Menoctone resistance in malaria parasites is conferred by M133I mutations in cytochrome b that are transmissible through mosquitos

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ABSTRACT. Malaria related mortality has slowly decreased over the past decade; however, eradication of malaria requires the development of new antimalarial chemotherapies that target liver stages and combat the emergence of drug resistance. The diminishing arsenal of anti-liver stage compounds sparked our interest in reviving the old and previously abandoned compound menoctone. In support of these studies we developed a new convergent synthesis method that was facile, required fewer steps, produced better yields, and utilized less expensive reagents than the previously published method. Menoctone proved to be highly potent against liver stages of *Plasmodium berghei* (IC_{50} = 0.41 nM) and erythrocytic stages of *P. falciparum* (113 nM). We selected for resistance to menoctone and found M133I mutations in cytochrome b of both *P. falciparum* and *P. berghei*. This same mutation has ben observed previously in atovaquone resistance and we confirmed cross-resistance between menoctone and atovaquone in vitro (*P. falciparum)* and in vivo (*P. berghei*). Finally we assessed the transmission potential of menoctone-resistant P. berghei and found the M133I mutant parasites were readily transmitted from mouse to mosquitos and back to mice. In each step M133I mutation in cytochrome b of menoctone resistance was confirmed. In summary this study is the first to show the mechanism of resistance to menoctone and that menoctone and atovaquone resistance is transmissible through mosquitos.

INTRODUCTION. Despite the decline in deaths caused by malaria in the last 10 years, the disease threats from infections with *Plasmodium* spp. still pose a significant global public health problem. In 2015 alone, the World Health Organization estimates that malaria caused 214 million clinical cases and 438,000 deaths (1). The increased

prevalence of drug resistant parasites and insecticide-resistant vectors continues to drive the need for new treatments for this disease. New drugs are urgently needed and in some cases old compounds with proven antimalarial activity can serve as the basis for lead optimization of new potent antimalarial drugs (2). Menoctone, also known as 2-hydroxy-3-(8-cyclohexyloctyl)-1,-4-naphthoquinone, was first synthesized by Fieser et al in 1948 (3). Initial studies with menoctone and analogs thereof found the drug reduced blod stage parasitemia, but failed to prevent recrudescence at safe, non-toxic levels of drug administration (3). In 1965, Howland used rat liver mitochondrial preparations to model how menoctone and various naphthoquinones inhibited the respiratory chain between cytochromes b and c_1 (4). Intriguingly, Skelton et al showed that menoctone, when tested *in vitro* at concentrations of 10 - 25 nM inhibited the nicotinamide adenine dinucleotide (NADH) peroxidase of *P. berghei* by 90% (5). Specific morphological changes such as swelling and thickening of the mitochondria as well as reduplication of mitochondrial membranes were observed in intraerythrocytic trophozoites of *P. berghei* infected mice exposed to menoctone (6, 7). Peters et al later reported the ED_{50} and ED_{90} values against the N-strain of *P. berghei*, as 0.7 and 1.5 mg/kg, respectively, and showed an effective causal prophylactic dose of 1-3 mg/kg against *P. yoelii nigeriensis* (4). Later studies with menoctone treatment of the pre-erythrocytic stages in rodent malaria models demonstrated efficacy 2.5 times greater than primaquine (8). All of the previous studies lead to menoctone progressing to clinical trials, but disappointingly it proved less effective against *P. falciparum* as compared to other antimalarials under development at the time. Thereafter, research on menoctone was discontinued due to the low curative activity and the potential for development of resistance reported in *P. berghei*. When

administered daily at doses ranging from 6.25 to 25 mg/kg for five consecutive days, the cure rate for menoctone was low, where only four of 72 mice remained parasite-free for 28 days (8). Since resistant parasites were readily selected for, Peters et al created a highly resistant line of *P. berghei* against menoctone through serial technique (4). The menoctone resistant line was not responsive to challenge by primaquine and cycloguanil; however, remained sensitive to mefloquine, chloroquine, pyrimethamine and sulfadoxine. When tested for transmission blocking activity, menoctone showed no sporontocidal activity as demonstrated by successful oocyst and sporozoite development (9).

Although menoctone is not currently used for malaria prevention or treatment, it is an effective treatment at low intravenous and intramuscular doses against *Theileria parva*, a parasitic protozoan that causes East Coast Fever in cattle (10). Intramuscular doses were preferred over intravenous administration and oral administration proved to be ineffective (11). The efficacy of menoctone against *T. parva* is quite remarkable, but the complexity of compound synthesis and high manufacturing costs rendered menoctone unmarketable as theileiosis treatment (12).

Previous studies with menoctone did not reveal a mechanism of action, although the chemical structure of menoctone reveals a similar core scaffold to atovaquone (Figure 1). Atovaquone-proguanil (AP) is a fixed-dose combination antimalarial that is currently used for prophylaxis as well as treatment of *Plasmodium falciparum*. Atovaquone affects the malaria parasite by binding to the quinol oxidation (Q_o) site of cytochrome b (*cyt b*) in the mitochondrial respiratory chain. Atovaquone-resistant isolates have been described following atovaquone or AP treatment (13-17). *In vitro* studies of atovaquone with *P. falciparum* have selected for point mutations at multiple sites in *P. falciparum*

cytochrome b (*pfcytb*); however, the predominant clinical resistance mutation seen in patient isolates is Y268S. In contrast, *in vivo* studies with *P. berghei* have selected for mutations in *pbcytb,* specifically M133I, a mutation commonly seen with *P. falciparum in vitro* resistance selections (18, 19).

