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Letter

Drug Interaction Studies of Cabamiquine:Ganaplacide Combination against Hepatic *Plasmodium berghei*

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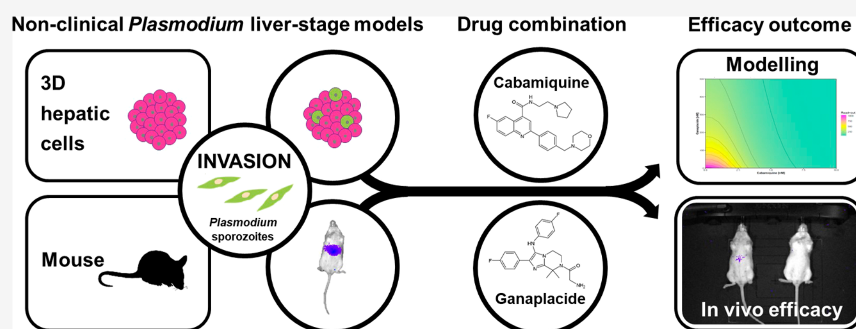

ABSTRACT: New antimalarial combination therapies with novel modes of action are required to counter the emergence and spread of *Plasmodium* drug resistance against existing therapeutics. Here, we present a study to evaluate the preventive activity of a combination of clinical antimalarial drug candidates, cabamiquine and ganaplacide, that have multistage activity against the liver and blood stages of *Plasmodium* infection. Cabamiquine (DDD107498, M5717) inhibits parasite protein synthesis, and ganaplacide (KAF156) inhibits protein trafficking, blocks the establishment of new permeation pathways, and causes endoplasmic reticulum expansion. The pharmacodynamic parameters of a combination of the two compounds were assessed employing a pharmacometrics approach in conjunction with *in vitro-in silico* checkerboard analysis. The *in vitro* study was performed on a previously established 3D infection platform based on human hepatic cell lines that sustain infection by rodent *P. berghei* parasites. The *in vivo* efficacy of this drug combination was assessed against the liver stage of the *P. berghei*. Our results show that the combination of both drugs at the tested concentrations does not interfere with the drugs respective mode of action or affect hepatocyte cell viability. The drug combination was fully effective in preventing the appearance of blood stage parasites when a systemic plasma $C_{av0\text{-}24}/EC_{50}$ ratio >2 for ganaplacide and >5 for cabamiquine was achieved. These findings demonstrate that chemoprevention using a combination of cabamiquine and ganaplacide has the potential to target the asymptomatic liver stage of *Plasmodium* infection and prevent the development of parasitemia.

KEYWORDS: *Antimalarial, Cabamiquine, Combination therapy, Ganaplacide, Preclinical, Liver stage infection*

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Malaria is one of the deadliest infectious diseases worldwide, having caused an estimated 608 000 deaths in 2022, 76% of which were children under 5 years old.[1] The disease is caused by *Plasmodium* parasites, transmitted to their mammalian host in the form of sporozoites injected by the bite of infected female *Anopheles* mosquitoes. Sporozoites home to the liver, where they multiply and differentiate into blood-infective merozoites. The obligatory liver stage of the *Plasmodium* life cycle in its mammalian host is asymptomatic and culminates in the release of thousands of merozoites into the bloodstream.[2−4] This marks the beginning of the blood stage of infection, during which merozoites cyclically infect, multiply in, and burst erythrocytes, thus causing the disease symptoms.[5] Since the development of *Plasmodium* sporozoites inside hepatocytes precedes the onset of the disease, this stage constitutes an attractive target for the development of antimalarial interventions to prevent clinical symptoms and disease manifestation, such as vaccines and prophylactic drugs.[2] The World Health Organization recently recommended two pre-erythrocytic malaria vaccines, RTS,S/AS01 (Mosquirix) and R21/Matrix-M for the prevention of *P. falciparum* malaria in children living in regions with moderate

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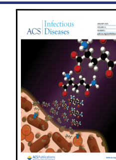

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to high malaria transmission.[1,6−8] Moreover, a recent study showed the feasibility of using monoclonal antibodies to prevent malarial infection.[9] Despite these advancements, the current demand for such therapeutics largely exceeds the available supply and their high cost of production limits their wide distribution among the endemic areas. Hence, chemotherapeutics remains an attractive alternative for both disease prevention and treatment.[9] However, improved prevention among vulnerable populations (e.g., pregnant women) is required. Moreover, the fast-developing drug resistance reported to the front-line therapies (e.g., artemisinin-based combination therapies) demands the use of novel approaches and combination therapies employing drugs with new mechanisms of action.[10−12]

Here, we present our results on the evaluation of the liver stage activity of a new combination of two liver stage active drugs, cabamiquine and ganaplacide, with modes of action distinct from those of currently marketed antimalarials. Cabamiquine (formerly known as DDD107498 or M5717 from the healthcare business of Merck KGaA, Darmstadt, Germany) is a long-lasting novel multistage antiplasmodial candidate under Phase II clinical development (in combination with pyronaridine) that selectively inhibits *P. falciparum* translation elongation factor 2 (*Pf*eEF2), essential for the parasite protein synthesis.[13−16] Ganaplacide (formerly known as KAF156 from Novartis AG, Basel, Switzerland) is an imidazolopiperazine that exerts multistage parasiticidal activity against both pre-erythrocytic and blood stage parasites by inhibiting protein trafficking, blocking the establishment of new permeation pathways, and causing endoplasmic reticulum expansion.[17−22] The latter drug is currently undergoing Phase III clinical development (in combination with lumefantrine) for the treatment of acute uncomplicated malaria. When used as a monotherapy, either drug showed causal prophylactic activities in mouse models. Furthermore, both monotherapies were assessed in a liver stage controlled human malaria infection (CHMI) model where blood stage parasitemia was used as a surrogate measure of efficacy, as human biomarkers to follow human malaria liver infection do not exist and the effect of the drugs can only be monitored indirectly by assessing delayed or negative blood stage infection.[14,15,17] The objective of this study was to evaluate whether both drugs have an additive effect on clearing liver stage parasites when used in combination in a validated preclinical mouse model. To this end, we evaluated the pharmacodynamic (PD) parameters (e.g., parasite growth) of the two compounds in combination and identified any possible drug−drug interaction effects using a pharmacometrics approach. In combination with *in silico* modeling, we leveraged a previously established *in vitro* 3D infection platform for the generation of spheroids of human hepatic cell lines to sustain the invasion and the development of rodent *P. berghei* parasites[23] and to perform a checkerboard interaction assay. We then confirmed our findings with a well-established rodent malaria model,[23,24] further proving the usefulness of the *in vitro* system for translating activity from *in vitro* to *in vivo* models.[9,13,23]

