>18</sup> SMT010 is an ART-susceptible field isolate from Mali.<sup>32</sup> SMT010 P413A, carrying the *pfk13* P413A mutation, is an ART-resistant strain obtained after ART pressure on SMT010.<sup>16</sup> The strain 3D7 R561H is a strain resistant to ARTs, obtained after gene editing of the 3D7 strain to incorporate the *pfk13* R561H mutation. 3D7 ctrl is its ART-susceptible twin (mock-edited),<sup>33</sup> and both strains were provided by Dr D. Fidock from Columbia University. IPC6610 (*pfk13* R539T), IPC8262 and IPC8461 (*pfk13* C580Y) are ART-resistant field isolates from Cambodia provided by Dr B. Witkowski from the Pasteur Institute in Cambodia. Therefore, all strains included in this study as ART-resistant fulfilled the WHO criteria for ART resistance, i.e. presence of a *pfk13* gene validated mutation associated with a survival rate above 1% in the ring stage survival assay (RSA).

### Parasite culture

Parasites were cultured according to Trager and Jensen<sup>34</sup> with slight modifications. All field isolates and the two 3D7 strains were cultured at 2% haematocrit in human red blood cells (RBCs) (EFS, French blood bank, France) in 'enriched conditions' corresponding to RPMI-1640 medium (with HEPES and L-glutamine, Dutscher, France) supplemented with 5% human serum (EFS, French blood bank, France), enriched with 0.55% Albumax II (Fisher Scientific, France), 0.4 mM hypoxanthine, 1 mM L-glutamine and 11 µg/mL gentamicin; parasites were incubated in hypoxia conditions with 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub> at 37°C. The F32-TEM and F32-ART strains were also grown in 'enriched condition' to be comparable to the other strains. In parallel, the strains F32-TEM and F32-ART were cultivated in 'basic condition', in which parasites were cultured at 2% haematocrit in human RBCs in RPMI-1640 medium supplemented with 5% human serum, and incubated with 5% CO<sub>2</sub> at 37°C in a humidified atmosphere,<sup>35</sup> leading to an O<sub>2</sub> level approximating to 18.5%.<sup>36</sup> This change in culture conditions for F32-TEM and F32-ART was done several days/weeks before starting experiments.

As extensively reviewed by Basco in 2023,<sup>37</sup> there is no consensus regarding the gas mixture required for *Plasmodium* cultivation. Although in humans parasites inside RBCs live in fluctuating O<sub>2</sub> levels mainly in a

hyperoxic environment,<sup>38</sup> it appears that the actual O<sub>2</sub> level is not critical for most parasite strain cultivation as long as the CO<sub>2</sub> level is limited to 5%.<sup>37,39</sup> Here, two culture conditions were compared: hyperoxia with 5% CO<sub>2</sub> in a humidified atmosphere leading to an O<sub>2</sub> level approximating to 18.5%,<sup>36</sup> close to the atmosphere found in candle jars, which contain approximately 3% CO<sub>2</sub> and 17%–18% O<sub>2</sub>,<sup>37</sup> and hypoxic conditions with 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>.

### Genotyping of drug resistance molecular markers

The genotype of the different strains regarding markers of resistance to common antiplasmodial drugs<sup>40,41</sup> and imidazolopiperazines was determined by Sanger sequencing and quantitative PCR (q-PCR). DNA was extracted from parasites using the High Pure PCR Template Preparation Kit (Roche Diagnostic, France). The genes of interest (*pfk13*, *pfprt*, *pfmdr1*, *pfmdr2*, *pfdhps*, *pfdhfr*, *pfcty*, *pfact*, *pfcarl* and *pfugt*) were amplified by PCR using the DreamTaq Hot Start DNA polymerase (Invitrogen, USA). Sanger sequencing was then performed by the Genoscreen Company (Lille, France) (the primers used are described in Tables S1 and S2; available as Supplementary data at JAC Online). Sequences were compared with the reference genome of the *P. falciparum* 3D7 strain using MEGA 11 software.<sup>42</sup> Assessment of *pfmdr1* gene amplification was done by q-PCR. Real-time amplification reactions were performed on a CFX Real Time PCR instrument (Biorad, USA) using the Biorad SsoFast EvaGreen Supermix. Threshold cycle analysis was used to calculate the relative amount of *pfmdr1* gene compared with the control gene (*pfβtubulin beta chain*).

