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Discovery of Potent Small-Molecule Inhibitors of Multidrug-Resistant *Plasmodium falciparum* Using a Novel Miniaturized High-Throughput Luciferase-Based Assay▽†

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Malaria is a global health problem that causes significant mortality and morbidity, with more than 1 million deaths per year caused by *Plasmodium falciparum*. Most antimalarial drugs face decreased efficacy due to the emergence of resistant parasites, which necessitates the discovery of new drugs. To identify new antimalarials, we developed an automated 384-well plate screening assay using *P. falciparum* parasites that stably express cytoplasmic firefly luciferase. After initial optimization, we tested two different types of compound libraries: known bioactive collections (Library of Pharmacologically Active Compounds [LOPAC] and the library from the National Institute of Neurological Disorders and Stroke [NINDS]) and a library of uncharacterized compounds (ChemBridge). A total of 12,320 compounds were screened at 5.5 μM. Selecting only compounds that reduced parasite growth by 85% resulted in 33 hits from the combined bioactive collection and 130 hits from the ChemBridge library. Fifteen novel drug-like compounds from the bioactive collection were found to be active against *P. falciparum*. Twelve new chemical scaffolds were found from the ChemBridge hits, the most potent of which was a series based on the 1,4-naphthoquinone scaffold, which is structurally similar to the FDA-approved antimalarial atovaquone. However, in contrast to atovaquone, which acts to inhibit the bc_1 complex and block the electron transport chain in parasite mitochondria, we have determined that our new 1,4-napthoquinones act in a novel, non-bc_1-dependent mechanism and remain potent against atovaquone- and chloroquine-resistant parasites. Ultimately, this study may provide new probes to understand the molecular details of the malaria life cycle and to identify new antimalarials.

The number of episodes of malaria worldwide is estimated to be around half a billion per year (30). Malaria caused by the parasite *Plasmodium falciparum* results in approximately 1 to 3 million deaths per year, mostly among young children under the age of 5. The recent and continuing emergence of parasite strains resistant to antimalarials is a major challenge and has been directly linked to the increase in the global mortality associated with this disease (29). As such, the discovery and development of novel antimalarial therapeutics is essential.

The development and pursuit of chemical high-throughput screening (HTS) methods can provide new bioactive molecules against the malaria parasite and also may help identify new "druggable" targets for future therapeutic development. HTS can utilize two basic approaches: (i) biochemical target-based screens or (ii) whole-organism-based screens. Biochemical screens must have a predefined target, a protein that typically is necessary for parasite survival. These targets normally must be recombinantly expressed (or purified from native sources) and utilized in a robust activity or binding assay to screen for inhibitors. One drawback in biochemical screening is that small-molecule hits may not be able to reach their protein target in a live cell assay because of poor cell permeability or the presence of drug efflux pumps. Whole-organism screening is advantageous because all small-molecule hits act directly against a live cell, in this case a parasite, and all possible targets expressed in that cell are included in a single screen. However, frequently the target of each hit is unknown and requires identification, which may involve complex purification protocols and/or genetic validation.

Currently, there are several methods used to screen for parasite viability in culture. To date, the main methods for detecting parasite growth are based on a measurement of DNA content. The most widely used growth assay is the [^{3}H]hypoxanthine incorporation assay (9). This assay relies on the incorporation of tritiated hypoxanthine into DNA during the development of daughter parasites. Several groups have adapted the use of fluorescent DNA stains such as SYBR green or 4′,6′-diamidino-2-phenylindole (DAPI) to measure

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parasite growth (1, 7, 19, 26, 28). A shortcoming of these fluorescence-based DNA detection screens is the modest signal-to-background ratio of 3- to 9-fold, which may result in a high number of false positives and false negatives. In addition, flow-cytometric assays have been developed that rely on measuring the DNA content of parasitized red blood cells (RBCs) using a fluorescent DNA stain such as propidium iodide or YOYO (23, 35). One shortcoming currently is that these flow cytometry-based assays are difficult to scale up for the HTS of many thousands of compounds.

Given this historical reliance on DNA-based screens to quantify parasite content, we set out to develop a new screen with optimal properties for high-throughput screening. We used a transgenic strain of parasites stably expressing cytoplasmic firefly luciferase (3D7-luc) (18) to measure the growth of intracellular parasites during their erythrocytic phase using luminescence as the readout. This luciferase assay is similar to one previously described (8). However, we now have adapted this luciferase-based assay to a 384-well plate HTS, providing a novel tool for *P. falciparum* screening that displays a high dynamic signal-to-noise range compared to that of traditional assays. We tested two compound collections, the Library of Pharmacologically Active Compounds (LOPAC) and the library from the National Institute of Neurological Disorders and Stroke (NINDS), that have known targets and/or confirmed bioactivity. We also used a collection of uncharacterized compounds from the ChemBridge DiverSet library to facilitate the discovery of novel antimalarial scaffolds. The ChemBridge library contains small sets of analogs based on common pharmacophores to aid in the determination of structure-activity relationships (SAR).

