Potent *Plasmodium falciparum* gametocytocidal activity of lead anti-malaria chemotype, diaminonaphthoquinones, identified in an anti-malaria compound screen

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Running title: Gametocytocidal activity of anti-malaria chemotypes

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Forty percent of the world’s population is threatened by malaria, which is caused by *Plasmodium* parasites and results in an estimated 200 million clinical cases and 650,000 deaths each year. Drug-resistance has been reported for all commonly used anti-malarials and prompted screens to identify new drug candidates. However, many of these new candidates have not been evaluated against the parasite stage responsible for transmission, gametocytes. If *P. falciparum* gametocytes are not eliminated patients continue to spread malaria for weeks after asexual parasite clearance. Asymptomatic individuals can also harbor gametocyte burdens sufficient for transmission and a safe, effective gametocytocidal agent could also be used in community wide malaria control programs. Here, we identify 15 small molecules with nanomolar activity against late stage gametocytes. Fourteen are diaminonaphthoquinones (DANQ) and one is a 2-imino-2-benzo[d]imidazole (IBI). One of the identified DANQs is a lead anti-malarial candidate, SJ000030570. In contrast, 94% of the 650 compounds tested are inactive against late stage gametocytes. Consistent with the ineffectiveness of most approved anti-malarials against gametocytes, of the 19 novel compounds with activity against known anti-asexual targets, only 3 had any strong effect on gametocyte viability. These data demonstrate the distinct biology of the transmission stages and emphasize the importance of screening for gametocytocidal activity. The potent gametocytocidal activity of DANQ and IBI coupled with their efficacy against asexual parasites provides leads for the development of anti-malarials with the potential to prevent both symptoms and the spread of malaria.
Introduction

Effective chemotherapy remains a critical component of current malaria control strategies and is essential to treat severe malaria (1). The introduction of artemisinin combination therapies (ACTs) has successfully lowered malaria mortality but does not effectively control disease spread because ACTs do not eliminate the sexual stages of the parasite that are required for malaria transmission (2, 3). As a consequence, patients remain infectious for over a week after asexual parasite clearance and the cessation of symptoms. Moreover, the identification of parasite lines with delayed parasite clearance following ACT treatment have spurred the effort to identify new anti-malarials (4). Several recent screens of novel small molecule libraries against asexual parasites have expanded the repertoire of potential candidates for treating acute malaria, but the analysis of their effects on the sexual stages is just beginning and has been focused on the 400 molecules included in the malaria box (5-13). Only 12 of the 260 anti-malaria compounds analyzed in this study are also present in the malaria box.

Both gametocytes and asexual parasites develop within the erythrocyte but have distinct developmental patterns that contribute to their differential sensitivity to common anti-malarials (14, 15). While *P. falciparum* asexual stages undergo 4-5 rounds of DNA replication to produce 16-32 new parasites over the course of 48 hrs, gametocytes differentiate through 5 morphologically distinct stages (I-V) into a single male or female gametocyte over 10-12 days (16). To completely block transmission, all these stages need to be eliminated during the course of treatment. The lack of DNA replication during gametocyte development provides resistance to drugs that target nucleic acid production, such as sulfadoxine/pyrimethamine, atovaquone, and dihydroorotate dehydrogenase inhibitors (17). Additionally, stage III-V gametocytes are no longer affected by compounds that block hemoglobin digestion, such as the 4-aminoquinolines and cysteine protease inhibitors (17, 18). Gametocytes are also resistant to sorbitol lysis,
suggesting a reduction in permeability pathways such as the plasmodial surface anion channel (PSAC) (19-21). The lack of PSAC could affect drug accessibility as shown for blasticidin and leupeptin (22). Likewise, gametocytes are not cleared by antibacterial agents that target the apicoplast, such as clindamycin and tetracycline analogs (23). Additional apicoplast-specific enzyme systems have not yet been evaluated in gametocytes (24). In contrast, proteasome and protein synthesis inhibitors are quite effective against all parasite stages (18, 25-27), including late stage gametocytes, which indicates the presence of shared pathways that could be targets of drugs with activities against both asexual and sexual stage parasites.

