

Searching for New Antimalarial Therapeutics amongst Known Drugs

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The need to discover and develop new antimalarial therapeutics is overwhelming. The annual mortality attributed to malaria, currently approximately 2.5 million, is increasing due primarily to widespread resistance to currently used drugs. One strategy to identify new treatment alternatives for malaria is to examine libraries of diverse compounds for the possible identification of novel scaffolds. Beginning with libraries of drug or drug-like compounds is an ideal starting point because, in the case of approved drugs, substantial pharmacokinetic and toxicologic data should be available for each compound series. We have employed a high-throughput screen of the MicroSource Spectrum and Killer Collections, a library of known drugs, bioactive compounds, and natural products. Our screening assay identifies compounds that inhibit growth of *Plasmodium falciparum* cultured in human erythrocytes. We have identified 36 novel inhibitors of *P. falciparum*, of which 19 are therapeutics, and five of these drugs exhibit effective 50% inhibitory concentrations within similar ranges to therapeutic serum concentrations for their recently indicated uses: propafenone, thioridazine, chlorprothixene, perhexiline, and azlocillin. The findings we report here indicate that this is an effective strategy to identify novel scaffolds and therefore aid in antimalarial drug discovery efforts.

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Malaria is a devastating disease with an annual morbidity of 300–500 million people and annual mortality of over 1 million. The

majority of deaths result from pediatric cases in developing countries (1). *Plasmodium falciparum* is the single-celled eukaryotic parasite responsible for the most lethal form of human malaria, cerebral malaria. The parasite is transmitted to humans by *Anopheles* mosquitoes. Endemic regions encompass approximately 40% of the global human population. The socioeconomic impact of malaria in endemic regions is estimated at a depressed annual growth rate of 1.5% over a 25-year period, when compared with other countries (2).

The current state of antimalarial chemotherapeutics is particularly bleak for those living in malaria endemic regions of the world because of the low economic incentive for drug development and the rise of resistant strains. Chloroquine-resistant strains of *P. falciparum* are now common in most malaria endemic regions (1), exacerbating the need for novel, cheap alternatives. Unfortunately, the success rate for new chemotherapeutics to move into clinical use is extremely low. This is true for any new drug; however, the situation is significantly worse for antimalarial therapeutics (3). Only one new antimalarial chemotherapeutic, malarone, developed by GlaxoWellcome (now GlaxoSmithKline, Brentford, UK), received FDA approval for clinical use in the last decade.

With the cost of new drug discovery exceeding \$750 million per new chemical entity (4), novel therapeutics for diseases endemic to the third world would seem out of reach. If academia is to play a role in the discovery and development of drugs for socially imperative but financially challenging conditions, new development paradigms must evolve. Because the cost of creating, maintaining, and screening large compound libraries is high, we have chosen the alternative approach of screening a smaller, focused compound library. We used a high-throughput cell-based assay that quantifies parasite growth cultured in human erythrocytes to screen the MicroSource Spectrum and Killer Collections. Together, these make up a library of 2160 known drugs, bioactive compounds, and natural products. This approach aims to leverage the extensive study that have already been performed with the known drugs to ensure their viability as human therapies. Another potentially advantageous aspect to screening this library of diverse compounds is the possibility of identifying novel scaffolds to optimize that are entirely unrelated to known antimalarial drugs. This is particularly important in light of a recent study that highlights the paucity of new antimalarial compound development around novel classes of compounds (5). Identification of new targets and pathways are a vital aspect of antimalarial discovery in which academia must play a key role (6).

In the MicroSource Spectrum and Killer Collections we have identified 19 drugs with previously unreported growth inhibitory activity for *P. falciparum* at micromolar or submicromolar concentrations.

A companion article in this Journal reports the results of screening the same compound collection against *Trypanosoma brucei* (7). Several compounds inhibit both *P. falciparum* and *T. brucei*. We discuss these broader spectrum antiparasitic agents and the novel antimalarial inhibitors that will merit further study. Particularly exciting, is the discovery that five drugs from the collection have anti-*P. falciparum* growth activity in similar ranges to current therapeutic serum concentrations. Follow-up assays of compounds related to these lead molecules suggest initial structure–activity relationships.

