Superior Pyronaridine Single-Dose Pharmacodynamics Compared to Artesunate, Chloroquine, and Amodiaquine in a Murine Malaria Luciferase Model

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ABSTRACT Many previous in vitro and in vivo preclinical malaria drug studies have relied on low-parasite-number drug inhibition numerically compared to the untreated controls. In contrast, human malaria drug studies measure the high-parasite-density killing near 100 million/ml. Here we compared the in vivo single-dose pharmacodynamic properties of artesunate and the 4-aminoquinolines pyronaridine, chloroquine, and amodiaquine in a Plasmodium berghei ANKA-green fluorescent protein GFP-luciferase-based murine malaria blood-stage model. Pyronaridine exhibited dose-dependent killing, achieving parasite reductions near 5 to 6 logs at 48 h, with complete cure at 10 mg/kg of body weight compared to artesunate, which exhibited a 48-h dose-dependent killing with a 2-log drop at the noncurative 250-mg/kg dose. Chloroquine, which was noncurative, and amodiaquine, which was partially curative, had nearly the same initial dose-independent killing, with a lag phase of minimal parasite reduction at all doses between 6 and 24 h, followed by a 2.5-log reduction at 48 h. In experiments with drug-treated, washed infected blood transfer to naïve mice, chloroquine and amodiaquine showed fewer viable parasites at the 24-h transfer than at the 8-h transfer, measured by a prolonged return to parasitemia, despite a similar parasite log reduction at these time points, in contrast to the correlation of the parasite log reduction to viable parasites with artesunate and pyronaridine. Artesunate in combination with pyronaridine exhibited an initial parasite reduction similar to that achieved with pyronaridine, while with chloroquine or amodiaquine, the reduction was similar to that achieved with artesunate. Single-oral-dose pyronaridine was much more potent in vivo than artesunate, chloroquine, and amodiaquine during the initial decline in parasites and cure.

KEYWORDS antimalarial agents, chemotherapy, malaria, pharmacodynamics

Humans with a 4% parasitemia have about 1 trillion parasites (160 billion infected erythrocytes per liter). Human malaria chemotherapy can be measured by a parasite reduction per time interval, such as the 48-h single Plasmodium falciparum life cycle (1, 2). The artemisinins are postulated to provide a 4-log killing rate each 48-h parasite cycle, quinolines a 3-log killing rate, antifolates a 2-log killing rate, and antibacterials a single next-cycle (delayed generational) 1-log kill rate (3). In contrast to this parasite reduction measurement in humans, drug screening in vitro with P. falciparum starts with 0.2 to 0.5% parasitemia at 2% hematocrit, or about a million per milliliter in a well, to compare percent inhibition with that for the untreated drug control at 72 h. Both the low erythrocyte concentration and the low protein concentration (10% serum) are not physiologic, which affects free drug concentrations. Drug-treated parasites that slowly increase a log, with untreated parasites increasing 2 logs, have 90% inhibition. Most mouse models of drug activity commonly utilize the

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Peters suppression test, which delivers a low parasite inoculum followed by high daily doses of drugs commencing hours after infection (4). The parasite number at 4 days on drug is compared to that in the no-drug controls, in which parasites multiply by almost a log each day. If drug treatment results in a slower growth of only 2 logs in 4 days, this is expressed as 99% inhibition even if the parasites have multiplied.

Batty and colleagues have recently investigated detailed pharmacodynamic relationships with some current antimalarial drugs in the high-parasitemia mouse model using the laborious blood film enumeration of *P. berghei*-parasitized erythrocytes (5, 6). They explored the dose-dependent single-log parasite reduction obtained with dihydroartemisinin in the range of 1 to 100 mg/kg of body weight (7) and the quinolines chloroquine at up to 30 mg/kg (8) and piperaquine (9) as well as dihydroartemisinin and quinoline combinations.

The aim of this study is a side-by-side comparison of the single-dose pharmacodynamic initial killing rate and 30-day cure achieved with three quinolines, pyronaridine, chloroquine, and amodiaquine, along with artesunate, in an adapted luciferase (Luc)-expressing mouse malaria model with a range of equivalent salt doses. The purpose was to explore both time-dependent and dose-dependent killing. Because the long-half-life quinolines preclude short-duration drug exposures, we also transferred drug-treated, washed infected erythrocytes into naive mice to measure the early (under 24 h) time-dependent killing obtained with artesunate and the quinolines. Finally, we measured the time course of artesunate-quinoline interactions in combination single-dose therapy.

