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Modified dosing schedule efficacy of fosmidomycin and clindamycin against murine malaria *Plasmodium berghei*

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ABSTRACT

Fosmidomycin and clindamycin target the *Plasmodium* apicoplast. Combination clinical trials have produced mixed results with the primary problem being the recrudescent infection frequency by day 28. Given that antibiotic efficacy against bacterial infections often depends on the constant drug presence over several days, we hypothesized that the antimalarial blood or liver stage efficacy of fosmidomycin and clindamycin could be improved by implementing a more frequent dosing schedule. A blood stage murine malaria *P. berghei* GFP-luciferase low and high parasitemia model was implemented to follow pharmacodynamics and cure for modified dose, schedule and duration of individual and combination fosmidomycin and clindamycin. *P. berghei* sporozoites were used to investigate fosmidomycin during the 48 h murine liver stage. Here we observed that the same total dose of fosmidomycin and clindamycin, alone and in combination, are more efficacious when scheduled in smaller, more frequent doses. Fosmidomycin added measurably small additional killing in combination with clindamycin. Despite dosing every 6 h during liver stages, fosmidomycin was inhibitory, but noncurative even with addition of atorvastatin to decrease hepatocyte production of mevalonate. We have also demonstrated *in vitro* efficacy of fosmidomycin and clindamycin against *P. falciparum* C580Y with IC_{50}s similar to those for drug sensitive *P. falciparum*. The dosing schedule of quinoline and artemisinin partner drugs fosmidomycin or clindamycin targeting the apicoplast should maximize time above minimum inhibitory concentration.

1. Introduction

Plasmodium parasites are susceptible to antibiotics such as fosmidomycin and clindamycin due to the presence of the apicoplast, which harbors prokaryote-like ribosomes and metabolic pathways (Chakraborty, 2016). Fosmidomycin was initially intended for the treatment of urinary tract infections and is known to inhibit a key enzyme in the nonmevalonate pathway for the synthesis of isoprenoid precursors, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Lell et al., 2003; Knak et al., 2022). Clindamycin is clinically used against many bacterial and protozoan microbes at schedules every 6–8 h and rarely every 12 h and targets the 23s rRNA and ribosomal translocation (Na-Bangchang et al., 2007; Chakraborty, 2016). Clinical trials of the combination of fosmidomycin and clindamycin utilized twice daily doses. Clindamycin at every 8 h intervals is in WHO guidelines for first trimester malaria treatment with quinine where artemisinins are not available(WHO, 2024). The recent study in Kenya evaluated quinine plus clindamycin versus artemether-lumefantrine for uncomplicated malaria in children dosed all drugs on a twice daily schedule(Obonyo et al., 2022) which, as pointed out in a following commentary, may have underdosed both quinine and clindamycin(Krishna and Kremsner, 2022).

Parasites exposed with clindamycin progress through the asexual cycle in which they are exposed. The inability of the apicoplast to successfully replicate results in parasite death in the second generation of parasites and leads to the phenomenon of delayed generational death. By contrast, fosmidomycin has been reported to have an immediate death phenotype (Uddin et al., 2018). A previous *P. falciparum* study found that fosmidomycin was synergistic with the lincosamide antibiotics clindamycin and lincomycin *in vitro* (Wiesner et al., 2002). Both Fosmidomycin and clindamycin in humans have a pharmacokinetic terminal half-life of approximately two-three hours(Murakawa et al., 1982; Flaherty et al., 1988; Ruangweerayut et al., 2008). Importantly, fosmidomycin also has a low oral bioavailability of approximately 33%

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(Jomaa et al., 1999).

Seven human clinical trials of fosmidomycin and clindamycin for the treatment of malaria have produced differing results. For each of the clinical trials, fosmidomycin and clindamycin were dosed at approximately 30 mg/kg and 10 mg/kg, respectively with two doses 12 h apart for three to five days. Two studies in Thai adult populations resulted in 28-day cure rates exceeding 90%; however, 28-day cure rates among pediatric populations ranged from 46% to 100% (Borrmann et al., 2004a, 2004b, 2006; Na-Bangchang et al., 2007; Oyakhirome et al., 2007; Ruangweerayut et al., 2008; Lanaspa et al., 2012). The geographic location of the study and the age and immune status of the children within the pediatric population may explain some of the variance in efficacy. In human clinical studies fosmidomycin-clindamycin combinations were administered every 12 h for 3–5 days, which represents four doses per 48-h erythrocytic cycle, for approximately two cycles (*P. falciparum* malaria). As noted below, Notwithstanding the different host and parasite biology this is comparable to doses every 6 h for two days in a murine model with a 24 h erythrocytic cycle.

Here we investigated the smaller time interval with more frequent fosmidomycin and clindamycin dosing schedules *in vivo* in a mouse blood stage low parasitemia model starting with low parasitemias and separately a high parasitemia parasite reduction model using *P. berghei* GFP-luciferase, which has previously been utilized to analyze quinine, chloroquine, and artesunate efficacy (Franke-Fayard et al., 2008). We also explored fosmidomycin liver stage action with atorvastatin added to inhibit host hepatocyte mevalonate production, a precursor to isoprenoid synthesis. Host cell cholesterol synthesis blockers inhibited intracellular toxoplasmosis(Nishikawa et al., 2011; Li et al., 2013). We also assessed the *in vitro* efficacy of fosmidomycin and clindamycin against *P. falciparum* bearing the Kelch-13 C580Y mutation, which is most commonly associated with delayed clearance to the artemisinins (Ashley et al., 2014). Broadly, our study aimed to couple the *in vivo* pharmacodynamics of several different dosing schedules of fosmidomycin and clindamycin against *P. berghei* with *in vitro* activity against a clinically relevant *P. falciparum* strain in order to better understand how to most effectively implement apicoplast targeting fosmidomycin and clindamycin for the treatment of malaria. In the murine blood-stage model fosmidomycin added little to the parasite reduction activity of clindamycin. The same total clindamycin dose delivered in more frequent (continuous) intervals led to a greater reduction in parasite number. Neither fosmidomycin nor the combination of fosmidomycin and atorvastatin eliminated murine liver stage parasites.

2. Materials and methods

2.1. Ethics

All experimentation was carried out under an approved protocol by the Animal Care and Use Committee of the Johns Hopkins University (MO15H319). Mice were 6 week females (The Jackson Laboratory) approximately 20 g housed 5 per cage with unlimited access to food and water.