Materials and Methods

MicroSource Collection

The MicroSource Collection, as referred to in this study, is the MicroSource Spectrum (2000 compounds) and Killer (160 compounds) Collections. This library is made up of known drugs, bioactives, and natural products. The compounds were obtained from the Bay Area Screening Center (BASC) at the California Institute for Quantitative Biology (QB3; San Francisco, California). BASC originally obtained these compounds from MicroSource Discovery, Systems, Gaylordsville, CT, USA. Compounds in neat dimethyl sulfoxide (DMSO) were delivered in 96-well polypropylene conical plates. All compounds were diluted to 100 μ L in 10% DMSO in 1X phosphate-buffered saline (PBS). About 5 μ L of compound in 10% DMSO were transferred to 200 μ L of parasite culture for the growth inhibition assay, yielding a final DMSO concentration of 0.24%. *Plasmodium falciparum* growth is highly sensitive to DMSO, thus requiring this low concentration.

Plasmodium falciparum inhibition assay

Growth inhibition of *P. falciparum* cultures was quantified using a fluorescent-active cell sorting (FACS) assay (8,9). Briefly, cultures of *P. falciparum* [3D7 (10) and W2 (11) strains] were grown in purified human erythrocytes and RPMI 1640 media supplemented with 0.25% Albumax II (Gibco, Life Technologies, San Diego, CA, USA), 2 g/L sodium bicarbonate, 0.1 mM hypoxanthine, 25 mM HEPES (pH 7.4), and 50 μ g/L gentamycin, at 37 °C, 5% O₂, and 6% CO₂. Synchronous cultures (0.8% ring-stage, 0.5% hematocrit) were plated onto 96-well tissue culture plates (Falcon, BD Biosciences, San Jose, CA, USA) and cultured in the presence of compounds (0.24% final DMSO concentration).

After a 72-h growth period, cultures were fixed for 1 h at room temperature (RT) in 1% paraformaldehyde and stained with 50 nM YOYO-1 (Molecular Probes, Carlsbad, CA, USA) for approximately 24 h in the dark at RT. Samples were analyzed on a Becton-Dickinson (BD Biosciences, San Jose, CA, USA) LSR2 fitted with a microplate reader. Uninfected erythrocytes were used to determine background autofluorescence. Parasite growth in each sample was determined relative to infected erythrocytes without test compound. Chloroquine was used as a control to monitor the quality of the assay.

Initial and follow-up screenings were performed at final drug concentrations of 10 μ M, 1 μ M and 100 nM. Dose–response curves were generated with 9-point, serially diluted concentration schemes: 6 μ M, 3 μ M, 1 μ M, 600 nM, 300 nM, 100 nM, 60 nM, 30 nM and 10 nM. Effective 50% inhibitory concentrations were determined using GRAPHPAD PRISM software.

Analysis of inhibition data

All data relevant to the project (plate and compound information, screening data, annotation info, etc.) was stored in multiple tables in a MySQL database (v. 4.1.7). Data were manipulated and analyzed using protocols written in PIPELINE PILOT 4.5.2 (Scitegic, Inc., San Diego, CA, USA). Our protocols automated the process of joining experimental data to compound information, flagging suspicious plates based on low Z-prime and Z-factors, extracting compounds with statistically significant activity, annotating hits with additional information (i.e. chemical similarity to known bioactive compounds, known genotoxic/cytotoxic molecules, or available compounds, and profiles from ADME models), and generating preliminary structure–activity relationships. The results from this analysis, available as a searchable html hierarchy, guided subsequent rounds of screening.

Results

The MicroSource Spectrum and Killer Collections (referred to hereafter as the MicroSource Collection) make up a library of 2160 known drugs, bioactive compounds, and natural products. We screened the entire MicroSource Collection against two representative laboratory strains of *P. falciparum*: 3D7 [drug-sensitive (10)] and W2 [drug-resistant (11)]. The growth inhibition assay we use consists of a 3-day incubation of each compound with *P. falciparum* cultured in human erythrocytes, followed by detection and quantification of parasite growth via flow cytometry, as detailed in the Materials and Methods section (8,9). Industrial high-throughput screening efforts normally use compound concentrations of 1–10 μ M for cell-based assays. We performed our initial screening at 10 μ M final concentration. The quality of the results was found to be quite high, with a median Z-prime factor of 0.75 [Z-prime is a measure of assay quality, with a maximum of 1.0 indicating an ideal assay, and >0.5 indicating a good, reliable assay (12)]. More than 50% of the compounds in this library exhibited some amount of malaria growth inhibition, presumably due to the nature of the library.