In this study, we report the screening of 12,320 compounds against the erythrocyte life cycle of the *P. falciparum* parasite using our luciferase-based assay. A number of compounds containing novel drug scaffolds were identified with potent activity against both standard drug-sensitive 3D7 (although somewhat resistant to sulfadoxine [34]) and a multidrug-resistant parasite line, TM90C2B.

MATERIALS AND METHODS

Parasite culture. 3D7 *P. falciparum* parasites expressing firefly luciferase under the control of the histidine-rich protein II (HRPII) promoter were obtained from Kirk Deitsch (Cornell University). *P. falciparum* parasites were cultured in complete medium consisting of RPMI 1640 medium containing L-glutamine (Invitrogen) supplemented with 50 mg/liter hypoxanthine (Sigma), 25 mM HEPES (Invitrogen/Gibco), 10 mg/liter gentamicin (Invitrogen/Gibco), 25 mM sodium bicarbonate (Invitrogen), and 0.5% albumax II (Invitrogen). Packed human RBCs were obtained from Biological Specialty Corporation (Colmer, PA), and the blood type was varied from week to week. Parasites were cultured in 4% hematocrit at 37°C with gas composed of 5% O_2, 5% CO_2 and was balanced with N_2 in a modular incubator chamber (Hot Box system; Billup-Rothernberg Inc.). Parasite line TM90C2B was a kind gift from Dennis Kyle (Department of Global Health, College of Public Health, Tampa, FL).

Compound libraries. The Penn Center for Molecular Discovery (PCMD) at the University of Pennsylvania provided the compound libraries used in this study. The LOPAC consists of 1,280 compounds from Sigma Aldrich with known activities classified as follows: cell signaling (9%), phosphorylation (8%), cell stress (4%), lipids (4%), ion channels (6%), G proteins (3%), apoptosis/cell cycle (2%), gene regulation (3%), hormone related (3%), and neuroscience related (58%). Compounds were stored at a concentration of 2 mM in 10 μl of dimethylsulfoxide (DMSO) in four 384-well plates. The NINDS library consists of 1,040 compounds from MicroSource Discovery Systems, Inc. Compounds were stored at a concentration of 2 mM in 10 μl of DMSO in four 384-well plates. The ChemBridge DiverSet is a commercially available compound library consisting of 10,000 diverse drug-like small-molecule compounds, covering a broad set of biologically relevant pharmacophore diversity spaces. Compounds were stored at a concentration of 2 mM in 10 μl of DMSO in 32 384-well plates.

HTS luciferase assay. Parasites expressing cytoplasmic luciferase were synchronized at the ring stage using 5% sorbitol solution (Sigma) for at least two cycles prior to the assay. The assay was optimized to use a starting parasitemia of 1% of late-ring-stage parasites at 3% hematocrit. Complete culturing medium (10 μl) was dispensed under sterile conditions using a Wellmate microplate dispenser (Matrix Technologies) into a 384-well white flat-bottom polystyrene well plate (Greiner 781080). An EP3 workstation (Perkin Elmer) then was used to pin tool 110 nl of compounds (from a stock plate at 2 mM in DMSO) into 10 μl of complete culturing medium in the assay plate for a final concentration of 5.5 μM. Subsequently, a volume of 30 μl of *P. falciparum*-infected RBCs was dispensed into each well of the 384-well plate in all columns (except columns 1 and 23, which served as the background) for a final hematocrit of 3%. In columns 1 and 23, 30 μl of complete medium containing a final hematocrit of 3% uninfected RBCs was dispensed to calculate the background signal. Assay plates then were covered with a Breath-Easy seal (Diversified Biotech) and a Kalypsys metal lid (Kalypsys, Inc.) and incubated for 48 h (approximately one life cycle) under controlled gas conditions as described above at 37°C. To assay the plates, the metal lid and seal were removed manually, and 40 μl of Bright-Glo luciferase substrate (Promega) was dispensed into each well. The plates were centrifuged at 200 rpm for 30 s, and luminescence was read in an EnVision 2102 Perkin Elmer luminometer. Using IDBS ActivityBase, the percent inhibition was calculated for each compound from the mean luminescent signal for each treated sample (designated signal), the mean luminescence of untreated, infected RBCs (control), and the mean luminescence of uninfected RBCs (background) using the equation % inhibition = $100 \times \{1 - [(\text{signal mean} - \text{background mean})/(\text{control mean} - \text{background mean})]\}$. A hit cutoff of 85% inhibition at 5.5 μM was defined.

Compound hit validation. For compounds scored as hits from the high-throughput screen, active compounds were cherry-picked and tested in dose-response tests (16 2-fold dilutions from 17 μM to 0.5 nM) in 384-well plates using the protocol described above. Fifty percent inhibitory concentrations (IC_{50}s) were determined by nonlinear regression (four-parameter logistic fit) using XLfit (IDBS, Guildford, United Kingdom). In addition, all validated hits from the screening were counterscreened to determine if the hits directly inhibited luciferase activity by using purified luciferase detected with CellTiter-Glo (Promega). The compounds were pintooled as described above in dose-responses into 10 μl of culture medium with 10 nM ATP and luciferase and incubated for 5 min, and then 10 μl of CellTiter-Glo was added. Resveratrol (Sigma), a known luciferase inhibitor, was used at 273 μM as a positive control. Luminescence was read on the EnVision microplate reader.