### Chemosensitivity assay

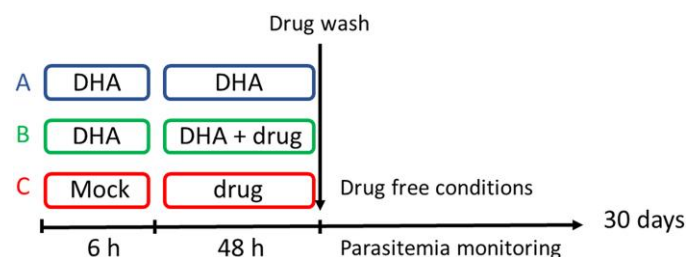
The SYBR Green I assay was carried out to evaluate the *in vitro* antimalarial activity of the different compounds.<sup>43</sup> Ring-stage parasites were synchronized with 5% D-sorbitol and adjusted at 1% parasitaemia before being exposed during 48 h at 37°C to different concentrations of the drugs in 96-well plates. Each concentration was tested in triplicate. Fluorescence was measured at 485 nm excitation/528 nm emission in the VICTOR Nivo plate reader (Perkin Elmer, USA). The IC<sub>50</sub> values were then calculated using GraphPad Prism 7 software.

### Ring-stage survival assay

The RSA<sup>0–3h</sup> was performed to check the ART-resistance phenotype of all studied strains.<sup>44</sup> Ring-stage parasites aged between 0 and 3 h post-invasion, obtained after a Percoll-sorbitol treatment, at 0.5% parasitaemia were exposed to 700 nM DHA or 0.1% DMSO (vehicle control) for 6 h in duplicate. Parasite pellets were then washed in RPMI-1640 before being placed in drug-free conditions for 66 h. The survival rate of DHA-treated parasites compared with the DMSO-treated ones was determined by counting the parasitaemia of each condition on 10 000 RBCs by two independent microscopists.

### Recrudescence assay

A recrudescence assay was performed on three strain sets: F32-ART versus F32-TEM, 3D7 ctrl versus 3D7 R561H, and SMT010 versus SMT010 P413A to compare the ability of each pair of ART-susceptible and -resistant strains to survive and proliferate after drug exposure.<sup>45</sup> Ring-stage parasites synchronized with 5% D-sorbitol, adjusted to 3% parasitaemia and 2% haematocrit, were treated in a 6-well plate with the drug to be tested. After 48 h of incubation, the drug was washed off with RPMI-1640 and the parasites placed in drug-free culture conditions. Blood smears were done to follow parasitaemia until the day when each parasite culture reached its starting parasitaemia, defined as the recrudescence day. If after 30 days, no parasite recrudescence was observed, the data were censored. Data analysis was performed using Kaplan–Meier survival curves. Statistical significance was ascertained by using a log-rank (Mantel–Cox) test using GraphPad Prism 7 software.



**Figure 2.** Experimental procedure of the quiescent-stage survival assay (QSA). DHA, dihydroartemisinin. Adapted from Reyser *et al.*, 2020.<sup>46</sup> This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

### Quiescent-stage survival assay

To determine the activity of KAF156, lumefantrine, and KAF156 + lumefantrine on ART-resistant parasites at the quiescent stage, a quiescent-stage survival assay (QSA) was performed as previously described<sup>46</sup> with slight modifications. This test was carried out on many ART-resistant strains to reflect the diversity of genotypes, in order to be more representative of the reality in the field: F32-ART, 3D7 R561H, SMT010 P413A, IPC6610, IPC8262 and IPC8461. Ring-stage parasites synchronized by 5% D-sorbitol treatment were adjusted to 3% parasitaemia and 2% haematocrit before being exposed during 6 h to 700 nM DHA to induce quiescence (Figure 2, conditions A and B) or to a mock (Figure 2, condition C). After that, the compound to be tested was added in conditions B and C for 48 h. Condition A was the control of recrudescence for quiescent parasites. The DHA treatment was kept in condition B to ensure that quiescence was maintained throughout the treatment with the compound of interest. Conditions A and B were compared to determine if the molecule is active on quiescent parasites. Condition C enabled checking that the concentration at which the molecule was tested is active on proliferating parasites. At the end of the treatment, the drugs were washed off with RPMI-1640 and parasites placed back in drug-free conditions. RBCs were smeared to follow parasitaemia until the day the cultures reached their initial parasitaemia, defined as the recrudescence day. If after 30 days no parasite recrudescence was observed, the data were censored. Data analysis was performed using Kaplan–Meier survival curves. Statistical significance was tested using a log-rank (Mantel–Cox) test using GraphPad Prism 7 software.