Compounds with Z-score of ≥ 1.65 , as determined by comparison with negative controls (corresponding to a p-value of ≤ 0.05), are considered statistically significant hits in biologic screening assays. We initially used this cut off to determine the hit list for active *P. falciparum* inhibitors in our screen. However, this cut off included only compounds with 100% inhibition activity against 3D7 and W2. We therefore relaxed the cut off to a Z-score of 1.50 (corresponding to a p-value of ≤ 0.067), which then included 126 compounds with inhibition activity >97.5% against 3D7 and 228 compounds with inhibition activity >93.7% against W2. About 103 compounds inhibited both 3D7 and W2 *P. falciparum* strains, making a total of 251 compounds for further investigation. This subset of compounds was screened again at lower concentrations of 1 μ M and 100 nM.

Seventy-two compounds exhibited significant activity (defined here as >70% growth inhibition relative to control) at 1 μ M and 19 compounds exhibited significant activity at 100 nM. The 72 compounds with >70% growth inhibition relative to control at 1 μ M were flagged for further investigation (see Table S1). Literature searches were conducted to determine current therapeutic uses, toxicity information, and any prior identification of antimalarial activity. Compounds within this set of 72 that lacked literature evidence of anti-*P. falciparum* activity were then investigated for dose–response behavior.

Thirteen of the 72 active inhibitors of *P. falciparum* were previously known antimalarial therapeutic agents. These compounds served as an excellent internal validation of our screening assay, as we were blinded to the identities of the compounds. The known antimalarial therapeutics we identified were all active at 100 nM and included: quinine (13), quinidine and hydroquinidine (14), chloroquine and hydroxychloroquine (15), amodiaquine (16), cinchonine (17), cinchonidine (18), mefloquine (19), atovaquone (20), pyrimethamine (15), dihydroartemisinin (21), and quinacrine (22). Six more members of the MicroSource Collection inhibited *P. falciparum* growth at a

concentration of 100 nM. These compounds have been studied previously and confirmed to have antimalarial activity: tetrandrine (23,24), actinomycin D (25), anisomycin (26), puromycin (26), methotrexate (27), and pentamidine (28,29). Seventeen additional known inhibitors of *P. falciparum* were identified with significant activity at a concentration of 1 μ M.

To our knowledge, 36 compounds have not been studied previously for antimalarial activity and inhibited *P. falciparum* growth at 1 μ M. Nineteen of these compounds were known therapeutics. This set included known antibiotics, topical anti-infectives, natural products, a known antitrypanosomal, antineoplastics, vasodilators, and antipsychotics. Effective 50% inhibitory concentration values (EC₅₀s) of these novel antimalarial inhibitors are listed in Table 1.

Discussion

One advantage to screening a library such as the MicroSource Collection, which contains already approved drugs, is the potential to identify novel inhibitors of *P. falciparum* that have been tested and formulated for safe therapeutic use in humans. Of course, not all FDA-approved drugs are appropriate for antimalarial treatment for many reasons. However, drug delivery route is one simple filter to differentiate out potential scaffolds for further optimization. We therefore group the novel inhibitors of *P. falciparum* by delivery route, excluding those that are simply natural products and bioactives, not therapeutics, and those that are not human therapies. The novel inhibitors that meet these conditions consist of nine topical, five i.v., and seven orally administered therapeutics (some of

these are available in multiple delivery methods, see Table 1). A flowchart following the distribution of antimalarial activity in the MicroSource Collection is illustrated in Figure 1.

Inhibitors that are topical therapeutics

Several novel inhibitors of *P. falciparum* are topically administered therapeutics. These compounds are mainly composed of antibiotics, anti-infectives, antiseptics, and antifungal drugs. Table 1 shows representative EC₅₀ values for these inhibitors. A number of these compounds inhibit *P. falciparum* growth in concentrations of the low hundreds of nanomolar range. However, topical treatments are inappropriate as potential antimalarial therapeutics, because the required antimalarial activity must take place within the bloodstream.

Inhibitors that are intravenous therapies

Intravenous therapies are better candidates for antimalarial treatment than topical drugs in that they have undergone clinical testing within the bloodstream, and therefore may make better starting point scaffolds. In particular, i.v. formulations of antimalarials are important for treatment of acute severe cerebral malaria and are part of the World Health Organization (WHO) product profile. However, i.v. administration does not satisfy the need for antimalarial therapeutics to be easily accessible for large populations for routine therapy or prophylaxis.