**RESULTS**

**Single-dose parasite reduction.** We sought to directly compare the pharmacodynamic killing of allometrically scaled doses of the four drugs (10, 11). Human daily doses and mouse allometric adjustment translated to 50 mg/kg for artesunate (4 mg/kg in humans) and 150 to 200 salt mg/kg for the quinolines (human pyronaridine at 12 mg/kg, chloroquine at 17 mg/kg, and amodiaquine at 13 mg/kg) based on salt doses. We chose to dose the mice at 10, 50, 100, and 250 salt mg/kg, corresponding to human-equivalent doses of 0.8, 4.2, 8.4, and 21 mg/kg, respectively, to encompass the 50- to 200-mg/kg salt range in mice (Table 1). Artesunate exhibited a dose-dependent reduction in parasites at 24 h with log reductions in parasites per microliter of 0.2, 1, 1.3, and 1.5 for the 10-, 50-, 100-, and 250-mg/kg doses, respectively, which increased to 0-, 0.4-, 1.1-, and 1.8-log reductions, respectively, at 48 h (Table 2). The numbers of days to return to initial parasitemia were 2, 3, 3, and 4, respectively (Fig. 1A). In contrast, pyronaridine in this same dose range exhibited a seemingly dose-independent log reduction of 3.5 for all doses at 24 h and a 6-log reduction at 48 h (Table 2). As pyronaridine was completely curative at all these doses, there was no return to parasitemia in 30 days. Chloroquine and amodiaquine showed a plateau phase after an initial 6-hour drop from 6 to 24 h postdosing, with an approximate single-log reduction in the first 24 h, no matter the dose from 10 to 250 mg/kg (Fig. 1). Mice dosed with 50 to 250 mg/kg showed a further dose-independent ~3-log parasite reduction, falling to near the limit of quantification (1,000 parasites/μl) at day 3 after the initial dose before reversing. The duration to return to initial parasitemia was measured and was found to be dose dependent, taking nearly 3 to 9 days for mice to return to initial parasitemia with increasing doses, a reflection of the longer dose-dependent drug exposure time.

<table>
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<tr>
<th>Drug</th>
<th>Daily human dose (mg salt)</th>
<th>Total treatment dose (mg salt)</th>
<th>Daily human dose (mg/kg salt)</th>
<th>Daily allometric mouse dose (mg/kg salt)</th>
<th>Base/salt mol wt ratio</th>
<th>Daily base mouse dose (mg/kg)</th>
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<td>720</td>
<td>4</td>
<td>48</td>
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<td>16.6</td>
<td>200</td>
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<td>13.2</td>
<td>160</td>
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<sup>a</sup>Chloroquine 900 mg base day 1, 300 mg base day 2 and 3.
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<td></td>
<td>Log reduction at 24 h</td>
<td>Log reduction at 48 h</td>
<td>No. of days to initial parasitemia</td>
<td>Log reduction at 24 h</td>
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**TABLE 2** Parasite log reduction ratio and time to return to initial parasitemia
FIG 1 Single-oral-dose killing profile of artesunate and the quinolines in a *P. berghei* ANKA-GFP-luciferase infection. (A to D) Single doses of artesunate (A), pyronaridine (B), chloroquine (C), and amodiaquine (D) of 10 mg/kg (empty diamonds), 50 mg/kg (open triangle), 100 mg/kg (×), and 250 mg/kg (open circles-solid lines) were administered orally to female BALB/cJ mice (*n* = 3) at time zero, which corresponds to about 10% parasitemia, which was confirmed by Giemsa blood film counting. Data are represented as the mean ± SEM. The initial parasitemia (IP) was normalized to 1 million parasites per μl. The limit of quantification (LQ) of 1,000 parasites per μl was above the limit of detection. For amodiaquine (D), the three open circles and solid lines represent individual mice, one of which had recrudescence. (E and F) Parasite reduction over 48 h with artesunate (the same symbols described above with a dotted line) and pyronaridine (the same symbols described above with a dashed line) (E) and amodiaquine (the same symbols described above with a dotted line) and chloroquine (the same symbols described above with a dashed line) (F) with the same 10-, 50-, 100-, and 250-mg/kg doses.
Two of three mice achieved a complete cure with amodiaquine at 250 mg/kg. A comparison over 48 h is depicted in Fig. 1E and F, showing the faster decline with pyronaridine and a two-step reduction with chloroquine and amodiaquine.

