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Identification of inhibitors for putative malaria drug targets amongst novel antimalarial compounds

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Abstract

The efficacy of most marketed antimalarial drugs has been compromised by evolution of parasite resistance, underscoring an urgent need to find new drugs with new mechanisms of action. We have taken a high-throughput approach toward identifying novel antimalarial chemical inhibitors of prioritized drug targets for *P. falciparum*, excluding targets which are inhibited by currently used drugs. A screen of commercially available libraries identified 5,655 low molecular weight compounds that inhibit growth of *P. falciparum* cultures with EC₅₀ values below 1.25 μM. These compounds were then tested in 384- or 1536-well biochemical assays for activity against nine *Plasmodium* enzymes: adenylosuccinate synthetase (AdSS), choline kinase (CK), deoxyuridine triphosphate nucleotidohydrolase (dUTPase), glutamate dehydrogenase (GDH), guanylate kinase (GK), N-myristoyltransferase (NMT), orotidine 5'-monophosphate decarboxylase (OMPDC), farnesyl pyrophosphate synthase (FPPS) and S-adenosylhomocysteine hydrolase (SAHH). These enzymes were selected using TDRtargets.org, and are believed to have excellent potential as drug targets based on criteria such as their likely essentiality, druggability, and amenability to high-throughput biochemical screening. Six of these targets were inhibited by one or more of the antimalarial scaffolds and may have potential use in drug development, further target validation studies and exploration of *P. falciparum* biochemistry and biology.

Keywords

target-based drug development; enzyme activity assays; antimalarial compounds

1. Introduction

Approximately 3 billion people, one half of the world's population, live in at-risk regions for malaria infection. This leads to about 250 million malaria cases every year and nearly one

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million deaths (World Malaria Report, WHO, 2009, http://www.who.int/malaria/world_malaria_report_2009/en/index.html). The etiologic agent *Plasmodium falciparum* is the organism responsible for the majority of deaths due to malaria, 90% of which occur in Africa with more than 85% in children under the age of 5. The presence of widespread drug resistance is hampering the effectiveness of most of the available drug arsenal [1–6] with the notable exception of artemisinin-based derivatives. Artemisinin-based combination therapies (ACT) are the recommended first-line treatments for *falciparum* malaria in all countries with endemic disease [7]. Though ACT remains effective, there are recent concerns that the efficacy of artemisinin-based derivatives has declined on the Thai-Cambodian border [8–10]. Potential for clinical resistance is closely monitored in areas of Southeast Asia where artemisinin had been used as a monotherapy for decades and where antimalarial drug resistance previously developed. The potential development of artemisinin resistance by the parasite and loss of ACT would be a crisis, as ACTs have been recommended worldwide and all new agents currently in phase 3 clinical trials are based on ACT. In the battle to fight malaria, introduction of new drugs with novel mechanisms of action is essential.

Data on the essentiality of putative drug targets for *Plasmodium* growth are limited to a few individual genes that have been tested in gene disruption studies [11–19]. However, chemical inhibition of a target with genetic essentiality does not always translate to pharmacological efficacy against the pathogen [20], nor are genetic data on essentiality available in a comprehensive genome-wide dataset for *Plasmodium*. Inhibition of *P. falciparum* replication through chemical inhibition of potential drug targets can, instead, provide chemical validation of these targets. To this end, we undertook an effort to provide chemical tools to explore the biology of prioritized *Plasmodium* targets, and to provide a starting point for chemical validation of these targets. We developed biochemical assays to test the enzymatic activity of nine prioritized targets. A set of compounds which had been pre-annotated as having inhibitory activity on *P. falciparum* proliferation in erythrocytes was tested for any inhibitory activity in each of these biochemical assays. We reasoned that screening compounds already known to have cell-based activity might, at least in part, address some of the issues which have plagued biochemical approaches. These include cellular permeability, efflux and metabolism of small-molecule inhibitors.