Since menoctone is structurally similar to atovaquone and displays activity against liver stages of *P. berghei* (8), we hypothesize that menoctone shares a similar target as atovaquone and may generate similar mutations in *pfcytb* that confer cross-resistance to atovaquone. In this study, we generated menoctone resistant parasites in both *P. berghei* and *P. falciparum* and sequenced *cytb* to better understand the mechanism(s) of resistance to menoctone and potential for this compound to serve as a lead for antimalarial drug development.

MATERIALS AND METHODS

Parasites and animals. We used *P. falciparum* (W2) that was cloned from an Indochina isolate by Oduola et al in 1988 (20). Human O^+ red blood cells and plasma for culturing *P. falciparum* were obtained from Interstate Blood Bank (Memphis, TN). All *in vitro* cultured parasites were maintained according to previously published methods (21).

Menoctone resistant *P. berghei* MEN (MRA-414) was obtained from Malaria Research and Reference Reagent Resource Center (MR4); the line was originally generated by Professor Wallace Peters (22, 23). *P. berghei* 1052 Cl1 (*Pb* 1052 Cl1) was obtained from C.J. Janse at Leiden University and was used for menoctone resistance selections. This line was generated from the reference ANKA clone c1115cyl and expresses both green fluorescent protein (GFP) and firefly luciferase (24). *Pb* 1052 Cl1

has two copies of the GFP-luc incorporated into its genome. One copy is regulated by a constitutively expressed *eef1aα* promoter, and the other is an *ama1* promoter (24, 25).

All mice used in these experiments were female Balb/C mice (average weight was approximately 20g) obtained from Harlan/EnVigo (Indianapolis, IN) and from Charles River (Wilmington, MA). Rodent malaria parasites were monitored via modified Giemsa stained blood from tail vein smears, and parasitemia was determined via light microscopy. Parasites were passaged by removing blood via cardiac venipuncture and inoculating the intraperitoneal cavity of a naïve mouse with parasite infected blood. Mice were humanely euthanized when parasitemia reached 40% or the animal showed severe symptoms of malaria. This study was conducted in compliance with the Guide for the Care and Use of Laboratory Animals of the National Research Council for the National Academies and the University of South Florida Institutional Animal Care and Use Committee approved the animal use protocols.

Drugs and chemicals. Atovaquone was obtained from Sigma Aldrich (St. Louis, MO). Menoctone was not commercially available at reasonable costs and the published synthesis methods required improvement, therefore a new method was devised and is described herein.

Susceptibility assessment of *P. berghei* liver stages *in vitro*.

Mosquito infections and sporozoite isolation. Mice were infected using previously described methods above. When parasites reached >4% parasitemia, mice were anesthetized using ketamine (100 mg/kg) and xylazene (10 mg/kg) and placed on cartons containing 100 naïve female, 3-4 day old *Anopheles stephensi.* Mosquitos fed for 25 minutes and were maintained on 10% sucrose *ad libitum* inside environmental chambers

at 22°C. On day 10 post-exposure (PE), 50 mosquitoes were checked for infection through the visualization of oocysts on the midgut. On day 21 PE, infected salivary glands were dissected and sporozoites were isolated and counted.

Liver stage drug susceptibility assay. HepG2 cells (17,500 cells/well) were seeded in black 384-well collagen coated plates with optically clear bottoms (Becton Dickson, Franklin Lakes, NJ). Cells were seeded using the Tecan Freedom Evo robotic system (Tecan Group Ltd, Männedorf, Switzerland) at 50 µl per well and were maintained at 37°C in 5% mixed gas humidified incubators. Complete media consisted of Eagle's Minimum Essential Medium, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine. Mosquito dissections were harvested as described above, and 4,000 sporozoites were added to each well. Plates were incubated at 37°C for three hours and then media was removed and serially diluted drugs that were previously prepared were added to the respective wells (26). After 44-hour incubation period we lysed the cells with 5 µl of Promega Cell Lysis Reagent Buffer and added 20 µl Promega Luciferase Assay Reagent (Promega, Madison, WI). Luciferase activity in parasite lysates was quantified by using the Perkin Elmer EnVision (Perkin Elmer, Waltham, MA). An IC_{50} dose response curve was generated from this data in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) to determine the efficacy of drugs against liver stage parasites.

***P. falciparum* asexual blood stage susceptibility.** Parasitemia was cultured and allowed to grow to approximately 5% parasitemia where it was then synchronized at ring stages by using sorbitol as previously described (27). Drug dilutions were prepared in 96-well plates and were tested in duplicate using a 1:3 dilution series with a starting concentration

of 10 µg/mL for menoctone and 62.5 ng/mL for atovaquone. Drugs were allowed to act on parasite development for 48 hours. [^{3}H]hypoxanthine was diluted 1:20 and added at 10 µl per well to the whole plate. Plates were placed into a specially contained incubator reserved only for hypoxanthine treated plates for another 24 hours. Plates were then removed and placed in an -80°C freezer for an additional 24 hours. Cells were harvested using the Perkin Elmer MicroBeta FilterMate-96 Harvester (Perkin Elmer, Waltham, MA). We used the TopCount Luminometer (Perkin Elmer, Waltham, MA) to read the plates and used the data to determine the fifty percent inhibition concentration (IC_{50}) (28).