Here, we used the gametocyte viability assay we developed (28) and validated (29) to screen a library of 260 lead-like compounds with activity against asexual parasites. The results indicate that the majority of the anti- sexual compounds tested were inactive (>80% viability after treatment), including novel inhibitors of hemozoin formation and pyrimidine synthesis. This finding is consistent with the limited gametocytocidal activity of commonly used antimalarials and also demonstrates the specificity of the assay for late stage gametocytes. However, nine percent of the compounds (23/260) did decrease gametocyte viability more than 50%, suggesting the presence of targets that are important for both asexual and sexual development. These 23 gametocytocidal compounds are members of five different chemotypes: the diaminonaphthoquinones (DANQ), dihydropyridines (DHP), bisphenylbenzimidazoles (BPBI), carbazoleaminopropanols (CAP), and iminobenzimidazoles (IBI). Two of these scaffolds, DANQ and DHP, have been identified as leads against asexual parasites (30). Follow up studies screened 390 additional compounds to define structure-gametocytocidal activity profiles identified 15 compounds with nanomolar EC50s.

Materials and methods

Chemical preparation
All compounds used in these studies were purchased from vendors and used without further purification. Prior to use, the identity of each compound was confirmed by UPLC/MS, and their purities were confirmed to be greater than 95% by UPLC/ELSD/UV/MS. Stock solutions were prepared at a nominal concentration of 10 mM in DMSO, and the concentrations were confirmed by CLND prior to use.

**P. falciparum gametocytocidal assay**

**AlamarBlue Viability Assay:** The gametocyte induction and gametocytocidal assays were performed using *P. falciparum* strain 3D7 as described (28). Briefly, parasite cultures were maintained in complete RPMI (RPMI 1640, 25 mM HEPES, 25 mM NaHCO₃ (pH 7.3), 100µg ml⁻¹ hypoxanthine, and 5 µg ml⁻¹ gentamycin (KD Biomedical, Columbia, MD) supplemented with 10% human serum (Interstate Blood Bank, Memphis, TN). Gametocyte cultures were set up at 0.2% parasitemia and a 6% hematocrit. On day three the hematocrit was reduced to 3% by increasing the media added during the daily feed. Following N-acetyl glucosamine (NAG, 50 mM) treatment on days 10-12 to eliminate asexual parasites, stage III/IV/V gametocytes were purified on a 65% Percoll gradient and returned to culture. The next day, the parasites were resuspended at 10% gametocytemia, 0.5% hematocrit and aliquoted into a 96-well plate containing the test compounds or positive (30 nM epoxomicin) and negative (DMSO) controls. After incubation at 37°C for 3 days, 1/10 volume of the fluorescent viability indicator dye, alamarBlue was added, and 24 hrs later the fluorescence was determined at 590/35 nm following excitation at 530/25 nm. For compounds that interfere with alamarBlue reduction, wash steps were added before alamarBlue addition to dilute the compound 2,500-fold. To do this, 100 µl of incomplete media was added to each well at the end of the incubation period instead of alamarBlue, resulting in a 2-fold dilution. After, centrifugation at 1,860 x g for 2 min, 150 µl of supernatant was removed from each well and 200 µl of incomplete media was added, resulting in
a 5-fold dilution. After centrifugation, 200 μl of supernatant was removed and replaced with 200 μl of incomplete media, resulting in another 5-fold dilution, and this procedure was repeated twice more. After the last centrifugation, 200 μl of supernatant was removed and 50 μl/well of complete media (10% human serum) was added, resulting in a 2-fold dilution, for a final dilution of 2,500 (2x5x5x5x5x2) before the addition of 1/10 volume of alamarBlue.