We were also interested in comparing the single-dose killing dynamics of artesunate given in a three-dose regimen of 50 or 200 mg/kg every 8 h (0, 8, and 16 h) to those of low-dose pyronaridine given at 1 to 10 mg/kg, respectively (Fig. 2). Artesunate has a short 6- to 8-h period of exposure in the rodent (12), so testing a multiple-dose regimen over 24 h creates a greater but intermittent drug exposure somewhat comparable to that for the quinolines but short of a constant time above the inhibition concentration. Mice that received the 3-dose regimen of artesunate at a 150-mg/kg total dose also showed a large log reduction in parasite numbers, with reductions of 2 logs at 24 h and 3.5 logs at 48 h, more comparable to the pyronaridine numbers. Artesunate at 200 mg/kg also showed a rapid reduction in parasites, with log reductions of 3 and 5 at 24 h and 48 h after the initial dose, respectively, similar to those achieved after a single dose of 10-mg/kg pyronaridine. Pyronaridine at a single dose of 1 mg/kg showed no reduction. However, 2.5 to 25 mg/kg had a dose-dependent kill rate at 24 h and 48 h after initial dosing. Strikingly, the results for the 5-mg/kg pyronaridine dose were similar to those for the 250-mg/kg artesunate single dose shown in Fig. 1. Two of three mice receiving single-dose pyronaridine at 7 mg/kg achieved a complete cure. However, single-dose pyronaridine at 10 mg/kg was the most potent, with a nearly 3-log kill at 24 h and a 5-log kill at 48 h, almost twice the log drop seen with 5 mg/kg and 7 mg/kg, after the initial dosing (Table 2). The duration to return to the initial parasitemia with low-dose pyronaridine was dose dependent and ranged from 3 to 11 days.

Parasite reduction following transfer to naive mice. Following determination of the single-dose dynamic killing profiles, we were interested in investigating a seldom used measure of killing in an experiment involving washed infected blood transfer to naive mice in order to effectively lower the drug concentration to below that for minimal inhibition so that the time-dependent killing of the quinolines could be compared to that of artesunate. For the standard curve, we isolated infected erythrocytes from a cardiac puncture and then serially diluted 100 million, 10 million, 1 million, and 0.1 million infected erythrocytes into 100 μl of heparinized uninfected blood, which was triply washed and injected into 3 naive mice. The time to the return to a million parasites per microliter was 2, 3, 4, and 5 days for the serial dilutions, respec-
Next we dosed infected mice with drugs and measured the parasite log reduction in the donor mice compared to the number of days to return to a million parasites per microliter. Pyronaridine at 50 mg/kg showed a linear correlation over time of exposure between the parasite log drop and the duration that it took to reach 1,000,000 viable parasites posttransfer. Low-dose (50 mg/kg) and high-dose (150 mg/kg) pyronaridine-treated blood took nearly the same number of days to reach viable parasites at the 8-h transfers with similar donor mean parasite log reductions. However, at 24 h there was both a log more donor parasite reduction and an additional log kill with the increase in dose from 50 to 150 mg/kg. Only two mice came up positive during the 48-hour transfer, and one mouse was positive during the 72-hour transfer (Fig. 3). Artesunate given in a 200-mg/kg multidose regimen had a 2-log parasite reduction in donor mice but a 4-day return to initial parasitemia at 8 h. Similarly at 24 h, a 3-log reduction resulted in a 6-day return to initial parasitemia, interpreted to mean that a larger proportion of detectable parasites was dead with artesunate than with

![Diagram](image-url)
pyronaridine. Chloroquine and amodiaquine, both at a 50-mg/kg single oral dose, had nearly the same parasite log reduction of about 1 and 1.5 at 8 and 24 h postdosing, respectively, with the duration to reaching initial parasitemia being 2 and 3 days, respectively (Fig. 3).