Selection of menoctone resistance in *P. falciparum in vitro* and mutation detection.

W2 menoctone resistance selection. Parasite line W2 was grown to 10% parasitemia, then 10^8 parasites were seeded in fresh parasite culture media containing ~10x IC_{50} concentration of menoctone (1.5 µM). Media was changed with fresh menoctone and blood smears were made twice per week, with fresh erythrocytes added as needed. Cultures were split 1:3 every 10 days, and cultured until parasite recovery was observed (2% parasitemia) in presence of menoctone.

DNA extraction. Parasite genomic DNA extraction was performed with an initial lysis step in 1x PBS+0.01% saponin, followed by extraction using the Qiagen MiniBlood Prep Kit according to manufacturer's instructions. Genomic DNA (gDNA) was assessed for concentration and purity on a NanoDrop spectrophotometer.

PCR Amplification and sequencing of Pf-cytochrome b. PCR primers were designed for amplification based on the annotated *P. falciparum* 3D7 cytochrome *b* sequence (MAL_MITO_3) on Plasmodb.org v27 as follows: Pf-cytb-PCRFOR: 5'—TGCCTAGACGTATTCCTG—3' and Pf-cytb-PCRREV 5'—

GAAGCATCCATCTACAGC—3'. PCRs were amplified using Phusion HS II High-Fidelity PCR Master Mix (ThermoFisher Scientific) with ~20 ng parasite gDNA template, according to manufacturer's instructions with the following program: 98°C—30s initial denaturation step, then 35 cycles: (98°C—10s, 54°C—40s, 72°C—30s) and a final extension of 72°C for 7 min. PCR products were confirmed as a single, discrete band of 1,382 bp length on a 1% agarose gel then subsequently purified using the Qiagen PCR Purification Kit according to manufacturer's instructions. Purified PCR products were prepared for Sanger sequencing service at Genewiz (Genewiz, South Plainfield, NJ) using the following sequencing primers: pf-cytb-SEQFOR1: 5'—GTGGAGGATATACTGTGAGTG—3', pf-cytb-SEQFOR2: 5'—TACAGCTCCCAAGCAAAC—3', pf-cytb-SEQREV1: 5'—GACATAACCAACGAAAGCAG—3', and pf-cytb-SEQREV2: 5'—GTTCCGCTCAATACTCAG—3'. PCR primers Pf-cytb-PCRFOR and Pf-cytb-PCRREV were also used for sequencing purposes.

Analysis of Pf-cytochrome b sequences for mutation detection. Sequence files were aligned to *P. falciparum* 3D7 cytochrome *b* sequence (MAL_MITO_3) using open source software: A Plasmid Editor (ApE v2.0.49). Trace files were checked at all mutant positions to validate any potential mutations found.

Generation of menoctone resistance in *P. berghei* and mutation detection. We used similar methods W. Peters used to generate a new menoctone resistant parasite from the well-characterized *P. berghei luc* ANKA (4). Infected mice were treated with increasing levels of menoctone at doses of 3 mg/kg, 30 mg/kg, and 300 mg/kg. Recrudescent

parasites were inoculated into malaria naïve mice, and subsequent treatment with higher doses of drug led to resistance at a dose of 300 mg/kg. Resistance was confirmed by the lack of response to subsequent inoculation of high doses of menoctone. A maintenance dose of 300 mg/kg was used to maintain a high level of parasite resistance.

DNA extraction. Parasite genotyping was performed by sequencing cardiac venipuncture blood containing parasites at high parasitemias (>40%) that were majority schizonts. Murine leukocytes were removed by treating the blood with Ficoll-Paque PLUS (GE Healthcare Life Sciences, Waltham, MA). Parasite genomic DNA extraction was then performed as described earlier.

PCR Amplification and sequencing of Pb-cytochrome b. PCR primers were designed for amplification based on the annotated *P. berghei* cytochrome *b* sequence (PBANKA_MITO1900) on Plasmodb.org v27 as follows: Pb-cytb-PCRFOR: 5'—TGCCTAGACGTATTCCTG—3' and Pb-cytb-PCRREV 5'—GCTGAGCATGTTAACTCG—3'. PCRs were amplified similarly with the following program: 98°C—30s initial denaturation step, 35 cycles: (98°C—5s, 54°C—10s, 72°C—39s) and a final extension of 72°C for 7 min. PCR products were confirmed as a single, discrete band of 1,307 bp length on a 1% agarose gel then subsequently purified and sequenced as described earlier, using the following primers: pb-cytb-SEQFOR1: 5'—GTGGAGGATACACTGTTAGTG—3', pb-cytb-SEQREV1: 5'—CATAACCTATAAAAGC—3', pb-cytb-SEQREV2: 5'—GTTTGCTTGGGAGCTGTA—3'. PCR primers Pb-cytb-PCRFOR and Pb-cytb-PCRREV were also used for sequencing purposes.

Analysis of Pb-cytochrome b sequences for mutation detection. Sequence files were aligned to *P. berghei* cytochrome *b* sequence (PBANKA_MITO1900) as described previously.

