A Novel Antiparasitic Compound Kills Ring-Stage *Plasmodium falciparum* and Retains Activity Against Artemisinin-Resistant Parasites

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Spreading antimalarial resistance threatens effective treatment of malaria, an infectious disease caused by *Plasmodium* parasites. We identified a compound, BCH070, that inhibits asexual growth of multiple antimalarial-resistant strains of *Plasmodium falciparum* (half maximal inhibitory concentration [IC$_{50}$] = 1–2 μM), suggesting that BCH070 acts via a novel mechanism of action. BCH070 preferentially kills early ring-form trophozoites, and, importantly, equally inhibits ring-stage survival of wild-type and artemisinin-resistant parasites harboring the PfKelch13:C580Y mutation. Metabolic analysis demonstrates that BCH070 likely targets multiple pathways in the parasite. BCH070 is a promising lead compound for development of new antimalarial combination therapy that retains activity against artemisinin-resistant parasites.

**Keywords.** antimalarial; artemisinin resistance; malaria; *Plasmodium falciparum*; ring-stage survival.

Malaria is caused by infection with *Plasmodium* parasites. Despite the existence of antimalarial drugs, malaria remains a significant global health burden, with >200 million cases and >400 000 deaths annually [1]. *Plasmodium falciparum* has developed resistance against all widely used antimalarials, threatening successful treatment and control of malaria. Artemisinin-based combination therapy is the recommended first-line treatment for *P. falciparum* malaria. However, resistance to artemisinin has been steadily emerging worldwide [1, 2]. Therefore, it is essential to develop new classes of antimalarials and identify novel druggable targets in *Plasmodium* parasites. In this report, we describe a new lead compound that maintains activity against multiple drug-resistant *P. falciparum* strains, including artemisinin-resistant parasites, and is most active during the early ring-form trophozoite stage of the asexual parasite life cycle, referred to as the ring stage.

**METHODS**

*Plasmodium falciparum* Culture

*Plasmodium falciparum* cultures were maintained as previously described [3]. The 3D7 strain was obtained from Alan Cowman (Walter and Eliza Hall Institute, Parkville, Australia); Dd2attB parasites were provided by David Fidock (Columbia University, New York, NY); and Dd2-ProRS:L550V, Dd2-ProRS:L482H, Dd2-ProRS:Y1356N, Dd2-ScDHODH, and 3D7-Kelch13:C580Y parasites were provided by Dyann Wirth (Harvard T.H. Chan School of Public Health, Boston, MA). Dd2-KnL parasite strain was generated via electroporation with pJDD251 reporter plasmid in Dd2attB parasites, selection with 2.5 μg/mL blasticidin, cloning by limiting dilution, and polymerase chain reaction confirmation of integration.

**Dose Response**

BCH070/analogs were obtained from MolPort or synthesized by W.H. and/or Q.Z. Schizont-stage parasites were purified with 60% Percoll. After 3 hours of reinvasion, ring-stage parasites were synchronized with 5% sorbitol, plated in triplicate at 1% parasitemia/2% hematocrit across serial drug dilutions or dimethyl sulfoxide (DMSO), and incubated at 37°C for 72 hours. SYBR lysis buffer (0.16% saponin, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1.6% Triton X-100, 1:1000 SYBR Green I or luminescence buffer and substrate was added to each well, and fluorescence and/or luminescence was read on a SpectraMax iD3. Half maximal inhibitory concentration (IC$_{50}$) was calculated on Prism 8 software.

**Flow Cytometry Analysis of Replication and Viability**

Zero- to three-hour ring-stage parasites were isolated as described above and plated in triplicate at 1% parasitemia/4% hematocrit. A total of 10 μM BCH070 or DMSO was added and washed out at indicated timepoints. Every 12 hours, 100 μL culture was washed with 0.5% bovine serum albumin (BSA)-phosphate-buffered saline (PBS), incubated with 1:1000 SYBR Green I and 0.6 μM MitoTracker Deep Red FM for 20 minutes at room temperature (RT), washed with 0.5% BSA-PBS, and resuspended in PBS.
were collected using a FACSCalibur with acquisition of 100,000 events/sample. Gating was done with unstained and uninfected erythrocytes, SYBR/MitoTracker staining alone, and double-stained 2 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)-treated samples as a MitoTracker negative control. Parasitemia was calculated using SYBR'*;MitoTracker' cells, and fraction of dead cells calculated as SYBR'*;MitoTracker'/SYBR' cells.