## Results and discussion

### KAF156 and lumefantrine are active on *P. falciparum* proliferative parasites independent of their drug resistance genotype

The different parasite strains were chosen based on their genotype related to ART resistance (Table 1). Concerning the *pfk13* gene, M476I is the mutation that allowed identification of the K13 polymorphism as a molecular marker of ART resistance,<sup>15</sup> and was also found in the field.<sup>47</sup> C580Y and R539T are frequent mutations in South-East Asia<sup>48</sup> whereas R561H is expanding in Africa.<sup>24</sup> P413A is an *in vitro*-validated resistance mutation located outside the propeller domain of the K13 protein.<sup>16</sup> Antiplasmodial activity of KAF156 and LUM was assessed in a standard 48 h chemo-susceptibility assay with DHA, ATQ and CQ as controls. The chemo-susceptibility analysis confirmed that DHA IC<sub>50</sub> values, ranging from 1.6 nM to 2.9 nM, did not allow discrimination between ART-resistant and ART-susceptible

**Table 1.** Chemo-susceptibility evaluation of dihydroartemisinin (DHA), atovaquone (ATQ), chloroquine (CQ), lumefantrine (LUM) and KAF156 on different *Plasmodium falciparum* strains

Culture conditions <sup>a</sup>	Strain	<i>pfk13</i> mutation	Mean of IC <sub>50</sub> ±SD (nM) <sup>b</sup>					RSA <sup>0-3h</sup> survival rate DHA
			DHA	ATQ	CQ	LUM	KAF156	
Enriched condition: 5% O <sub>2</sub> , 5% CO <sub>2</sub> and 90% N <sub>2</sub> at 37°C, RPMI 1640 enriched medium	F32-ART	M476I	2.5±0.6	1.8±0.4	35±5	89±23	5±1	13.4%
	F32-TEM	WT	2.2±0.3	1.5±0.2	35±2	73±13	7±1	0%
	3D7 R561H	R561H	1.6±0.6	2.4±1.3	24±11	69±14	9±4	7.5%
	3D7 ctrl	WT	2.9±0.1	2±0.7	36±1	60±24	11±1	0.3%
	SMT010 P413A	P413A	1.8±0.4	0.9±0.1	37±10	14±1	5±1	4%
	SMT010	WT	1.9±0.6	0.8±0.4	34±1	16±1	9±2	0.3%
	IPC8461	C580Y	2.6±0.6	1.2±0.3	287±162 <sup>c</sup>	30±11	3±2	3.1%
	IPC8262	C580Y	2.5±0.7	0.8±0.4	111±57 <sup>c</sup>	22±11	6±5	3.1%
	IPC6610	R539T	1.8±0.2	0.7±0.2	55±28	21±16	4±2	31.4%
Basic condition: 5% CO <sub>2</sub> at 37°C, RPMI 1640 medium	F32-ART	M476I	1.6±0.9 <sup>d</sup>	1.3±0.5 <sup>e</sup>	34±0	27±6	8±3	8.4±5% <sup>e</sup>
	F32-TEM	WT	1.7±0.5 <sup>d</sup>	1.2±0.5 <sup>e</sup>	33±0.7	33±13	10±5	0.03±0.03% <sup>e</sup>

<sup>a</sup>RPMI 1640 medium is always supplemented with 5% human serum.

<sup>b</sup>IC<sub>50</sub> values are the mean (±SD) of at least two independent experiments for each strain. A ring-stage survival assay (RSA) was performed once for each strain.

<sup>c</sup>Mean of four independent experiments.

<sup>d</sup>Mean of seven independent experiments.

<sup>e</sup>Mean of five independent experiments.

**Table 2.** Genotypic traits of the *P. falciparum* strains used in this study compared with the reference strain Pf3D7 regarding genomic variations associated with resistance to common antiparasmodial drugs and imidazolopiperazines

