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# Chemical genetics of *Plasmodium falciparum*

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Author contributions

WAG and RKG designed and coordinated the project. AAS wrote the algorithms for the data analysis and generated the figures. Assays were conceived, performed and analyzed by WAG and DB (*P. falciparum* phenotypic screen), MC (human cell lines), DCS (*T. brucei*), PHD and DSR (*T. gondii*), JSL and ERS (*L. major*), AKT and DJS (hemozoin inhibition), GJC and WCVV (thermal melt experiments) MAP, PKR and FEM (PfDHOD), JWF and PKR (*P. falciparum* dihydrofolate reductase), JG and PJR (FP-2), IF and MKR (cytochrome bc 1), JC (*P.falciparum* mutant drug sensitivity). EBW, SD, JLD and VMA (independent antimalarial *in vitro* experiments), FZ (*in vitro* pharmacokinetics), MBJD, MSM, IAB and SF (*in vivo* pharmacokinetics and efficacy), IB (coordination of technology development and network development), SC and PLM (resynthesis). WAG, AAS and RKG wrote the manuscript. All authors contributed to the design of the experiments.and the preparation of the manuscript.

Supplementary information

The supplementary information provides a summary of all relevant data arising from the phenotypic screen and all secondary screens including relevant diagnostics and details about the following: cell-based, enzyme, and thermal shift screens, data processing, Bland-Altman analysis, and the algorithm to generate chemical structure network graph. Chemical structures annotated with assay data and high resolution PDF's of the figures may be downloaded from http://www.stjuderesearch.org/guy/data/malaria/

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## Abstract

Malaria caused by *Plasmodium falciparum* is a catastrophic disease worldwide (880,000 deaths yearly). Vaccine development has proved difficult and resistance has emerged for most antimalarials. In order to discover new antimalarial chemotypes, we have employed a phenotypic forward chemical genetic approach to assay 309,474 chemicals. Here we disclose structures and biological activity of the entire library, many of which exhibited potent *in vitro* activity against drug resistant strains, and detailed profiling of 172 representative candidates. A reverse chemical genetic study identified 19 new inhibitors of 4 validated drug targets and 15 novel binders among 61 malarial proteins. Phylochemogenetic profiling in multiple organisms revealed similarities between *Toxoplasma gondii* and mammalian cell lines and dissimilarities between *P. falciparum* and related protozoans. One exemplar compound displayed efficacy in a murine model. Overall, our findings provide the scientific community with new starting points for malaria drug discovery.

The wide-spread resistance of *P. falciparum* to many antimalarial drugs, the dependence of all new combinations on artemisinins (for which resistance may have emerged),1 ,2 and new efforts to eradicate malaria all drive the need to develop new, effective, and affordable antimalarial drugs.3 Although our understanding of the parasite's biology has increased with sequencing of the genome4 and the development of new technologies to study resistance acquisition,5 ,6 ,7 few new drug targets or classes of drugs have been clinically validated.8 The lack of publicly-accessible antimalarial chemotypes with differing modes of action has significantly hindered efforts to discover and develop new drugs.9 In order to address this urgent need, we have developed a forward chemical genetic approach to identify novel antimalarials (Supplementary Fig. 1).

## The forward chemical genetic screen

A library containing 309,474 unique compounds, designed at the scaffold level to provide diverse, comprehensive coverage of bioactive space,10 ,11 was screened against *Plasmodium falciparum (Pf3D7)* at a fixed concentration of 7 μM (Supplementary Information).12 Fidelity of the assay was examined by receiver operator characteristic (ROC) analysis and other metrics (Supplementary Fig. 2 and 3), demonstrating good discriminatory power (AUC ~0.85) and suggesting that a cutoff of ≥ 80% activity would retain the majority of true positives. The strength of the assay was further determined by