***In vivo* antimalarial efficacy against blood stages of *P. berghei* life cycle (modified Thompson test).** In breif, five mice were randomly assigned to each dosage group. *P. berghei luc* ANKA, as well as, *Pb* MEN-R were inoculated at $2x10^6$ parasites per mouse. Menoctone was administered orally at 3 mg/kg, 30 mg/kg and 300 mg/kg and atovaquone was administered at 1 mg/kg, 3 mg/kg and 10 mg/kg. Both drugs were suspended in polyethylene glycol 400 (PEG 400) (Sigma Aldrich, St. Louis, MO) and administered via oral gavage at 100 µl per mouse. Dosing by oral gavage occurred once daily for 3 days on days 6 to 8. Blood films were made on days 3, 5, 6, 9, 12, 15, 18, 21, 24, 27, and 30 post-infection from tail vein blood to monitor parasite recrudescence.

Elucidation of mutation selection by atovaquone or menoctone. Sensitive and resistant parasites were inoculated into mice and challenged with either atovaquone or menoctone at different concentrations. Atovaquone was administered to mice at dosages of 1 mg/kg, 3 mg/kg, and 10 mg/kg, and menoctone was given at 3 mg/kg, 30 mg/kg, and 300 mg/kg respectively. Parasitized blood was then removed and sequenced for *cytb* mutations.

***P. berghei* MEN (MRA-414) assessment of atovaquone cross-resistance.** Mice were infected with *P. berghei* MEN and allowed to grow to >4% parasitemia at which point they were challenged with atovaquone at 3 mg/kg, 30 mg/kg and 100 mg/kg. Subsequent administration of atovaquone at 100 mg/kg was used as confirmation of atovaquone resistance.

Transmission of menoctone resistant *P. berghei* to mosquitoes. Resistant parasites were passaged and maintained using methods described above. Once the parasitemia reached 3-5%, the mice were anesthetized with a solution of xylazene (10 mg/kg), ketamine (100 mg/kg), then placed on cartons of 200, 3-4 day old, naïve female *Anopheles stephensi* mosquitoes. Mosquitoes were allowed to feed for approximately 25 minutes and maintained on 10% sucrose inside environmental chambers at 22°C. On day 10 PE, 50 mosquitos were checked for infection through the visualization of oocysts on the midgut wall. Oocysts were scored based on two different characteristics: the percentage of midguts infected with oocysts and the quality of infection. The percentage of midguts infected was calculated by dividing the number of infected midguts by the total number of midguts dissected, then multiplying the result by 100. Each midgut was also graded by the following scale to determine the quality of infection: 0-50 oocysts = +; 51-100 oocysts = ++; 101-150 oocysts = +++, 151-200 oocysts = ++++; and greater than 200 oocysts in a midgut = +++++. On day 21 PE, the salivary glands were dissected from mosquitoes to determine the presence sporozoites.

***P. berghei luc* menoctone resistant sporozoite liver and blood stage infection.** On day 21 PE, we harvested sporozoites and infected five female mice with 10,000 sporozoites per mouse via tail vein injection. Once parasites were visualized in peripheral blood smears, two of the five mice were treated with 300 mg/kg of menoctone to confirm parasite resistance, while the remaining three were kept as controls. Cardiac venipuncture was used to collect blood from all five mice and leukocytes were removed prior to parasite genotyping.

RESULTS

Synthesis of menoctone. Although first identified as an antimalarial by Fieser and co-workers in collaboration with Sterling-Winthrop Research Institute in 1948 (29), it wasn't until 1969 that Lorenz patented a synthetic route for menoctone starting from 5-phenylvaleric acid (30). Dolan et al reported that this synthesis had many shortcomings requiring costly raw materials, harsh reaction conditions, and some low yielding synthetic steps (10). In an effort to design a facile synthetic route for menoctone, we developed a novel convergent approach involving modern and classical transformations (Figure 1). Isochroman-1,4-dione **1** was synthesized from 2-acetylbenzoic acid, which was first brominated and then cyclized. The copper mediated alkane synthesis of 1-bromo-7-chloroheptane and allylmagnesium bromide afforded chloroalkene **2** (31). Substitution of the chloride with iodide via a Finkelstein reaction afforded iodoalkene **3** that was then treated with cyclohexylmagnesium bromide in another copper mediated alkane synthesis to afford the allylcyclohexane **4**. Lemieux-Johnson oxidation of allyl **4** yielded aldehyde **5**. Condensation of aldehyde **5** with isochroman-1,4-dione **1** afforded 3-substituted isochroman-1,4-dione **6**, which was rearranged to menoctone using sodium methoxide in methanol (Figure 1). This new synthetic route was facile, required fewer steps, produced better yields, and utilized less expensive reagents than the previously published method.

Efficacy of menoctone against exoerythrocytic and blood stage parasites. Previous studies demonstrated menoctone is efficacious against asexual blood stage as well as liver stage parasites, yet these studies were done *in vivo* before the advent of quantitative *in vitro* susceptibility assays. Therefore we first profiled the activity of menoctone against asexual blood stages of *P. falciparum in vitro*. The menoctone IC_{50} was 113 nM against

W2, a clone that is resistant to chloroquine, pyrimethamine, and sulfadoxine. Next we assessed the activity of menoctone against *P. berghei* liver stages infecting HepG2 hepatoma cells *in vitro*. In these assays menoctone proved to be extremely efficacious (IC_{50} = 0.41 nM) and was approximately three fold more potent than atovaquone (Figure 2).