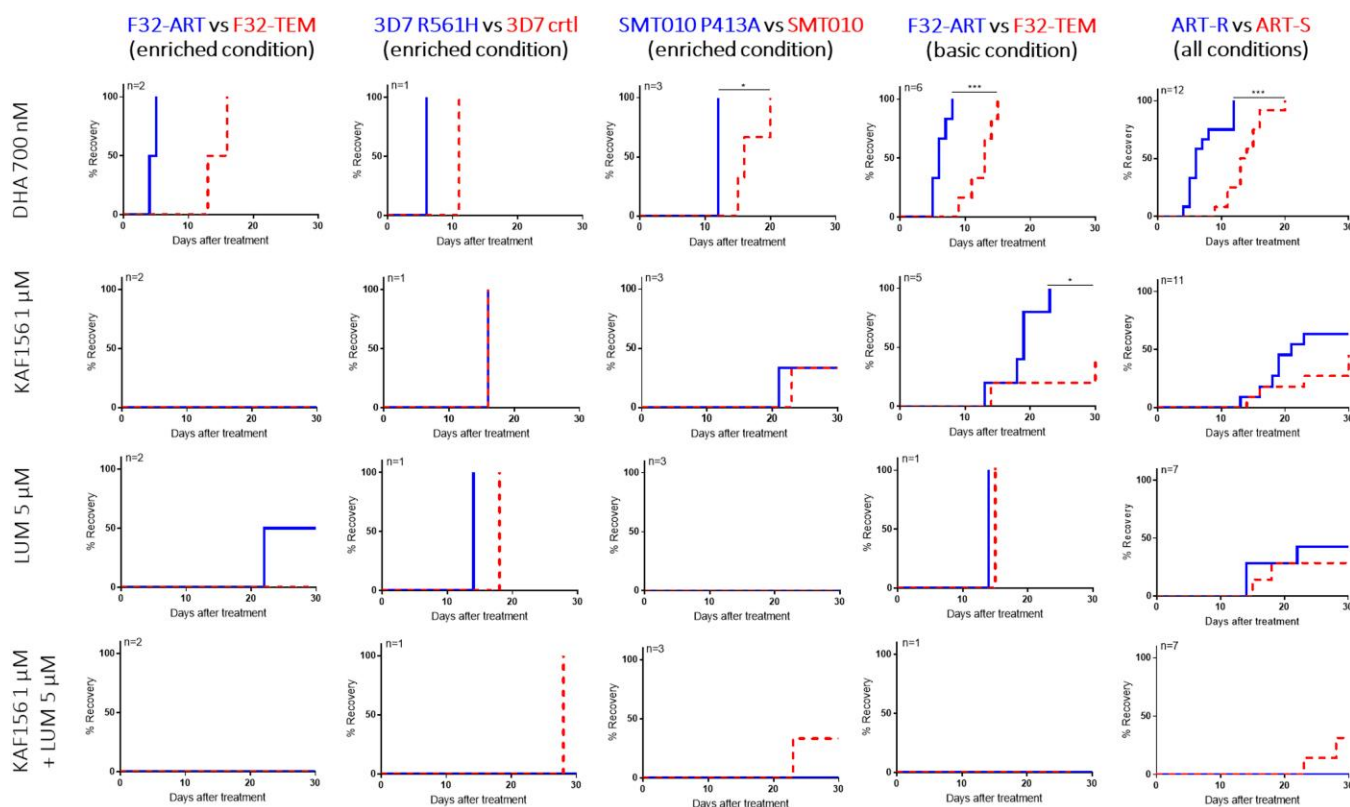
Strain	<i>pfk13</i>	<i>pfcr1</i>	<i>pfmdr1</i>	<i>pfmdr2</i>	<i>pfdhps</i> <sup>a</sup>	<i>pfdhfr</i>	<i>pficytb</i>	<i>pfcarl</i>	<i>pfact</i>	<i>pfugt</i>
F32-ART	M476I	WT	Y184F	F423Y	S436A, 437G	WT	WT	WT	WT	WT
F32-TEM	WT	WT	Y184F	F423Y	S436A, 437G	WT	WT	WT	WT	WT
3D7 R561H	R561H	WT	WT	WT	437G	WT	WT	WT	WT	WT
3D7 ctrl	WT	WT	WT	WT	437G	WT	WT	WT	WT	WT
SMT010	WT	WT	N86Y, Y184F	F423Y	G437A	WT	WT	WT	WT	WT
SMT010 P413A	P413A	WT	N86Y, Y184F	F423Y	G437A	WT	WT	WT	WT	WT
IPC8461 <sup>b</sup>	C580Y	M74I, N75E, K76T	Y184F+CNV <sup>b</sup>	F423Y	437G, K540N, A581G	N51I, C59R, S108N	WT	WT	WT	WT
IPC8262	C580Y	M74I, N75E, K76T	Y184F	F423Y	437G, K540N, A581G	N51I, C59R, S108N	WT	WT	WT	WT
IPC6610	R539T	M74I, N75E, K76T	Y184F	F423Y	S436A, 437G, K540E	N51I, C59R, S108N	WT	WT	WT	WT

<sup>a</sup>In the Pf3D7 reference strain, dihydropteroate synthase (DHPS) have a glycine at the position 437 (437G) but this amino acid is associated with sulfadoxine resistance<sup>49</sup> (alternative amino acid: alanine). Consequently, the strains with the mutation G437A are sulfadoxine-susceptible.

<sup>b</sup>The IPC8461 strain carries *pfmdr1* copy number variation (CNV) (Table S3).

parasites.<sup>18</sup> In contrast, the reference test used for this purpose (RSA<sup>0-3h</sup>)<sup>44</sup> highlighted survival rates of all ART-susceptible strains below 1% whereas survival rates of ART-resistant strains ranged from 3.1% to 31.4% (Table 1). ATQ showed strong antiplasmodial activity across all strains, with IC<sub>50</sub> values from 0.7 nM to 2.4 nM, in accordance with the WT *pficytb* genotype of all strains (Table 2). For CQ, the Cambodian parasite strains had IC<sub>50</sub> values above the 100 nM threshold set for CQ resistance with the exception of strain IPC6610.<sup>50</sup> These highest values comparable to those of the other strains are consistent with *pfcr1* mutations M74I, N75E and K76T identified in IPC8262, IPC8461 and IPC6610 strains and associated with CQ resistance (Table 2).<sup>51</sup> Indeed, for the other strains showing a CQ-sensitive