**Gametocytocidal confirmation assays:** Zero, 12, 24, 48 and 72 hr after the addition of the indicated compound, samples (5% gametocytemia, 2-3% hematocrit) were washed 3 times with complete RPMI and analyzed using alamarBlue, Giemsa-stained smears, or MitoProbe DiIC1(5), a membrane-potential-sensitive cyanine dye (Life Technologies). Samples probed with alamarBlue were incubated for 24 hrs before the fluorescent signal was determined as previously described. For MitoProbe DiIC1(5) staining 20 μl of the washed, compound-treated sample was diluted to 200 μl with buffer containing 1.67 mg ml⁻¹ glucose; 8 mg ml⁻¹ NaCl; 8 mM Tris-Cl (pH 8.2) and incubated with 50nM MitoProbe DiIC1(5) for 30 min prior to flow cytometry (AccuriC6, BD). Uninfected RBCs incubated with MitoProbe DiIC1(5) and unstained *P. falciparum* infected RBCs were used as controls to determine the threshold for MitoProbe DiIC1(5) positive, single, intact cells (640 nm laser excitation and FL4 emission filter (675/25 nm). All experiments were done in triplicate.

**Exflagellation assay:** Twenty four to 48 hrs after Percoll purification, gametocytes were diluted to 10% parasitemia using fresh human RBCs. Parasites and resuspended to 0.5% hematocrit with complete RMPI 1640 media containing 10% human serum and the indicated compound concentration or carrier alone. The cultures were gassed with 90% N₂, 5% O₂, 5% CO₂ and allowed to incubate at 37°C for 72 hrs. To measure exflagellation, a 500 μl aliquot was pelleted by centrifugation (900 x g) and resuspended in 10 μl room temperature human serum with 100
µM xanthurenic acid. Following a 15 minute incubation, 5 μl was applied to a hemocytometer and the number of exflagellation centers counted in 50 fields using a 40x objective.

*P. falciparum* asexual growth assay: Asynchronous parasites were maintained in culture based on the method of Trager (31). Parasites were grown in presence of fresh group O-positive erythrocytes (Key Biologics, LLC, Memphis, TN) in Petri dishes at a hematocrit of 4-6% in complete RPMI 1640 supplemented with 0.5% AlbuMAX II (Life Technologies). Cultures were incubated at 37°C in a gas mixture of 90% N₂, 5% O₂, 5% CO₂. For EC₅₀ determinations, 20μl of RPMI 1640 with 5μg ml⁻¹ gentamycin were dispensed per well in a 384-well assay plate (Corning 8807BC). An amount of 40 nl of compound, previously serial diluted in a separate 384-well white polypropylene plate (Corning, 8748BC), was dispensed to the assay plate by hydrodynamic pin transfer (V&P Scientific Pin Head, FP1S50H) and then an amount of 20 μl of a synchronized culture suspension (1% rings, 4% hematocrit) was added per well, thus making a final hematocrit and parasitemia of 2% and 1%, respectively. Assay plates were incubated for 72 hr, and the parasitemia was determined by a method previously described (32): Briefly, an amount of 10μl of the following solution in PBS (10X SYBR Green I, 0.5% v/v Triton X-100, 0.5 mg ml⁻¹ saponin) was added per well. Assay plates were shaken for 1min, incubated in the dark for 90min, then read with the EnVision spectrophotometer at Ex/Em of 485nm/535nm. EC₅₀s were calculated with the robust investigation of screening experiments (RISE) with four-parameter logistic equation.

**Drug susceptibility assay on human cell lines**

BJ and HepG2 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured according to their recommendations. Cell culture media were purchased from ATCC. Cells were routinely tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). Exponentially growing cells (BJ:1000 cells/25
μl/well; HepG2: 400 cells/25 μl/well), were plated in Corning 384 well white custom assay plates and incubated overnight at 37º C in a humidified, 5% CO2 incubator. DMSO inhibitor stock solutions were added the following day to a maximum final concentration of 25 μM, 0.25% DMSO and then diluted 1/3 for a total of ten testing concentrations. Cytotoxicity was determined following a 72 hr incubation using Promega CellTiter Glo Reagent according to the manufacturer’s recommendation. Luminescence was measured on an Envision plate reader (Perkin Elmer).