Menoctone resistance selection in *P. falciparum*. Based upon structural similarity between menoctone and known mitochondrial electron chain inhibitors, we hypothesized that cytochrome *bc1* is the site of action of menoctone. A common way to identify mechanisms of action is the selection of resistance to an inhibitor followed by sequence of the putative target or whole genome. We used standard methods to select for resistance to menoctone in *P. falciparum* (W2). We grew 10^8 asexual erythrocytic stage parasites and exposed them to constant menoctone drug pressure (1.5 μM). Initial exposure of W2 to menoctone resulted in pyknotic forms and parasites outside of erythrocytes after 48 hours, followed by a gradual reduction of parasites observed in thin smears. However, on day 23 of menoctone exposure, parasites recovered to healthy morphology and reached 2% parasitemia, which were expanded further under continued menoctone drug pressure. We sequenced the parasites to identify mutations in cytochrome *b* and found the menoctone resistant progeny had a non-synonymous SNP (G to C at nucleotide 399) that resulted in a M133I mutation (Table 1).

Menoctone resistance selection in *P. berghei*. In previous studies Wallace Peters and colleagues found that resistance to menoctone was selected rapidly in *P. berghei in vivo* (4); however, these parasites have not been fully characterized or genotyped. Therefore, we aimed to select for resistance in *P. berghei luc* ANKA by using a stepwise selection

317 method. First we infected mice with asexual erythrocytic stages and treated with a single
318 dose (3 mg/kg) of menoctone and no decrease in parasitemia was observed post treatment
319 (Figure 3A). Blood from these mice was then inoculated into two malaria naïve mice and
320 once patent infections ensued, we treated with a single dose of menoctone (30 mg/kg). In
321 these mice parasitemia decreased to <1% three days post treatment, but recrudesced to
322 approximately 30% six days later. Once again blood from the recrudescent infections
323 (day 6) were inoculated into malaria naïve mice and the treatment dose was escalated to
324 300 mg/kg. As noted in Figure 3C, parasitemia reduced rapidly after treatment, yet
325 recrudesced a few days later. Parasitemia in these mice was low so we immediately sub-
326 inoculated the infected blood into more malaria naïve mice and repeated the single high
327 dose of menoctone (300 mg/kg). In these mice parasitemia continued to increase at a
328 rapid rate despite treatment, thus indicating selection of highly resistant parasites to
329 menoctone. The menoctone resistant *P. berghei luc* ANKA parasite is hereafter referred
330 to as *Pb* MEN-R (Figure 3).

331 **Confirmation of menoctone resistance in *Pb* MEN-R infected mice.** Next we aimed
332 to assess the efficacy of menoctone and atovaquone in the standard Thompson efficacy
333 assay. In these studies, mice were inoculated with parasites and after parasitemia reached
334 ~1% mice were treated once a day for three days with drug. Efficacy was assessed by
335 reduction of parasitemia on day 9 (one-day post drug treatment) as well as cure rate
336 (survival to day 30 of the study). The doses used were 1, 3, and 10 mg/kg for atovaquone
337 and 3, 30, and 300 mg/kg for menoctone. In these studies, we confirmed that atovaquone
338 was more efficacious than menoctone against asexual blood stages of *P. berghei luc*
339 ANKA as well as *Pb* MEN-R. Interestingly there was evidence of atovaquone cross-

340 resistance in Pb MEN-R as evidenced by higher survival rates in *Pb* MEN-R versus *Pb*
341 ANKA following treatment with atovaquone (Figure 4). These studies confirmed that *Pb*
342 MEN-R is highly resistant to menoctone when compared to the response of the parental
343 line *P. berghei luc* ANKA (Figure 4).

344 **Menoctone resistance genotype in *P. berghei*.** Next we compared the cytochrome *b*
345 genotype of the parent *P. berghei luc* ANKA line and the menoctone resistant *Pb* MEN-R
346 progeny. Sanger sequencing revealed the same M133I mutation in *Pb* MEN-R that we
347 observed in menoctone resistant *P. falciparum* (W2 MEN-R) (Table S1). The SNP
348 identified (G to T, nt 399) is located outside of the quinol oxidation site (Table S1) and is
349 the same SNP as observed in previous studies in which atovaquone was used to select for
350 resistance (18). Although we did not have access to the parental line, we obtained the *P.*
351 *berghei* MEN (MRA-414) line selected for resistance to menoctone by W. Peters (4) and
352 sequenced cytochrome *b*. Interestingly in *Pb* MEN (MRA-414) we observed a G to T
353 mutation at nucleotide position 397 that confers a M133L mutation (Table 1). These data
354 suggest that menoctone resistance is conferred by similar mutations in cytochrome *b* in
355 both *P. berghei* and *P. falciparum* and that these mutations confer cross-resistance with
356 atovaquone.

357 A previous study suggested rapid selection of menoctone resistance in *P. berghei*,
358 therefore we next aimed to evaluate if M133I is readily selected in a single passage. In
359 this study we challenged both sensitive and resistant menoctone parasites to single dose
360 exposures of different doses of menoctone or atovaquone. After one round of atovaquone
361 or menoctone treatment, we found WT *cytb* in all of the menoctone sensitive parasites.