genotype status, IC<sub>50</sub> values for CQ were similar, ranging from 24 to 37 nM whatever the culture conditions (Table 1). The IC<sub>50</sub> values obtained for LUM ranged from 14 nM to 89 nM. This variability may be due to the lipid composition of the different batches of human serum used in parasite culture media,<sup>52</sup> whereas no significant difference was reported between either of the culture conditions for F32-ART and F32-TEM strains. LUM activity may also depend on the *pfmdr1* gene.<sup>53</sup> The lowest LUM IC<sub>50</sub> values were obtained for SMT010 and SMT010 P413A strains, both carrying the *pfmdr1* N86Y mutation, which was previously associated with increased susceptibility to LUM (Table 2; N86Y mutated strains versus other strains, Mann-Whitney *P* value=0.01).<sup>54</sup> No difference in LUM IC<sub>50</sub> values was observed in



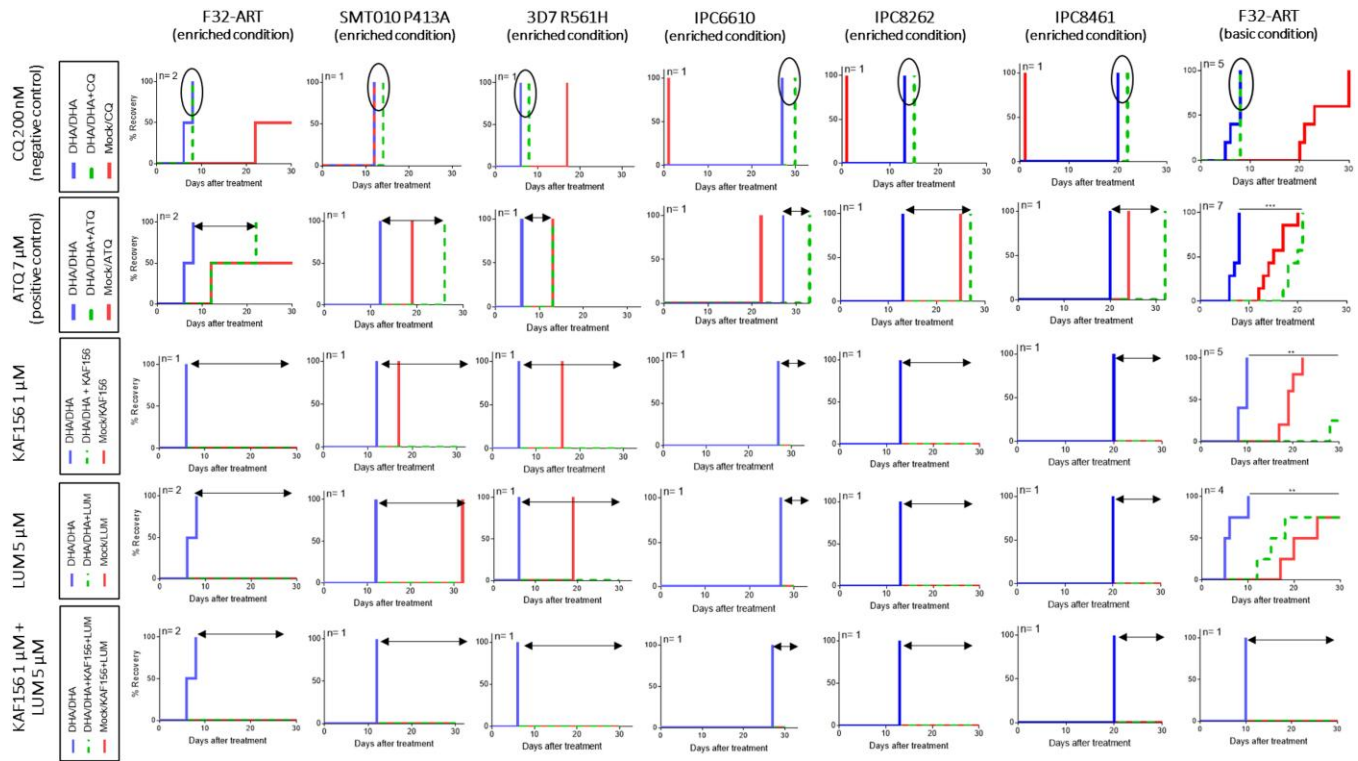
**Figure 3.** Kaplan-Meier analysis of the recrudescence day in different pairs of ART-resistant (ART-R) and ART-susceptible (ART-S) parasite strains (F32-ART versus F32-TEM, 3D7 R561H versus 3D7 ctrl, and SMT010 P413A versus SMT010) after a 48 h treatment with 700 nM dihydroartemisinin (DHA, control condition), 1  $\mu$ M KAF156, 5  $\mu$ M lumefantrine (LUM) or KAF156 1  $\mu$ M+LUM 5  $\mu$ M. Results with ART-resistant strains are represented by blue lines and data from ART-S strains are noted by red dotted lines. The final event was defined as the time necessary for treated cultures to attain initial parasitaemia. Observations were censored if no recrudescence was observed at Day 30. When the number of independent experiment ( $n \geq 3$ ), statistical significance was tested using a log-rank (Mantel-Cox) test (\* =  $P$  value  $< 0.05$ ; \*\*\* =  $P$  value  $< 0.001$ ). Basic condition: RPMI 1640 medium (supplemented with 5% human serum) at 5%  $\text{CO}_2$  in humidified atmosphere; enriched condition: RPMI 1640 medium (supplemented with 5% human serum) enriched with Albumax, hypoxanthine and L-glutamine, at 5%  $\text{O}_2$ , 5%  $\text{CO}_2$  and 90%  $\text{N}_2$ . The last column combines data obtained in all ART-R and ART-S strains, whatever the culture conditions. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