**Data analysis**

Dose-response curves were calculated from normalized percent activity values and log10-transformed concentrations using the proprietary Robust Interpretation of Screening Experiments (RISE) application written in Pipeline Pilot (Accelrys, v. 8.5) and the R program (33) (http://www.R-project.org/). Briefly, non-linear regression was performed using the R drc package with the four-parameter log-logistic function (LL2.4) (34). The median value from replicates for each compound was fit three separate times by varying the parameters that were fixed during regression: (1) all parameters free, (2) high response fixed to 100, (3) low response fixed to 0. The best fit from these three nested models was selected using the anova.drc function. Confidence intervals of 95% were produced based on this fit. Dose-response curves were assigned a quality score according to the following heuristic. Compounds that failed to fit to any curve, or with curves having efficacy <25% or >150% or hill slope <0.5 or >25 were designated class ‘D1’. Compounds passing this first criteria with curves having efficacy <50%, calculated EC50 > the highest concentration tested, lower and upper EC50 confidence limits > 10-fold EC50, or slope at the highest concentration tested >75% (non-saturating) were designated class ‘C1’. Compounds passing previous criteria with curves having lower and upper EC50 confidence limits >5-fold EC50 or slope at the highest concentration tested >25% (not completely saturating) were
designated class ‘B1’. All remaining curves were designated ‘A1’, which is indicative of ideal, well-behaved sigmoidal response. In general only A-class curves were assigned potencies for this manuscript. Curves that were inverted (activity decreased as concentration increased) were prefixed with the letter ‘N’, such as ‘NA1’. In tabulating data, a single EC$_{50}$ was reported only for A1 and B1 class curves. C1 and D1 curves were assigned an arbitrary value of greater than the highest concentration tested.

Results

Primary screening with anti-asexual compounds

For primary screening, 260 anti-malarial compounds were selected from 309,474 compounds in the St. Jude chemical library tested against asexual \textit{P. falciparum} parasites (5). These 260 compounds inhibited asexual growth $>$80% at a concentration of 2 $\mu$M in the original screen. To evaluate their efficacy against late stage III-V gametocytes, they were tested at a single concentration (10 $\mu$M), and 24 compounds were found to decrease viability to $<$55% (Fig. 1 & Supplemental Table S1). Importantly, gametocytes were insensitive ($>$80% viability) to the majority of the 260 anti-asexual compounds (200/260) demonstrating the distinct biology of late stage gametocytes as well as the specificity of the assay for gametocytes (Fig. 1 and Supplemental Table S1). The library included 19 compounds that have targets previously shown not to affect gametocyte viability (dihydroorotate dehydrogenase, dihydrofolate reductase, cytochrome \textit{bc}$\_1$ complex or hemozoin formation) (5, 35) and only one of these, bisphenylbenzimidazole (SJ000111341), an inhibitor of hemozoin formation, decreased gametocyte viability to $<$22% (Supplemental Fig. S1). Twelve of the 260 compounds were included in the malaria box and none of these decreased viability to $<$70% (Supplemental Table
In all, derivatives from just 3 molecular scaffolds, DANQ, IBI, and BPBI decreased gametocyte viability to <22% at 10 µM. The 8 most effective compounds were DANQ derivatives and will be discussed separately below. Based on the first screen, 390 additional compounds were selected as structural analogs to five chemotypes (Supplemental Table S2). Fifteen derivatives, at 6.25 µM, decreased viability to <55% (Supplemental Table S2). In total, the structure activity relationship of 479 unique compounds were analyzed, 22 (4.6%) of which decreased gametocyte viability to <50% (Table 1, Supplemental Fig. S2).