Resistant parasite drug assays. Secondary screening to determine the efficacy of confirmed hits against the resistant parasite line TM90C2B was assessed by the measurement of fluorescence after the addition of SYBR green I as previously described by Smilkstein et al. (28). Drug IC_{50}s were calculated from the log of the dose-response relationship and fit with Grafit software (Erithacus Software, Kent, United Kingdom). Results are given as the means from at least three separate experiments.

Preparation of *P. falciparum* cell-free extracts. Parasite extracts were prepared by saponin lysis and freeze-thawing as described previously (2, 3).

Bovine mitochondrial membrane preparation. Bovine mitochondrial membranes (Keilin-Hartree particles) were prepared as described by Kuboyama et al. (21).

Measurement of bc_1 activity. Cytochrome *c* reductase activity measurements were assayed in 50 mM potassium phosphate, pH 7.5, 2 mM EDTA, 10 mM KCN, and 30 μM equine cytochrome *c* (Sigma Chemical, Poole, Dorset, United Kingdom) at room temperature (12, 13). Cytochrome *c* reductase activity was initiated by the addition of decylubiquinol (50 μM). The reduction of cytochrome *c* was monitored in a Cary 4000 UV-visible spectrophotometer (Varian, Inc., Palo Alto, CA) at 550 and 542 nm. Initial rates (computer fitted as zero-order kinetics) were measured as a function of the decylubiquinol concentration. Decylubiquinol was prepared based on the method of Fisher et al. (13). Inhibitors of bc_1 activity were added without prior incubation. DMSO in the assays did not exceed 0.3% (vol/vol). IC_{50}s were calculated using the four-parameter logistic method (Grafit).

NADH:ubiquinone oxidoreductase activity. Selected compounds were screened for inhibitory activity against the *P. falciparum* respiratory enzyme type II NADH:ubiquinone oxidoreductase (PfNDH2). Membrane preparations of transformed *Escherichia coli* F571 containing recombinant PfNDH2 were prepared and assayed as described by Fisher et al. (11). Inhibitors were added without prior incubation. DMSO in the assays did not exceed 0.3% (vol/vol).

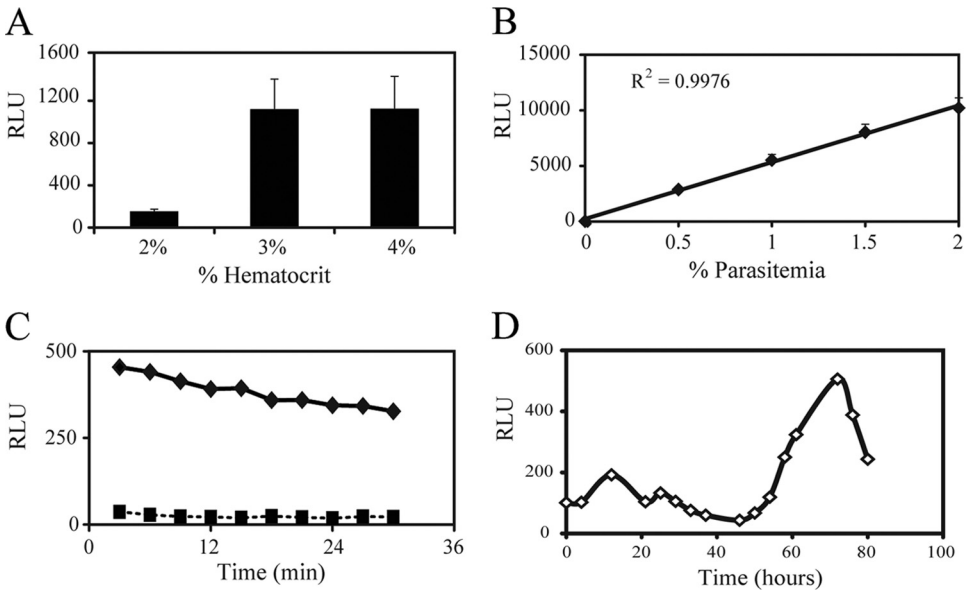


FIG. 1. *P. falciparum* luciferase assay optimization. The *y* axis shows the signal in luminescence units. (A) Optimal hematocrit concentration determination using the luciferase assay at different levels of hematocrit (2 to 4%). (B) Linear regression of luciferase production at different levels of parasitemia (0 to 2%). (C) Stability of luminescence reading following the addition of BrigthGlo. Symbols: ◆, signal (infected RBC); ■, background (noninfected RBC). (D) Determination of highest expression of luciferase during the erythrocytic life cycle (0 to 80 h) of *P. falciparum* (◇).

Cytotoxicity assay. A cellular cytotoxicity assay was conducted with human aortic endothelial cells (HAEC) and human hepatocellular liver carcinoma cells (HepG2) as previously described (27) to measure viability after 24 h of incubation.