Ring-Stage Survival
Zero- to three-hour ring-stage 3D7 or 3D7-PfKelch13:C580Y parasites were isolated as described above, plated in triplicate at 1% parasitemia/2% hematocrit, and incubated at 37°C for 6 hours with 700 nM dihydroartemisinin (DHA), 100 µM BCH070, and/or DMSO. After drug removal, parasites were incubated at 37°C for 66 hours, then parasitemia was assessed with flow cytometric analysis of SYBR Green I and MitoTracker Deep Red FM [4]. Ring-stage survival was calculated as drug-treated parasitemia/DMSO parasitemia.

BCH070-Resistant Parasites
Dd2-KnL parasites were plated at 0.5% parasitemia/2% hematocrit with 10 µM BCH070 for 6 days, then allowed to recover without drug until parasites were visible via Field's staining. This process was repeated 3 times.

Metabolomic Analysis by Mass Spectrometry
Metabolic analysis was performed as previously described using 10 µM BCH070 against trophozoite-stage 3D7 P. falciparum [5].

Plasmodium berghei Liver Stage
Liver-stage assessment was performed as previously described [6].

Clonal Viability
Clonal viability was assessed as described previously, with modifications (see [7]). Ring-stage Dd2-KnL parasites were synchronized with 5% sorbitol, plated in triplicate at 1% parasitemia/1% hematocrit, treated with BCH070 serial-dilutions, DMSO, or 70 nM mefloquine, and incubated at 37°C for 72 hours. Parasitemia of DMSO-treated sample was determined via Field's staining, and growth of remaining samples was estimated using dose-response best-fit equation. Approximately one parasite/well of a 384-well plate was plated and incubated at 37°C for 4 weeks. Luminescence buffer was added to each well and plate was read on SpectraMax iD3. The DMSO/mefloquine controls were used to set luminescence cutoffs. The median lethal dose (LD$_{50}$) was calculated using Prism 8 software.

Sequencing
Sequencing libraries were prepared, aligned, and filtered as previously described [8].

Trypanosoma cruzi IC$_{50}$ Determination
Normal human dermal fibroblasts (NHDF) were grown at 37°C with 5% CO$_2$ in Dulbecco's modified Eagle medium supplemented with 25 mM glucose, 2 mM glutamine, 100 U/mL penicillin/streptomycin, and 10% or 2% fetal bovine serum for uninfected or infected cultures, respectively. The NHDF were seeded 24 hours before infection. Trypomastigotes were allowed to infect at multiplicity of infection of 1.25 for 2 hours followed by washes to remove uninvaded trypomastigotes. At 18 hours post-invasion (hpi), compound was added to infected cultures. At 66 hpi, growth medium was removed, CellTiter-Fluor was added, and fluorescence was measured using EnVision plate-reader. Subsequently, Beta-Glo was added and luminescence was measured after 30-minute incubation at RT.

Trypanosoma cruzi Amastigote Enumeration and Epimastigote Growth
The NHDF were seeded on coverslips 24 hours before infection. Trypomastigotes were allowed to invade for 2 hours and cells were washed twice. At the indicated timepoints, infected cells were fixed with 1% paraformaldehyde/PBS and stained with 0.1% Triton X-100 PBS with 100 ng/mL 4,6-diamidino-2-phenylindole. For epimastigote assays, epimastigotes were seeded (2 × 10$^6$/well), treated with compound, and maintained at 28°C. After 120 hours, 10 µL/well were moved to a fresh plate with Beta-Glo. Luminescence was measured after 30-minute incubation at RT.

RESULTS

Nano-Luciferase Reporter
To obtain a high-throughput, reliable system to screen for novel antimalarial compounds, we generated a bioluminescent reporter strain of P. falciparum named Dd2-KnL. We fused nLuc to the signal sequence of PfKAHRP such that nLuc is exported into the parasitophorous vacuole. To generate Dd2-KnL parasites, this expression cassette was integrated into the attB locus of Dd2attB parasites [10], a derivative of the multidrug-resistant Dd2 strain. We see equivalent baseline growth of Dd2-KnL compared with parental Dd2attB parasites, and dose-response assays performed with Dd2-KnL are approximately equal when assessed with either bioluminescence or SYBR Green I fluorescence (Supplementary Figure 1a).