362 All of the *Pb* MEN-R parasites, regardless of the treatment maintained the M133I
363 mutation (Table 1).

364 **Assessment of atovaquone cross-resistance on *P. berghei* MEN resistant parasites.**
365 Upon initial treatment with 3 mg/kg of atovaquone, parasitemia decreased to <1% in *Pb*
366 MEN infected mice; however, parasites never completely cleared. Parasites recrudesced
367 by day six post-treatment and 10% parasitemia was reestablished by day nine. Blood was
368 passaged into naïve Balb/C mice and a higher dose of 30 mg/kg atovaquone decreased
369 the parasitemia to <1%. Resistant parasite populations recrudesced faster, four days post-
370 treatment when compared to the first drug exposure, 9 days. Again, a higher dose of
371 atovaquone (100 mg/kg) brought the parasitemia down to <1%, and parasites recrudesced
372 by day seven post-treatment. Successive treatment with a high dose of atovaquone (100
373 mg/kg) failed to clear parasites from peripheral blood (Figure 5).

374 **Mosquito transmission of menoctone resistance.** Recent studies have suggested
375 resistance to atovaquone that is mediated by cytochrome *b* mutations is not transmissible
376 through mosquitos (32). We aimed to test that hypothesis with the *Pb* MEN-R parasites
377 generated in this study. First we inoculated mice ip with *Pb* MEN-R- or *Pb* ANKA-
378 infected erythrocytes, collected blood for cytochrome *b* genotyping, and then fed adult
379 female *A. stephensi* mosquitos on the infected animals. On day 10, midgut dissections
380 revealed 32.6% of mosquito midguts were infected with oocysts of *Pb* MEN-R (Table 2).
381 Mercurochrome staining of midguts showed healthy, round oocysts with definitive
382 borders. Infected midguts contained approximately 130 oocysts and produced a
383 qualitative score of +++. On day 21 (D21), salivary glands were dissected and yielded
384 approximately 200,000 sporozoites of *Pb* MEN-R. In contrast, infections with *Pb luc*

ANKA produced higher rates of oocyst development and sporozoites per mosquito, as compared to *Pb* MEN-R. Some sporozoites were used to extract gDNA and the rest were used to inoculate iv into five malaria naïve Balb/C mice. Three of the mice infected with *Pb* MEN-R were not treated with drug and produced patent blood stage infections 12 days PI. The two *Pb* MEN-R infected mice that were treated with menoctone (300 mg/kg) demonstrated menoctone resistance as evidenced by continued growth despite treatment with the highest dose of drug.

As noted above, at each stage of the study we collected gDNA and then sequenced parasites to elucidate the cytochrome *b* genotype (Table 2). Genotype analysis confirmed that *Pb* MEN-R parasites used for mosquito infections had the M133I mutation as did sporozoites dissected from the salivary glands of the mosquitos D21 post feed. Furthermore, the M133I mutation was maintained in subsequent sporozoite challenge and menoctone resistant erythrocytic stages of *Pb* MEN-R maintained the resistance phenotype and M133I genotype. In simultaneous studies with the parental line *Pb luc* ANKA, we found the WT cytochrome *b* sequence throughout the transmission study. These studies demonstrate that menoctone and atovaquone resistance associated with M133I mutations in cytochrome *b* are transmissible through mosquitos. The only differences observed were somewhat lower oocysts and sporozoite yields with *Pb* MEN-R as compared to *Pb luc* ANKA (Table 2). Mosquito transmission studies with menoctone resistant *P. falciparum* were not possible since neither W2 nor W2 MEN-R produce viable gametocytes for mosquito infections.

DISCUSSION

In previous studies we have shown that old, potent drug scaffolds can be optimized into clinical candidates (2). Menoctone represents an interesting case where there was evidence of efficacy against multiple stages, although much was not known since the drug was abandoned shortly after its discovery. Early studies were limited mostly to in vivo evaluation in rodent models and a few biochemical analyses. In addition the limited reports date back almost 70 years, laying down initial groundwork for determining efficacy, yet the mechanism of action of menoctone against malaria was still not fully understood. Therefore the major aim of this study was to better profile the parasitological efficacy of menoctone and to generate resistance to better understand the mode of action. Limited availability of the compound prompted us to develop a new synthesis of menoctone using contemporary synthetic approaches. In comparison to the original menoctone synthesis (29, 30) our new synthetic route is convergent and requires fewer synthetic steps. At the synthetic scale we prepared menoctone, the overall yield of our synthetic approach also surpassed the overall yield of the original synthesis. Availability of menoctone then enabled us to generate encouraging efficacy data, especially the fact that menoctone is much more active against liver stages of *P. berghei* than the approved drug atovaquone. We also were successful at generating menoctone resistant malaria parasites that has allowed us to evaluate the possible mechanism(s) of action exhibited by menoctone on *Plasmodium* species.

In this study we demonstrated the high potentcy of menoctone against liver and blood stage parasites. We tested menoctone in a quantitative dose response assay for *P. berghei* liver stages and found that menoctone was approximately three fold more potent

than atovaquone. Further investigation with human blood stage parasites demonstrated nanomolar activity against *P. falciparum* (W2). These results for a putative cytochrome *b* inhibitor are different than most other scaffolds we have studied. Usually potency against blood and liver stages are more similar in fold differences when compared to atovaquone. In contrast, menoctone is much more potent against liver stages than blood stages. Further studies to better understand the <1nM potency of menoctone for liver stages is warranted.