We first screened GNF's non-proprietary compound collection and identified a subset of 5,655 compounds which have potent inhibitory activity on *P. falciparum* growth *in vitro* as described in [21]. These compounds are non-proprietary and most are available commercially, facilitating broad exploration of their mechanism of action by the academic community. We have made the structures of these compounds available at <http://www.ebi.ac.uk/chembl/db/> and <http://www.TDRtargets.org/>. We have included in this dataset dose-response data for activity on two strains of *P. falciparum*, 3D7 and W2, as EC₅₀ values, as well as cytotoxicity information on the human hepatocellular carcinoma cell line Huh7, and a measure of the promiscuity of the compounds across other historical high-throughput screens conducted at GNF. Where available, the compounds were tested as re-ordered powders from the supplier. This collection was then screened in high-throughput biochemical assays against nine *Plasmodium* enzymes considered to be promising drug targets based upon data compiled at TDRtargets.org [22] and other literature data. The results of these nine target-based biochemical screens are discussed below. Notably we identified chemical inhibitors of 6 of the 9 targets tested from this sub-library of only a few thousand compounds, preselected to have cellular activity.

2. Materials and methods

2.1. Identification of antimalarial compounds

A high-throughput screen of GNF's non-proprietary compound collection was performed using an erythrocyte-based *P. falciparum* strain 3d7 infection assay essentially as described elsewhere [21]. Compounds were screened at 1.25 μM ; those hits inhibiting growth by $\geq 50\%$ were reconfirmed in dose-response format to determine EC_{50} values, and cellular cytotoxicity (quantified as CC_{50}) was assessed in the Huh7 hepatocellular carcinoma cell line. Compounds with a selectivity index of < 5 ($\text{CC}_{50}/\text{EC}_{50}$) were excluded from further consideration, as were compounds found to quench fluorescence. Reconfirmed hit compounds (5,655 in total) were re-ordered as powders and 3,086 compounds were available for purchase from suppliers.

2.2. Target selection

In selecting enzymes to screen (Table 1), we employed TDRtargets.org [22] and other sources to identify *Plasmodium* enzymes as candidates for compound screening using the following criteria: i) the enzyme is likely to be required for survival of the parasite; ii) the enzyme has a small-molecule binding pocket that might be exploited with a drug-like molecule to inhibit enzymatic activity; iii) there are exploitable differences between the parasite and human enzymes and/or the pathways to which they belong so that a drug might be developed with minimal adverse effects on the host; iv) the enzyme is amenable to recombinant expression (for which there was precedent for all selected targets [23–30]; and v) the enzymatic activity can be measured in a high-throughput format. Of these criteria we focused especially on i, iv, and v because druggability (ii) and selectivity (iii) are currently difficult to predict. (As an example of the latter, difluoromethylornithine inhibits both the human and *T. brucei* ornithine decarboxylases, yet selectively kills *T. brucei* cells, apparently because of the more rapid turnover of the human enzyme [31].) The nine enzymes chosen by these criteria were adenylosuccinate synthetase (AdSS), choline kinase (CK), deoxyuridine triphosphate nucleotidohydrolase (dUTPase), farnesyl pyrophosphate synthase (FPPS), glutamate dehydrogenase (GDH), guanylate kinase (GK), N-myristoyltransferase (NMT), orotidine 5'-monophosphate decarboxylase (OMPDC), and S-adenosylhomocysteine hydrolase (SAHH).

2.3 Expression and purification of recombinant Plasmodium proteins

Recombinant histidine-tagged enzymes from *Plasmodium falciparum* and *P. vivax* were expressed in *Escherichia coli* and purified by immobilized metal affinity chromatography, essentially as described previously [32].

2.4 Biochemical assay details

Specific activities of the nine enzymes studied have been reported previously [33], along with comparisons to literature values where possible.

Enzyme activity assays were miniaturized and optimized for 1536-well plates and test compounds screened in dose-response format over 12 points in $\frac{1}{2}$ -log serial dilutions (0.0005 – 100 μM), the exception being SAHH, which was screened in single-point format in 384-well plates. Incubations were at room temperature unless otherwise noted. All reactions included a final [DMSO] of 1%. The initial collection of antimalarial compounds was available as a set of DMSO solutions pre-existing in the GNF compound library (available formats: 5,655 compounds, 1536-well dose-response format; 5,543 compounds, 384-well single point format), and 6 targets were screened using these collections (Table 3). As the original stocks of antimalarial compounds became depleted, the three remaining targets (FPPS, dUTPase, and NMT) were screened using DMSO solutions reconstituted

from the 3,086 compounds available for repurchasing at the time of these screens. Hits were reconfirmed using re-ordered purified powders where available from the vendor and tested in dose-response format.