Gametocytocidal activity and human cell cytotoxicity of DANQ derivatives

In the initial 260 compound screen, the 8 most potent compounds were all DANQ derivatives (Supplemental Table S1). However, the fluorescent signals were lower than positive control wells that contained 30 nM epoxomicin, a potent gametocytocidal agent, raising concern that the compounds were affecting the alamarBlue indicator. Consequently, a series of wash steps were included in the protocol and two approaches were taken to confirm the gametocytocidal activity of the compounds (Fig 2). Gametocyte viability was tested immediately after drug addition using the modified alamarBlue protocol that included wash steps and a new flow cytometry protocol using a membrane-potential sensitive dye (MitoProbe DiIC1(5)) (Fig 2). In contrast to the 24 h incubation period needed for alamarBlue, MitoProbe DiIC1(5) staining only requires 30 minutes allowing more rapid screening of gametocyte viability. The results indicate that gametocyte viability remains high 12 hrs after drug addition and then gradually decreases until viable gametocytes are no longer detected at 72 hrs. At 24 hrs both assays detected a ~20-30% reduction in viability for SJ000030570 (71±3% viability, alamarBlue & 80±5% viability DiIC1(5)) & SJ000024933 (70±2% viability, alamarBlue & 71±6% viability DiIC1(5)), indicating that following the wash steps at early time points alamarBlue could detect viable gametocytes even when using high DANQ concentrations SJ000030570 (18 µM) &
SJ000024933 (23 µM). In contrast, at 72 hrs none of the gametocytes were viable in either assay or Giemsa-stained smears. The time course of gametocyte elimination was similar for SJ000030570, SJ000024933 and epoxomicin and this modified alamarBlue protocol was then used to determine the structure-activity relationship of the DANQ scaffold using 21 DANQ derivatives (Table 2, Fig 3). The asexual parasite EC\textsubscript{50}s were also determined using the SYBR green assay (Table 2). Derivative SJ000030570 showed the best potency against both gametocytes and asexual stage parasites (Gametocytocidal activity, EC\textsubscript{50} = 0.061 µM) (Table 2), while three additional derivatives had EC\textsubscript{50}s of 0.1 µM. The dose response curves are shown in Supplemental Fig S3.

All 21 DANQ derivatives were also assayed for cytotoxicity against 2 mammalian cell lines, BJ and HepG2 (Table 2). Four of the five most effective compounds (SJ000030570, SJ000024933, SJ000022283, SJ000024948, SJ000032726) were over 55-fold more potent against gametocytes than BJ or Hep2G cells with the most effective compound, SJ000030570, demonstrating 180- and 80-fold selectivity, respectively. These five compounds also had nanomolar activity against asexual parasites (Table 2) indicating a potential to be used to both treat patients and block malaria transmission. However, there was poor correlation between the anti-gametocyte and anti-axial potency of the compounds suggesting different modes of action in these two intraerythrocytic parasite stages (Table 2).

**Structure/activity relationship DANQ derivatives**

Analysis of the structures of gametocytocidal DANQ derivatives shows two structural variations of the DANQ scaffold that display gametocytocidal activity (Fig. 3 and Table 2). The compounds with the most potent activity are analogs of asexual lead compound SJ000030570, which are defined by an acetyl group on the aniline nitrogen and a dimethylamine moiety (Fig. 3A). For this series, anti-malarial activity is retained with either electron-donating...
substitutions on the aniline ring, suggesting the electronics of this ring are not important. The tertiary amide DANQ scaffold retained potency when the dimethylamine was replaced with a morpholine moiety (Fig. 3B). For the morpholine series, the hydrocarbon chain length of the aniline ether significantly influenced gametocytocidal activity: reduction from a five carbon chain (SJ00032726) to a four carbon chain (SJ00032719) decreased activity 10-fold for the morpholine analogs. Hydrocarbon chain length did not have a consistent effect on potency against asexual stage parasites. A fluorine substitution in the \textit{para} position on the aniline (SJ000001054) was found to retain gametocytocidal and asexual activity, while replacement of the aniline with an alkyl substituent negatively affected both gametocytocidal and asexual activity as shown by SJ000154238.

A second DANQ scaffold characterized by a non-acetylated secondary amine aniline nitrogen with a piperidine moiety as the second amine substituent also displayed activity against both sexual and asexual stage parasites (Fig. 3C). Following the previously described series, gametocytocidal compounds with long-chain alkyl ether substitutions in the \textit{para} position of the aniline (SJ000032721 and SJ000032714) were identified. In addition, analogs containing electron-withdrawing substitutions (SJ000294509, SJ000021272 and SJ000044720) and cyclohexane the substituted analog (SJ000294518) showed gametocytocidal activity. Another variation on the second series of the DANQ scaffold containing benzotriazole substitutions, SJ000244625 and SJ000244627, also provided compounds with gametocytocidal and asexual potency (Table 2).