Active compounds with an i.v. administration route include an antibiotic, a vasoconstrictor, a progestogen, and two antineoplastic therapies. The fact that two of these (dihydroergotamine and hydroxyprogesterone) are available not only in i.v. form, but also oral delivery, shows that the possibility exists to pursue these further. The effective therapeutic ranges for these two compounds are unfortunately orders of magnitude lower than the EC₅₀ values observed here against cultured malaria (30); however, this is not the case for azlocillin. The EC₅₀ values for azlocillin are approximately 1.5 and 2.5 μ M against 3D7 and W2, respectively. While the raw values seem high, these inhibitory concentrations are well within the range of minimum inhibitory concentrations of azlocillin for the treatment of severe infections (4–16 μ M; 30).

Mitoxantrone is an antineoplastic with particularly good inhibition activity: approximately 100 nM EC₅₀ values against both strains of *P. falciparum*. However, therapeutic serum levels are unknown for this antineoplastic (30). The same is true for aclarubicin.

Inhibitors that are oral therapies

Novel inhibitors of *P. falciparum* that are oral therapeutics are ideal for potential antimalarial drug development. These compounds have already been clinically tested and formulated for the preferred delivery method. Seven members of the MicroSource Collection fall under this category. Two of these drugs are antipsychotics (chlorprothixene and thioridazine), one is a vasoconstrictor (dihydroergotamine) and one is a vasodilator (perhexiline), one is an anthelmintic (hycanthone), one is a progestogen (hydroxyprogesterone), and one is an antiarrhythmic (propafenone). The EC₅₀ values for these drugs

Table 1: Representative EC₅₀ values and distribution of MicroSource antimalarial inhibitors with topical, i.v., and oral delivery routes

Compound	3D7 (μ M)	W2 (μ M)
Topical (9)		
Acriflavinium	0.03 \pm 0.005	0.04 \pm 0.003
Benzalkonium	0.2 \pm 0.01	0.3 \pm 0.02
Benzethonium	0.3 \pm 0.02	0.2 \pm 0.02
Cetrimonium	0.9 \pm 0.05	0.6 \pm 0.05
Cetylpyridinium	0.4 \pm 0.05	0.5 \pm 0.05
Ciclopiroxolamine	1.2 \pm 0.5	0.7 \pm 0.03
Dequalinium	<0.01	0.04 \pm 0.002
Methylbenzethonium	0.3 \pm 0.02	0.2 \pm 0.02
Tannic acid	1.6 \pm 0.1	1.3 \pm 0.1
Intravenous (5)		
Aclarubicin (aclacinomycin A1)	0.4 \pm 0.03	0.4 \pm 0.02
Azlocillin	1.5 \pm 0.3	2.5 \pm 0.3
Dihydroergotamine	3.0 \pm 0.9	3.0 \pm 0.9
Hydroxyprogesterone	1.0 \pm 0.1	1.3 \pm 0.4
Mitoxantrone	0.1 \pm 0.006	0.1 \pm 0.006
Oral (7)		
Chlorprothixene	1.7 \pm 0.7	1.0 \pm 0.2
Dihydroergotamine	3.0 \pm 0.9	3.0 \pm 0.9
Hycanthone	1.9 \pm 1.1	0.3 \pm 0.02
Hydroxyprogesterone	1.0 \pm 0.1	1.3 \pm 0.4
Perhexiline	1.1 \pm 0.1	0.6 \pm 0.02
Propafenone	1.0 \pm 0.1	0.2 \pm 0.02
Thioridazine	2.6 \pm 0.3	1.9 \pm 0.2