Parasite reduction in combination therapy with artesunate and 4-aminoquinolines. We were interested in investigating the effect of single-dose combination therapy with artesunate at 250 mg/kg with pyronaridine, chloroquine, or amodiaquine administered orally alone (open triangles-dotted lines) in replicate biological experiments and in combination with 250 mg/kg i.p. artesunate (filled triangles-dashed lines) to female BALB/cJ mice (n = 3) at 0 h are shown. Artesunate alone (open circles-solid lines) was also dosed at 250 mg/kg i.p. Data are represented as the mean ± SEM.

DISCUSSION

Batty and colleagues developed a pharmacodynamic high-parasitemia parasiticidal model of *P. berghei*, looking at chloroquine, dihydroartemisinin, doxycycline, and piperaquine by Giemsa blood film analysis (5, 6). While chloroquine was used at a lower human-equivalent dose of ~300 mg, dihydroartemisinin (an ~4-mg/kg dose for humans or a 50-mg/kg dose for mice) was used at 2.5 mg/kg human. They observed that it took 3 to 5 days to return to 2% parasitemia with 10 to 50 mg/kg chloroquine and 2 days with dihydroartemisinin. The luciferase measurements provide the ability to quickly process data from many more time points with a greater robustness in parasite counting at lower parasitemias with a larger range of quantification. The luciferase is
not prone to the inherent errors in counting blood films described in many reports, such as overdispersion (13) and variability in slides and counting (14, 15). A disadvantage to the luciferase measurements is not visualizing an altered morphology caused by drugs or altered stage ratios. However, discrete time points can be inspected for these drug morphological effects by traditional Giemsa staining. Others have touted the mouse-adapted immunocompromised *P. falciparum* model, which does allow for drug dosing in a physiologically altered animal. Ideally, a direct comparison of the pharmacodynamic killing data from *P. falciparum* in immunocompromised SCID mice to our pharmacodynamic killing data should also be performed (16).

Here we extend this important body of work to higher single doses of the quinolines in the range of 100 and 250 mg/kg mouse as well as the 10- to 50-mg/kg doses in a direct comparison of artesunate, pyronaridine, chloroquine, and amodiaquine all near their respective human-equivalent doses. The main observations in this luciferase-measured model are a rapid dose-dependent decrease by pyronaridine and artesunate. Chloroquine and amodiaquine both exhibited a slower dose-independent initial decrease, which is compatible with the infrequent observation of a delayed effect with the quinolines. Patel et al. noted a 12-h lag in monotherapy with piperaquine in *P. berghei*-infected subjects (17). A *P. falciparum* study in 1992 in Brazil noted a 50% decrease in the geometric mean parasite density at 24 h and a 98% decrease at 48 h with chloroquine (28). A 1999 study in the Philippines noted an 80% decrease in *P. falciparum*-infected mice treated with chloroquine at 24 h (18). Sanz et al. investigated by serial dilution the in vitro kill rates of asynchronous *P. falciparum*, noting a delayed kill with atovaquone, pyrimethamine, and the antibiotics but did not see a lag in killing with the quinolines, measuring at 0 and 24 h (19). Investigations into synchronous cultures of *P. falciparum* observed no arrest of ring stages by the quinolines but the arrest of rings and trophozoites by the artemisinins (20). In a recent review, White looked at quinine, chloroquine, and artesunate, counting blood films every 6 h and observing a slight lag with quinine but no lag with chloroquine at 6 h (21). An interpretation of these mixed results is an inability to measure true percent rings as a fraction of the total parasite biomass for *P. falciparum*. In *Plasmodium* infections, whether they are caused by *P. falciparum* or *P. berghei*, if the percent rings is high, there is a predicted delay in killing with chloroquine and amodiaquine, which is more specific to the trophozoite stage. Pyronaridine may have off-hemozoin-target effects to achieve the more rapid consistent killing.

In order to compare the parasite reduction of the long-half-life quinolines with that of the short-half-life artemisinins, we adapted a washed blood transfer experiment to measure viable parasites at 8 and 24 h. We estimate that a starting parasitemia of 5 million per ml represents close to 10 billion parasites, which, when transferred in serial dilutions, demonstrated a successive day extension with each 10-fold dilution, translating to an additional log kill with drugs. Pyronaridine demonstrated a correlation with donor mouse parasite reduction before transfer to the extended duration of time to return to 10% of the initial parasitemia. At 8 and 24 h, chloroquine and amodiaquine demonstrated a 2- to 3-log kill at 8 and 24 h, despite a similar donor parasite reduction. Artesunate had a 4- to 6-log kill at 8 and 24 h, which was slightly superior to that of pyronaridine with 3- and 6-log kill. The blood transfer experiments observed the fast-killing artesunate effects masked in serial luciferase measurements in the comparison to pyronaridine.