During the lead optimization of SJ000030570 it was observed that compounds containing dialkylamine moieties display an inherent sensitivity to light. In the case of dimethylamine analogs (Fig 3A), prolonged exposure to light results in the photolysis of one of the methyl groups on the amine resulting in the formation of the monomethylamine analog SJ000541602.
Furthermore, this analog is capable of cyclizing to form the corresponding imidazolium analog SJ000561981. The resulting secondary amine and the corresponding imidazolium have shown to be more stable to light and provide elevated levels of antimalarial activity against asexual stages of malaria. To test this directly, SJ000030570 and two photostable derivatives were re-evaluated taking precautions to decrease light exposure. In both the alamarBlue and MitoProbe DiIC1 assay light-protected SJ0000305070 (EC\textsubscript{50} 0.319 ± 0.050 µM and 0.235 ± 0.093 µM, respectively) was less active than derivatives SJ000561981 (EC\textsubscript{50} 0.093 ± 0.027 µM and 0.090 ± 0.064 µM, respectively) and SJ000541602 (EC\textsubscript{50} 0.220 ± 0.034 µM and 0.240 ± 0.041 µM, respectively) when tested against stage III-V gametocytes (Fig. S3). Importantly, all three compounds completely inhibited exflagellation; the derivatives at concentrations >0.1 µM and light-protected SJ0000305070 at >0.3 µM concentrations, confirming the biological activity of these compounds.

Validation of the activity data with light protected SJ000030570 and its photostable analogs has validated the gametocytocidal activity of the DANQ series. However, the potential exists for the other active compounds, which contain a dialkylamine moiety, to be partially degraded at the time of analysis. Therefore, the reported activity for these compounds may be overestimated by the presence of more potent degradation products. Conversely, the potency may be underestimated by the degradation of the active constituent. Moving forward, further analysis will be conducted on analogs from the photostable series.

**IBI activity and cytotoxicity**

A second class of compounds currently being investigated for anti-sexual activity also consistently inhibited late stage gametocyte viability (Table 1 & Supplemental Fig S2). Over half of the five 2-imino-benzo[d]imidazole derivatives (IBI) tested had >50% gametocytocidal activity with EC\textsubscript{50} ranging between 1 - 4 µM. The most effective compound (SJ000016864) had
a similar EC\textsubscript{50} against asexual parasites and was > 44-fold less toxic to the BJ human fibroblast
cell line. Additional compounds from this promising chemotype will have to be screened to
better define the structure activity relationship.

Discussion

Fifteen compounds with gametocytocidal activity in the nanomolar range were identified
in a screen of a total of 650 compounds, including 260 lead-like anti-malarial compounds
discovered in a whole cell screen against the asexual stages of the parasite life cycle. These 15
gametocytocidal compounds were derived from just 2 scaffolds: 14 were DANQ derivatives and
one was an IBI. The most effective compound, SJ000030570, was initially found to be >100-fold
more effective against gametocytes than the BJ human fibroblast cell line (EC\textsubscript{50} >11 µM). In fact
all fourteen DANQ compounds had a therapeutic window >7 times when compared to BJ cells.
DANQ is one of the 3 scaffolds selected from the St. Jude Chemical Library for lead
optimization as new anti-malarials and possesses the best gametocytocidal potency of the 3 lead
compounds (30).