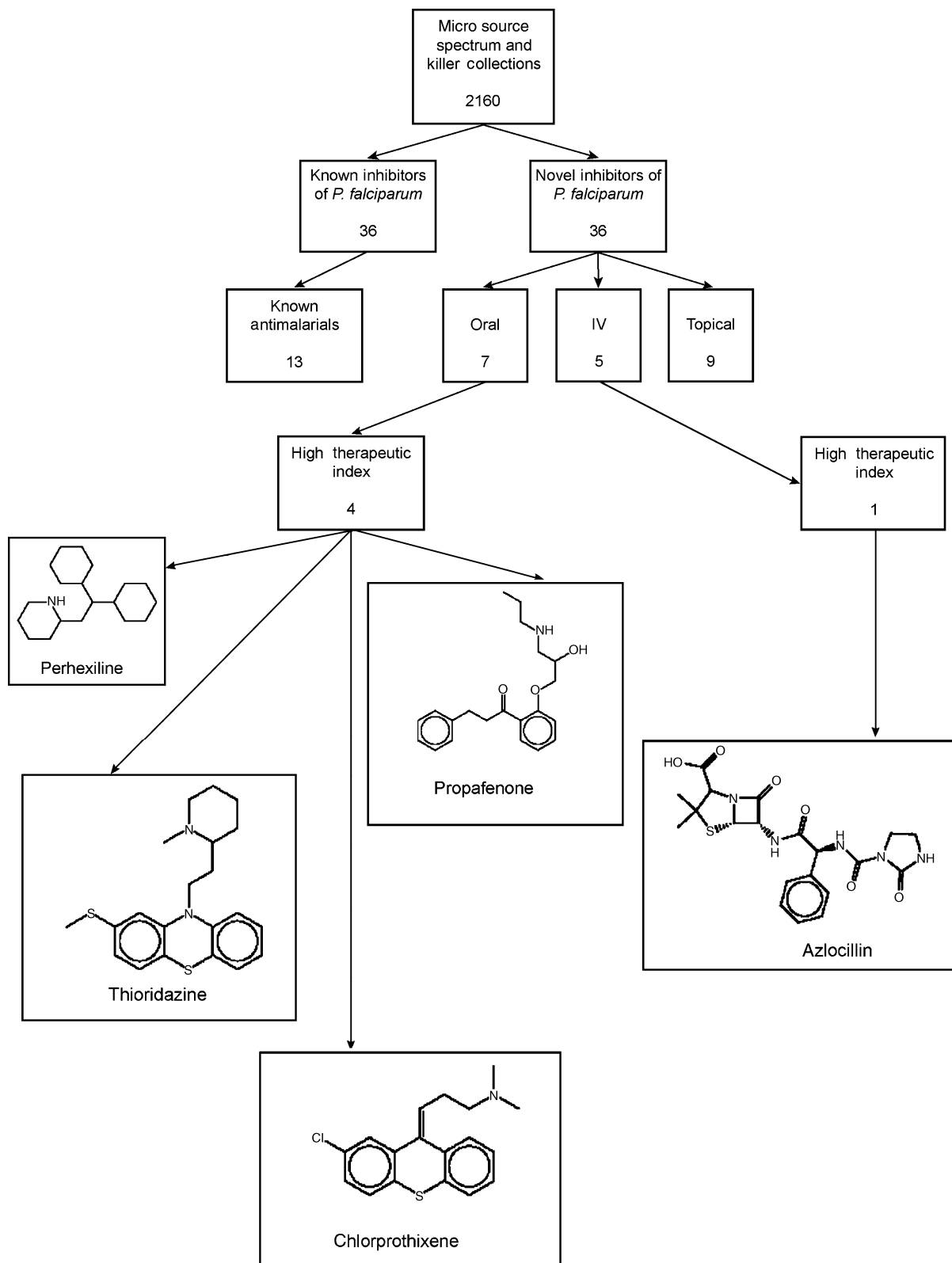


Figure 1: Distribution of antimalarial inhibition activity in the MicroSource Spectrum and Killer Collections. Thirty-six novel compounds exhibited $\geq 70\%$ growth inhibition of *Plasmodium falciparum* cultures at $1 \mu\text{M}$ final concentration. Of these 36 novel antimalarial compounds, nine are topical therapeutics, five are i.v. therapeutics, and seven are oral therapeutics. Five drugs are identified with a high therapeutic index; 50% effective inhibitory concentrations in the same range as therapeutic serum levels in patients.

against drug-sensitive and drug-resistant strains of *P. falciparum* are shown in Table 1.

None of the seven orally administered drugs has remarkably low effective 50% inhibitory concentrations against these two strains of the parasite. However, the EC_{50} values of some of these drugs fall into the therapeutic range (detected in serum levels) for their indicated therapies. Propafenone, with submicromolar EC_{50} values against 3D7 and W2, is one of these drugs. Therapeutic serum levels are extremely variable from patient to patient, and effective treatments cover a concentration range of 0.1–8.0 μM (30). The dangers of cardi-

otoxicity and seizures associated with propafenone overdose require administration to be initiated under hospital supervision (30). However, the negative side-effects associated with propafenone may not be associated with the attributes that could make it an effective inhibitor of malaria growth, and optimization may prove fruitful. As an initial probe of the potential for propafenone optimization, we tested five analogs of propafenone (Chembridge, Inc., San Diego, CA, USA). These compounds and their EC_{50} values are illustrated in Figure 2. The wide range of EC_{50} s in this small set of propafenone analogs increases the likelihood that these compounds are making specific interactions, and not merely inhibiting growth by non-specific