RESULTS

Development of 384-well plate HTS assay. *P. falciparum* has a 48-h intraerythrocytic life cycle comprised of three major phases: the ring, trophozoite, and schizont phases. In this study, a *P. falciparum* parasite line expressing the cytoplasmic firefly luciferase gene under the malarial HRPII promoter (which is active during the ring stage of the intraerythrocytic life cycle) was used in the HTS optimization and HTS screen. Parasites were allowed to grow in the presence of the inhibitor for an entire life cycle from the ring stage through egress and reinvasion, thus providing a readout for assessing compound effects on any part of the erythrocytic cycle.

To adapt this luciferase-based assay for small-molecule HTS using *P. falciparum*, we further optimized the parameters used in the 96-well plate format (not shown) to a 384-well plate format. To determine the suitability of the erythrocytic *P. falciparum* luciferase assay in the 384-well format, the hematocrit, parasitemia, and growing conditions were optimized. A satisfactory luminescence signal above the background was obtained using 3% hematocrit (Fig. 1A) and 1% parasitemia (Fig. 1B). We found a linear relationship between initial parasitemia levels and final luminescence values, indicating that luminescent signal in this assay provides a readout directly proportional to parasite growth over a range of standard parasitemia levels. Bright-Glo luciferase substrate provided stable detection for 30 min after the addition of the reagent (Fig. 1C). Using synchronized cultures, we determined the timing of maximal luciferase signal during the parasite life cycle. Maximum activity occurred at the late ring stage of parasite development, 18 to 20 h postinvasion (shown during the second cycle at 70 h after initial infection in Fig. 1D). Since the compound collections were dissolved in DMSO, we determined the maximum DMSO tolerance of the parasite to be approximately 0.2% (not shown).

To determine plate uniformity and to confirm the absence of edge effects, a quality-control plate was evaluated with parasites treated with DMSO only or a single concentration of artesunate (see Fig. S1A and B in the supplemental material). As a final validation of this assay, five known antimalarials were tested to determine their IC_{50}s using concentrations ranging from 0.17 nM to 5.5 μM (Table 1). The measured IC_{50}s corresponded well with known values in the literature (1).

We screened 12,320 compounds from the LOPAC, NINDS, and ChemBridge libraries. Statistics for the high-throughput screen using the three compound collections demonstrated a high signal-to-background (S/B; 147 to 430), low percent coefficient of variation (% CV), and acceptable Z factors of >0.6 in accordance with accepted standards for HTS campaigns (Table 2) (17, 18).

TABLE 1. IC_{50} activity of known antimalarials in *P. falciparum* luciferase assay[a]

Compound	3D7-Luc IC_{50} (nM)
Chloroquine	22 ± 1.0
Artesunate	3.3 ± 0.60
Mefloquine	2.1 ± 0.10
Dihydroartemisinin	0.60 ± 0.065
Atovaquone	0.41 ± 0.021

[a] Data shown are means from three replicates ± standard errors of the means.

TABLE 2. Statistical parameters of HTS of compound libraries using *P. falciparum* luciferase assay

Library	No. of compounds	S/B	Z factor	% CV	Total hits of $IC_{50} < 20$ μM	Hit rate (%)
NINDS	1,040	338	0.76	7.6	16	1.5
LOPAC	1,280	430	0.64	9.8	17	1.3
ChemBridge	10,000	147	0.66	16.8	130	1.3

Bioactive collection analysis: LOPAC and NINDS HTS. Of the 1,280 LOPAC compounds screened, a total of 67 compounds displayed >85% inhibition of parasite growth within the 48-h treatment period (5.2% hit rate) in the primary HTS. Using dose-response testing to eliminate false positives, 17 compounds were confirmed active (1.3% hit rate for confirmed hits), with IC_{50}s ranging from 1 nM to 1.2 μM (Table 2). In a counterscreen, none of these 17 compounds displayed any direct inhibition of luciferase activity when tested at 10 μM. The detection of known antimalarial quinine derivatives as hits confirmed the validity of the HTS assay to detect active antimalarial compounds. Microtubule disruptors such as vincristine and vinblastine were scored as hits, as were protein translation inhibitors such as emetine. Other hits were similar to those found in a recent antimalarial HTS study (see Table S1 in the supplemental material) (36).

Out of 1,040 compounds screened from the NINDS library, a total of 87 compounds were active at >85% inhibition (8.4% hit rate in the primary HTS). The false-positive rate for this compound collection was higher, as only 16 hits were confirmed via IC_{50} retesting, resulting in a 1.5% confirmed hit rate (Table 2). These 16 compounds displayed no direct luciferase inhibitory activity when tested at 10 μM. One well-established antimalarial was found, cinchonine, a quinine derivative with an IC_{50} of 5.2 nM. Four additional compounds previously reported to show antimalarial activity were found: tyrothricin, pentamidine isothionate, astemizole, and hycanthone. Sulfamerazine is an antibacterial agent that we found to be hemolytic (not shown). The other 10 hits from this library were distributed into various classes, including antiinfective, antiinflammatory, and antifungal agents (see Table S1 in the supplemental material).