2.2. P. berghei blood-stage and liver-stage in naïve mice

Blood-stage assays used a *P. berghei,* strain (ANKA) 676m1cl1 expressing GFP-luciferase (MRA-868) that was obtained through BEI Resources Repository, NIAID, NIH and previously contributed by Chris J. Janse and Andrew P. Waters (Franke-Fayard et al., 2008). *P. berghei,* strain (ANKA) 676m1cl1 was passaged through naïve female BALB/cJ mice and periodically through *Anopheles stephensi* mosquitoes. A frozen stock was used to generate a donor mouse for each set of experiments and the number of passages in mice did not exceed 5 from mosquito emergence. Liver stage assays used either the transgenic *P. berghei* mCherry parasites (2204cl5) (Hopp et al., 2015) expressing probe during sporozoite stage for Balb/c mice or wild type *P. berghei* ANKA for C57Bl/6 mice.

2.3. Drug preparation for in vivo dosing

Fosmidomycin, sodium salt (FR-31564, Thermo Fisher Scientific) and clindamycin phosphate (1138008, USP) were dissolved in nuclease-free water and administered via intraperitoneal injection in a volume of 200 μL. For fosmidomycin, a 100 mg/kg intraperitoneal injection is equivalent to a 300 mg/kg oral dose as measured by achievable blood levels, as only 33% of fosmidomycin is absorbed (Murakawa et al., 1982). In contrast, clindamycin has good bioavailability of greater than 85% (Bouazza et al., 2012). Atorvastatin (PHR1422, Sigma) was dissolved 100 mg/mL in DMSO and diluted to 4 mg/mL in sterile water/1% Tween 80 for oral dosing by gavage cannula. D-luciferin (88292, Pierce D-Luciferin, Thermo Fisher Scientific) was used at 250 μM.

2.4. Determination of in vivo drug activity

For the low parasitemia assay, female mice were infected with 1,000,000 infected *P. berghei,* strain (ANKA) 676m1cl1 erythrocytes and drug treatment with fosmidomycin, clindamycin, or combination was initiated 24 h after infection. For malaria blood stage infections and drug responses, there are not sex differences in Balb/c mice. As male and female Balb/c mice display differences in tissue iron levels, female mice were used for consistency throughout all experiments(Hahn et al., 2009). The high parasitemia assay was used for subsequent determination of *in vivo* drug efficacy. Mice were infected with 500,000 infected *P. berghei,* strain (ANKA) 676m1cl1 erythrocytes and drug treatment was initiated 4–8 days following infection once a high parasitemia (~5–10%) was reached. Mice were monitored for recrudescent parasitemia for up to 30 days and were euthanized when parasitemia levels exceeded that of the parasitemia on the day of drug treatment initiation or when a 20% decrease in body weight was detected.

2.5. Luciferase assay and analysis

Sensitive blood stage parasitemia was demonstrated initially after sporozoite infection in mice(Zuzarte-Luis et al., 2014). Generation of a standard curve translating the luciferase signal (photons per second) into number of parasites per well was later described (Walker and Sullivan, 2017). At each time point, 5 μL of tail blood was drawn from each mouse and deposited into a 96 well plate containing 45 μL of lysis buffer (Franke-Fayard et al., 2008). Luciferase activity was measured in the IVIS Spectrum *In Vivo* Imaging System and analyzed using Living Image v. 4.4 software. Data analysis was carried out using GraphPad Prism 5 software.

2.6. Liver stage assay

Anopheles stephensi, 6–10 days old were fed on infected mice to generate mosquitoes infected with *P. berghei* mCherry parasites 2204cl5 (Hopp et al., 2015), which allows fluorescent sorting of salivary gland infected mosquitoes in the day 20–22 day window after feeding (Flores-Garcia et al., 2019). Infected mosquitoes were fed on Balb/c mice. Separately 3,000 wild *P. berghei*-ANKA sporozoites inoculated by tail vein injection initiated the C57Bl/6 mice. C57Bl/6 have higher measurable malaria liver loads than Balb/c mice(Scheller et al., 1994; Li et al., 2017; Flores-Garcia et al., 2019).

2.7. Maintenance of P. falciparum culture

In vitro experimentation involved either *P. falciparum* CamWT (MRA-1250) or *P. falciparum* CamWT_C580Y (MRA-1251), which were obtained through BEI Resources, NIAID, NIH and were previously contributed by David A. Fidock. Parasite cultures were maintained under a modification of the Trager and Jensen method (Trager and

Jensen, 1976). *P. falciparum* cultures were synchronized to ring stage by incubation in 5% sorbitol. IC_{50} values were determined using a modified version of the [3H]-hypoxanthine incorporation assay (Desjardins et al., 1979). Chloroquine diphosphate salt (C6628, Sigma-Aldrich) was dissolved in nuclease-free water. Dihydroartemisinin (D7439, Sigma-Aldrich) was dissolved in acetone. Twenty-four hours before harvesting the parasite cultures, 0.5 μCi of [3H]-hypoxanthine was added to each well in 5 μL of hypoxanthine-free culture media. The 96-well plates were harvested onto glass fiber filters (GF/C, Brandel) by a cell harvester (MB48, Brandel). IC_{50} curves and IC_{90} values were generated using nonlinear regression analysis (log (inhibitor) vs. response) in GraphPad Prism software.

2.8. Data analysis

All the data analyses and representation were performed with Graph Pad Prism 5 Software. Data are represented as mean ± SD. The statistical methods employed include the following: Two-Way ANOVA and/ or *t*-test (non-parametric) were used for comparing two groups. For groups more than two, One-way Analysis of Variance (ANOVA) with Tukey Multiple comparison post-hoc test were used for comparison of groups as appropriate with alpha significance level $p < 0.05$.