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testing a set of bioactive compounds including known antimalarials, all of which were re-identified (Supplementary Table 3), demonstrating that the method was very likely to identify any molecule acting by a known mechanism. The primary screen gave 1152 compounds with activity > 80%. These 1152 compounds and the true positives from the ROC set were serially diluted and tested against both the chloroquine-sensitive *Pf3D7* and the chloroquine-resistant *PfK1* strain, giving 1300 validated hits that had saturated dose-response curves. Chemical structure analysis of validated hits by topology mapping and clustering10 revealed a wide distribution of chemotypes in the active chemical space, with several displaying promising structure-activity relationships (Fig. 1). While all known antimalarial scaffolds (aminoquinolines, quinolones, bis-amidines) present in the screening collection were identified, providing positive controls for the screen, most of the chemotypes identified were novel. 561 of the validated hits had $EC_{50}$ values ≤ 2 μM against *either Pf3D7* or *PfK1* and a therapeutic window ≥ 10-fold against two mammalian cell lines (HepG2 & BJ). From this set, 228 structurally distinct, pure compounds were re-purchased. Antimalarial potencies of ~75% of these compounds (172) were re-confirmed to within 10-fold (Bland-Altman analysis, Supplementary Fig. 4) by three laboratories using distinct methods providing the cross-validated hit set utilized for all subsequent experiments.

## Combination with antimalarial drugs

Due to rapid resistance acquisition, the WHO recommends combination therapy.13 The agonistic and antagonistic synergies of the cross-validated set were therefore quantified by measuring $EC_{50}$ shifts in the presence of a fixed fraction of potency ($EC_{10}$) concentration of chloroquine, mefloquine, artemisinin, and atovaquone. Most cross-validated compounds were additive in effect or had minor synergies with existing drugs. Two classes demonstrated strong synergies ($EC_{50}$′s reduced by ≥ 10-fold): the diaminonaphthoquinones with artemisinin, and the dihydropyridines with mefloquine (Fig. 2). One diaminonaphthoquinone and a cycloguanil analog displayed antagonism with chloroquine and mefloquine, respectively.

## Reverse chemical genetics

The advantages of phenotypic screens for the identification of novel chemotypes are that no *a priori* assumptions are made concerning drug targets and that active compounds inherently have cellular bioavailability. Because insight into mechanism of action is helpful for drug development, we also investigated the interaction of the cross-validated set with 66 potential targets using enzyme inhibition assays and thermal-melt shift assays (to detect binding).

Three high-priority, well characterized biological targets were evaluated in activity assays (Fig. 3, left): *P. falciparum* dihydroorotate dehydrogenase (*Pf*DHOD), hemozoin formation, and falcipain-2 (*Pf*FP-2). *Pf*DHOD, catalyzes the oxidation of dihydroorotate to orotate in *de novo* pyrimidine biosynthesis, which is essential for parasite viability.14 ,15 Three compounds inhibited this enzyme: two triazolopyrimidines, structurally related to known *Pf*DHOD inhibitors with comparable potencies,14 and a dihydropyridine, structurally related to the calcium blocker felodipine. The potency of these compounds against *Pf*DHOD strongly correlated with their antimalarial activities (Supplementary Table 5). Furthermore,

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these compounds were inactive against transgenic parasites expressing *Saccharomyces cerevisiae* dihydroorotate dehydrogenase (Supplementary Table 6). Next, hemozoin formation inhibition was investigated. The parasite digests host hemoglobin to provide amino acids, detoxifying the resulting heme molecules by conversion to a crystallized form known as hemozoin. Heme detoxification is believed to be the target of many antimalarial drugs.16 Twelve compounds exhibited appreciable efficacy in an *in vitro* hemozoin formation assay,17 including analogs of quinazoline, benzofuran, benzimidazole, and carbazole as well as amodiaquine, a known hemozoin formation inhibitor present in our library. The correlation between enzyme inhibitory potency and antimalarial potency was similar to that displayed by the positive controls quinine and amodiaquine (Supplementary Table 5). The third enzyme assayed was *Pf*FP-2, which plays a critical role in hemoglobin degradation.18 Falcipains are redundant in *P. falciparum*, with four known homologs including two (falcipain-2 and falcipain-3) that appear to play key roles in erythrocytic stage parasites.19 Three weakly FP-2 inhibitors were identified. Thus 19 compounds (11%) were inhibitors of validated antimalarial targets.