We further investigated two scaffolds from the bioactive collection that previously had been tested as drugs: amperozide and zolantidine (also found in a separate HTS screen [36]). Amperozide has been tested in humans and is currently in use as a veterinary drug to reduce aggression and stress in pigs (22). Zolantidine is an H2 antagonist (acid

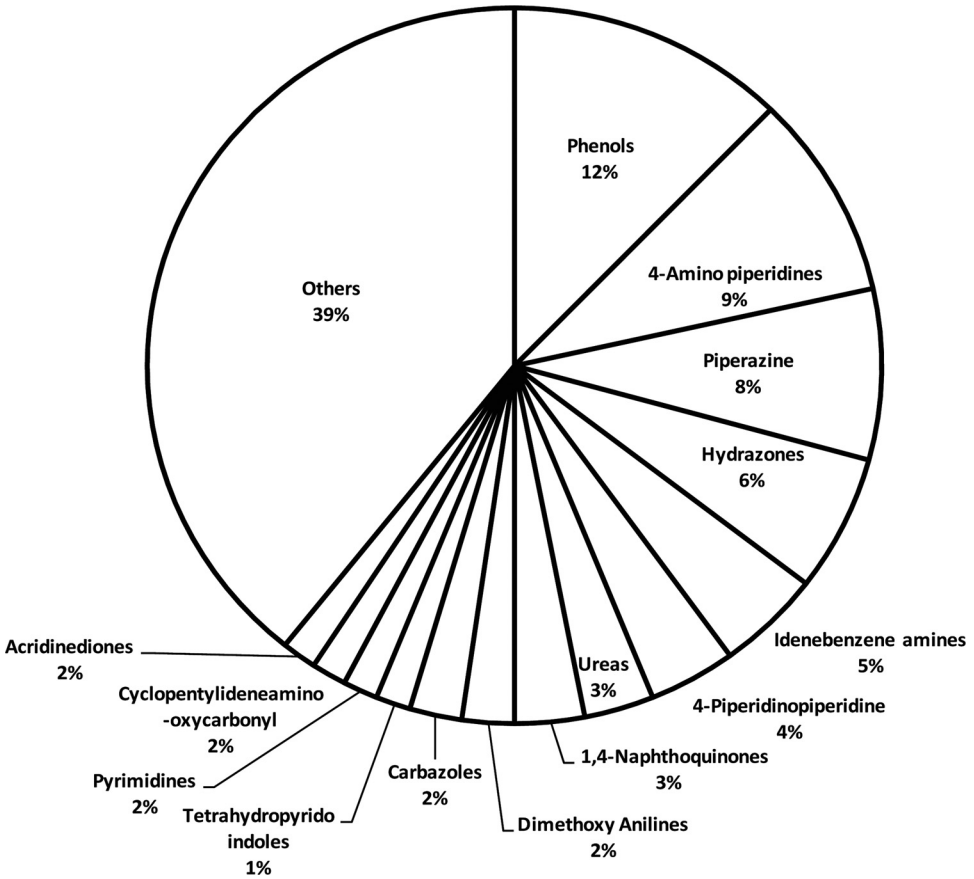
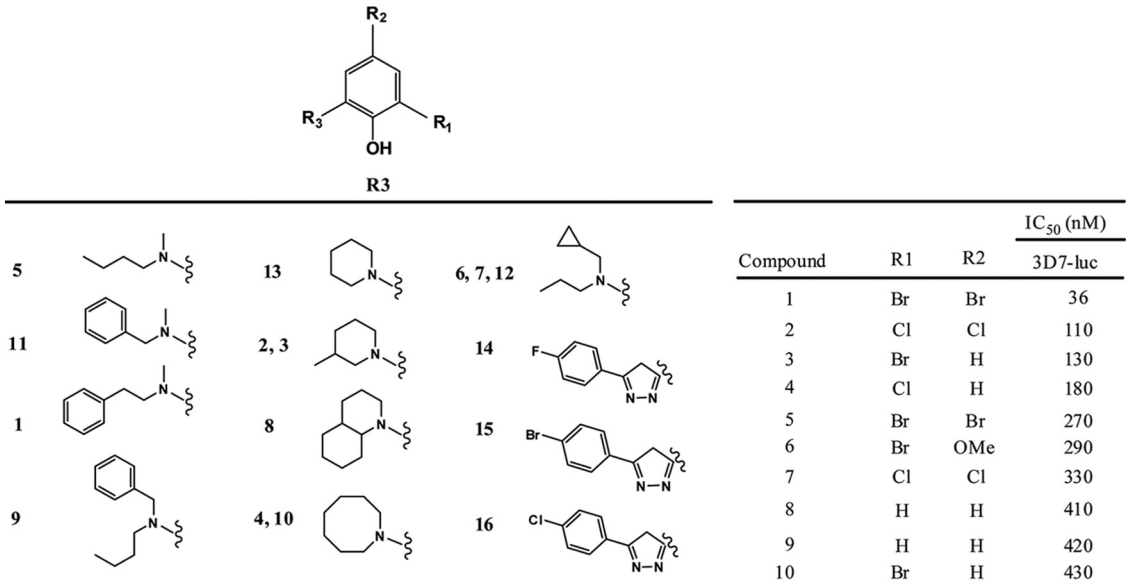


FIG. 2. Distribution of ChemBridge library hits obtained in HTS using the *P. falciparum* luciferase assay, clustered by scaffold and shown as percentages of the total 130 hits. Data shown are percentages of compounds with the assign scaffold obtained after the single measurement of compounds' IC_{50}s in confirmation screening.