Chemical reactions and detection strategies for each enzyme are shown in Table 2; additional details are noted below. In general, assay concentrations of most substrates were approximately 3 times their respective K_m 's. Compounds, in general, were added to enzyme and buffer reagents 10 minutes prior to initiation of reaction by addition of substrate. If compounds hit in multiple target-based assays they were triaged due to lack of specificity (i.e., likely false positives) and not included in the final hit list (Supplementary Table S1).

AdSS was assayed in a buffer of 50 mM Tris, pH 7.0, with 5 mM DTT, 0.1% BSA, 0.001% Tween-20, and 2.5 mM $MgCl_2$. Final concentrations were 500 ng/mL AdSS (10 nM), 30 μ M GTP, 60 μ M IMP, and 1 mM aspartate. Incubation time was 40 minutes, after which the GDP produced by AdSS was detected with the Transreener GDP detection mix (Cisbio), used according to the manufacturer's instructions. Homogeneous time-resolved fluorescence (HTRF) of the GDP produced was monitored via excitation at 337 nm and dual emission at 620 and 665 nm.

CK was assayed in a buffer of 100 mM HEPES, pH 7.5, with 150 mM NaCl and 1 mM $MgCl_2$. Final concentrations were 312.5 ng/mL CK (5 nM), 10 μ M ATP, and 280 μ M choline. Incubation time was 30 minutes, after which the ADP produced by CK was detected with the Transreener ADP detection mix (Cisbio), as for AdSS.

dUTPase was assayed in a buffer of 25 mM MOPS, pH 8.0, with 10 mM KCl, 1.25 mM $MgCl_2$, 0.1 mg/mL BSA, 0.005% Triton X and 20% glycerol. Final concentrations were 2 ng/mL dUTPase (97 pM) and 14 μ M dUTP. Incubation time was 30 minutes. Production of PP_i was monitored with the PPLight kit (Lonza) via conversion to ATP and measurement of luminescent signal corresponding to [ATP]. The PP_i detection mix was diluted 1:4 with reaction buffer prior to use.

FPPS was assayed in a buffer of 50 mM HEPES, pH 7.5, with 0.625 mM $MgCl_2$, 1 mM NaCl, 0.25 mM TCEP, 5% BSA, 10% glycerol, and 0.01% Triton X-100. Final concentrations were 1.3 μ g/mL FPPS (45 nM), 25 μ M geranyl diphosphate, and 25 μ M isopentenyl diphosphate. Incubation time was 30 minutes. Production of PP_i was monitored with the PPLight kit (Lonza), as for dUTPase.

GDH was assayed in a buffer of 100 mM Tris, pH 8.0, with 1 mM DTT, 0.1 mg/mL BSA, and 0.005% Triton X-100. Final concentrations were 0.5 μ g/mL GDH (10 nM), 400 μ M $NADP^+$, and 10 mM glutamate. Incubation time was 45 minutes. The fluorescence of the NADPH produced was monitored via excitation at 355 nm and emission at 486 nm.

GK was assayed in a buffer of 50 mM Tris, pH 7.5, with 50 mM KCl, 2 mM $MgCl_2$ and 0.1 mg/mL BSA. Final concentrations were 1.5 ng/mL GK (130 pM), 7 μ M GMP, and 10 μ M ATP. Incubation time was 20 minutes, after which ADP and GDP produced by GK were detected with the Transreener ADP detection mix (Cisbio), as for AdSS.

NMT was assayed in a buffer of 50 mM Tris, pH 7.5, with 6.25 mM NaCl, 0.625 mM $MgCl_2$, 10% BSA, and 0.005% Triton X-100. Final concentrations were 1.5 μ g/mL NMT (30 nM), 15 μ M myristoyl-CoA, and 90 μ M GSSYSRKNK, a synthetic peptide obtained from Peptides International or GenScript with order parameters of at least 85% purity and no N- or C-terminal modifications. This peptide was based on the N-terminal sequence of adenylate kinase 2, a substrate of N-myristoyltransferase from *P. falciparum in vivo* [34]. Incubation time was 45 minutes at 30 °C, after which ThioGlo [35] was added to a final

concentration of 15 μM to detect the free $-\text{SH}$ groups of CoA produced by NMT. ThioGlo fluorescence was monitored via excitation at 360 nm and emission at 540 nm.