INTERACTION STUDIES OF CABAMIQUINE-GANAPLACIDE COMBINATION AGAINST HEPATIC *P. BERGHEI*

The isobologram (Supplementary Figure S1) and the *in vitro-in silico* checkerboard methods, recently developed for blood stage infection and adapted here for the liver stage,[24] were employed to assess potential PD interactions of the cabamiquine:ganaplacide combination against the hepatic *P. berghei* infection *in vitro*. For both methods, the effective concentrations (ECs) of 20, 50 and 80% of the monotherapies were extrapolated from the previously generated dose−response curves (Table 1).

Table 1. Effective Concentrations of Cabamiquine and Ganaplacide Were Determined in HepG2 Spheroids Infected by *P. berghei* Parasites[a]

	Cabamiquine		Ganaplacide	
Effective Concs	nM	SD	nM	SD
EC_{20}	0.38	0.08	17.87	10.55
EC_{50}	1.28	0.25	45.78	15.38
EC_{80}	3.78	1.38	170.43	26.79
$10 \times EC_{50}$	12.81	2.46	457.83	153.78
$20 \times EC_{50}$	25.62	4.91	915.66	307.56

[a]The effective concentrations presented refer to 20, 50, and 80% of parasite inhibition and 10 and 20 times their respective EC_{50}, determined *in vitro* for a period of 24 h of drug incubation. EC: effective concentration; nM: nanomolar; SD: standard deviation.

3D hepatocyte cultures were infected with *P. berghei* parasites and exposed to different concentrations of cabamiquine and ganaplacide, in monotherapy or in combination, at 24 hours post infection (hpi). Solutions for each drug were prepared at final concentrations equal to the previously determined EC_{20} and EC_{80}. In addition, concentrations of 10 and 20 times the respective EC_{50} were used to determine each drug maximal parasite killing effect (Table 1). Parasite viability (infection rate) was assessed at different time points of the hepatic infection after drug exposure (30, 44, and 48 hpi). Of note, no hepatic cell toxicity was observed at 48 hpi at any of the drug concentrations employed.

Time-dependent drug effects were assessed by longitudinal monitoring of the parasite viability by luciferase bioluminescence (Figure 1). The parasite killing kinetics from four individual experiments could be adequately described by the developed model assuming Bliss Independence as additivity criterion (Figure 1 and Supplementary Figure S2). No deviation from additivity was quantifiable, i.e. no synergistic or antagonistic interaction was found. The final parameter estimates of the nonlinear mixed effects model are presented in Table 2. The EC_{50} of cabamiquine was determined at 3.58 nM, and the EC_{50} of ganaplacide was 371 nM (Supplementary Figure S3). Of note, the interexperiment variability of the EC_{50} of ganaplacide was 101.5% (relative standard error (RSE) of the parameter estimate: 44%) while no interexperiment variability could be quantified for the EC_{50} of cabamiquine. The maximum killing effect for both drugs was slightly higher than the growth rate, which was in line with the slight decline of the signal over time (Table 2). These results were consistent with the observations obtained from the isobologram analysis, which indicated a nondetrimental effect between the two drugs (Supplementary Figure S1). However, since both positive and negative deviations were observed, an additive effect could not be concluded from these experiments (Supplementary Figure S1).

The pharmacometric analysis indicated additivity, with the combination data being well described by the Bliss Independence model (Figure 2). The simulations obtained from the final pharmacometric model were visualized in a 2D