Compound	R1	R2	IC_{50} (nM) 3D7-luc
1	Br	Br	36
2	Cl	Cl	110
3	Br	H	130
4	Cl	H	180
5	Br	Br	270
6	Br	OMe	290
7	Cl	Cl	330
8	H	H	410
9	H	H	420
10	Br	H	430
11	Br	Br	680
12	Br	Br	710
13	H	OMe	790
14	Cl	H	1,500
15	Me	H	4,700
16	Cl	H	15,000

FIG. 3. Antimalarial activity (IC_{50}) of confirmed hits with a phenolic scaffold. Data shown are from a single IC_{50} determination.

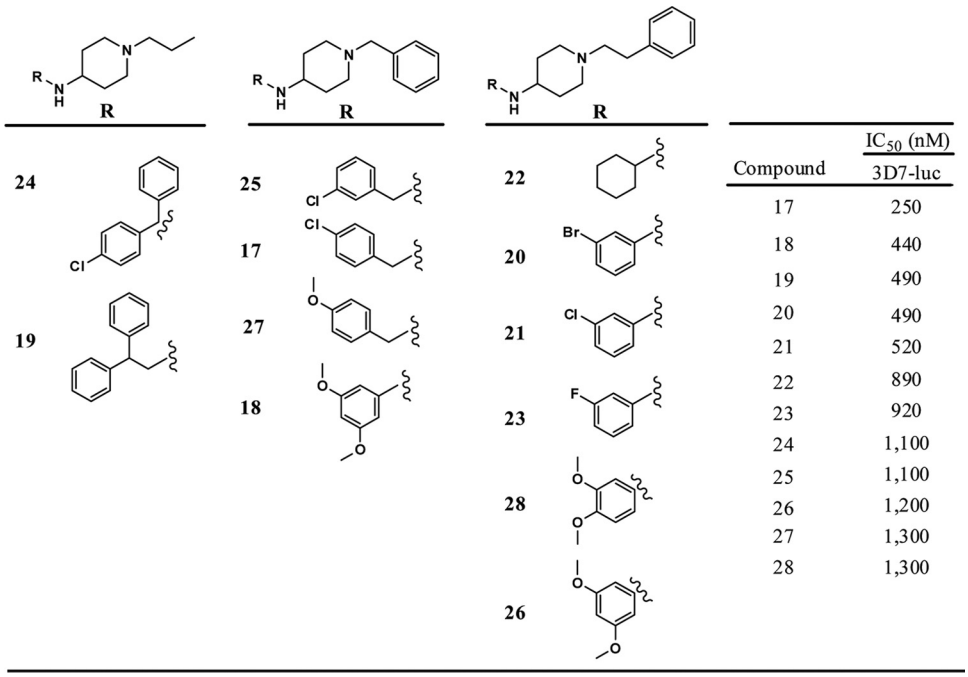
blocker). Although it is not clinically used, several related compounds are readily available as over-the-counter drugs. We then screened several available amperozide and H2 antagonists and found that the best amperozide analog had an IC_{50} of 770 nM, and that zolantidine was the most potent H2 antagonist against *P. falciparum*, with an IC_{50} of 660 nM (see Tables S2 and S3 in the supplemental material). The further optimization of these scaffolds would be necessary to pursue these inhibitors as antimalarials, but this is beyond the scope of this work.

Uncharacterized compound collection analysis: ChemBridge HTS. From the ChemBridge collection, dose-response testing confirmed the activity of 130 out of 10,000 compounds screened (1.3% confirmed hit rate). IC_{50}s ranged between 27 nM and 15 μM (see Table S4 in the supplemental material). None of the 130 compound hits displayed any luciferase inhibition when tested at 10 μM. To help facilitate the analysis of this library, the hits from the ChemBridge library screen were stratified by scaffold to identify structure-activity relationships. The structural classification of the 130 confirmed hits revealed two major clusters and a few minor clusters (Fig. 2).

Phenol cluster. A 2-aminomethylphenol moiety was found frequently in the screening hits; similar compounds have been reported previously to have antimalarial activity (Fig. 2) (4, 10, 32). This scaffold constitutes more than 50% of the top ChemBridge hits; activities ranged from 36 nM to 15 μM (Fig. 3). The top phenolic compounds varied in their substituent moieties in the 2, 4, and 6 positions around the phenolic ring. Compounds possessing bromines at positions R1 and R2 with an aromatic moiety at the R3 position were the most potent. It also is noteworthy that one of the LOPAC hits, the 5-HT1A serotonin receptor antagonist S(−)-UH-301, with an activity of 4.7 nM in the primary screening (see Table S1 in the supplemental material), possesses a similar structural motif.