OMPDC was assayed in a buffer of 100 mM Tris, pH 8.0, with 50 mM NaCl, 1 mM MgCl_2 , 1 mM DTT, and 0.3 mg/mL BSA. Final concentrations were 3 ng/mL OMPDC (77 pM), 2 μM OMP, 1 μM ATP, and 1 $\mu\text{g/mL}$ human CMP kinase [36], which was used to convert UMP (produced by OMPDC) + ATP to UDP + ADP. Incubation time was 180 minutes, after which the ADP was detected with the Transreener ADP detection mix (Cisbio), as for AdSS.

SAHH was assayed in a buffer of 50 mM potassium phosphate, pH 8.0. Final concentrations were 5 $\mu\text{g/mL}$ SAHH (100 nM), 4 μM S-adenosylhomocysteine, and 2.5 $\mu\text{g/mL}$ adenosine deaminase from *P. falciparum* (added to consume the adenosine produced by SAHH, promoting continued progress of the forward reaction). Incubation time was 90 minutes, after which ThioGlo [35] was added to a final concentration of 25 μM to detect the free $-\text{SH}$ groups of homocysteine produced by SAHH. ThioGlo fluorescence was monitored via excitation at 360 nm and emission at 540 nm. The SAHH screen was unique in that compounds were initially tested at a single concentration of 8.3 μM ; therefore, initial hits were defined as compounds inhibiting SAHH by $\geq 25\%$ at this concentration.

3. Results

3.1. Identification of antimalarial compounds

We identified a set of 5,655 compounds that inhibit growth of *P. falciparum* in erythrocytes as described in [21]. $\sim 7,500$ primary screen hits were tested in 8-point dose-response curves for reconfirmation of antimalarial activity and EC_{50} value determination against two drug-resistant strains: a sulfadoxine-resistant 3D7 strain and a multi-drug resistant W2 strain (resistant to chloroquine, quinine, pyrimethamine, sulfadoxine, and cycloguanil). Known inhibitors in the compound collection (e.g., artemisinin, mefloquine, dihydrofolate reductase inhibitors, quinine) were identified, providing internal validation of the assay sensitivity. Counterscreens included fluorescence quencher assessment and cytotoxicity in the Huh7 human hepatocellular carcinoma cell line. Compounds with an $\text{EC}_{50} < 1.25 \mu\text{M}$ against *P. falciparum* 3d7 and W2 strains and a selectivity index (SI) over the Huh7 cell line of at least 5-fold and that did not exhibit fluorescence quenching were retained for further study.

3.2. Choline kinase

CK catalyzes a step in the synthesis of phosphatidylcholine, which is essential for intraerythrocytic growth of the Plasmodium parasite [26, 37, 38]. The K_m of CK for choline was determined as $\sim 200 \mu\text{M}$, consistent with the value of 140 μM reported in [26]. The chemical inhibitor hexadecyltrimethylammonium bromide (HDTAB) can be used as a positive control for CK inhibition [37]; however, the physicochemical properties of HDTAB interfered with the homogeneous time resolved fluorescence (HTRF) signal detection.

As listed in Table 2, the CK screen resulted in 69 hits for a 1.2% hit rate. IC_{50} values ranged from 0.06 μM to 11 μM while *P. falciparum* EC_{50} values ranged from 0.013 μM to 1.25 μM . 23 powders were available for reconfirmation as powders, nine of these reconfirmed in triplicate with IC_{50} values ranging from 2.9 μM to 19.0 μM . Corresponding *P. falciparum* EC_{50} values were at least 10-fold more potent. Of the nine hits identified (see Supplementary Table S1), compound CK-1 (a 3-chlorobenzothiophene amide; shown in Figure 1A) has a chemically tractable structure and may be a suitable starting point for chemical optimization. CK-2 and CK-3 (Fig. 1A) are analogs in the same scaffold. While activities on the enzyme are weaker (20-fold) than the cell-based activities, the rank orders

in potency of these two compounds are the same between the cell-based and target-based assays.