Each of these 3 anti-malaria leads [DANQ, DHP and dihydroisoquinoline (DHIQ)], as
well as IBI, are hypothesized to have novel mechanisms of action because they are structurally
distinct from previous anti-malarials and do not inhibit or bind to known asexual targets
including PfDHOD, PFDHFR, cytochrome \textit{bc}\textsubscript{1}, falcipain 2 or hemozoin (5). In contrast to the
DANQs, the hydroxynaphthoquinone, atovaquone, inhibits cytochrome \textit{bc}\textsubscript{1} and does not reduce
gametocyte viability even at 10 µM (36). The dual anti-asesual and gametocytocidal activity of
DANQ and IBI suggest they interfere with pathways that are essential for both these
intraerythrocytic stages, while DHP and DHIQ target critical asexual-specific pathways.
However, the lack of correlation between the asexual and gametocytocidal potency of the DANQ
derivatives suggests their modes of action may differ in these two intraerythrocytic parasite
stages. The structural differences between the DANQ derivatives could directly influence the binding of the compound to a specific target or alter access of the compound to the parasite or host red blood cell. RBC permeability has been shown to be enhanced in asexual-infected RBCs, but not gametocyte infected RBCs (19, 20), and this difference could lead to differential uptake of distinct compounds. Marked phenotypic and transcriptomic differences exist between the two life cycle stages (37-41). For example, late stage female gametocytes contain a large set of translationally repressed transcripts that are not expressed until the gametocyte is taken up in a blood meal by a mosquito (42). A number of *P. falciparum* genes also have stage-specific homologues, including diaminopeptidase (DPAP2) and plasmepsins (VI-IX) (39, 41). It is possible that homologues expressed at different stages could have subtly different affinities for compounds such as the DANQ derivative series that result in different activity profiles. The presence of distinct targets in different parasite stage would also suggest that both genes would have to acquire mutations for the parasite to become completely resistant to the compound.

The lack of gametocytocidal activity of the majority of the anti-asexual compounds tested (237/260, 91%) further demonstrates the distinct sensitivities of late stage gametocytes and asexual parasites and confirms the gametocyte specificity of the assay. As previously reported, pathways involved in hemoglobin digestion, hemozoin formation, DNA replication, apicoplast activity and increased RBC permeability were shown not to be essential for gametocyte maturation (14, 17). Elucidating the mechanisms of action of these 237 novel anti-asexual specific compounds will further increase the understanding of pathways required for asexual growth, but not gametocyte viability. In contrast, the targets of DANQ and IBI are expected to be required for the viability of both asexual and sexual stage parasites. Both stages develop within the confines of an erythrocyte, and in silico profiling of proteomic data into broadly defined functional classes indicate the presence of common pathways, including glutathione metabolism.
and protein expression and degradation (43). Additional screening of the remaining compounds from the St. Jude chemical library without asexual activity will be of interest to reveal gametocyte specific compounds and their corresponding targets.

In summary, two scaffolds, DANQ and IBI that effectively block both asexual growth and late stage gametocyte viability have been identified. One of the DANQs, SJ000030570, has already been selected for anti-malaria lead optimization (30) resulting in the identification of two photo stable analogs (SJ000541602 and SJ000561981) that were also found to have potent gametocytocidal activity. In contrast, 625 other novel compounds were inactive against late stage gametocytes (>50% viability). Differences between asexual and sexual stage parasites were also observed in the structure-activity analysis of DANQ derivatives, as well as the other 6 chemotypes that had measurable activity against both parasite stages. Whether these structural differences reflect stage-specific targets or access to the parasite remain to be determined. The results clearly demonstrate the need to test both asexual and sexual stages to identify compounds with the potential to inhibit the symptoms and spread of malaria.

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References


Figure Legends

Figure 1: Gametocyte viability.

Late stage gametocyte viability after incubation with the indicated compounds (10 µM) was assayed using fluorescent indicator, alamarBlue as described in Methods. Gametocyte viability is presented as the percent of carrier (DMSO) only control signal after subtracting the background fluorescence signal remaining after treatment with 30 nM epoxomicin. The dashed green line indicates 80% gametocyte viability, the red line indicates 55% gametocyte viability and the dashed red line indicates 22% gametocyte viability.

Figure 2: Gametocytocidal activities of DANQ derivatives
Cultures were assayed for viability using flow cytometry (solid bars), alamarBlue fluorescence (striped bars) (A) and Giemsa-stained blood smears (B) at the indicated times after the addition of epoxomicin (light gray bar), SJ000024933 (blue bars), and SJ000030570 (dark gray bars). The data is presented as the percent of the DMSO vehicle control value.