Identification of BCH070
We identified a series of quinazoline-based compounds with activity against blood-stage P. falciparum in vitro (Supplementary Figure 2). The quinazoline class is a known privileged scaffold with many bioactive derivatives used for an array of medicinal applications [11]. After performing extensive structure-activity relationship analysis on commercially available derivatives, we concluded that BCH070 (Figure 1a) is among the most potent derivatives and focused on this compound for further analysis.

BCH070 is active against blood-stage P. falciparum (Dd2-KnL IC$_{50}$ 0.4 ± 0.2 µM) (Figure 1b) and liver-stage P. berghei in vitro.
BCH070 is more inhibitory on growth of Dd2-KnL parasites than on other parasites derived from Dd2 or 3D7 lineages (Figure 1c, Supplementary Figure 1b).

BCH070 Maintains Efficacy Against Antimalarial-Resistant Plasmodium falciparum

Drugs that target the same molecular pathway or belong to a similar chemical class are prone to cross-resistance, but novel drugs can evade pre-existing resistance mechanisms. To determine whether BCH070 works via a unique pathway, we performed a BCH070 dose-response assay across a panel of P. falciparum strains containing resistance-associated mutations [8, 12–14]. BCH070 maintained similar efficacy across this panel, suggesting a novel mechanism of action (Figure 1c).

Mutations in PfKelch13 have been linked to delayed clearance and increased ring-stage survival after artemisinin treatment [2, 15]. Given the increasing emergence of artemisinin-resistant parasites, we assessed whether BCH070 is active against
parasites that contain one of these resistance-associated mutations, PIKelch13-C580Y. We performed a ring-stage survival assay [4] on wild-type 3D7 parasites and 3D7-PIKelch13-C580Y parasites with DHA, BCH070, or both. A 6-hour pulse of BCH070 equally inhibited survival of early rings in wild-type 3D7 parasites and 3D7-PIKelch13-C580Y parasites. In addition, BCH070 in combination with DHA decreased ring-stage survival of 3D7-PIKelch13-C580Y parasites compared with DHA alone (Figure 1d). This finding suggests that BCH070 could be an effective combination therapy with DHA, even against DHA-resistant parasites. As artemisinin-resistance continues to steadily emerge, and artemisinin-based combination therapy is the recommended first-line treatment for 
P. falciparum malaria, it is vital that we discover novel drugs, such as BCH070, that remain effective in combination with DHA in artemisinin-resistant strains.

**BCH070 Preferentially Kills Ring-Stage Plasmodium falciparum**

Given that BCH070 hinders ring-stage survival, we hypothesized that BCH070 is active against early ring-stage parasites. To test this hypothesis, we treated synchronized Dd2-KnL parasites with either BCH070 or DMSO as follows: early rings (0–12 hours postinvasion), late rings (12–24 hpi), rings (0–24 hpi), late trophozoites (24–36 hpi), rings + late trophozoites (0–36 hpi), schizonts (36–48 hpi), late trophozoites + schizonts (24–48 hpi), or rings + late trophozoites + schizonts (0–48 hpi) (Figure 2a). It is interesting to note that ring-stage parasites treated with BCH070 (0–24 hpi) phenotypically stall and remain rings even up to 72 hpi, 2 days after BCH070 has been washed out (Figure 2c). Unlike DHA-treated rings, BCH070-treated ring-stage parasites are not pyknotic.

Traditional dose-response assays measure growth, but they do not distinguish between cidal and static effects. To overcome this, we assessed parasite viability using a mitochondrial membrane potential dye, MitoTracker Deep Red FM [4]. Viable parasites stain positive for both SYBR and MitoTracker, and inviable parasites stain SYBR-positive but MitoTracker-negative. We used an oxidative phosphorylation uncoupler, FCCP, as a control for mitochondrial inhibition.