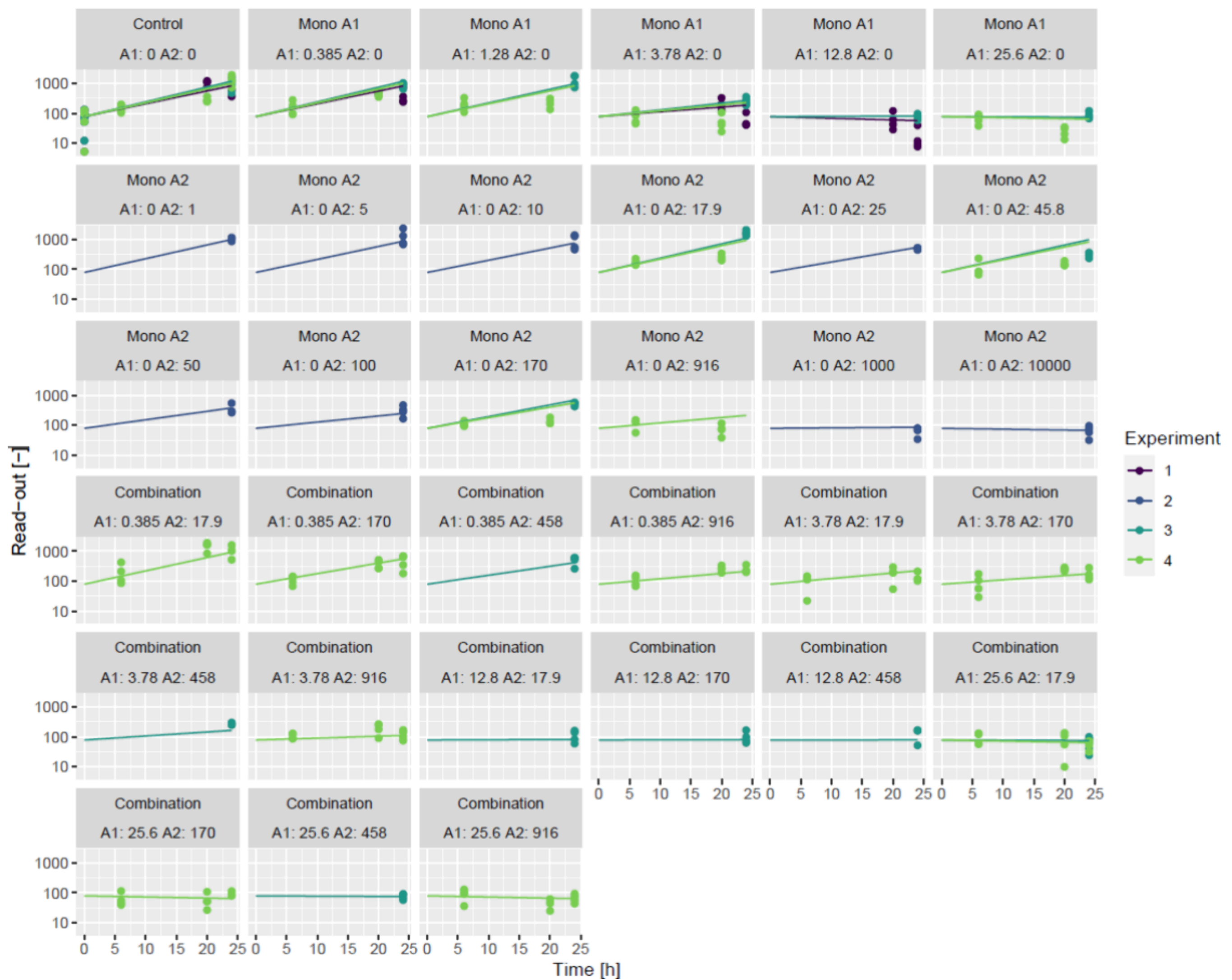

Figure 1. Individual model fits for cabamiquine:ganaplacide combination for the*in vitro* dug-drug-PD interaction assay data using *P. berghei* parasites. Model predictions from the final model (lines) and original data (points) for each study (colors); each facet displays a studied scenario for cabamiquine (A1) and ganaplacide (A2). The numbers in each facet present the concentration of either cabamiquine or ganaplacide in nM in each well.

checkerboard heat map (Figure 2). As also seen in the isobologram of the raw data (Supplementary Figure S1), the calculated isoboles of identical effect are curved due to the different Hill factors for cabamiquine (Hill: 2.48) and ganaplacide (Hill: 0.908) in the Bliss Independence model.

IN VIVO PRE-ERYTHOCYTIC ACTIVITY OF THE CABAMIQUINE:GANAPLACIDE COMBINATION

Pharmacodynamic Interaction. To further evaluate the pre-erythrocytic efficacy of cabamiquine and ganaplacide *in vivo* and to confirm *in silico-in vitro* predictions, monotherapy and drug combinations were assessed using a transgenic *P. berghei* ANKA reporter line (mCherry$_{hsp70}$Luc$_{ef1\alpha}$), which expresses luciferase under the control of the eef1α (PBANKA_113330) promoter and, in addition, mCherry under the control of the hsp70 (PBANKA_071190) promoter, allowing the quantification of the parasite load in the liver and potentially ensuing blood stages (Figure 3A). Effective monotherapy dose ranges in the *P. berghei* mouse model were already established in previous work,[21,23] and to limit animal use, we decided to combine the lowest effective dose for both drugs (1.5 mg/kg for cabamiquine, 5 mg/kg for ganaplacide), suboptimal doses for both drugs (0.6 mg/kg for cabamiquine, 2 mg/kg for ganaplacide) and infective doses for both drugs (0.3 mg/kg for cabamiquine, 1 mg/kg for ganaplacide). All infected control mice either developed a liver infection at 24 and 48 hpi (Figure 3B) or developed a pronounced blood stage parasitemia within 6−7 days (Supplementary Figure S4). The standard-of-care drug employed as a curative control (atovaquone, 10 mg/kg), dosed at 24 hpi, cured all treated mice with no parasites detected in the liver at 48 hpi or in the blood for at least 33 days postinfection (dpi).[23] Oral administration of 1.5 mg/kg of cabamiquine at 24 hpi resulted in no observable liver parasitemia at 48 hpi (Figure 3) and no development of blood stage infection until 37 dpi (Supplementary Figure S4A). However, monotherapy treatment with either 0.3 or 0.6 mg/kg of cabamiquine was not fully curative. Although liver infection could not be detected when 0.6 mg/kg of cabamiquine was employed, one mouse developed blood stage parasitemia at 10 dpi. This highlights the need, when

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Table 2. Plasma Parameter Estimates of the Nonlinear Mixed Effects Model[a]

	Parameter (RSE)
Fixed effect parameters	
N_0 [-]	78.8 (6%)
k_{growth} [h^{-1}]	0.107 (5%)
$Emax_{cabamiquine}$ [h^{-1}]	0.116 (4%)
$EC_{50,cabamiquine}$ [nM]	3.58 (7%)
$h_{cabamiquine}$[-]	2.48 (27%)
$Emax_{ganaplacide}$ [h^{-1}]	0.117 (5%)
$EC_{50,\ ganaplacide}$ [nM]	371 (57%)
$h_{ganaplacide}$ [-]	0.908 (13%)
Interexperiment variability	
$\omega\ k_{growth}$ [%CV]	5.7% (51%)
$\omega\ EC_{50,ganaplacide}$[%CV]	101.5% (44%)
Prop. σ [%]	55.1% (5%)

[a] N_0: read-out at initiation of the experiment, k_{growth}: growth rate, Emax: maximum killing rate, EC_{50}: concentration stimulating 50% of Emax, h: Steepness of the concentration-effect relationship, Prop. σ: residual error of the individually predicted parasite time course vs observations, RSE: relative standard error of the parameter estimates.