4-Aminopiperidine cluster. A series of compounds containing a 4-aminopiperidine core showed good activity against *P. falciparum*. A total of 12 compounds with this scaffold had activity ranging from 0.25 to 1.3 μM (Fig. 4). While related compounds are known to have activity against plasmepsin 2 (24), none of the compounds found in our study have been reported as inhibitors of this enzyme. From the preliminary SAR, compounds with aromatic groups attached to the piperidine nitrogen were favored (Fig. 4).

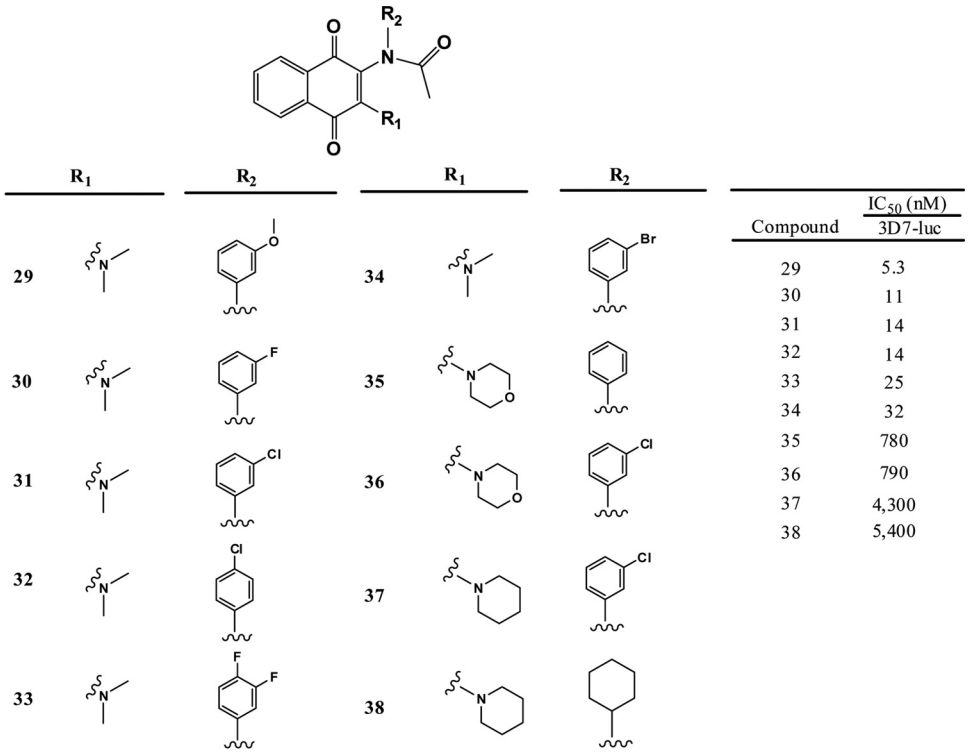
1,4-Naphthoquinone cluster. One of the most interesting clusters from this screen contained a 1,4-naphthoquinone scaffold, which is structurally similar to atovaquone, an FDA-approved antimalarial drug used in malarone. The best hit from the luciferase screen was a 2,3-diamino-1,4-naphthoquinone hit with an IC_{50} of 27 nM, while the other three compounds in this series had more moderate activities (1.8 and 2.7 μM) (see Table S1 in the supplemental material). This led us to further evaluate the 2,3-diamino-1,4-naphthoquinone scaffold. IC_{50}s were generated for repurchased compounds by using the luciferase assay with an additional 10 compounds with this core scaffold, which revealed a number of more potent hits (Fig. 5). The best hit



Compound	IC_{50} (nM) 3D7-luc
17	250
18	440
19	490
20	490
21	520
22	890
23	920
24	1,100
25	1,100
26	1,200
27	1,300
28	1,300

FIG. 4. Antimalarial activity (IC_{50}) of confirmed hits with a 4-amino piperidine scaffold. Data shown are from a single IC_{50} determination.

(compound 29) was highly potent, indicating that a small amino group is favored over piperidine or morpholine at the R1 position. In addition, this compound was only moderately cytotoxic, with a 50% lethal dose (LD_{50}) of 10.7 ± 0.7 μM against HAEC cells and an LD_{50} of 12.5 ± 0.5 μM against HepG2 cells (Table 3). This demonstrated 460- to 620-fold selectivity for parasite killing over human cells bodes well for future therapeutic design with this scaffold.



Compound	IC_{50} (nM) 3D7-luc
29	5.3
30	11
31	14
32	14
33	25
34	32
35	780
36	790
37	4,300
38	5,400

FIG. 5. Antimalarial activity (IC_{50}) of confirmed hits with a 2,3-diamino-1,4-naphthoquinone scaffold. Data shown are from a single IC_{50} determination.

TABLE 3. Cytotoxicity (IC_{50}) of selected hits from HTS against HAEC and HepG2 cells[a]

Compound	IC_{50}[b] (μM)	
	HAEC	HepG2
29	11 ± 0.70	13 ± 0.50
Doxorubicin	13 ± 4.3	69 ± 14
32	41 ± 9.3	8.1 ± 0.90
31	45 ± 21	5.1 ± 1.9
Zolantidine	75 ± 16	76 ± 21
Amperozide	83 ± 15	95 ± 9.5

[a] Data shown are means from three replicates ± standard errors of the means.
[b] Doxorubicin was used as the positive control.