To expand the pool of potential targets, the compounds were tested for binding to 61 recombinant malarial proteins (95% purity or better after affinity and size exclusion chromatography, Supplementary Table 1) in a thermal-melt shift assay.20 Fifteen compounds displayed reproducible thermal shifts with seven malarial proteins (Fig. 3, right; $K_d$'s in Supplementary Table 2): 6-phosphogluconolactonase, 6-pyruvoyltetrahydropterin synthase, choline kinase, D-ribulose-5-phosphate 3-epimerase, dUTPase, glycogen synthase kinase 3, and thioredoxin. 2 compounds bound multiple proteins. 2 out of the 7 proteins are in essential malarial pathways: phosphatidylcholine synthesis21 (choline kinase) and redox metabolism22 (thioredoxin). The remaining 5 protein targets potentially represent novel antimalarial drug targets.

## The potential for cross resistance

To evaluate the potential for cross-resistance with existing drugs, the cross-validated compounds were tested against a panel of *P. falciparum* strains with different chemosensitivities to known antimalarials, including *Pf3D7* (chloroquine sensitive), *PfK1, PfW2, PfV1/S* and *PfDd2 (*all resistant to both chloroquine and to antifolates), *PfSB-A6 and PfD10_yDHOD* (both chloroquine sensitive and atovaquone resistant). All strains were profiled for sensitivity to a set of antimalarial drugs to normalize activity (Supplementary Table 3). 58 cross-validated compounds displayed similar potencies ($EC_{50}$ shift ≤ 3-fold) against *Pf3D7, PfK1, PfV1/S* and *PfSB-A6*, suggesting that these compounds do not share mechanisms of resistance with chloroquine, atovaquone, or sulfadoxine/pyrimethamine. A subset of the 172 compounds that were inactive against drug resistant *P. falciparum* strains with known mutations in target proteins were tested against *Pf*3D7 dihydrofolate reductase and *P. yoelii* cytochrome $bc_1$ complex in biochemical assays. Two inhibitors were identified for each protein (Supplementary Table 5 and 6).

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## Phylochemogenetic profiling

To understand relationships between chemical sensitivity of *Plasmodium* and related parasites, the cross-validated set was tested against three additional protozoan parasite species: *Toxoplasma gondii* (*Tg*), which belongs to the same phylum as *Plasmodium* (Apicomplexa); *Leishmania major* (*Lm*) and *Trypanosoma brucei* (*Tb*), which are both Kinetoplastida, unrelated to the Apicomplexa; and an expanded panel of human cell lines including a Burkitt's lymphoma line (Raji) and embryonic kidney fibroblast cells (HEK-293). Phylogenetic criteria predict that chemical sensitivity should correlate with evolutionary history, due to homology between key protein targets, as is known to be the case for many antiparasitic drugs.23 ,24 Although a few compounds showed activity in other parasites, most were highly selective for *Plasmodium* (Fig. 4), while *Toxoplasma* exhibited a chemosensitivity pattern more similar to human cell lines. Similarly, the highly potent anti-leishmanial benzothiazoles were only weakly active against the related *Trypanosoma*. These findings suggest that chemical sensitivity of pathogens is regulated by a combination of pathogen genetics, physiology, and relationships to host and vector species *in vivo*.