using these models, to assess both the liver and blood stage infections to determine with accuracy the curative potential of prophylactic antimalarial treatment regimens. In the lowest 0.3 mg/kg dose of cabamiquine, liver infection was quantifiable at 48 hpi and blood stage parasites were detected at 6- and 10-dpi (Supplementary Figure S4B). Mice administered with the highest dose of ganaplacide (5 mg/kg) did not display a detectable liver infection at 48 hpi (Figure 3) or blood stage parasites at 33 dpi (Supplementary Figure S4A). A lower dose of 2 mg/kg of ganaplacide lowered the liver load below the detection limit, preventing us from visualizing the liver infection. Nevertheless, both mice developed blood stage parasitemia on dpi10 or 20, respectively (Table 3, Supplementary Figure S4C). At 1 mg/kg, ganaplacide did not prevent liver or blood stage infection, with both mice developing blood parasitemia within 10 dpi. Thus, both experimental compounds displayed a dose-dependent response when administered as monotherapies. The combination of curative doses of the monotherapies (1.5 mg/kg cabamiquine and 5 mg/kg ganaplacide) was also curative, as neither liver nor blood stage parasites were detected (Figure 3B and Supplementary Figure

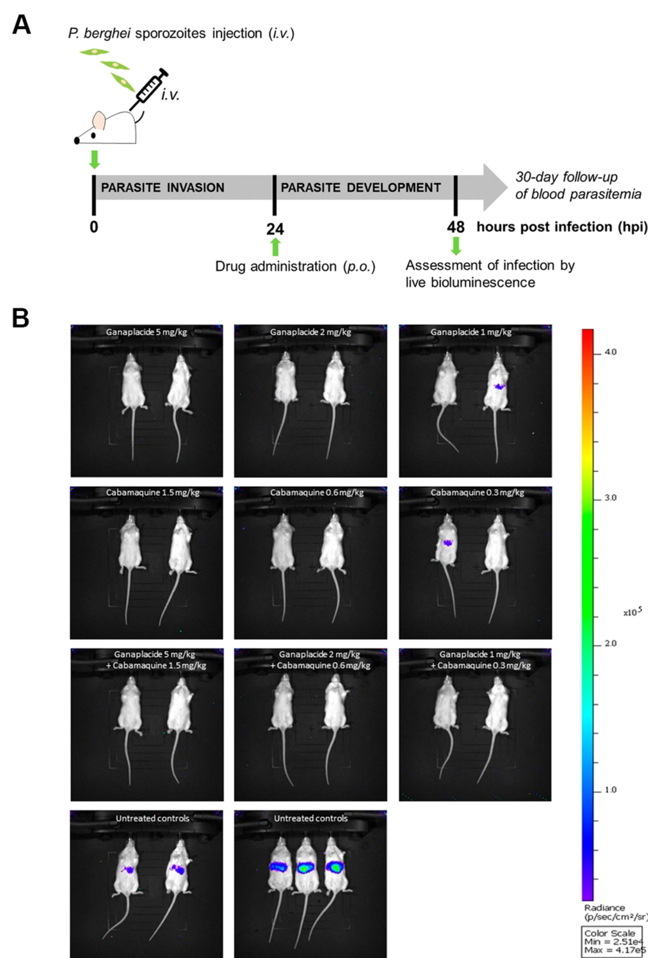

Figure 3. Evaluation of ganaplacide and cabamiquine drug combination in the *P. berghei* mouse model. (A) Schematic representation of the infection strategy and mode of exposure to the drug combination. (B) Representative ventral view images of NMRI mice at 48 h after administration of *P. berghei* sporozoites and after 24 h exposure to cabamiquine and ganaplacide as monotherapies and in combination (as indicated). Heat maps of mice represent intensity of bioluminescence, radiance as indicated by pseudocolor scale.

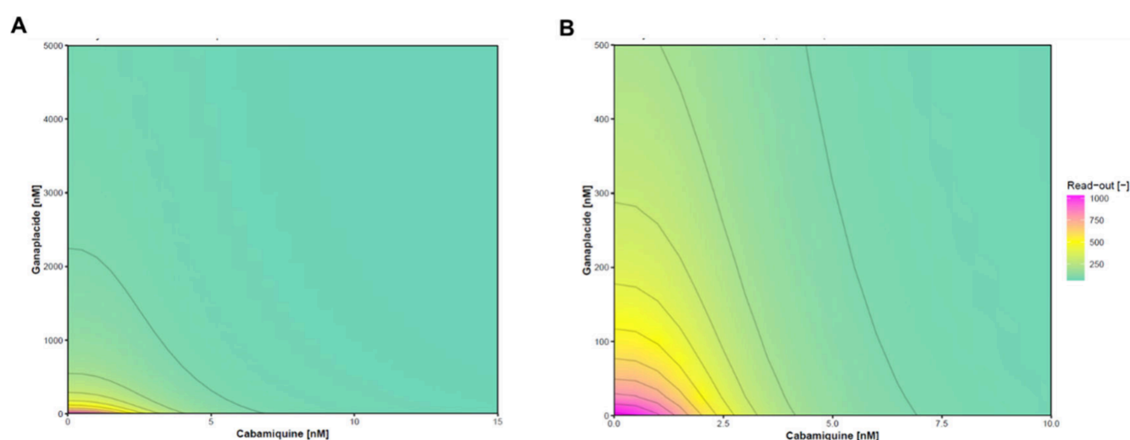

Figure 2. Heat map of the additive interaction (Bliss Independence) of cabamiquine and ganaplacide. (A) Read−out−heat map and (B) Zoomed read−out−heat map. Color gradient visualizes the assay read out at 24 h, the gray lines represent the isoboles of identical effects.