the IPC8461 strain carrying *pfmdr1* gene amplification (Table S3). Whatever the strain, KAF156  $\text{IC}_{50}$  values ranged from 3 nM to 11 nM (Table 1) and were similar to those previously published for KAF156-susceptible parasites.<sup>7,8</sup> These data are in accordance with previous studies against ART-susceptible ( $\text{IC}_{50}$  ranging from 6 to 17 nM on 15 different strains)<sup>5,31</sup> or ART-resistant parasite strains ( $\text{IC}_{50}$  ranging from 4.3 to 7.2 nM on field isolates carrying K13 R539 T, C580Y or G449A mutations).<sup>26</sup> KAF156 activity was in accordance with the *pfcarl*, *pfact* and *pfugt* WT genotypes of the tested parasite strains. Interestingly, KAF156 activity was not impaired by any amplification of the *pfmdr1* gene or mutations in *pfk13*, *pfcr1*, *pfmdr1*, *pfmdr2*, *pfdhps* and *pfdhfr* genes (Table 2, Table S3).

### No cross-resistance is evidenced between ARTs and the combination KAF156 + lumefantrine

As the classical chemo-susceptibility assay cannot distinguish between ART-resistant and ART-susceptible parasites, it is unable to identify cross-resistance between ARTs and other drugs.<sup>18,44</sup>

Hence, the possible cross-resistance of any drug with ARTs was determined by comparing the ability of ART-resistant and ART-susceptible parasites to recover after a 48 h drug exposure.<sup>45</sup> This assay was conducted on three strain couples: F32-ART/F32-TEM, 3D7 R561H/3D7 ctrl and SMT010 P413A/SMT010. All drugs were used at pharmacologically relevant concentrations (close to their plasma peak concentrations in patients).<sup>27,55</sup> As expected, when treated with DHA at 700 nM, all ART-resistant parasites recovered faster than ART-susceptible ones (Figure 3). No difference in recrudescence time after KAF156 treatment alone was observed for the three pairs of ART-resistant and ART-susceptible strains in enriched conditions, suggesting no cross-resistance with ARTs. Surprisingly, in the F32-ART/F32-TEM pair under basic conditions, we observed a significant delay in the recrudescence time between these two strains. This difference in recrudescence times observed with F32 strains between both conditions was found only in ‘long-term’ experiments (read-out at up to 30 days) and could be explained by the fact that the longer an experiment lasts, the more the culture conditions can have an impact on parasite



**Figure 4.** Evaluation of the activity of chloroquine (CQ, targeting haem detoxification) as negative drug control and atovaquone (ATQ, targeting mitochondrial respiration) as positive drug control, KAF156, lumefantrine (LUM) and of the combination KAF156 + LUM towards artemisinin-resistant parasites in the quiescent-stage survival assay (QSA). Kaplan-Meier survival analysis of recrudescence days after different drug treatments. Dihydroartemisinin (DHA) was used at 700 nM, ATQ at 7  $\mu$ M, CQ at 200 nM, KAF156 at 1  $\mu$ M and LUM at 5  $\mu$ M, close to their respective plasma peak concentrations in patients. The final event was defined as the time necessary for treated parasite cultures to reach their initial parasitaemia. Observations were censored if no recrudescence was observed at Day 30. If at this time some parasites were observed, the experiment was continued. When the number of independent experiment ( $n$ )  $\geq$  3, statistical significance was tested using a log-rank (Mantel-Cox) test between the conditions DHA/DHA and DHA/DHA + compound (\*\* =  $P$  value  $<$  0.01). The black ellipses and the black arrows highlight the absence or the presence respectively of a delay in recrudescence time between the conditions DHA/DHA and DHA/DHA + other drug. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