3. Results

3.1. Bloodstage dose and 24h schedule killing

The dose of 100 mg/kg was chosen as a benchmark for the first experiments as a rough approximation of the effective human equivalent doses of fosmidomycin using interspecies allometric scaling and a Km factor of 12 (Reagan-Shaw et al., 2008). We first assessed the significance of single 100 mg/kg dose versus every 6 h doses of 25 mg/kg over 24 h for fosmidomycin, clindamycin and the combination in an *in vivo P. berghei* GFP-luciferase suppression low parasitemia model. Fosmidomycin in the single 100 mg/kg dose exerted zero reduction in parasite number compared to control while the every 6 h dose showed a small

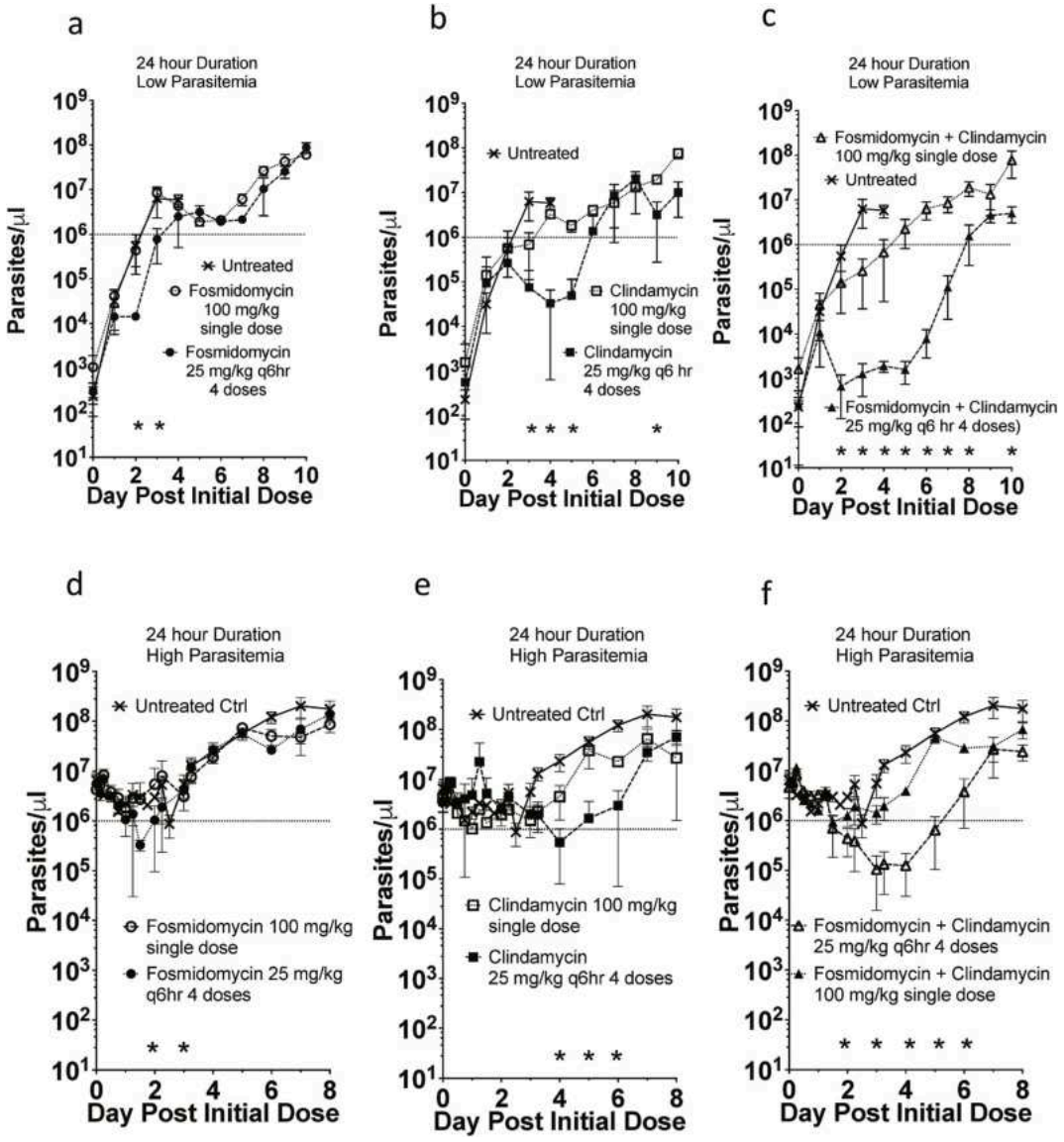

Fig. 1. Single day dosing frequency of fosmidomycin and clindamycin in the low parasitemia (a–c) and high parasitemia(d-f) model. Inhibitory effects of fosmidomycin (circles) (a,d), clindamycin (squares) (b,e), and both drugs in combination (triangles) (c,f) were assessed against untreated controls (X). Identical total 100 mg drug dose was administered as a single dose of 100 mg/kg (empty symbol-dotted line) or four doses of 25 mg/kg given in 6 h intervals (filled symbol-dashed line). Each drug treatment group contains 3 mice. Horizontal dotted line is one million parasites/μL in a-f. Data is represented as mean ± SD. Two-Way ANOVA and/ or *t*-test (non-parametric) were used for comparing two groups with alpha significance level $p < 0.05$ marked by (*).

drop on day 2 and 3. In contrast clindamycin had a small parasitemia drop with the single 100 mg dose at day 3 with a more pronounced reduction with the every 6 h dosing. The combination of fosmidomycin and clindamycin was measurably lower from day 2–8 at both dosing regimens (Fig. 1a,b,c).

Next, we sought to verify the importance of the same dosing frequency over 24 h in the higher parasite density cytocidal model which measures parasite reduction after parasitemia is more than one million parasites/μL. The single dose of 100 mg/kg of fosmidomycin and clindamycin had minimum parasite reduction or cytocidal activity, while