## Early leads for drug development

In order to understand the potential for development of the novel chemotypes, the pharmacokinetic properties of the cross-validated set were assessed. The majority are reasonably drug-like, with 78% of compounds have no violations of the Lipinski Rule of 5, and 99% have one or fewer violations.25 Within the cross-validated set were embedded 3 chemical series that had multiple members that together gave structure-activity relationships that spanned 1000-fold potency differences, had consistent activity in drug resistant strains, had very good cellular therapeutic windows, and had at least one member with an $EC_{50}$ more potent than 50 nM. An exemplar compound was selected from each series and fully profiled using standard models of *in vitro* and *in vivo* adsorption, distribution, metabolism, and toxicity (Supplementary Table 7). Each possessed reasonable characteristics for developable hits, indeed each comes close to passing MMV criteria for "late leads." The compound from these exemplars with the best pharmacokinetic profile, was further evaluated to measure *in vivo* antimalarial activity and displayed efficacy in a murine malaria model infected with *P. yoelii.* A twice-daily administration of 100 mg/kg for 3 days resulted in a 90% suppression of the parasitemia (Supplementary Fig. 5). Although it is not suggested that any of the compounds discussed herein are *bone fide* preclinical candidates all provide reasonable starting points for drug development.

## Conclusions

Drug therapy remains a key component in controlling malaria. Current challenges of rapid acquisition of resistance, cross-resistance, and dependence upon a limited number of chemical classes of antimalarials highlight the need to enhance our understanding of the "chemical space" that can fruitfully be brought to bear on malaria treatment. Solving this problem requires understanding the relationships between the structures of compounds active against malaria parasites and their potency, selectivity, and targets. We have identified a number of novel compounds and defined these relationships. We expect that

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these findings will provide novel paths for drug development and hope that making this set of well characterized, non-proprietary lead antimalarials publicly available to the global research community will help to reinvigorate drug discovery for malaria.

## Methods summary

The primary screen was carried out by comparing quantities of DNA in treated and control cultures of *Plasmodium falciparum* in human erythrocytes after 72 h incubation with a fixed concentration of 7 μM of the test compounds.. The secondary potency determination was made by using the same assay in a dose-response mode with 12 concentrations varying from 10 μM to 5 nM. Chemical sensitivities of the human cell lines and *T. brucei* were determined by measuring their ATP content (Cell Titer Glo, Promega**).** *T. gondii* parasites expressing luciferase were cultured and drug sensitivity was determined by luminescence; *L. major* promastigotes drug susceptibility (Alamar Blue assay, Promega). Chemicals were assayed for hemozoin formation inhibition17, PfDHOD15, FP-227 activities based on previously described methods. Thermal shift assays were done at compound concentrations of 25 μM and protein concentrations of 100 μg/ml. All data processing and visualization, and chemical similarity and substructure analysis was performed using custom programs written in the Pipeline Pilot platform (Accelrys, v.7.0.1) and the R program.26 A complete description of the methods can be found in Supplementary Information.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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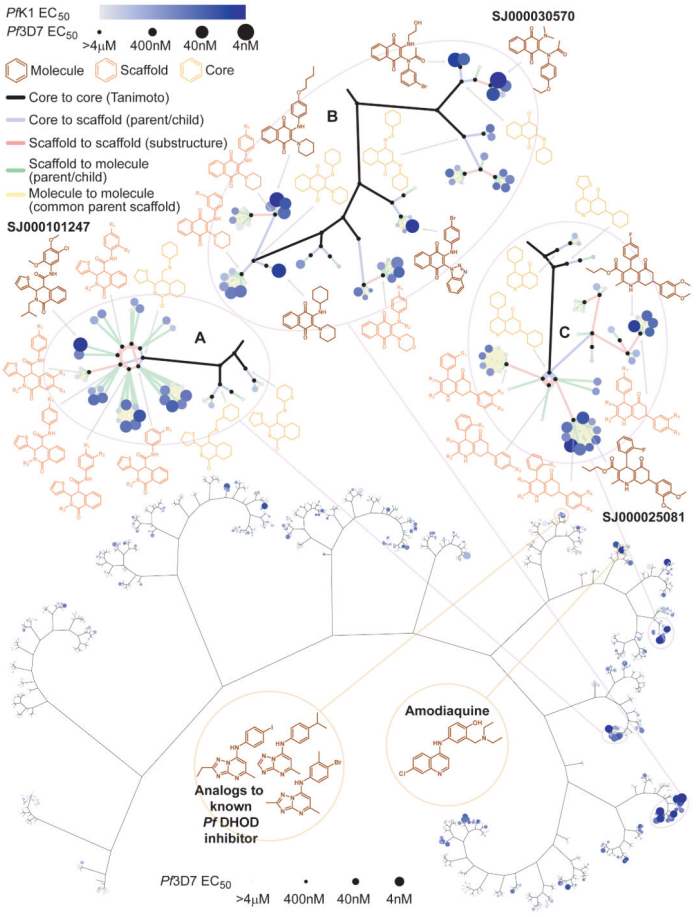