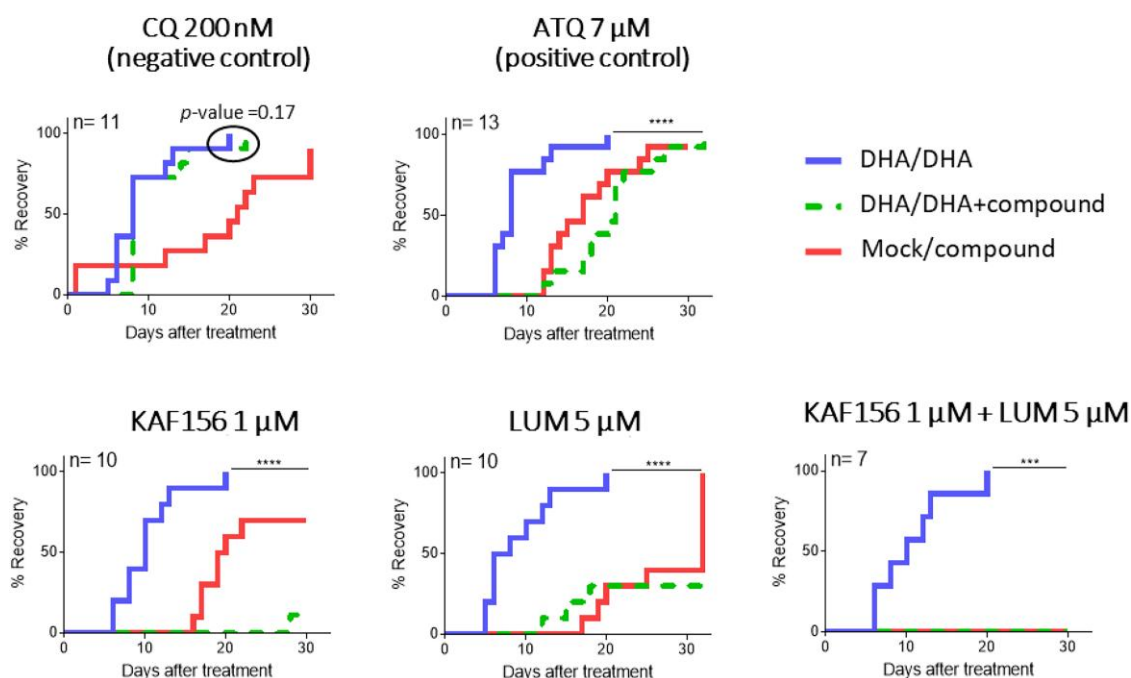
proliferation. However, according to these results, a risk of cross-resistance between ART and KAF156 cannot be ruled out.

In parasites treated with LUM alone or with the combination KAF156 + LUM, no cross-resistance was observed, whatever the parasite strains and culture conditions. Only the ART-susceptible parasites 3D7 ctrl and SMT010 strains were able to survive KAF156 + LUM exposure but with a late recrudescence (Figure 3). These results highlight the potentiation of the combination KAF156 + LUM to kill proliferating parasites resistant or not to ART, compared with the compounds alone. The combining of the recrudescence data obtained in the different parasite strains and in different culture conditions showed no significant cross-resistance between ARTs and KAF156, LUM or KAF156 + LUM (Figure 3, right column).

### **KAF156, LUM and KAF156 + LUM retain their efficiency when used in combination with DHA**

During ACT treatment, due to the mismatched pharmacokinetic properties of each compound, parasites are first exposed to ARTs, and then to the partner drug of the combination. In

ART-resistant parasites that leads to the quiescence of a subset of the parasite population.<sup>17,18</sup> This quiescent state markedly reduces the parasite metabolism<sup>56</sup> and thus may impair the activity of the partner drug. Indeed, molecules that are active in proliferative forms are not necessarily active in quiescent parasites.<sup>46</sup> For example, we previously demonstrated that CQ (targeting haem detoxification) is highly active against ART-resistant parasites in a proliferative state but loses its activity against ART-induced quiescent parasites.<sup>46</sup> Thus, in the current context of ART resistance, a gold-standard partner drug of ART should be able to kill all ART-resistant parasites, including those in a quiescent state, to prevent any parasite recrudescence that could lead to treatment failure. Previously, activity of the KAF156 analogue, GNf179, against quiescent parasites was evidenced in a short-term experimental design in which DHA-treated parasites were exposed to 100 nM of GNf179 for 24 h and parasite viability was followed for up to 3 days.<sup>57</sup> Here, the efficacy on quiescent ART-resistant parasites was evaluated using the QSA,<sup>46</sup> in which DHA-treated parasites were exposed to a pharmacologically relevant dose of KAF156 for 48 h, concomitantly with DHA. Then, parasite recovery was assessed