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Table 3. Summary of Pharmacokinetic and Pharmacodynamic Parameters in *P. berghei* Mouse Liver Stage Infection Model of Cabamiquine-Ganaplacide Drug Combination Based on a 30-Day Follow-up of Blood Stage Parasitemia[c]

Cabamiquine								
Test agents		Blood parameters				Plasma parameters		
Cabamiquine(mg/kg)	Ganaplacide(mg/kg)	C_{max} (nM)	AUC_{0-24} (nM·h)[a]	C_{24h} (nM)	C_{av0-24} (nM)	C_{av0-24} (nM)	C_{av0-24}/EC_{50}	PD (Cure)[b]
0.3	-	18	304	8	13	3.6	2.8	0/2
0.3	1	23	403	14	17	4.8	3.7	1/2
0.6	-	31	508	11	21	6.0	4.7	1/2
0.6	2	40	717	20	30	8.5	6.6	2/2
1.5	-	191	3042	96	127	36.0	28.1	2/2
1.5	5	127	1953	59	81	23.1	18.1	2/2
Ganaplacide								
Test agents		Blood parameters				Plasma parameters		
Ganaplacide(mg/kg)	Cabamiquine(mg/kg)	C_{max} (nM)	AUC_{0-24} (nM·h)[a]	C_{24h} (nM)	C_{av0-24} (nM)	C_{av0-24} (nM)	C_{av0-24}/EC_{50}	PD (Cure)[b]
1	-	122	1282	29	53	34.3	0.7	0/2
1	0.3	185	1780	25	74	47.6	1.0	1/2
2	-	367	3039	12	127	81.3	1.8	0/2
2	0.6	380	2954	24	123	79.0	1.7	2/2
5	-	1016	12886	77	537	345	7.5	2/2
5	1.5	961	11238	130	468	301	6.6	2/2

[a]AUC_{0-24h}: area under the concentration−time curve from time 0 h (dosing) to 24 h after dosing; C_{max}: maximum observed concentration; C_{24h}: maximum observed concentration at 24 h; C_{av0-24}: average plasma concentration over 24 h; EC_{50}: half maximal effective concentration. [b]PD represents the number of mice cleared from blood stage parasitemia relative to the total number of mice in the same group. Cure is defined as no emergence of blood stage parasites at day 30 or equivalent. (2/2) represents complete cure; (1/2) represents partial cure and (0/2) represents no cure. [c]The mouse blood:plasma ratio for cabamiquine and ganaplacide was 3.52[15] and 1.56, respectively; the *in vitro* potency (EC_{50}) for cabamiquine and ganaplacide was 1.28 and 45.78 nM, respectively (Table 2.

S4A). Interestingly, suboptimal doses of combined 2 mg/kg ganaplacide and 0.6 mg/kg cabamiquine completely eliminated both liver stage parasites (Figure 3B) and subsequent blood stage parasitemia (Supplementary Figure S4C), which was not observed when each drug was employed individually at such doses (Table 3). The lowest combination regimen with 0.3 mg/kg cabamiquine and 1 mg/kg ganaplacide reduced the liver infection below the limit of quantification but did not prevent the development of blood stage parasitemia in one out of two mice (Figure 3B).

Pharmacokinetic (PK) Interaction. The PK/PD analysis of blood stage monitoring via Giemsa-stained blood smears (until 34 dpi) is described in Table 3.

Following a single oral dose of cabamiquine (0.3 0.6, or 1.5 mg/kg) in mono- and combination therapy in *P. berghei* liver stage infected mice, the C_{max} and AUC_{0-24} increased approximately 10-fold with a 5-fold increase in dose (Table 3). Exposure to a combination of cabamiquine with ganaplacide was comparable to exposure to cabamiquine monotherapy (ratio of PK parameters ranged between 0.6- and 1.4- fold across the doses studied). Following a single oral dose of ganaplacide (1, 2, or 5 mg/kg) in mono- and combination therapy, the C_{max} and AUC increased approximately 8- to 10-fold with a 5-fold increase in dose. Further, ganaplacide exposure in combination with cabamiquine was comparable to its exposure measured as monotherapy (ratio of PK parameters ranged between 0.9- and 1.5-fold across the doses studied). To correlate the *in vivo* exposures obtained at various oral doses (as monotherapy or as combination) and the *in vivo* efficacy observed under such treatments, the AUC_{0-24} was converted to an average plasma concentration of the compounds (Cav_{0-24}) over 24 h and was compared to the respective EC_{50} determined *in vitro*. All mice for which the drug was fully effective in preventing the appearance of blood stage parasites achieved systemic plasma C_{av0-24}/EC_{50} ratios of >2 for ganaplacide and >5 for cabamiquine. A C_{av0-24}/EC_{50} ratio of <1 for ganaplacide and <3 for cabamiquine was observed when employing the dose that did not prevent the early development of blood stage parasites. A more delayed emergence of blood stage parasitemia was seen when the C_{av0-24}/EC_{50} ratio was increased to 1−2 for ganaplacide and 3−5 for cabamiquine.

In conclusion, these *in vitro-in silico* findings support the suitability of cabamiquine:ganaplacide combination as a valuable pre-erythrocytic therapy targeting hepatic *Plasmodium* infection.

DISCUSSION

The antimalarial drug pipeline primarily targets the symptomatic blood stage of *Plasmodium* infection. Our study presents a combination of two drugs that target the liver stage, cabamiquine and ganaplacide. Other preclinical *in vitro* and *in vivo* analyses indicated that both drugs have been efficacious against multiple lifecycle stages of both asexual and sexual blood stage parasites.[13]

Recently, we published the results of a Phase Ib, randomized, double-blind, placebo-controlled, adaptive, dose-finding, CHMI study of cabamiquine.[14] The outcomes unveiled the effect of cabamiquine on the liver stage of *Plasmodium* infection by successfully restricting the emergence of blood stage parasites, thereby establishing the distinction between early and late dosing. A single dose of cabamiquine (100 mg or higher) exhibits 100% protection with causal chemoprophylactic activity.[14] Together with previously demonstrated results from our first-in-human study, the safety, pharmacokinetics and antimalarial activity had been established where a single dose of 800 mg of cabamiquine cleared parasitemia with no recrudescence.[13,25]

Cabamiquine was assessed in combination with pyronaridine, a hemozoin inhibitor targeting the blood stage of infection, and was shown to be an effective therapy against the erythrocytic stage of *Plasmodium* infection.[26] Complementing this work, our group evaluated the activity of the same drug combination against the liver stage of *P. berghei* infection. Our results confirmed the suitability of the cabamiquine:pyronaridine combination to inhibit *P. berghei* hepatic infection, and we reported a slightly enhanced pre-erythrocytic activity of this drug candidate when in combination compared to its monotherapy, employing both human hepatic cell line spheroids and a murine model of *P. berghei* infection.[10] A similar study has been reported for the combination of atovaquone and proguanil, synergistically effective against the blood stage of infection and discovered later to be equally effective against the hepatic development of *Plasmodium* parasites.[27]