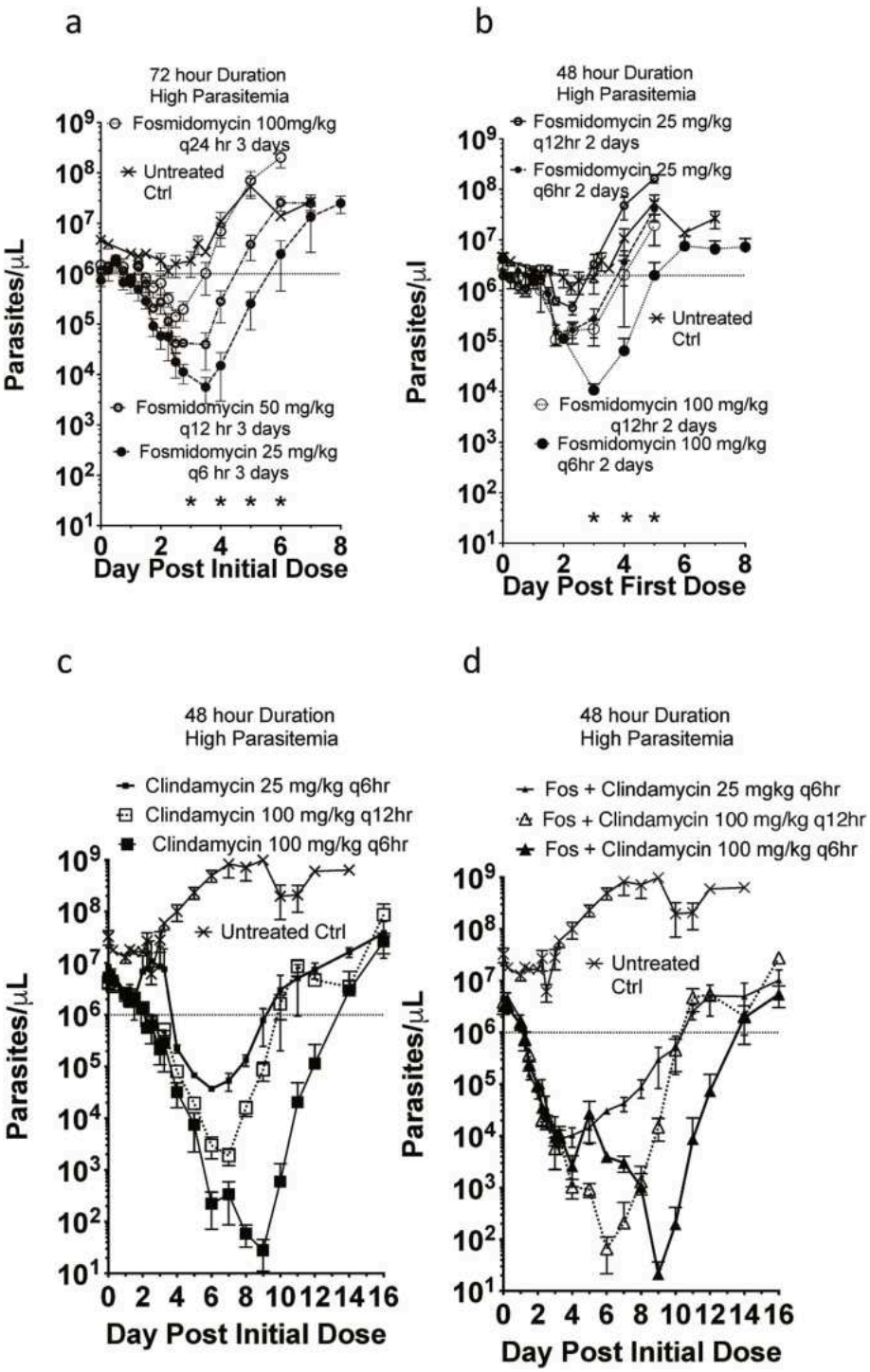

Fig. 2. Two to three day drug dosing and frequency. (a) Fosmidomycin at constant total dose of 300 mg over 72 h was administered 100 mg/kg every 24 h (empty circle-dashed line), 50 mg/kg every 12 h (empty thick circle-dashed line) and 25 mg/kg every 6 h (filled circle-dashed line) compared to untreated controls (X). (b) Fosmidomycin at 25 mg/kg (small circles) or 100 mg/kg (large circles) was given every 6 (filled circles) or 12 (empty circles) hours over 48 h. (c) Clindamycin at 25 mg/kg (small squares) or 100 mg/kg (large squares) was given every 6 (filled squares) or 12 (empty squares) hours over 48 h 25 mg/kg at 12 h was not dosed. (d) Both fosmidomycin and clindamycin at 25 mg/kg (small triangles or 100 mg/kg (large triangles) was given every 6 (filled triangles) or 12 (empty triangles) hours over 48 h 25 mg/kg at 12 h was not dosed. Horizontal dotted line is one million parasites/μL. Data is represented as mean ± SD. Two-Way ANOVA and/ or *t*-test (non-parametric) were used for comparing two groups with alpha significance level $p < 0.05$ marked by (*).

the every 6 h schedule provided more cytocidal activity as evidenced by the lower nadir parasitemia for the former treatment schedules and a delay in return to initial more than one million parasites/μL for the latter (Fig. 1d and e). For the combination, the delay in return to initial parasitemia before treatment at 5 days was similar to clindamycin alone for both the single 100 mg/kg dose and the four 25 mg/kg doses indicative of little additional killing from fosmidomycin (Fig. 1f).

3.2. Fosmidomycin 3 day constant total dose killing

We sought to measure killing of varying fosmidomycin doses and frequencies over a longer duration. Three drug schedules totaling 300 mg/kg were compared over three days: 25 mg/kg every 6 h, 50 mg/kg every 12 h, and 100 mg/kg every 24 h. Relative to the 100 mg/kg every 24 h treatment group, the every 12 h and every 6 h dosing resulted in a one or 2 $\log_{10}$ greater parasite reduction respectively in the nadir with a shift by successive days in return to initial parasitemia (Fig. 2a, Table 1).

3.3. Fosmidomycin, clindamycin and combination 2 day killing

To investigate the significance of the dose and interval fosmidomycin, clindamycin and combination, we administered 25 versus 100 mg/kg over 2 day at every 6 h or every 12 h intervals. Fosmidomycin 25 mg/kg at every every 6 h or the 100 mg/kg at both intervals increased the delay in return to million parasite/μL from 2 days to 4 days (Fig. 2b–Table 1). Clindamycin and the combination were also investigated except at the 25 mg/kg dose every 6 h. Clindamycin without or with fosmidomycin at same dose had a similar day to return to one million parasites/μL at the 25 mg/kg every 6 h or 100 mg/kg every 12 h at ~10, 10 and 13 days. Further investigation of the addition of fosmidomycin to clindamycin compared a single fosmidomycin 25 mg/kg dose versus 4 fosmidomycin doses in a 24 h period with clindamycin 25 mg/kg for a 24 or 48 h duration noted little change in the day to return of one million parasites/μL of 5 or 10 days (Fig. 3a). The parasite $\log_{10}$ reduction over 24 h was less than 1, and at 48 h less than 2 with notable little difference of Clindamycin and fosmidomycin every 6 h dosed either 25 or 100 mg/kg (Table 1).

A dose regimen of 25 mg/kg of both fosmidomycin and clindamycin in combination for 4.5 days or 450 mg/kg total dose of each drug was curative with no return to parasitemia at 28 days (Fig. 3b) while clindamycin cured 2 of three mice in the same period.