**Figure 5.** Cumulative data of the evaluation of the activity of atovaquone (ATQ), chloroquine (CQ), KAF156, lumefantrine (LUM) and of the combination KAF156+LUM towards artemisinin-resistant parasites (F32-ART in both culture conditions, 3D7 R561H, SMT010 P413A, IPC8461 and IPC8262) in the quiescent-stage survival assay (QSA). Kaplan–Meier survival analysis of recrudescence times after different drug treatments. Dihydroartemisinin (DHA) was used at 700 nM, ATQ at 7  $\mu$ M, CQ at 200 nM, KAF156 at 1  $\mu$ M and LUM at 5  $\mu$ M, close to their respective plasma peak concentrations in patients. The final event was defined as the time necessary for treated cultures to reach initial parasitaemia. Observations were censored if no recrudescence was observed at Day 30. If at this time some parasites were observed, the experiment was continued. Statistical significance was tested using a log-rank (Mantel–Cox) test between the conditions DHA/DHA and DHA/DHA+compound (\*\*\* =  $P$  value <0.001, \*\*\*\* =  $P$  value <0.0001),  $n$  (number of independent experiment). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

for up to 30 days (Figure 4). We used CQ as negative drug control and ATQ as a positive one.<sup>46</sup> As expected, independently of the strains and the culture conditions, recrudescence times of parasites treated with DHA/DHA+CQ were similar to those of parasites treated with DHA/DHA (Figure 4, black ellipses). CQ alone (condition Mock/CQ) led to a delayed recrudescence, confirming its efficacy on proliferative parasites in the CQ-susceptible strains F32-ART, SMT10 P413A and 3D7 R561H, whereas parasite strains IPC8461, IPC8262 and IPC6610 reached initial parasitaemia only 1 day after the end of the treatment, in accordance with their CQ-resistance profile (Tables 1 and 2). Conversely, a delay in recrudescence time between parasites treated with DHA/DHA+ATQ and with DHA/DHA was observed in all strains and culture conditions (Figure 4, arrows). Altogether, these results confirm the loss of CQ activity in quiescent ART-resistant parasites but the persistence of ATQ efficacy, in accordance with the maintenance of mitochondrial activity in quiescent ART-resistant parasites<sup>46</sup> (Figure 4). In QSA conditions, exposure of DHA-treated parasites to KAF156, LUM or the KAF156+LUM combination led, for all parasite strains and culture conditions, to a delayed recrudescence or no recrudescence in the time frame of the experiment, highlighting the efficiency of these treatments on quiescent parasites (Figures 4 and 5). It is noteworthy that parasite cultures treated with DHA alone reached their initial parasitaemia between 5 and 20 days after the end of the drug exposure, depending on the parasite strains (Figure 4), consistent with the

influence of the genetic background in their response to DHA treatment.<sup>58</sup> Also of note, the IPC6610 strain, treated with DHA alone, had a very late recrudescence on Day 27 despite a very elevated survival rate in the RSA<sup>0–3h</sup> (31.4%, Table 2). This discrepancy could be explained by the difference in sensitivity according to the age of ring-stage parasites at the time of DHA exposure (0–3 h post-invasion in the RSA<sup>0–3h</sup> versus 0–24 h in the QSA), leading us to exclude this strain in further analyses.

The combining of the QSA data obtained in each ART-resistant parasite strain (except for IPC6610) indicated a strong effect of KAF156, LUM and KAF156+LUM in the elimination of DHA-induced quiescent parasites. Indeed, we observed a significant delay in recrudescence, comparable to parasites treated with DHA alone, with a  $P$  value <0.001 (Figure 5), just like ATQ. Interestingly, the strong activities of KAF156, LUM and KAF156+LUM against ART-resistant parasites in a quiescent state were not impaired by the presence of different markers of antimalarial drug resistance such as amplification of *pfmdr1* gene and mutations in *pfcr1*, *pfmdr1*, *pfmdr2*, *pfhps* or *pfahfr* genes.

These *in vitro* results are a very encouraging sign to explore the possibility of using KAF156+LUM as partner drugs to ART in a TACT, in accordance with different modelling studies in which TACTs outperformed most other drug-policy interventions in slowing the spread of ART and ACT resistance and improving the chances of malaria elimination.<sup>59–61</sup>



## Conclusion

We report here that genetic mutations known to be involved in resistance to several antimalarial drugs have no impact on the *in vitro* efficacy of KAF156, although the risk of slight cross-resistance between ART and KAF156 cannot be excluded. As such parasites are currently circulating in the field, these results reinforce our confidence in the future of the ganaplacide/lumefantrine combination as a new antimalarial therapy. Moreover, KAF156 effectiveness, alone or in combination with lumefantrine, in killing quiescent ART-resistant parasites highlights the possibility of using it in a triple combination with an ART derivative, in order to prevent treatment failure due to recrudescence of quiescent parasites in infected patients.

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## Transparency declarations

None to declare.

## Supplementary data

Tables S1 to S3 are available as [Supplementary data](#) at JAC Online.

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