Ganaplacide, currently under Phase III clinical evaluation, inhibits asexual blood stages, hepatic-stages, and sexual-stages of *Plasmodium* and displayed transmission-blocking activity *in vitro* (including against artemisinin-resistant strains)[28] and *in vivo* in rodents.[18] Early phase clinical studies demonstrated prophylactic efficacy in a CHMI model[17] with clearance of *P. falciparum* and *P. vivax*, including artemisinin-resistant parasites, when administered as monotherapy,[18] showing promising single-dose antimalarial activity with no evident safety concerns.[22] A single oral dose of 100 mg or higher (300 and 800 mg) of ganaplacide provided 100% protection with chemoprophylactic activity.[17] Ganaplacide has been studied in combination with a lumefantrine-solid dispersion formulation (SDF) in patients with uncomplicated *P. falciparum* malaria. In a Phase II study, ganaplacide 400 mg and lumefantrine-SDF 960 mg given to fasted patients for 3 days achieved drug exposures considered to provide optimal efficacy for adults, adolescents, and children.[29]

These and other studies highlight the importance of better understanding the targets and efficacy of drug combinations, with particular attention to the pre-erythrocytic stage of *Plasmodium* infection, an attractive target for malaria chemoprevention.[30,31]

In this study, we evaluated a novel drug combination against the liver stage of *Plasmodium* infection. We employed our 3D hepatic infection model previously developed for the preclinical evaluation of antiplasmodial drugs.[10] Cabamiquine and ganaplacide were combined and their pre-erythrocytic activity was assessed following two methodologies: the isobologram method, employed in the assessment of PD interaction of two antiplasmodial drugs, and a recently described *in vitro-in silico* checkerboard analysis.[32] Given the isobologram method is valid only for drugs whose effects are mutually exclusive,[33] we chose to assess our findings using checkerboard analysis. Our results indicate that neither drug affects cell viability, either as monotherapy or in combination, at the tested concentrations. Moreover, our results point to a nondetrimental effect between both drugs, indicating that they do not interfere with their respective modes of action.

The EC_{50} of cabamiquine was determined to be 3.58 nM and was in line with previously reported values.[34] Ganaplacide's EC_{50} was 371 nM and may denote a lower potency to the rodent over the human *Plasmodium* species[21] and subject to more variability (101.5%). As a result, PD interactions within this variability may have been missed.

Additionally, it was observed that the maximum killing effect for both drugs was slightly higher than the growth rate, in line with the shallow decline of the assay signal over time. Hence, regardless of the ratio used between the drugs, no detrimental pharmacodynamic effects were observed.

In the *P. berghei* mouse model, we established that drugs were fully effective in preventing the appearance of blood stage parasites, at a systemic plasma $C_{av0\text{-}24}/EC_{50}$ ratios of >2 for ganaplacide and >5 for cabamiquine. $C_{av0\text{-}24}/EC_{50}$ ratios of <1 for ganaplacide and <3 for cabamiquine were observed with the noncurative dose (that did not prevent the development of blood stage parasites). For partially preventing the development of blood stage parasitemia, $C_{av0\text{-}24}/EC_{50}$ ratios of 1−2 for ganaplacide and 3−5 for cabamiquine were required. Causal prophylaxis, no blood-stage parasitemia was observed with doses of 0.6 mg/kg for cabamiquine in combination with 2 mg/kg for ganaplacide, for which the corresponding monotherapies were suboptimal.

Overall, the potency against the pre-erythrocytic stage of *Plasmodium* infection is maintained in combination for both drugs, suggesting its possible use in clinical settings. Hence, the cabamiquine:ganaplacide combination offers the potential toward efficacy against the pre-erythrocytic stage of *Plasmodium* infection and possibly limits the emergence of drug resistance. Moreover, since both drugs present a multistage activity toward malaria parasites, their combination could be effective for both malaria prevention and treatment.

■ METHODS

Ethics Approval Statement. Animal experimentation procedures carried out at Instituto de Medicina Molecular João Lobo Antunes (iMM-JLA, Lisbon, Portugal) were approved by the institute's animal ethics committee (ORBEA) and were performed in strict compliance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). Animal experiments that were carried out at the Swiss Tropical and Public Health Institute (Swiss TPH, Basel, Switzerland) were in agreement with local and national regulations of laboratory animal welfare in Switzerland (awarded permission no. 2693). Protocols were regularly reviewed and revised following approval by the local authority (Veterinäramt Basel Stadt).

Using Test Product or Compounds. Cabamiquine and ganaplacide were supplied in salt forms by the healthcare business of Merck KGaA, Darmstadt, Germany, and Novartis AG, Basel, Switzerland, respectively. Stock solutions of cabamiquine and ganaplacide were prepared in dimethyl sulfoxide (DMSO) at 10 and 25 mM, respectively.

Mice, Cell Sources, and Parasites. *In vitro*: For sporozoite production, male Balb/c mice (Charles River Laboratories), 7−12 weeks of age, were housed in specific pathogen-free (SPF) conditions at an iMM-JLA's rodent facility. Uninfected female *Anopheles stephensi* mosquitoes reared at iMM-JLA were allowed to feed on anaesthetized mice infected with *P. berghei* ANKA-Luci-GFP parasites for 30 min. Blood-fed mosquitoes were subsequently fed on an aqueous solution of 8% fructose and 0.5% para-aminobenzoic acid (PABA) for 21 days. For sporozoite isolation, salivary glands were collected into Roswell Park Memorial Institute Medium (RPMI) 1640 medium (Thermo Fisher Scientific), macerated, and filtered through a 40 μm cell strainer. *In vitro* studies were performed by employing the HepG2/C3A cell line purchased from ATCC (CRL/10741). *In vivo*: Female

NMRI mice (Janvier, Le Genest-Saint-Isle, France) were infected with *P. berghei* ANKA reporter line (mCherry$_{hsp70}$Luc$_{ef1\alpha}$), donated by the Insitute of Cell Biology (ICB, Bern), and mantained in standard laboratory husbandry conditions.