Figure 3: DANQ Structure-Activity analysis

The gametocytocidal EC₅₀s of three series of DANQ derivatives were determined using the alamarBlue viability assay to allow the comparison of chemical structure and activity.
Table 1. Gametocytocidal activities of selected chemotypes. The chemotype (group) and backbone structure (backbone) are listed in addition to the number of derivatives of each chemotype that were tested and reduced gametocyte viability <50% or 50-70%.

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<th>Group</th>
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<th>Tested</th>
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<th>50%-70% viability</th>
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</table>
Table 2. Biological activities of 1,4-dioxo-1,4-dihydronaphlene (DANQ) derivatives.

| SJ000001054 | O | O | F | 0.077 | 0.44 | 3.14 | 3.87 |
| SJ000030569 | O | O | H | 5.6   | 4.29 | >26.0 | 22.6 |
| SJ0000327219 | O | O | H | 0.220 | 1.84 | >31.2 | 3.89 |
| SJ000032726 | O | O | H | 0.072 | 0.18 | >36.1 | 22.5 |
| SJ000032725 | O | O | H | 0.18  | 0.98 | >25.3 | 9.76 |
| SJ000032718 | O | O | H | 0.459 | 0.80 | >28.4 | 13.7 |
| SJ000019400 | O | O | H | 0.014 | 6.67 | >29.2 | >29.2 |
| SJ000021272 | O | O | I | 0.052 | 0.74 | >20.0 | >20.0 |
| SJ000294509 | O | O | H | 0.021 | 0.95 | >25.9 | 21.8 |
| SJ000022283 | O | O | H | 0.057 | 0.83 | >20.0 | >20.0 |
| SJ000024948 | O | O | H | 0.030 | 0.10 | 3.83  | 0.71 |
| SJ000030570 | O | O | H | 0.032 | 0.061 | >30.0 | >26.0 |
| SJ000244625 | O | O | H | 0.006 | 0.88 | >24.8 | >24.8 |
| SJ000244627 | O | O | H | 0.038 | 0.6  | >24.5 | >24.5 |

Continued on next page
Table 2. Biological activities of 1,4-dioxo-1,4-dihydronaphlene (DANQ) derivatives.

<table>
<thead>
<tr>
<th>DANQ</th>
<th>R</th>
<th>R₁</th>
<th>R₂</th>
<th>Asex EC₅₀ (µM)</th>
<th>Gcyt EC₅₀ (µM)</th>
<th>Cytotoxicity EC₅₀ (µM)</th>
<th>BJ</th>
<th>HepG2</th>
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<tr>
<td>SJ000044720</td>
<td>-N</td>
<td>-H</td>
<td>-H</td>
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<tr>
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<td>O</td>
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</table>
Figure 1: Gametocyte viability. Late stage gametocyte viability after incubation with the indicated compounds (10 μM) was assayed using fluorescent indicator, alamarBlue as described in Methods. Gametocyte viability is presented as the percent of carrier (DMSO) only control signal after subtracting the background fluorescence signal remaining after treatment with 30 nM epoxomicin. The dashed green line indicates 80% gametocyte viability, the red line indicates 55% gametocyte viability and the dashed red line indicates 22% gametocyte viability.
Gametocytocidal activity of DANQ derivatives

Figure 2: Gametocytocidal activities of DANQ derivatives. Cultures were assayed for viability using flow cytometry (solid bars), alamarBlue fluorescence (striped bars) (A) and Giemsa-stained blood smears (B) at the indicated times after the addition of epoxomycin (light gray bar), SJ000024933 (23 μM, blue bars), and SJ000030570 (18 μM, dark gray bars). The data is presented as the percent of the DMSO vehicle control value.
Figure 3: DANQ Structure-Activity analysis. The gametocytocidal EC50s of three series of DANQ derivatives were determined using the alamarBlue viability assay to allow the comparison of chemical structure and activity.