3.3. S-Adenosylhomocysteine hydrolase

SAHH, a component of the methylation cycle in which methyl groups are donated to lipids, nucleic acids, proteins, and other molecules, is almost certainly essential and is highly druggable (Table 1). The similarity of the parasite and human active sites is a concern, with only one residue of the adenosine binding site – Cys59 for PfSAHH, Thr60 for HsSAHH – differing between the two [39]. The K_m of SAHH for S-adenosylhomocysteine was determined to be $\sim 2 \mu\text{M}$, close to the literature value of $1.2 \mu\text{M}$ [24]. The SAHH screen resulted in 38 hits and a hit rate of 0.68% (Table 2). 20 powders of the 38 hits were available, and of these, seven compounds reconfirmed in dose-response format and two were triaged based on overlap with other target screens (AdSS, below), suggesting that these were promiscuous inhibitors. IC_{50} values ranged from $0.1 \mu\text{M}$ to $9.1 \mu\text{M}$ and the corresponding *P. falciparum* EC_{50} values ranged from $0.9 \mu\text{M}$ to $7.6 \mu\text{M}$. Of the five reconfirmed and specific hits identified (Supplementary Table S1), SAHH-1 (Fig. 1B) is an interesting 8-hydroxyquinoline-based hit that might serve as a potential starting point for medicinal chemistry. Other hits that may be of interest as tool compounds, though not chemically attractive for drug discovery, include SAHH-2, a quinazolin, a known vasodilator with α -1 adrenergic receptor as its target [40] and SAHH-3, known as phanquinone, an antimicrobial agent [41].

3.4. Enzymes involved in nucleotide metabolism (AdSS, dUTPase, GK, and OMPDC)

With AdSS, part of the purine salvage pathway, we obtained K_m values of $14 \mu\text{M}$ for IMP, $105 \mu\text{M}$ for GTP, and 0.05 mM for aspartate, somewhat different from the previously reported values of $22.8 \mu\text{M}$, $4.8 \mu\text{M}$, and 1.4 mM , respectively [29]. The AdSS screen resulted in 9 hits and a 0.2% hit rate (Table 2). Hadacidin was used as the positive control inhibitor [42], its IC_{50} value being $62 \mu\text{M}$. Of these hits, six were available as powders and five reconfirmed. Two compounds were triaged as they also inhibited SAHH activity, leaving 3 candidate AdSS inhibitors (Supplementary Table S1). However, all three of these compounds are unlikely starting points for medicinal chemistry because of chemically labile functionalities such as sulfonylureas (AdSS-1), imines derived from aldehydes (AdSS-2) and quaternary aromatic amines (AdSS-3). AdSS-1 (Fig. 1C), an N-sulfonylurea, may be useful as a tool compound; however, publicly available information cites a cytostatic mechanism of action in human cells (in PubChem: 120394059 120394064). AdSS-2 (Fig. 1C), a benzaldehydederived imine, is relatively specific for malaria inhibition; this compound may also be useful as a tool compound for AdSS.

GK was selected as a target based on its involvement in nucleic acid synthesis. GK converts (d)GMP to (d)GDP, which in turn is used to make (d)GTP, which is essential for nucleic acid synthesis. The likely essentiality of GK is, however, in doubt, as the *Plasmodium* thymidylate kinase (2.7.4.9) can also convert dGMP to dGDP [43, 44]. An additional enzyme, ribonucleoside-diphosphate reductase (1.17.4.1), can convert dGDP to GDP. Thus, even if GK is blocked, (d)GDP might still be made from dGMP. Nevertheless, the expression of GK in the liver stage of the *Plasmodium* life cycle [45] suggests its possible importance in this stage and thus its possible usefulness as a target complementing those most prominent in the erythrocyte stages.