Three-Dimensional (3D) Cell Culture (Spheroids). HepG2 were expanded in T-flasks in static conditions for 2 weeks in low glucose Dulbecco's modified eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 1% (v/v) penicillin/streptomycin and 10% (v/v) filtered fetal bovine serum (FBS) prior to inoculation in the spinner. Cell passage was performed twice per week at a cell inoculum of 5 × 1 × 10^4 cell/cm^2. For generation of hepatic spheroids, single cell suspensions of HepG2 cells were inoculated at 3 × 10^5 cell/mL in 125 mL spinners (Corning, the healthcare business of Merck KGaA, Darmstadt, Germany) in culture medium with 5% filtered FBS and maintained under dynamic conditions from 40 to 120 rpm (magnetic stirring, 2mag AG), in an incubator with humidified environment at 37 °C and 5% CO_2. Fifty % of the culture medium was replaced with fresh culture medium supplemented with 5% (v/v) filtered FBS every 3 days toward 100% weekly medium renovation.

Assessment of Spheroid Viability and Concentration. The viability of spheroid cultures was assessed as previously described.[23] Briefly, spheroids were incubated with a cell-permeant dye (fluorescein diacetate, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at 10 μg/mL and a DNA dye (propidium iodide, Sigma-Aldrich) at 1 μg/mL for the detection of viable and dead cells, respectively. Visual inspection of the spheroids was performed using an inverted fluorescence microscope (Leica DMI6000). The cell density of the 3D cultures was determined by the Trypan blue exclusion method, as previously described.[35]

***In Vitro* Hepatic *Plasmodium* Infection.** For infection with *P. berghei* sporozoites, HepG2 spheroids were seeded in ultralow attachment 96-well plates (Corning, the healthcare business of Merck KGaA, Darmstadt, Germany) at 5 × 10^5 cell/mL in infection medium (low glucose DMEM supplemented with 1% (v/v) penicillin/streptomycin, 5% (v/v) filtered FBS, 1:300 amphotericin B (250 μg/mL) and 1:200 gentamicin (50 mg/mL), all from Thermo Fisher Scientific). Sporozoites were added to the spheroid cultures at a 1:2 cell:sporozoite ratio and sedimented by a centrifugation step at 1800*g* for 5 min, after which plates were maintained in static conditions at 37 °C and 5% CO_2 for 48 h. Infection rates were assessed by bioluminescence (Infinite 200 PRO, Tecan Trading AG), as a measurement of luciferase activity.

***In Vitro* Assessment of *Plasmodium* Infection by Bioluminescence.** The infection load of *P. berghei*-infected cells was assessed by employing a commercially available Firefly Luciferase Assay Kit from Biotium, following the manufacturer's instructions. Briefly, spheroids were washed twice with phosphate buffer saline (PBS) and later incubated in lysis buffer diluted 1:4 in Milli-Q water. Samples underwent several freeze−thaw cycles, alternated with agitation steps at 500 rpm, until complete cell lysis was confirmed by visual inspection. Bioluminescence was measured using a microplate reader, and the light reaction of each well was measured for 100 ms.

Checkerboard Interaction Assay. Drug stock solutions were diluted in culture medium (low glucose DMEM supplemented with 1% (v/v) penicillin/streptomycin and 5% (v/v) filtered FBS, all from Thermo Fisher Scientific) to have a final concentration corresponding to the predetermined EC_{20} and EC_{80} values of each drug. Concentrations corresponding to total parasite kill (maximum effect) were also employed (10 and 20 times the previously determined EC_{50}). All the concentrations were combined as described for the checkerboard method,[36] and the infection rate (parasite viability) was assessed at 30, 44, and 48 hpi. Hepatocytes viability was also assessed at the end of the drug incubation period (48 hpi) by a PrestoBlue assay. All *in vitro* assay data from four individual experiments were analyzed simultaneously using nonlinear mixed effects modeling in the NONMEM software (ICON development service, Gaithersburg, MD, version 7.5), while assuming constant concentrations of cabamiquine and ganaplacide.

In the first step, the parasite growth parameters were estimated with an exponential growth model. The growth model is defined as follows:

$$\begin{cases} \dfrac{dN}{dt} = k_{growth} \cdot N \\ N(t=0) = N_0 \end{cases} \tag{1}$$

where $N(t)$ represents the model-predicted parasites at time t and k_{growth} is the first-order growth rate and N_0 is the initial condition (assay read-out at t = 0 h).

The raw assay read-out Y was related to N by a proportional residual error model as follows:

$$Y = N \cdot (1 + \varepsilon) \tag{2}$$

with ε quantifying the deviation from Y from N at each time point of the assay read-out.

Subsequently, the monotherapy drug effects were estimated from the wells containing only single drugs using a sigmoidal maximum effect model:

$$\frac{dN}{dt} = k_{growth} \cdot N - \frac{E_{\max} \cdot C^h}{EC_{50}{}^h + C^h} \cdot N \tag{3}$$

where E_{max} represents the maximum kill rate, EC_{50} the concentration stimulating half-maximum kill, and the hill coefficient h the steepness of the concentration-effect relationship.

Thereafter, the combined drug effects for both cabamiquine and ganaplacide were modeled assuming Bliss Independence, given that both drugs exhibit their individual effects on distinct and different targets:[33]

$$\frac{dN}{dt} = (k_{growth} - E_{comb}) \cdot N \tag{4}$$

where:

$$\begin{cases} E_{Cabamiquine} = \dfrac{E_{\max,Cabamiquine} \cdot C_{Cabamiquine}{}^{h_{Cabamiquine}}}{EC_{50,Cabamiquine}{}^{h_{Cabamiquine}} + C_{Cabamiquine}{}^{h_{Cabamiquine}}} \\ E_{Ganaplacide} = \dfrac{E_{\max,Ganaplacide} \cdot C_{Ganaplacide}{}^{h_{Ganaplacide}}}{EC_{50,Ganaplacide}{}^{h_{Ganaplacide}} + C_{Ganaplacide}{}^{h_{Ganaplacide}}} \\ E_{comb} = E_{Cabamiquine} + E_{Ganaplacide} - \dfrac{E_{Cabamiquine} \cdot E_{Ganaplacide}}{\max(E_{\max,Cabamiquine}, E_{\max,Ganaplacide})} \end{cases} \tag{5}$$

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Potential pharmacodynamic interactions were evaluated using the general pharmacodynamic interaction model quantified as shifts of EC_{50} or E_{max}.[37]

Interparasite variability was evaluated on all model parameters assuming a log-normal distribution. Model fit was assessed by goodness of fit plots, inspection of the individual model fits per each experimental scenario, and visual predictive checks. In the case of competing models, the likelihood ratio test (alpha = 0.05, df = 1) was used for nested models, whereas the Akaike Information Criterion (AIC, lower value indicates superior model) was used for non-nested models.