Since this series of 1,4-napthoquinones was potent, drug-like, and structurally related to atovaquone, we further pursued its mechanism of action (14). Atovaquone is thought to act by inhibiting the quinol oxidation site (Q_o) of the bc_1 complex of the parasite mitochondria (15, 20, 31, 33). We thus investigated whether the 2,3-diamino-1,4-naphthoquinones identified from HTS had a similar mode of action. We tested all potent 2,3-diamino-1,4-naphthoquinones against both drug-sensitive (3D7) and chloroquine/atovaquone-resistant (TM90C2B) parasite lines using the DAPI-based method (Table 4). All of the newly identified test compounds demonstrated potent activity against the atovaquone-resistant parasite line, indicating that it is unlikely that they share a mechanism with atovaquone. This observation was confirmed, as compound 29 demonstrated no inhibitory activity at ≤14 μM against both the parasite bc_1 complex and beef-heart bc_1. Furthermore, 29 did not show any inhibitory activity (at ≤14 μM) against the other quinone-dependent respiratory enzyme, NADH:ubiquinone oxidoreductase (PfNDH2) (2, 6). Taken together, these data indicate that the potent 2,3-diamino-1,4-naphthoquinone series identified from this HTS is likely to possess a novel mechanism of action distinct from that of atovaquone, although it contains a similar core scaffold. We currently are pursuing the identification of potential targets for these compounds to further elucidate the mechanism of action of this novel class of antimalarials.

Other clusters. The remaining clusters contained a diverse set of structures and also 59 singletons (sets containing only a single member) (see Table S4 in the supplemental material).

DISCUSSION

In this work, we have established a novel high-throughput screening assay and applied it to assay more than 10,000 small molecules for antiparasitic activity against *P. falciparum*. We have determined from a primary HTS effort, secondary IC_{50} validation screen, and luciferase counterscreen that 163 compounds have validated and potent antimalarial activity. In addition, several of our top hits showed little toxicity against several human cell lines, indicating that they are worth pursuing for antimalarial development.

One key advantage of using bioactive compound collections is that the compounds are already-known drugs or drug-like and likely can be more readily modified to increase potency while retaining good oral bioavailability. From the bioactive collection (LOPAC and NINDS) hits, the H2 antagonist zolantidine may be worthy of further medicinal chemistry efforts to optimize its antimalarial potency in culture and in mouse models. H2 receptor antagonists usually are well tolerated, have long half-lives (lasting hours), and are orally bioavailable (5). It is known that zolantidine crosses the blood brain barrier (25), which is essential for treating cerebral malaria. It is interesting that there are no H2 histamine receptors (the target for zolantidine in humans) in the *P. falciparum* genome or in RBCs; thus, there must be another target, perhaps a basic amino acid transporter of parasite origin.

TABLE 4. Drug sensitivity profile of *in vitro* growth of multidrug-resistant (TM90C2B) and drug-sensitive (3D7) *Plasmodium falciparum*[a]

Compound	IC_{50} (nM)	
	P. falciparum TM90C2B	*P. falciparum* 3D7
29	31 ± 9.8	35 ± 8.9
31	72 ± 39	140 ± 41
30	74 ± 21	110 ± 15
34	94 ± 23	130 ± 21
33	110 ± 46	170 ± 67
36	510 ± 150	1,800 ± 300
35	530 ± 170	1,600 ± 130
Atovaquone	12,000 ± 1,700	0.80 ± 0.20
Chloroquine	51 ± 3.2	7.2 ± 0.80

[a] Data shown are means from three replicates ± standard errors of the means.

We also studied a series of 2,3-diamino-1,4-naphthoquinones that are structurally similar to the approved antimalarial atovaquone. Surprisingly, we observed that these compounds acted by a novel mechanism from atovaquone, having no activity against bc_1 and PfNDH2 *in vitro*. Moreover, the best hits showed potent activity against a multidrug-resistant parasite line that was resistant to atovaquone and chloroquine. One of the longstanding problems with atovaquone is its poor bioavailability; however, the greater polarity of the 2,3-diamino-1,4-naphthoquinone hits in this screen ameliorate this problem. One potential concern with the 2,3-diamino-1,4-naphthoquinone scaffold is the presence of an aniline moiety, which has been associated with some toxicity. While the manuscript was in the process of resubmission, another screen was published that showed that a related diaminonaphthoquinone was potent against *P. falciparum* and demonstrated low toxicity against the host cells (16). This evidence is in agreement with our data, which show very little toxicity against several different human cell lines, indicating a good therapeutic index. If, in future animal studies, toxicity does occur, further medicinal chemistry will be needed to overcome this issue, perhaps through the guanidinylation of the aniline. In this vein, we now are pursuing further medicinal chemistry efforts to optimize the potency while reducing any potential toxicity of this scaffold and to create an affinity reagent to biochemically purify and identify its target(s).

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