Pyronaridine induces a faster initial rate of parasite reduction and a very low rate of cure at 10 mg/kg compared to 250 mg/kg of artesunate. This is in contrast to studies in humans, where the artemisinins induce a greater reduction in parasitemias (21). Other studies have shown a similar rate of parasite clearance with chloroquine and pyronaridine (22). Fidock and colleagues also noted superior clearance with pyronaridine against artemisinin-tolerant *P. berghei* (23). In a murine model, pyronaridine and artemisinin were additive against chloroquine-resistant *P. yoelii* but synergistic against the artemisinin-resistant *P. yoelii* and also against the pyronaridine-resistant *P. yoelii* (24). Here we just measured early kill pharmacodynamics and did not use the more
complicated checkerboard for measurement of synergy with multiple doses of artesunate with a range of doses of pyronaridine. Human studies have shown no antagonistic killing with pyronaridine and artesunate (25). The mean parasite clearance of the combination has varied by region tested, with faster times in Africa than in Cambodia (26).

In conclusion, here we expanded on the pharmacodynamic cytocidal effects of three quinolines compared to artesunate in a high-parasitemia mouse model. Pyronaridine showed dose-independent very rapid 5- to 6-log kill in 48 h compared to the dose-dependent artesunate, which showed a single-dose 2-log decrease at 48 h with a 250-mg/kg maximum dose. Three doses within 24 h of 200-mg/kg artesunate administration increased the killing log reduction to 5.5. Amodiaquine and chloroquine had a slower dose-independent killing, with a small lag phase being noted from 6 to 24 h. In a washed infected erythrocyte transfer experiment, artesunate given in three 200-mg/kg doses in 24 h killed twice as many parasites with a similar log reduction in parasitemia compared to lower doses of pyronaridine. In summary, while pyronaridine was almost 10 times as potent in parasite reduction as chloroquine, amodiaquine, and artesunate, multiple high doses of artesunate within the single 24-h P. berghei cycle had killing similar to that of pyronaridine.

MATERIALS AND METHODS

Ethics statement. Mouse experimentation was performed under a protocol (MO15H319) approved by the Johns Hopkins University Animal Care and Use Committee.

Drug preparation and dosing. The antimalarial drugs artesunate and the 4-aminoquinolines pyronaridine, chloroquine, and amodiaquine were obtained from Sigma-Aldrich. The quinoline salts used were pyronaridine tetraphosphate (molecular weight [MW] = 910 and 56% free base), chloroquine diphosphate (MW = 515 and 62% free base) and amodiaquine dihydrochloride (MW = 464 and 76% free base). Artesunate is 100% parent drug. For all the experiments, the quinolines were dosed in the salt form. The conversion of the base/salt weight percentage is shown in Table 1 and indicates the actual measurement of free base in the salt dosage employed. Artesunate was dissolved in 3% sodium bicarbonate, and pyronaridine tetraphosphate, chloroquine diphosphate, and amodiaquine dihydrochloride were each dissolved in deionized water (dH2O). Fresh drug solution was used for each experiment.

In vivo cytocidal model of murine malaria. For all the experiments, female BALB/cJ mice from The Jackson Laboratory were used in the rodent model. The mice were at least 6 weeks of age and weighed approximately 20 g each. For each study done, replicates of three mice were used for each drug dose regimen tested. P. berghei ANKA 676m1cl1-green fluorescent protein (GFP)-luciferase (Luc), obtained from ATCC (catalog number MRA-868), constitutively expresses the luciferase at all stages in the life cycle. The P. berghei ANKA-GFP-Luc clone enables precise measurements of parasite dynamics over 4 to 8 h and a 3-log range in an IVIS Spectrum in vivo imaging system using the plate reader function. For each experiment, female BALB/cJ mice were infected intraperitoneally (i.p.) with 10,000 erythrocytes infected with P. berghei ANKA-GFP-Luc from a donor mouse at between 5 and 10% parasitemia (in the first or second passage). A parasite isolate in frozen blood obtained from BEI ATCC was inoculated into naïve mice. At the appropriate time, mosquitoes were fed to produce infective sporozoites with a passage through mosquitoes, followed by infection via mosquitoes to cycle the parasite through the liver and into blood. These completely passaged infected donor mice were exsanguinated, with infected blood being aliquoted as low-passage-number (less than three) isolates for use in all experiments. Parasitemia in the experimental mice was monitored using Giemsa-stained thin blood film counting and luciferase measurements until approximately 3 to 4% parasitemia, translating to millions of parasites per milliliter, as will be described in the luciferase assay protocol below, was reached before initiating drug dosing.