The K_m of our *P. vivax* GK for GMP was $3.5 \mu\text{M}$, lower than a value of $22 \mu\text{M}$ reported for the *P. falciparum* GK [28]. The GK screen resulted in 17 hits (0.3% hit rate) as shown in Table 2. The positive control iodoacetamide had an IC_{50} value of $36.5 \mu\text{M}$ under the assay conditions used. The range of guanylate kinase IC_{50} values across the 17 hits is $0.5 \mu\text{M}$ – 7.8

μM . Seven compounds were available as powders; four of these reconfirmed with 3 showing specificity for PvGK (Supplementary Table S1) and are shown in Figure 1D. GK-1 has two nitro groups and as such does not represent a chemically attractive scaffold unless analogs without these nitro groups remain active. Nevertheless, this compound is relatively specific and does not appear to be a promiscuous inhibitor in historical HTS run at GNF. Interestingly, most of the screens with overlapping activity were pathogen-based screens which may be related to the presence of the nitro groups. GK-2 and GK-3 (Figure 1D) are potentially useful as tool compounds to interrogate malaria parasite biology and to confirm PvGK is indeed the target of these compounds. However, GK-2 is an ammonium salt with low likelihood for oral bioavailability, and GK-3 is an imine of a naphthoquinone which may have stability issues and trap electrophiles; thus, neither is an ideal starting point for medical chemistry efforts.

OMPDC is a pyrimidine biosynthesis enzyme downstream of the well-validated target dihydroorotate dehydrogenase [46]. The approximate K_m for orotidine 5'-monophosphate (OMP) was $\sim 0.2 \mu\text{M}$, much lower than a previously reported value of $13.4 \mu\text{M}$ (PMID 15683248). The OMPDC screen resulted in 26 hits for an approximate hit rate of 0.5% (Table 2). IC_{50} values ranged from $0.22 \mu\text{M}$ to $10 \mu\text{M}$ while *P. falciparum* EC_{50} values ranged from $0.176 \mu\text{M}$ to $1.7 \mu\text{M}$. 11 powders were available for reconfirmation and of these four reconfirmed, although two were identified to be fluorescence quenchers and therefore false positives. Unfortunately, the most attractive hit from a chemical standpoint, OMPDC-1 (Fig. 1E), is only a weak inhibitor of OMPDC (IC_{50} $19.7 \mu\text{M}$), although more potent in the cellular *P. falciparum* assay. OMPDC-1 may be useful as a tool compound for investigation of the effects on the parasite of OMPDC inhibition.

dUTPase protects DNA integrity by preventing the buildup of dUTP through conversion to dUMP, which is then converted to dTTP and used in DNA synthesis [47]. The K_m of dUTPase for dUTP was measured as $4.5 \mu\text{M}$, somewhat higher than the literature value of $1.9 \mu\text{M}$ [48]. The positive control used for dUTPase inhibition was 2'-deoxy-5'-O-(triphenylmethyl)uridine, which had an IC_{50} value of $2.4 \mu\text{M}$ under our assay conditions. The dUTPase screen resulted in 95 hits (3.1% hit rate) and the positive control inhibitor performed as expected. 91 compounds that were available as powders were retested in dose-response for reconfirmation of screen IC_{50} values. In parallel, hits were counterscreened for the potential to inhibit components of the PP_i detection kit made by Lonza. The counterscreen tested putative hits for their ability to inhibit direct detection of $\sim 5 \mu\text{M}$ PP_i , using the same detection reagents as the primary dUTPase. The positive control showed the expected inhibition profile in the dUTPase assay without any inhibition in the counterscreen. However, all hits were also inhibitors in the counterscreen. In conclusion, we did not identify any hits as inhibitors of dUTPase.

3.5. Enzymes involved in pathways for adding lipid groups to proteins (NMT and FPPS)

NMT transfers myristate (saturated C_{14} fatty acid) groups to N-terminal glycine residues of substrate proteins, increasing their hydrophobicity and promoting associations with membranes and other proteins [49]. NMT had a K_m of $7 \mu\text{M}$ for myristoyl-CoA and $21 \mu\text{M}$ for the synthetic peptide GSSYSRKNK.