3.4. Effect of schedule and atorvastatin on liver stage killing

In the Balb/c mice liver stage infection model, the liver stage parasite burdens are about ten fold lower than in C57Bl/6 mice(Scheller et al., 1994; Li et al., 2017; Flores-Garcia et al., 2019). We first tested fosmidomycin alone Balb/c mice with a lower liver burden. Increasing the number of fosmidomycin doses every 6 h from 2 to 6 total doses from 12 to 36 h after infection in the 48 h liver stage window for *P. berghei* in Balb/c mice did not impact cure rate with a similar delay to patency of 2–3 days compared to untreated controls (Table 2). However, the increase to 8 doses (0, 6, 12, 18, 24, 30, 36 and 42 h post infection) with constant fosmidomycin exposure over the entire 48 h cured 4 of 6 Balb/c mice. To look at the combination interaction of atorvastatin which theoretically decreases host production of the host mevalonate precursor dosed with fosmidomycin, we used the C57Bl/6 mice which have higher liver stage parasite burdens. There was a slight decrease in delay of 2 days to patency with near a log drop in liver parasite number, but none of the mice were cured in this more stringent drug action model (higher burden of parasites to kill) with higher parasite loads in the liver of C57bl/6 mice (Fig. 4, Table 2).

3.5. In vitro P. falciparum inhibitory drug assay

Following the *in vivo* studies, we assessed the efficacy of fosmidomycin and clindamycin on two clinically relevant *P. falciparum* isolates: CamWT, an artemisinin sensitive strain, and CamWT_C580Y, a strain displaying delayed clearance to the artemisinins. In both the 72 h and 144 h dihydroartemisinin assay, CamWT and CamWT_C580Y resulted in IC_{50} values less than 10 nM (Table 3). For clindamycin, the 72 h assay did not generate an IC_{50} for either strain as the highest concentration tested (10 μM) barely inhibited parasite growth 25%. By contrast, the clindamycin 144 h assay resulted in a CamWT IC_{50} 19.5 nM and a CamWT_C580Y $IC_{50}$of 25.6 nM. For fosmidomycin, the 72 h and 144 h assays displayed similar results with CamWT IC_{50} values of 1.5 μM and 1.27 μM and CamWT_C580Y IC_{50} values of 2.7 μM and 1.3 μM. Overall, there were no statistically significant differences in the IC_{50} or the IC_{90} values between the two strains.

4. Discussion

Antibacterials are recognized to possess different pharmacokinetic and pharmacodynamic parameters that affect their clinical efficacy. Bactericidal activity can be divided into two groups: concentration-dependent killing, in which the higher the drug concentration, the greater amount of bacteria are killed, and time above minimal concentration-dependent killing, in which the longer the bacteria are exposed to the minimal drug concentrations, the more bacteria that are killed (Craig, 1998). This concept has also been applied to the antimalarial drug artemisinin as *in vitro* studies have demonstrated the efficacy of artemisinin is driven by exposure to peak concentrations (Bakshi et al., 2013). Additional work in the hollow fiber culture model with the antibacterial antimalarials clindamycin, tetracycline, chloramphenicol, and ciprofloxacin demonstrated time above minimal inhibition concentration killing(Caton et al., 2019). Taken together, our *in vivo* data suggest that the anti-plasmodial activity of fosmidomycin and clindamycin is driven principally by time above minimal

Table 1
Dose and schedule effects on blood stage parasite reduction.

Fos	Clin	Dose mg/kg	Schedule (qhr)	Dose #	Days of drug	Total mg/kg	Days to nadir	Parasite (x1000)/ μL nadir	Days to one million par/ μL	Parasite $\log_{10}$ reduction ratio at 24 h	Parasite $\log_{10}$ reduction ratio at 48 h	Parasite $\log_{10}$ reduction ratio at 72 h
x		100	24	3	3	300	2.5	141	4	0.3	0.3	0.9
x		50	12	6	3	300	3	39	5	0.2	0.6	1.4
x		25	6	12	3	300	3.5	6	6	0.0	1.1	1.8
x		25	6	8	2	200	3	167	4	0.5	1.6	1.8
x		100	6	8	2	800	3	10	5	0.4	1.6	2.5
	x	25	6	8	2	200	6	37	9	0.5	0.1	0.0
x	x	25	6	8	2	200	3	87	10	0.4	1.8	2.6
	x	100	12	4	2	400	7	2	10	0.2	0.5	1.0
x	x	100	12	4	2	400	6	0.07	10	0.4	1.6	2.8
	x	100	6	8	2	800	9	0.03	13	0.3	0.6	1.4
x	x	100	6	8	2	800	9	0.02	13	0.3	1.5	2.5

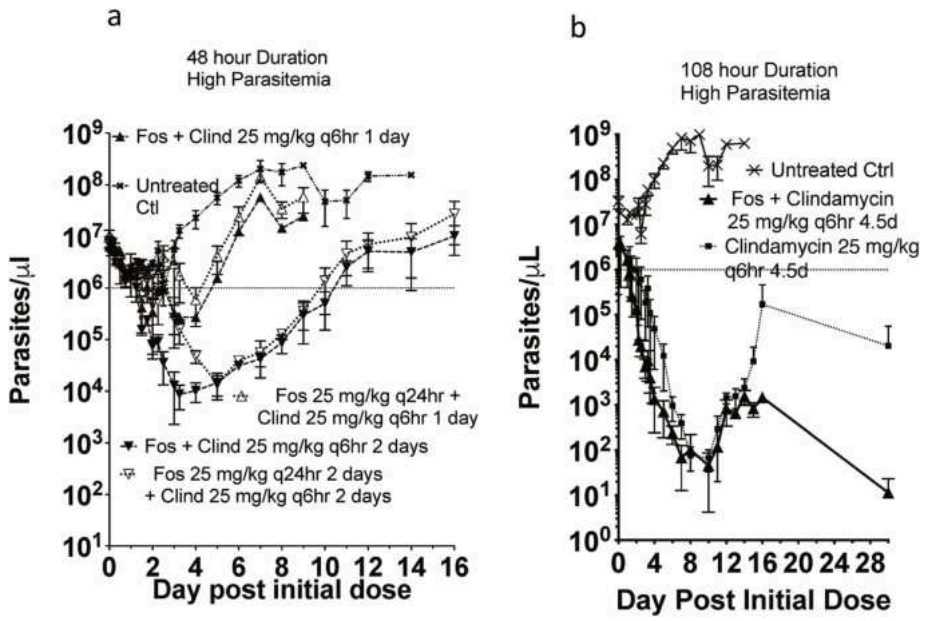

Fig. 3. Variable fosmidomycin schedules in addition to every 6 h clindamycin for 1, 2 or 4.5 days. (a) Fosmidomycin at 25 mg/kg over 48 h given as single 24 h dose (empty triangles) or every 6 h (solid triangles) with every 6 h clindamycin for one day (triangles) or two days (inverted triangles) and (b) 4.5 days of clindamycin alone (filled squares) or clindamycin and fosmidomycin combination (filled triangles) for 450 mg total dose. None of the combination mice had recurrent parasitemia while one of three clindamycin mice had recurrent parasitemia. Horizontal dotted line is one million parasites/μL. Data is represented as mean ± SD. There was no statistical difference in the one or two days of clindamycin comparing the two fosmidomycin schedules after day 5 in (a) or the two groups in (b).