In the course of generating the menoctone resistant parasite line *Pb* MEN-R, we demonstrated that WT *P. berghei* under menoctone pressure generates a mutation at M133I, which is identical to previous reports of *P. berghei* under atovaquone pressure (18). Since the mutation is located directly outside of the Q_o site, the known binding site for atovaquone, we believe that menoctone shares the same mechanism of action as atovaquone and that is collapsing the mitochondrial membrane potential and disrupting pyrimidine biosynthesis, leading to parasite death (33). The stability of resistance in *Pb* MEN-R following passage in untreated mice has not been verified beyond three passages, but resistance remains stable following cryopreservation, which is similar to observations of *Pb* MEN after cryopreservation (4). *Pb* MEN demonstrated cross-resistance with atovaquone and when sequenced, selected for a point mutation allowing for parasite survival. Interestingly, *Pb* MEN selected for the same guanine to tyrosine nucleotide change as seen for *Pb* MEN-R, however, the mutation was located at nucleotide position 397. This confers an amino acid change from methionine to leucine. Although *Pb* MEN and *Pb* MEN-R differ in nucleotide mutations, it is interesting that the resultant amino acids in resistant parasites are isomers of one another. The data suggest the M133I or

M133L mutations inhibit the activity of menoctone or atovaquone to the binding site. Our results show, for the first time, the menoctone resistance genotype and suggest a potential mechanism of action of menoctone.

Furthermore, we used a modified Thompson model to demonstrate the *in vivo* activity of menoctone against sensitive and resistant erythrocytic stages of rodent malaria (34). A summary of the *in vivo* results is presented in supplementary table 1. In the menoctone drug administered groups, all mice inoculated with sensitive parasites exhibited low survival outcomes, and menoctone administration did not provide protection for mice inoculated with *Pb* MEN-R. Alternatively, atovaquone at 10 mg/kg, 3 mg/kg and 1 mg/kg offered almost complete protection against sensitive *P. berghei* throughout the 30 day study. It is interesting to note that even though 12 out of the 15 mice survived *Pb* luc ANKA infection after atovaquone administration, some of the surviving mice did present with low levels of parasitemia on day 30 of the study. Overall, atovaquone imparted better protection against both *Pb* luc ANKA as well as *Pb* MEN-R when compared to menoctone, and only high dosages of menoctone, 30 mg/kg and 300 mg/kg respectively, offer low levels of protection against sensitive *P. berghei* infection (Figure 4). This data bolsters the initial findings by Fieser et al that menoctone fails to prevent recrudescence of blood stage parasites (3). In addition, our study confirms that menoctone resistance confers cross-resistance to atovaquone, which is characterized by the healthy and uninterrupted growth of *Pb* MEN-R in the presence of atovaquone *in vivo*. When comparing menoctone and atovaquone administration against resistant *Pb* MEN-R, there was a slight delay in parasite recrudescence for atovaquone treated mice, indicating perhaps that atovaquone clears more parasites than menoctone, thus requiring

more time for the parasites remaining in peripheral circulation to recrudesce. This finding was expected since the parasites were resistant to menoctone at high levels. Our results demonstrate that cross-resistance is clearly present between the two drugs and the mechanism by which atovaquone and menoctone act on the parasite are very similar if not affecting the same target.

Previously Coleman et al demonstrated menoctone failure to block transmission and successfully produced both oocysts and sporozoites in *P. berghei* and *P. falciparum* at a dose of 100 mg base drug/kg mouse body weight (9). More recently a study has shown that atovaquone resistance can't be transmitted via mosquitoes (32). This study reported that atovaquone drug pressure selects for the M133I *cytb* mutation in *P. berghei*, which is consistent with our data on menoctone drug pressure. Furthermore they found that the *P. berghei* with M133I or other *cytb* mutations could not be transmitted through mosquitoes. In contrast, we found that *P. berghei* MEN-R was easily transmitted through mosquitoes. We confirmed that the M133I mutation existed in the pre-feed *Pb* MEN-R infected blood that after feeding to *An. stephensi* produced healthy oocysts and infective sporozoites in the mosquito. Genotyping of the resulting sporozoites uncovered the same M133I point mutation, which we then compared to its sensitive parent parasites, displaying WT *cytb*. These sporozoites carrying the M133I mutation then successfully produced blood stage infection in the next generation of mice. We confirmed that the mutation is fully transmittable from sporozoites to mice since all sporozoite-infected mice produced blood stage infection and carried the M133I mutation. To further demonstrate that the resistance phenotype was maintained, we administered high doses of menoctone to two of the sporozoite inoculated mice to observe parasite growth; despite

500 treatment with high dose of menoctone (300 mg/kg) parasitemia continued to rise and
501 healthy parasites propagated despite treatment with menoctone. Parasites from these mice
502 also maintained the M133I mutation. We conclude that menoctone and atovaquone
503 resistant parasites containing the M133I mutation in *cytb* are indeed transmittable by the
504 mosquito vector. At present we do not understand why *P. berghei* with M133I mutations
505 were transmitted in our study, but not in the recent study by Goodman et al (32). The
506 difference could be due to inherent transmissibility of the different *P. berghei* strains or
507 mosquitoes used in the respective studies. Another possibility is that yet-to-be identified
508 mutations in the nuclear genome may contribute to resistance and transmission potential.
509 The transmission potential of parasites resistant to mitochondrial electron chain inhibitors
510 warrants additional studies.