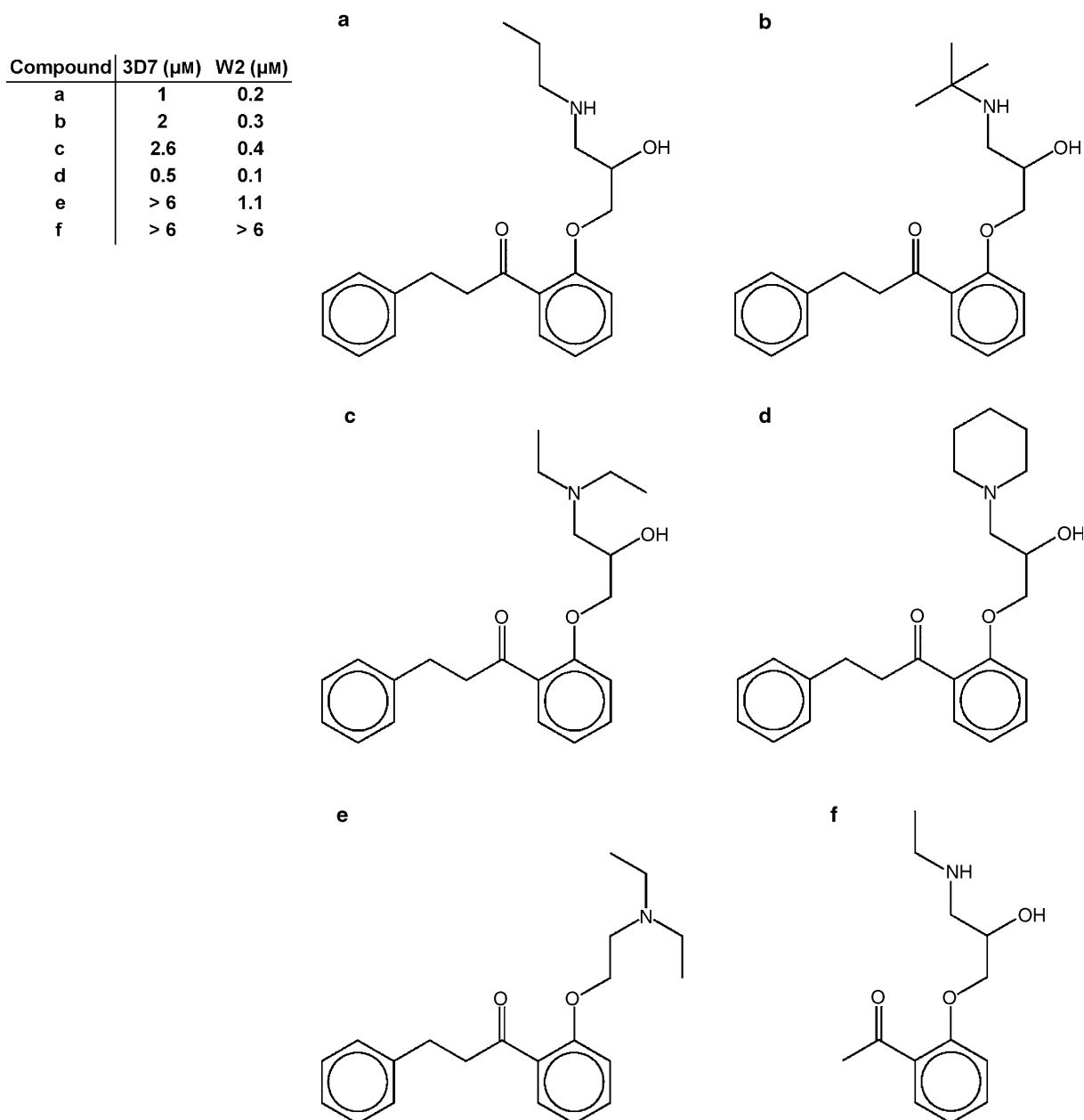


Figure 2: The oral antiarrhythmic drug propafenone (a) and five analogs of propafenone. The structures and EC_{50} values of this series suggest that *Plasmodium falciparum* growth inhibition is accomplished by specific interactions, and that optimization of antimalarial activity is possible.

interactions. In addition, one compound is twice as potent as propafenone ($EC_{50} = 0.5$ and $0.1 \mu\text{M}$ for 3D7 and W2, respectively). This preliminary data are very promising for selectivity, and even further optimization of the antimalarial effects of the propafenone series.