Table 2
Dose and schedule effects on liver stage parasite reduction q is abbreviation for quaque meaning every.

Mouse	Infection route	Drug	Dose (mg/kg)	Schedule (hr) X Number of Doses	Mouse #	# cured	Delay in Patency (days)
Balb/c	7 mosquitoes	Fosmidomycin	25	q6 x 2	6	1	2
Balb/c	7 mosquitoes	Fosmidomycin	25	q6 x 4	3	1	3
Balb/c	7 mosquitoes	Fosmidomycin	25	q6 x 6	3	1	2
Balb/c	7 mosquitoes	Fosmidomycin	25	q6 x 8	6	4	3
Balb/c	7 mosquitoes	No drug control	0	0	3	0	0
C57Bl/6	3000 sporozoites	Fosmidomycin	25	q6 x 8	5	0	1
C57Bl/6	3000 sporozoites	Atorvastatin	40	q24 x 7	3	0	0
C57Bl/6	3000 sporozoites	Fosmidomycin Atorvastatin	25 40	q6 x 8 q24x7	6	0	2
C57Bl/6	3000 sporozoites	Fosmidomycin Atorvastatin	25 40	q6 x 8 q24x2	3	0	2
C57Bl/6	3000 sporozoites	No drug control	0	0	3	0	0

concentration-dependent killing in the high-density mouse malaria model. Liver stage murine malaria action required continuous fosmidomycin exposure for 48 h.

For reference most of the human dosing interval is every 6–8 h for many bacterial and protozoan diseases (Table 4). Specifically anaerobic bacterial infections, gram positive infections caused by *Staphylococcus* or *Streptococcus, P. falciparum* infections during pregnancy and *Babesia* therapy in combination with quinine and is a second line therapy for pneumocystis and toxoplasmosis, the latter of which is caused by the apicomplexan *Toxoplasma gondii* (Dhawan and Thadepalli, 1982; Kaplan et al., 2009; Wei et al., 2015). We recognize that the half-life of fosmidomycin and clindamycin in rodents is approximately 1 h(Murakawa et al., 1982; Yang and Lee, 2007) or about half of that in humans. The six half-lives represent less than 2% of drug remaining in mice at 6 h. In humans with a 3 h half-life 25% is left at 6 h and 6% at 12 h. The numbers reduce to 12.5% left at 6 h and 1.5% at 12 h with a 2 h half-life. Our goal in this work was not to perfectly replicate human pharmacokinetics in the mouse, but to demonstrate the greater parasite reduction with more frequent continuous dosing in the mouse model with both fosmidomycin and clindamycin. While others have looked at dosing frequency in the mouse model for the artemisinins(Bakshi et al., 2013; Patel et al., 2013), doxycycline(Batty et al., 2007), spiroindolones (Lakshminarayana et al., 2015) and piperaquine(Moore et al., 2008), here we characterized two drugs-fosmidomycin and clindamycin used in human clinical trials at possibly less than optimal dosing schedules.

The significance of dosing frequency for the antibiotics fosmidomycin and clindamycin was assessed in the low parasitemia and high parasitemia *P. berghei* luciferase models. In the low parasitemia model, the single drug data indicated that a single dose of 100 mg/kg was less effective than four doses of 25 mg/kg administered 6 h apart. In the high parasitemia parasite reduction model the drugs showed the same trend of smaller, more frequent doses being more efficacious; however, the statistical significance was not as strong as it was in the low parasitemia assay. We believe this is due to a larger dose of drug being required to kill parasites in a higher density infection. It has previously been noted that total parasite burden is a main determinant of therapeutic response (White, 2013). Taken together, these data also underscore the importance of evaluating both low parasitemia and high parasitemia cytocidal drug efficacy, as has been previously discussed for the quinoline compounds' interaction with heme crystals (Gorka et al., 2013). The parasite reduction for both clindamycin and fosmidomycin was modest at less than one or two logs at 24–48 h, in contrast to the artemisinins and quinolines which drop parasite numbers by 2–3 logs at 24 and 48 h in the murine model(Okoth et al., 2018). A previous study also showed