Using the time-course scheme described above (Figure 2a), we stained parasites every 12 hours and measured viable parasitemia as well as the proportion of dead parasites. Although all BCH070-treated conditions had significantly lower parasitemia at 72 hpi than controls, we observed the biggest impact to parasitemia when BCH070 was present during the ring-stage (Figure 2b, Supplementary Figure 3). In contrast to the majority of antimalarial drugs that target metabolically active late-stage trophozoites or schizonts, BCH070 is not only active during the ring-stage, but it is most efficacious during the ring-stage.

At the time of drug washout, we see a significantly higher proportion of inviable parasites when BCH070 is present during the ring-stage than either the late trophozoite or schizont stage, or both (Figure 2e). This suggests that parasites, especially ring-stage parasites, die during BCH070 exposure. In addition, parasites do not recover after BCH070 washout (Supplementary Figure 4). For example, at 72 hpi, in the ring-stage (0–24 hpi) condition, ~70% of SYBR-positive parasites are MitoTracker-negative (Figure 2d), demonstrating that 2 days after BCH070 has been removed, only ~30% of parasites are viable. To further distinguish between death and growth arrest, we assessed clonal viability of Dd2-KnL parasites after BCH070 treatment [7]. We determined the LD₅₀ to be ~6.7 µM (Supplementary Figure 5), which is consistent with cidal activity.

**BCH070 Targets Multiple Parasite Pathways**

To identify molecular target(s), we selected for BCH070-resistant parasites after several pulse-recover cycles. We “pulsed” Dd2-KnL parasites with 10 µM BCH070 for 6 days, then removed drug pressure and allowed parasites to recover, repeating this process 3 times. Selected parasites have a ~3-fold increase in BCH070 resistance compared with the parental Dd2-KnL strain (Supplementary Figure 6a). We performed whole-genome sequencing on clonal parasites, but we did not identify sequence changes linked to BCH070 resistance, and we saw no changes in copy number across the genome. Although surprising, the precedent for nongenetic antimalarial resistance has been set by halofuginone [12].

To elucidate the mechanism of BCH070, we performed metabolomic profiling by liquid chromatography-mass spectrometry [5]. Upon BCH070 treatment, we saw changes in metabolites associated with inhibition of the mitochondrial electron transport chain and hemoglobin catabolism (Supplementary Figure 6b), suggesting that BCH070 targets multiple pathways in the parasite. This metabolic response is unique compared with many previously established responses determined for 
P. falciparum.

**BCH070 Is Active Against Trypanosoma cruzi**

To determine whether BCH070 has broad antiparasitic activity, we tested BCH070 against the kinetoplast 
T. cruzi, an evolutionarily distinct pathogen that causes Chagas disease. Treatment options for 
T. cruzi infection are limited and associated with significant side effects. It is interesting to note that BCH070 is active against 
T. cruzi amastigotes (IC₅₀ ~1 µM) (Supplementary Figure 7a), but only minimally active against the epimastigote stage (IC₅₀ ~10–20 µM) (Supplementary Figure 7b). Unlike the cidal effect we observe in 
P. falciparum, 
T. cruzi amastigotes partially recover upon drug washout, suggesting that BCH070 has static effects in 
T. cruzi (Supplementary Figure 7c).

**DISCUSSION**

BCH070 maintains activity in parasites that are resistant to existing antimalarials, including artemisinin. Most notably,
Figure 2. BCH070 preferentially kills ring-stage *Plasmodium falciparum*. (a) Schematic of experimental procedure. Synchronized parasites were treated with 10 µM BCH070 for indicated amount of time followed by BCH070 washout. (b) BCH070 is most effective when present during the ring stage. Parasitemia was measured at 72 hpi. N = 3; mean ± standard error of the mean (SEM). Analyzed with ordinary one-way analysis of variance (ANOVA). Dashed line represents initial parasitemia. (c) BCH070-treated parasites stall as rings and (d) do not have intact mitochondrial membrane potential, even up to 48 hours after drug has been washed out. (e) BCH070 treatment selectively kills ring-stage parasites but not trophozoites or schizonts. N = 3; mean ± SEM. Analyzed with ordinary one-way ANOVA. *, *P* < 0.0332; **, *P* < 0.0021; ***, *P* < 0.0002; ****, *P* < 0.0001. hpi, hours post-invasion. Dashed line at dimethyl sulfoxide (DMSO)-treated values.
however, we demonstrate that BCH070 can be used in combination with artemisinin to decrease ring-stage survival in artemisinin-resistant parasites. With additional optimization and testing, a future derivative of BCH070 could be added to existing first-line artemisinin combination therapies.