Thioridazine is another drug with similar therapeutic and effective 50% inhibitory concentration ranges against *P. falciparum*. Phenothiazines (thioridazine) and thioxanthenes (chlorprothixene) are antimicrobials that have traditionally been used to manage psychoses; however, various members of these classes, including chlorpromazine, have been shown effective in killing *P. falciparum* cultures (31–33). The therapeutic range of thioridazine in serum is difficult

to estimate, because of the large variations from patient to patient, as with propafenone. However, one source reports an estimated therapeutic range of roughly 0.5 – $6.4 \mu\text{M}$ (34). The antimalarial activity demonstrated against cultured *P. falciparum* is well within this concentration range. However, the onset of toxic effects because of thioridazine is indicated at serum levels $>2.5 \mu\text{M}$, which is close to the EC_{50} values against the two strains of malaria tested here. A preliminary test of five analogs of thioridazine (Figure 3) shows a range of EC_{50} values, and thus like propafenone, a real possibility for optimization. The major difficulty in development of thioridazine or chlorprothixene analogs lies in the need to decrease the effects on the central nervous system while improving antimalarial effects. The antipsychotic therapeutic range of chlorprothixene is approxi-

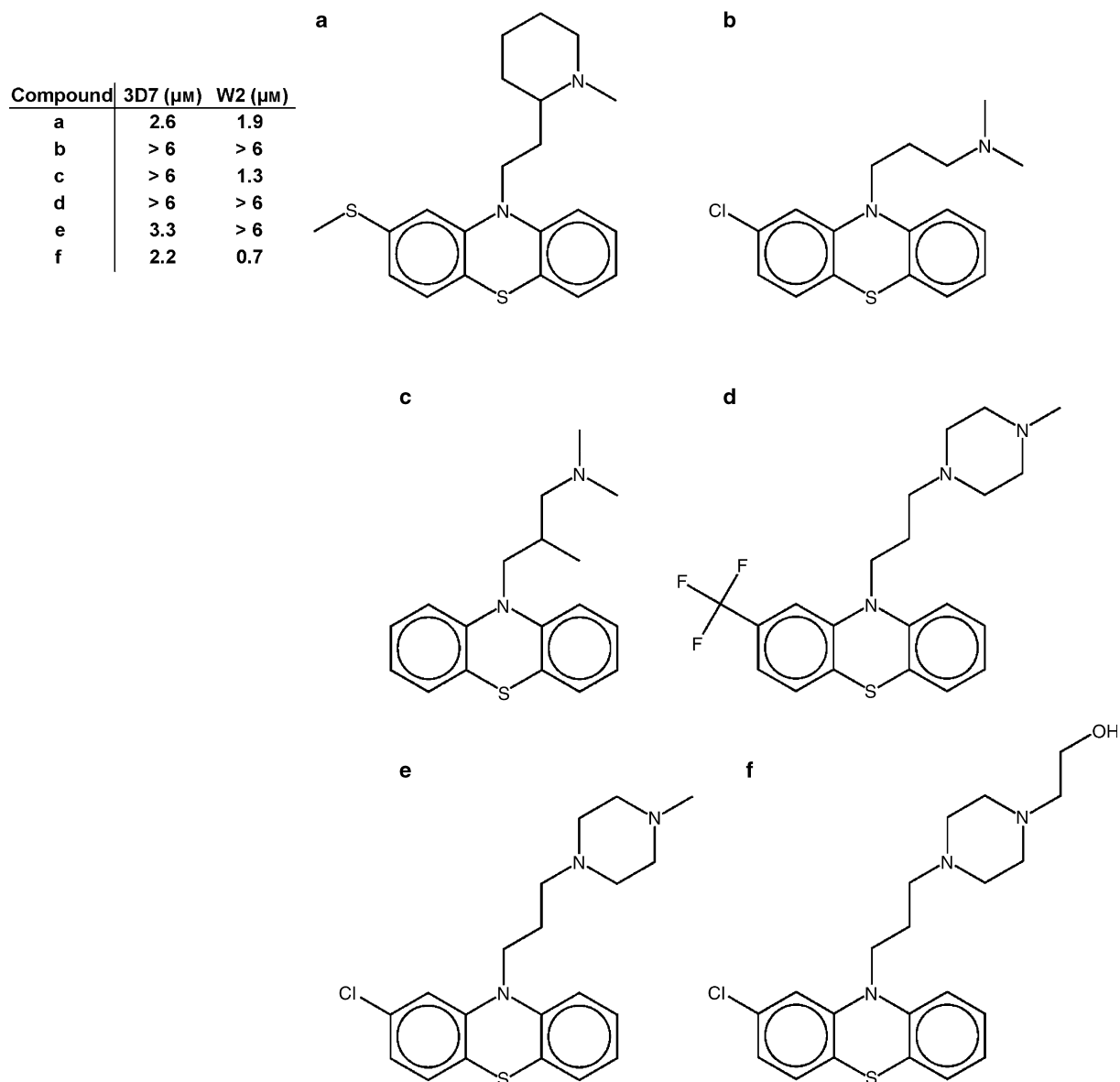


Figure 3: The oral antipsychotic drug thioridazine (a) and five analogs of thioridazine. Like propafenone, the structures and EC_{50} values of this series suggest that *Plasmodium falciparum* growth inhibition is accomplished by specific interactions, and that optimization of antimalarial activity is possible.

mately 60–600 nM (30), which is close to the EC₅₀ values of 1–2 μM against strains of *P. falciparum*.

Perhexiline is the final orally delivered therapeutic in this set with a therapeutic range comparable to the effective 50% inhibitory concentration values. Therapeutic dosing results in serum levels of 0.4–1.5 μM (30), and the EC₅₀ values against *P. falciparum* strains 3D7 and W2 are roughly 1.0 and 0.6 μM, respectively.

The therapeutic dosing ranges for hydroxyprogesterone, dihydroergotamine, and hycanthone are all orders of magnitude lower than the activity observed against *P. falciparum*.

Inhibitors common to *P. falciparum* and *T. brucei*

Eighteen compounds in the MicroSource Collection inhibit growth of both *P. falciparum* and *T. brucei* at 1 μM final concentration [see companion article (7)]. These compounds are listed in Table S2. Therapeutics that are efficacious against both of these species are interesting as possible broad spectrum antiparasitic agents. This is important due to the fact that the regions of the world where these parasites are endemic do indeed overlap, and dual infections are plausible and sometimes likely. Therapies that are able to treat both of these diseases would be particularly attractive to those afflicted.