511 The diminishing arsenal of anti-liver stage compounds sparked our interest in
512 reviving the old and previously abandoned compound menoctone. In summary,
513 menoctone is a potent liver stage antimalarial with corresponding erythrocytic stage
514 efficacy. The compound readily selects for *cytb* M133I mutation in *P. falciparum* and *P.*
515 *berghei*; this is the same mutation often selected for under atovaquone drug pressure.
516 This leads us to hypothesize that menoctone most likely targets the mitochondrial
517 respiration chain of *Plasmodium*. In these studies, we have demonstrated for the first time
518 that *cytb* mutations are transmittable from mouse to mosquito to mouse and have
519 provided insight to a potential mechanism of action of menoctone. In addition we
520 developed a new synthesis method for menoctone that could be used in medicinal
521 chemistry optimization of the scaffold. Further exploratory investigations would provide

support towards whether menoctone may serve as a lead for novel antimalarial drug development.

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644 **Table 1.** Single dose treatments not sufficient for mutation selection. Sensitive and
645 resistant parasites were challenged with either menoctone or atovaquone at different
646 concentrations to observe whether single dose treat was favored. Single rounds of drug
647 are not sufficient to select for M133I point mutation and resulted in WT genotypes at
648 *cytb*, identical to the parent strain, *Pb luc* ANKA. All menoctone resistant parasites
649 demonstrated the M133I point mutation. Remarkably when genotyped, sporozoites
650 produced from menoctone resistant blood stage parasites also carried the M133I point
651 mutation, while sporozoites harvested from the sensitive parasite line displayed WT *cytb*.
652

Parasite	Nucleotide	Mutation	Genotype
P. berghei luc ANKA (blood stages) + Atovaquone or Menoctone (single dose)	399	none	WT
P. berghei MEN-R (blood stages) + Atovaquone or Menoctone (single dose)	399	G->T	M133I
P. berghei luc ANKA (sporozoites)	399	None	WT
P. berghei MEN-R (sporozoites)	399	G->T	M133I
Pb MEN (MRA-414) (blood stages)	397	G->T	M133L
W2-MEN-R (blood stages)	399	G->C	M133I

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656 **Table 2.** Menoctone resistance point mutation capable of mosquito developmental stage
657 transmission and produces next generation blood stage infection. Menoctone resistant
658 point mutation was identified in pre-mosquito fed blood stage parasites. The blood meal
659 produced positive oocysts and sporozoite infections in mosquitoes. Sporozoites produced
660 next generation blood stage infection. M133I point mutation was fully maintained
661 throughout the transmission in menoctone resistant parasites while the sensitive parasites
662 demonstrated WT *cytb*.

663

	Pre-mosquito Feed	**Post-mosquito Feed**				**Mice post-sporozoite inoculation**	
Parasite strain	***Cyt b* Genotype**	**No. infected midguts (%)**	**Midgut infection score***	**Sporozoites per mosquito**	***Cyt b* Genotype**	**Mice with patent infections (%)**	***Cyt b* Genotype**
Pb luc ANKA	WT	37/50 (74%)	++	21,071	WT	3/3 (100%)	WT
Pb MEN-R	M133I	19/54 (35.2%)	+++	1,593	M133I	5/5 (100%)	M133I

664 *: 0-50 oocysts= +; 51-100 oocysts = ++; 101-150 oocysts = +++, 151-200 oocysts =
665 ++++; > 200 oocysts = +++++
666
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Figure 1. Synthesis of menoctone.

Figure 2. Menoctone and atovaquone efficacy against liver stages of *P. berghei luc* ANKA (A, B) and blood stages of *P. falciparum* in vitro (C).

Figure 3. Generation of menoctone resistance in *P. berghei luc* ANKA. Blood from recrudescent infections was serially passaged in malaria naïve mice following treatment with 3 (A), 30 (B), and 300 mg/kg (C, D). Mice were treated on day 0 of the study and 3 mice were used per group; data shown are the mean parasitemia of each group.

Figure 4. Survival curves of sensitive (A, C) versus menoctone resistant *P.berghei* (B, D) following treatment with menoctone or atovaquone. Menoctone offers some protection to *Pb* luc ANKA infected mice (A), but demonstrates a marked decrease in protection against menoctone resistant parasite *Pb* MEN-R (B). Atovaquone provided significantly more efficacy than menoctone (C), however even at the highest dose of 10 mg/kg, atovaquone was not effective at preventing recrudescence of *Pb* MEN-R (D).

Figure 5. Assessment of atovaquone cross-resistance with menoctone with *P. berghei* MEN (MRA-414). *P. berghei* MEN infected mice were treated with atovaquone at 3 (A), 30 (B), and 100 mg/kg (C, D) on day 0 (n=2 per group). Parasitemia decreased to <1% parasitemia following atovaquone treatment after each treatment; however, atovaquone failed to completely clear all of the parasites after each round and low concentrations of parasites were observed. Blood from recrudescent parasitemia mice treated with 100

693 mg/kg atovaquone (C) was inoculated into malaria naive mice and treated with 100

694 mg/kg (D); these infections also confirmed resistance with atovaquone.

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