**Figure 1. Chemical structure network graph and antimalarial potencies of the 1300 validated hits**

Topologically similar molecules cluster together in the branches of the network. To construct the graph, molecules were first abstracted to scaffolds and then further to cores using the Murcko algorithm.10 Each of these structural entities is represented as a node, and nodes are connected via edges according to topological relationships. Molecular nodes are coded to reflect potency against *Pf*K1 (low = white, high = blue) and *Pf*3D7 (low = small, high = large). The highly branched structure of the full network graph (bottom half of the figure) indicates that the 1300 compounds are organized into clusters of clusters: cores are well sampled by multiple scaffolds, and the cores themselves are grouped into families of related chemotypes. Previously reported antimalarial compounds are highlighted. The top half of the figure provides greater detail on three potent chemotypes with well-developed structure activity relationships: (A) tetrahydroisoquinoline, (B) naphthoquinone, and (C) dihydropyridine. Data in supplementary file (‘Guiguemde_Excel_SI’, ‘Structure’ tab).

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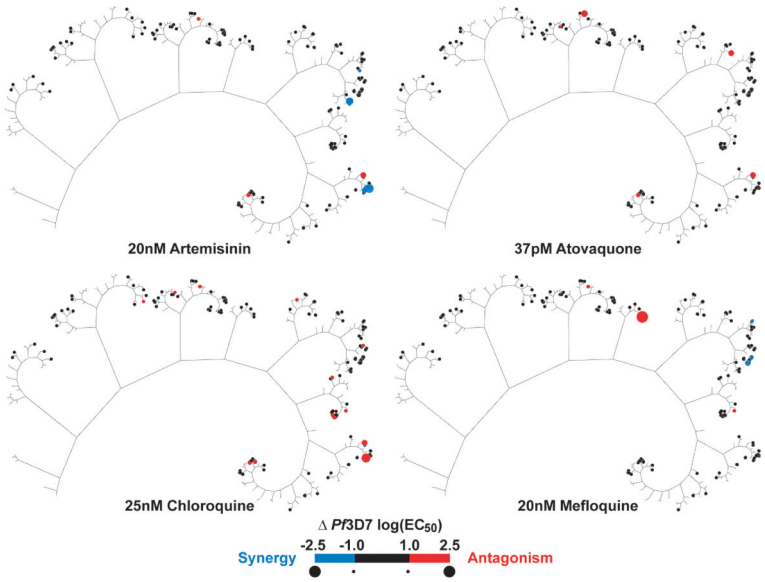


**Figure 2. Reduced representation of the network map showing synergistic activities with clinically relevant antimalarials**

The size of the nodes reflect the magnitude of the logarithmic difference between EC50 in the presence and absence of EC10 of exemplar antimalarial drugs. Absolute differences less than one log unit were not considered significant. Synergistic and antagonistic compounds are uniformly color-coded blue and red, respectively. Highly synergistic compounds can be seen with artemisinin and mefloquine. Data in supplementary file (‘Guiguemde_Excel_SI’, ‘Synergy’ tab).