 Luciferase assay and analysis. A dilution series was performed using P. berghei-infected blood to establish a standard curve for the translation of the luciferase signal (total flux) into the number of parasites per well (27). Five microliters of blood was drawn from 24 mice with parasitemias ranging from approximately 6% to 12% and added to 45 µl of lysis buffer. Twofold dilutions in lysis buffer (20 mM Tris [pH 7.5], 5 mM EDTA, 0.008% [wt/vol] saponin, 0.08% [vol/vol] Triton X-100) were performed 15 times. Samples were then processed for the luciferase assay, with the number of parasites per well ranging from 300 to 600,000. A total of 0.5 µl of whole blood was analyzed per well in the luciferase assay. During the drug treatment, 5 µl of blood was collected from the tail of each mouse at regular intervals and deposited into 45 µl of lysis buffer in a 96-well plate. Samples were stored at −80°C until they were processed. A total of 5 µl of blood-lysis buffer (whole-blood equivalent of 0.5 µl) was transferred to a black, opaque 96-well plate, and 95 µl of luciferase buffer (20 mM Tricine, 100 µM EDTA, 1.07 mM K2CO3, 2.67 mM MgSO4, 17 mM dithiothreitol, 250 µM ATP, 250 µM o-luciferin) was added. Luciferase activity was measured in an IVIS Spectrum in vivo imaging system and analyzed using Living Image (v.4.4) software. The raw luciferase activity is reported as radiant flux (in number of photons per second). Less than 1,000 photons/s was below the limit of detection. Total radiant flux was compared to the number of parasites per well using GraphPad Prism (v.5) software and the standard curve equation derived in...
Fig. 1. Blood film parasitemia was compared to the results of the luciferase assay when parasitemia was detectable by microscopy. A radiant flux above 52,000 photons/s, corresponding to 300 parasites per well or 600 parasites per μl was the robust limit of quantification. μl was the robust limit of quantification.

Blood transfer experiment. Female BALB/Cj mice infected with P. berghei ANKA-GFP-Luc were dosed with specific single and multidose regimens of 4-aminoquinolines and artesunate, respectively. At each transfer time point, 100 μl of infected blood was withdrawn by tail bleeding from each treated donor mouse and diluted in 1 ml sterile phosphate-buffered saline (PBS) solution. The blood suspension was then washed thrice with 1 ml sterile PBS by centrifugation (3,000 × g). After the third wash, the infected erythrocytes were suspended in a final volume of 210 μl and 5 μl was placed in 45 μl lysis buffer for quantification of the transferred cells using the luciferase assay, and 200 μl was injected i.p. into uninfected naive mice in order to effectively lower the plasma drug concentration below the level for minimal inhibition to determine cell viability. The following malaria metrics were followed: parasite growth curve; time to a parasite number of 10,000 and 100,000 parasites per μl after each transfer, and the time to the start of parasitemia to compute the number of killed parasites. A dilution curve for untreated infected mice at time zero served as the control.

Data analysis. All the data analyses and representations were performed with GraphPad Prism (v.5) software. Data are represented as the mean ± standard error of the mean (SEM). The luciferase raw data log₁₀ total flux (number of photons per second) was transformed to the number of parasites per microliter (y) using the equation y = (10log(phot) – 6.55)/0.05). All data were normalized to the geometric mean parasitemia of 5 million parasites per μl at the time of drug treatment. The parasite log reduction was achieved by getting the difference between the log₁₀ initial parasitemia (6.7) and the parasitemia at time points of 6 h, 12 h, 24 h, and 48 h. The statistical methods employed included the following: two-way analysis of variance (ANOVA) and/or a post hoc test was used for comparison of groups as appropriate, with an alpha significance level of α < 0.05 being used.

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