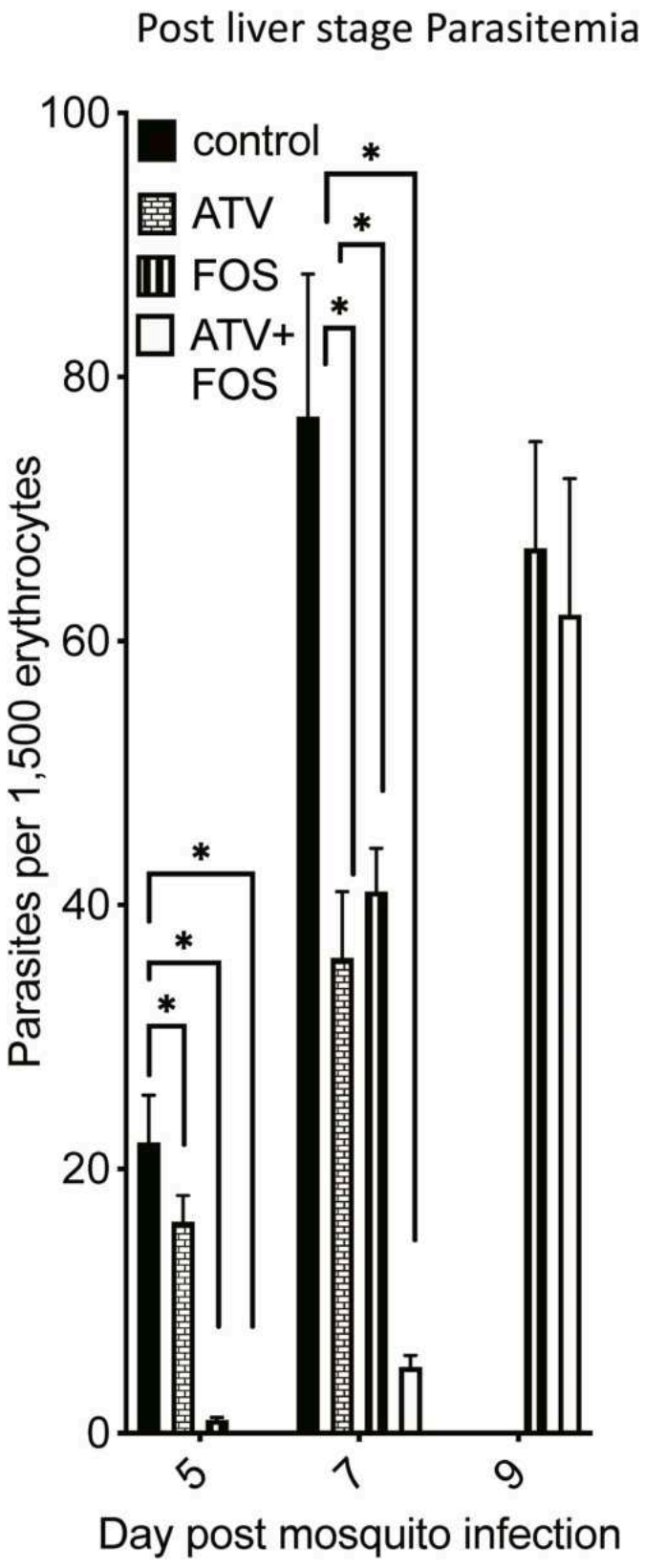

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Fig. 4. Atorvastatin and fosmidomycin at liver stages. 3,000 infectious *P. berghei* ANKA sporozoites were given by tail vein injection on day 5 after starting atorvastatin (horizontal small square bars, n = 3), atorvastatin day 5 and fosmidomycin 25 mg/kg IP q 6 h day 5 and 6 (empty bars, n = 6), fosmidomycin 25 mg/kg IP every 6 h day 5 and 6 (vertical stripe bars, n = 5) or nontreated controls (solid bars, n = 6). No parasites were seen on day 5 for the atorvastatin and fosmidomycin controls but were visible on day 7 at 5 per 1,500 erythrocytes with 41 for fosmidomycin, 36/1500 for atorvastatin and 77/1500 for controls on day 7. Control and atorvastatin mice were euthanized on day 7. Mean and SD by Giemsa blood film counting. * is P < 0.05 between groups marked.

similar large log drop with quinolines and artemisinins and more modest parasite reduction with doxycycline in the first two days after drug dosing(Batty et al., 2007). Doxycycline like clindamycin here had a greater drop by day 3–4 in parasites.

Clinical trials of fosmidomycin and clindamycin also support the notion that increased dosing frequency may result in slightly enhanced efficacy against cases of uncomplicated *P. falciparum*. While a clinical trial in Thailand comparing two dosing schedules of fosmidomycin 900 and clindamycin 300 mg every 6 h versus fosmidomycin 1,800 and clindamycin 600 mg every 12 h for three days found similar cure rates of 91.3% (n = 23) and 89.7% (n = 78), respectively(Ruangweerayut et al., 2008), the authors noted that the every 12 h schedule resulted in large fluctuations of the peak and trough plasma concentrations of fosmidomycin. The authors concluded that fosmidomycin (900 mg) and clindamycin (300–600 mg) in a every 6 h h dosing schedule for 5 days would be necessary for achieving greater than a 95% cure rate with these two apicoplast inhibitors (Ruangweerayut et al., 2008). However, most clinical trials have focused on twice daily dosing. In a clinical trial of asymptomatic schoolchildren in Gabon, 30 mg/kg of fosmidomycin and 4 mg/kg of clindamycin dosed twice daily for five days resulted in a 100% 28-day cure for the 11 patients (Borrmann et al., 2004a). Similarly, in Thailand, combination therapy of 900 mg and 600 mg clindamycin dosed twice daily for seven days was 100% curative at day 28 for 12 patients (Na-Bangchang et al., 2007). However, a shorter treatment duration in Mozambican children, 30 mg/kg of fosmidomycin and 10 mg/kg of clindamycin dosed twice daily for three days resulted in a poor 28-day cure in 17 of 37 patients (Lanaspa et al., 2012). Taken together, these three studies demonstrate the importance of treatment duration for fosmidomycin and clindamycin. However, there have also been some clinical trials demonstrating the efficacy of twice daily dosing in shorter duration treatments (Oyakhirome et al., 2007). The underwhelming clinical trial data almost eliminated the fosmidomycin and clindamycin combination from further clinical malaria trials consideration. However, clindamycin is still in use and should be dosed every 6 h for malaria. The recent clinical drug study in Kenya comparing quinine plus clindamycin versus artemether-lumefantrine for uncomplicated malaria in children (Obonyo et al., 2022) underdosed both quinine and clindamycin (Krishna and Kremsner, 2022).

With drug resistance a primary concern for malaria control efforts, it is worth mentioning that resistance to fosmidomycin has been generated *in vitro* in *E. coli*; however, these mutations have not been identified *in vivo* in *P. falciparum* (Armstrong et al., 2015). Analysis of the previously mentioned clinical trial in Mozambique determined that the 20 recrudescent infections were not due to the acquisition of fosmidomycin resistance by the parasites (Lanaspa et al., 2012; Guggisberg et al., 2016). This suggests that the failure of the twice daily dosing and three day duration of fosmidomycin and clindamycin combination therapy was due to inadequate dosing and not drug resistance.

Targeting both host cholesterol synthesis pathway and parasite isoprenoid synthesis in *Toxoplasma* and *Cryptosporidium* led to significant inhibition of these apicomplexan parasites(Bessoff et al., 2013; Li et al., 2013). Continuous fosmidomycin during the liver stage was better than a short interval of no drug in the lower liver stage parasite burden Balb/c model. The combination does result in a delay in parasitemia after liver

Table 3
P. falciparum IC_{50} Values.

Drug	Drug Exposure	Parasite	IC_{50}	n
Dihydroartemisinin	72 h	CamWT	8.6 nM (3.7–20.2)	2
	72 h	C580Y	6.3 nM (2.7–14.9)	7
	144 h	CamWT	5.3 nM (1.8–15.3)	2
	144 h	C580Y	4.9 nM (1.6–15.4)	4
Clindamycin	72 h	CamWT	Ambiguous	3
	72 h	C580Y	Ambiguous	7
	144 h	CamWT	19.5 nM (7.1–53.8)	2
	144 h	C580Y	25.6 nM (12.8–51.2)	4
Fosmidomycin	72 h	CamWT	1.5 μM (0.4–6.0)	3
	72 h	C580Y	2.7 μM (1.1–7.0)	6
	144 h	CamWT	1.27 μM (0.23–7.1)	2
	144 h	C580Y	1.3 μM (0.5–3.4)	4

stages, but is not curative in the higher liver stage parasitemia C57Bl/6 model.