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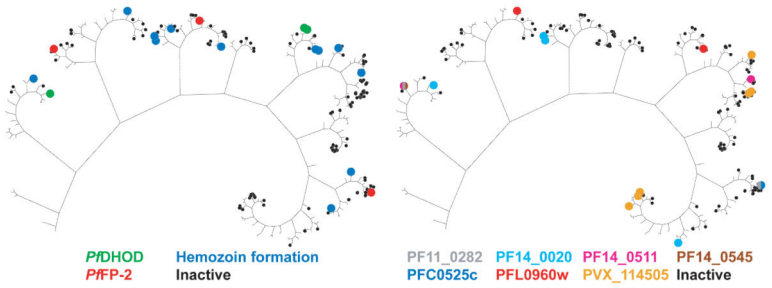


**Figure 3. Reduced representation of the network map showing the interaction of the cross-validated hits with potential biological targets**

The network map on the left displays compounds targeting well validated protein targets as measured in inhibition assays ($EC_{50} \leq 15$ μM). The map on the right shows compounds that bind to purified malarial proteins according to thermal-melt shift experiments. The size of nodes representing active or binding compounds is increased for clarity. Note that few of the compounds discovered in this study are potent inhibitors of known targets or bind potently to putative targets. Data in supplementary file (‘Guiguemde_Excel_SI ’, ‘Sensitivity’ tab).

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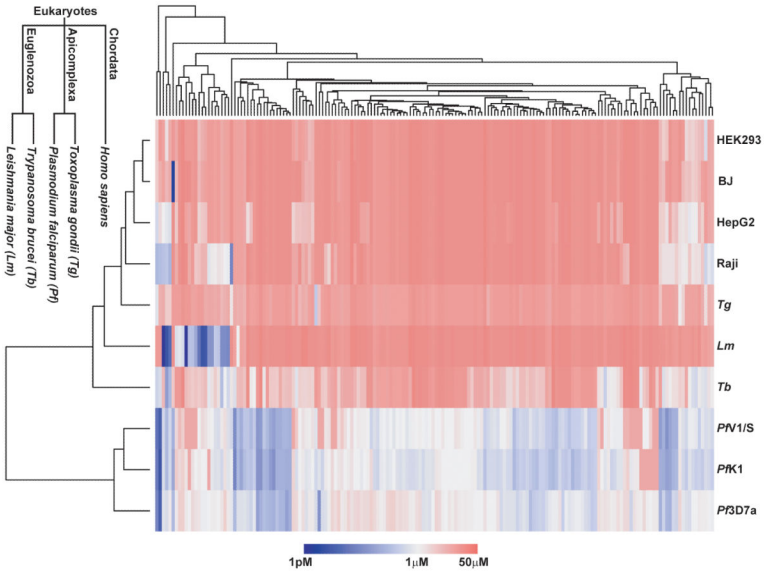


**Figure 4. Phylochemogenetic profiling**

Phylochemogenetic analysis of the cross-validated compounds using a two-way hierarchical clustering. of growth inhibition against *P. falciparum* strains (*Pf3D7*, *PfK1*, *PfV1/S*), other eukaryotic parasites (*Toxoplasma gondii* [Tg], *Trypanosoma brucei* [Tb], *Leishmania major* [Lm]), and human cell lines (HEK293, BJ, Raji, and HepG2). Columns represent single compounds and are clustered according to potency against the cell lines and organisms. Neighboring compounds share a similar potency spectrum. Rows represent a single cell line or organism and are clustered according to their chemosensitivity to the compounds in the study. A phylogenetic tree of the organisms in this study is provided for reference. Note that despite the many known examples of taxonomically-conserved pathways, on a global level, phylogeny is a poor predictor of chemical sensitivity profiles: *Toxoplasma* responses more closely parallel human than their evolutionary siblings, *Plasmodium*. Data in supplementary file (‘Guiguemde_Excel_SI’, ‘Phylo’ tab).