The final model was used for simulations to derive a heat map visualizing the assay signal in a two-dimensional checkerboard for both drugs.

***In Vivo* Drug Assays.** *P. berghei In Vivo Liver Stage Infection Model.* Naïve female NMRI mice were infected with 1×10^5 sporozoites of *P. berghei* by intravenous injection (*i.v.*) in the tail vein as previously described.[15,23] At 24 hpi, a single oral (*p.o.*) dose drug treatment with cabamiquine, ganaplacide, or a combination of both agents (n = 2−3 mice/group) as detailed in Table 4 (summarizing previous mono- and combination treatment regimens) was administered. All compounds were formulated in 1% (w/v) methylcellulose and 0.1% (w/v) Tween-80 in double distilled water and administered by oral gavage. *Plasmodium* liver infection was assessed *in vivo* at 24 and 48 hpi. For *in vivo* imaging, mice were injected intravenously (i.v.) with 150 mg/kg D-luciferin (PerkinElmer; #122799), anaesthetized with gaseous isoflurane (2.5% (v/v) in oxygen) and placed in an IVIS Lumina II *in vivo* imaging system (Caliper Life Science, PerkinElmer). Ventral images were acquired to measure bioluminescence using LivingImage v.4.3 software while continuing anesthesia. Exposure time was set to 1 min. Blood stage parasitemia was measured 6, 7, 8, 10, 13, 16, 20, 24, 27, 31, 35, and 37 days post-infection by light microscopy on Giemsa-stained blood smears, and blood stage positive mice were euthanized. Mice without detectable parasitemia up to day 37 post-infection (>30 days post-treatment) were considered as cured and were euthanized. Blood samples were collected at 2, 4, and 24 h post-treatment to assess the drug exposure level.

Table 4. Dose Groups of Mice Treated with Cabamiquine, Ganaplacide or a Combination of Both Agents

Agent(s)	Animal number	Dosage mg/kg: 1× (24 h post infection)
-	3	-
Cabamiquine	2	1.5
Cabamiquine	2	0.6
Cabamiquine	2	0.3
Ganaplacide	2	5
Ganaplacide	2	2
Ganaplacide	2	1
Cabamiquine + Ganaplacide	2	1.5 + 5
Cabamiquine + Ganaplacide	2	0.6 + 2
Cabamiquine + Ganaplacide	2	0.3 + 1
Atovaquone (positive control)	2	10

Pharmacokinetic Analysis. PK blood sampling from the tail vein (20 μL) was collected 2, 4, and 24 h post-treatment. Blood samples were extracted using a protein precipitation method by adding 120 μL of the extraction solution (acetonitrile containing 62.5 ng/mL labetalol hydrochloride as internal standard). The concentrations of compounds at various time points were determined by high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Blood from control mice was used for calibration and QC samples. Liquid chromatography was performed using a Shimadzu Nexera X2 LC-30 AD system with a Phenomenex Polar RP column (50 mm × 2 mm, 2.5 μM particle size) at an over temperature of 40 °C, coupled with a Sciex QTRAP 6500 mass spectrometer. Blood PK parameters were determined from the raw blood concentration time data by noncompartmental analysis using a Phoenix WinNonLin instrument (version 8.3, Certara, Cranbury, NJ). For PK/PD analysis, the average blood concentration from time 0 to 24 h ($C_{av0\text{-}24}$) was derived by dividing the $AUC_{0\text{-}24}$ by 24. The plasma average compound concentration ($C_{av0\text{-}24}$) was derived from blood partitioning. The ratio of $Cav_{0\text{-}24}$ over the *in vitro* potency against the liver stage *P. berghei* parasites was calculated, and these ratios were compared to the *in vivo* efficacy.

Data Analysis and Statistics. Nonlinear regression analysis of the normalized results for the determination of EC_{50} values and statistical analysis were performed employing GraphPad Prism version 6 for Windows (GraphPad software, La Jolla California U.S.A.). Outliers were identified by the robust regression and outlier removal (ROUT) method. Normality was assessed by the Shapiro-Wilk normality test. Significant differences were determined using a parametric or non-parametric *t* test, considering paired conditions when subjected to the same batch of sporozoites, or using one-way ANOVA. *P* values are presented for statistically significant results (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ****$P < 0.0001$), as indicated in each figure legend.

■ ASSOCIATED CONTENT

Data Availability Statement

Any requests for data by qualified scientific and medical researchers for legitimate research purposes will be subject to the healthcare business of Merck KGaA, Darmstadt, Germany's, Data Sharing Policy. All requests should be submitted in writing to the healthcare business of Merck KGaA, Darmstadt, Germany's data sharing portal https://www.merckgroup.com/en/research/our-approach-to-research-and-development/healthcare/clinical-trials/commitment-responsible-data-sharing.html. When the healthcare business of Merck KGaA, Darmstadt, Germany, has a coresearch, codevelopment, or comarketing or copromotion agreement, or when the product has been out-licensed, the responsibility for disclosure might be dependent on the agreement between parties. Under these circumstances, the healthcare business of Merck KGaA, Darmstadt, Germany will endeavor to gain agreement to share data in response to requests.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.4c00563.

Supplementary Methods and Supplementary Figures 1, 2, 3, and 4. (PDF)

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Notes

The authors declare the following competing financial interest(s): C.D.G. and T.S. are employed by Ares Trading S.A., Eysins, Switzerland, a subsidiary of Merck KGaA, Darmstadt, Germany. J.B. and S.B.L. are employed by Novartis. All other authors declare no competing interest.

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