After identifying superior dosing schedules of fosmidomycin and clindamycin *in vivo*, we wanted to assess their efficacy against *P. falciparum* delayed clearance isolates. In both the 72 h and 144 h assays, the fosmidomycin IC_{50} values for CamWT and CamWT_C580Y were in agreement with a recently published IC_{50} for drug sensitive *P. falciparum* D10 of 600 nM-1.2 μM(Uddin et al., 2018). For clindamycin, as expected, the 72 h assay did not reveal an IC_{50} for clindamycin at the tested concentrations, which did not exceed 10 μM. However, IC_{50} values for CamWT and CamWT _C580Y in the 144 h assay were determined to be 19.5 nM and 25.6 nM, which were also in agreement with recently published values of 30–50 nM for drug sensitive *P. falciparum* D10 (Uddin et al., 2018). Thus, we believe there is value in the *in vitro* demonstration that *P. falciparum* CamWT_C580Y exhibits similar susceptibility to fosmidomycin and clindamycin as *P. falciparum* isolates lacking delayed clearance to the artemisinin compounds.

While our murine data support more frequent dosing with greater parasite reduction, a limitation we have not answered is whether four clindamycin doses in either the human or murine 48 or 24 h lifecycle are equivalent. Another limitation is the duration of the scheduled doses base upon the mouse 24 h erythrocyte life cycle versus 48 h *P. falciparum* is also hard to extrapolate as the availability of a narrow or broad enzyme or metabolic process within the life cycle may not directly correlate to the entire life cycle. The liver stage is 48 h in the murine model and about 6 days in *P. falciparum*. Another limitation is mouse hepatocytes have many more cytochrome P450 enzymes which complicates direct extrapolation to humans. The recent mouse line extensively humanized for the cytochrome P450 gene superfamily ("8HUM") may bridge this gap(MacLeod et al., 2024).

We recognize the push to constrain antimalarial drug schedules to once or twice daily dosing for three days in order to increase the ease of treatment delivery and patient compliance; however, these data indicate that fosmidomycin and clindamycin have the potential to be safe and efficacious antimalarials if dosed more frequently than twice daily and for longer than three days. Of course, patient adherence is a primary concern for antimalarial schedules and a better understanding of the prevalence and impact of patient adherence is necessary, especially with longer treatment durations. It was recently shown that it is possible to model the relationship between treatment adherence and treatment failure with the common ACT, artemether-lumefantrine (Challenger et al., 2017). Additionally, our data suggesting that a more complex treatment schedule may be necessary in order to achieve sustained antimalarial efficacy is not alone. In an effort to preserve the future efficacy of ACTs throughout Africa, sequential use of two different ACTs is currently under investigation for superiority over a single three-day regimen (Schallig et al., 2017). Fosmidomycin did not add much extra parasite reduction activity to clindamycin indicating clindamycin without fosmidomycin should remain in alternative partner drug therapy in special circumstances like pregnancy(WHO, 2024). Broadly, our data reinforce the notion that dose, frequency of dosing, and treatment duration are essential components of establishing successful chemotherapy regimens.

Table 4
Human Disease Dosing for Clindamycin from Micromedex. q is abbreviation for quaque meaning every.

Disease	Dose	Schedule	Total daily	Role
Babesiosis, mild	600	q8hr	1800	combination
Babesiosis, severe	600	q6 hr	2400	combination
Bacterial vaginosis	300	every 12 hrr	600	single
Bite wound infection	300, 600	q 6, 8hr	900, 1800	single
Diabetic foot infection	300, 450	q 6, 8hr	900, 1350	single
Hidradenitis suppurativa	300	every 12 hrr	600	combination
Malaria	450	q8hr	1350	combination
Mastitis	300, 450	q6, 8hr	1200, 1350	single
Neutropenic fever	600	q8hr	2400	combination
Osteomyelitis IV	600, 900	q6, 8hr	1800, 2700	single
Osteomyelitis oral	600	q8hr	1800	single
Pelvic inflammatory disease, IV	900	q8hr	2700	combination
Pelvic inflammatory disease, oral	450	q6 hr	1800	combination
Pneumocystis jirovecii pneumonia, IV	600, 900	q6,8	2600, 2700	combination
Pneumocystis jirovecii pneumonia, oral	450, 600	q6,8	1800	combination
Aspiration pneumonia	300, 450	q8hr	900, 1350	single
methicillin, resistant *S. aureus* pneumonia	600	q8hr	1800	single
Postpartum endometritis	900	q8hr	2700	combination
Prosthetic joint infection, IV	600, 900	q8hr	1800, 2700	single
Prosthetic joint infection, oral	300, 450	q6 hr	1200, 1800	single
Rhinosinusitis	300	q6,8hr	900, 1200	combination
Septic arthritis due to *Staphylococcus aureus*	600	q8hr	1800	single
Skin and soft tissue infection, IV	600, 900	q6, 8hr	1800, 2700	single
Skin and soft tissue infection, oral	300, 450	q6,8hr	1800	single
Streptococcus, group A, bloodstream infection	900	q8hr	2700	combination
Streptococcus, group A, pharyngitis	300	q8hr	900	single
Streptococcus, group A, chronic carriage	300	q8hr	900	single
Streptococcus, group B, maternal prophylaxis	900	q8hr prn	900	single
Surgical prophylaxis	900	q6 hr prn	900	single
Toxic shock syndrome, toxin production suppression	900	q8hr	2700	combination
Toxoplasma gondii encephalitis and pneumonitis	600	q6 hr	2400	combination

CRediT authorship contribution statement

Leah A. Walker: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Conceptualization. **Vision Bagonza:** Writing – review & editing, Validation, Investigation, Formal analysis. **Bryce Bobb:** Writing – review & editing, Validation,

Investigation, Formal analysis. **David J. Sullivan:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Transparency declarations

DJS is co-Inventor on Johns Hopkins University patent for itraconazole for angiogenesis inhibition, co-Inventor on provisional patent for cethromycin for malaria liver stage and cofounder, scientific officer with stock interest in Aliquantum Rx which licensed cethromycin for liver stage malaria.

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