Conclusions and Future Directions

Whole cell screening of diverse compound libraries is an essential part of the malaria drug discovery effort. The results of our high-throughput growth inhibition screen are encouraging and show that progress may be made through this kind of approach. Within this one set of 2160 compounds, we have identified 36 novel inhibitors of *P. falciparum*, of which 19 are known drugs. Five of these drugs exhibit growth inhibition activity against cultured malaria in the same concentration range as exhibited in patient serum levels at therapeutic dosing for their indicated uses, and two of these drugs, propafenone and thioridazine, show preliminary evidence of specific inhibition with potential for scaffold optimization.

The preliminary identification of novel inhibitors of antimalarial growth *in vitro* is promising and strongly supports the high-throughput screening of additional compound libraries. The capacity we regularly screen with our flow cytometric assay is roughly 500–1000 compounds per week with a single concentration pass against two strains of malaria. This is far from high-throughput by industry standards. The throughput capacity of screening large libraries could certainly be increased by the incorporation of additional robotics into our system and possibly switching to a faster plate fluorescence platform. Regardless, the screening of more libraries with diverse scaffolds is an important strategy to continue exploring.

The mode of action of the novel inhibitors described in this study is a question that has not been addressed. While this is beyond the scope of this study, it is extremely important, and we are currently following up on the top leads. Along these lines, questions of pro-

tein binding, drug resistance, and combination activity are all avenues we will explore, as they will illuminate the relevance of future study on optimization of these lead compounds.

Another direction to explore in future study is the transcriptome response to the antimalarial inhibitors identified in this study. Transcriptome response to these known drugs will aid in the determination of novel pathways to target for drug design in the *P. falciparum* genome. Combination drug therapies are imperative for effective antimalarials due to the high efficiency of the parasite to develop mechanisms of resistance to drugs currently in use. Continuation of the kind of library screening that we have presented here and transcriptome analysis of proposed therapeutics are potentially effective strategies to help fight the worsening malaria plight.

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Supplementary Material

The following material is available for this article

Table S1: Compounds with greater than 70% inhibition activity against *Plasmodium falciparum* at 1 μM

Table S2: Antimalarial inhibitors identified in this study with activity against *T. brucei*

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