**CONCLUSIONS**

BCH070 is a strong lead candidate, and through structure-activity relationship analysis, we have laid the groundwork for development of an optimized version of BCH070 that could be used in combination with artemisinin to treat multidrug-resistant malaria.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

**Supplementary Figure 1.** Dd2-KnL reporter strain. (a) IC_{50} calculations with SYBR Green I and nano-luciferase after 72-hour dose-response assays are approximately equal. Dd2-KnL reporter parasites were simultaneously plated in 2 separate plates. After 72 hours, growth was assessed with either luminescence of the nano-luciferase or SYBR Green I fluorescence. N = 3; mean ± SD. (b) BCH070 shows higher activity against Dd2 parasite strains (IC_{50} = 0.8 µM) than 3D7 strains (IC_{50} = 2.7 µM). Growth was assessed with SYBR Green I fluorescence. N = 3; mean ± SD.

**Supplementary Figure 2.** Structure-activity relationship (SAR) studies. SAR studies highlight critical chemical features of BCH070 and demonstrate which residues can be altered without sacrificing efficacy. (a) Potency of compounds was assessed in Dd2-KnL parasites in 72-hour dose-response assay. Parasite growth was measured with nanoluminescence. All compounds are commercially available except 17-22-2, which was synthesized. Mean ± SD of 2 replicate experiments. (b) Conclusions about SAR data from 2 replicate experiments.

**Supplementary Figure 3.** BCH070-treated rings show a higher proportion of parasites without intact mitochondrial membrane potential than trophozoites or schizonts. Dd2-KnL parasites were treated with 10 µM BCH070 for the indicated times before washing the drug out. Flow cytometry was performed at 72 hpi. The proportion of parasites without intact mitochondrial membrane potential was assessed. The median lethal dose (LD_{50}) was calculated to be ~6.7 µM. N = 3; mean ± SD.

**Supplementary Figure 4.** BCH070-treated rings do not recover mitochondrial membrane potential after drug has been washed out for 24 hours. Dd2-KnL parasites were treated with 10 µM BCH070 for 0–24 hpi before washing the drug out. Flow cytometry was performed at indicated time points with SYBR Green I and Mitotracker Deep Red FM. All samples were gated using FCCP-treated control.

**Supplementary Figure 5.** Clonal viability of BCH070-treated parasites. (a) Dd2-KnL parasites were treated with varying concentrations of BCH070 for 72 hours, then clonal viability was assessed. The median lethal dose (LD_{50}) was calculated to be ~6.7 µM. N = 3; mean ± SD. 

**Supplementary Figure 6.** BCH070-resistant parasites suggest noncanonical drug resistance mechanisms. (a) BCH070-resistant parasites were selected with 3 pulse-recover cycles and demonstrate ~3-fold shift in IC_{50} values. N = 3; mean ± SD. (b) Metaprint analysis of BCH070-treated trophozoites suggest that BCH070 inhibits the mitochondrial electron transport chain and hemoglobin catabolism. (c) Genomic deoxyribonucleic acid (DNA) from BCH070-resistant clones was sequenced. Mutation calls for each clone are shown here. 0/0 indicates presence of parental allele only, 0/1 or 0/2 indicates mixed reads (parental and alternate), and 1/1 or 2/2 indicates presence of alternate allele only. (d) Sequencing statistics.

**Supplementary Figure 7.** BCH070 activity in *Trypanosoma cruzi*. (a) BCH070 is active against *T. cruzi* amastigotes. Luminescence was measured at 66 hpi, before differentiation and egress. (b) BCH070 has minimal activity against *T. cruzi* epimastigotes. Luminescence was measured after 120 hours. (c) The effects of BCH070 are partially reversed in *T. cruzi* amastigotes after drug is washed out. Transgenic *T. cruzi* parasites in human foreskin fibroblasts. BCH070 was added at 18 hpi, after invasion and differentiation into amastigotes, but before division. BCH070 was washed out at 42 hpi. The number of amastigotes per cell was counted with DAPI staining and microscopy at 42 hpi and 66 hpi. Median shown. Non-parametric test, P < .0001.

**Notes**

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