The positive control inhibitor for NMT inhibition, aurintricarboxylate, had an IC_{50} of $\sim 3 \mu\text{M}$. The NMT screen resulted in 7 hits and an overall hit rate of 0.2%. The positive control inhibitor, aurintricarboxylate, performed as expected. 5 compounds were available and reconfirmed for inhibition. NMT-1 (Fig. 1F) did not score in past screens performed at GNF, suggesting a rather specific activity on NMT. While there is a single nitro group, with concomitant risk for mutagenic potential [50], this compound may be a useful tool compound to validate NMT as a viable drug target. None of the other structures are

chemically attractive for drug discovery, as they contain too many structural alerts, such as nitro groups in NMT-1 and NMT-3 and ammonium salts in NMT-2, 4, and 5 (see Supplementary Table S1), but they can possibly be used as tool compounds for NMT inhibition.

FPPS is involved in the post-translational modification of proteins through protein farnesylation. FPPS produces farnesyl diphosphate (a.k.a. farnesyl pyrophosphate or FPP), which is then used by protein farnesyltransferase (PFT), a validated target in *Plasmodium* [51], in the attachment of farnesyl groups to proteins. K_m 's of FPPS were 8 μM for geranyl diphosphate and 9 μM for isopentenyl diphosphate. The positive control inhibitor (1-hydroxy-2-phenylethylidene)bis-phosphonic acid has an IC_{50} of 7.7 μM against FPPS and an EC_{50} value of ~ 7.05 μM against *P. falciparum* *in vitro*. The FPPS screen resulted in 147 hits. Unfortunately, with the exception of the positive control, (1-hydroxy-2-phenylethylidene)bis-phosphonic acid, all hits that reconfirmed in the FPPS assay also were hits in the counterscreen (conducted as described above for dUTPase). In conclusion, no compounds hit with specificity against FPPS with the exception of the positive control inhibitor.

3.6. Glutamate dehydrogenase

GDH provides NADPH for biosynthesis and for glutathione reductase, an oxidative stress-fighting enzyme thought to be essential [25]. In contrast to the mammalian GDH enzyme, PfGDH is highly specific for NADP^+ and NADPH, suggesting exploitable differences between the mammalian and parasite enzymes. K_m 's were estimated to be 16 μM for NADP^+ and 0.8 mM for glutamate, consistent with the literature values of 20 μM and 1 mM, respectively [25]. The GDH screen resulted in 164 hits, or a 2.9% hit rate; however, activity of most hits against GDH was weak. 78 powders were available and tested for reconfirmation but no hits reconfirmed under the assay conditions used. To decrease the assay stringency and increase sensitivity to inhibitors, the concentration of the substrate [NADP^+] was reduced from 400 μM to 50 μM , but still no hits reconfirmed. In conclusion, no hits were identified against GDH.

4. Discussion

The availability of large compound collections and automated robotic systems for ultra high-throughput screening is now commonplace for many pharmaceutical companies, but less frequent in academia. Here we describe a collection of antimalarial chemical inhibitors identified by screening non-proprietary compound collections. In this first application, we attempted to link some of our antimalarial compounds to their possible protein targets. The cell-active compounds were tested in high-throughput assays against enzymes believed to have potential as drug targets based on considerations of essentiality, druggability, selectivity, and amenability to high-throughput screening (section 2.2 and Table 1). These enzymes included several involved in nucleotide metabolism (AdSS, dUTPase, GK, and OMPDC) and attachment of lipid groups to proteins (NMT and FPPS) as well as components of other key metabolic pathways (CK, GDH, and SAHH). While we were unable to detect inhibitors of dUTPase, FPPS and GDH, we identified inhibitors of AdSS, GK, OMPDC, NMT, CK, and SAHH as potential starting points for medicinal chemistry or as tool compounds for these *Plasmodium* enzymes. In our view, these results neither validate nor disprove the wisdom of our target selection strategy. We were disappointed not to find more potent inhibitors of the enzymes chosen; however, a screen of a larger chemical library might have retrieved compounds suitable for development into leads with good activity against both the enzymes and parasite cells.

IC₅₀ values of preliminary hits against enzymes were checked with repurchased compounds (Table 3 and Supplementary Table S1). Reversible inhibitors generally act on their intracellular targets with an IC₅₀ lower than the EC₅₀ against the parasite. However, the compound concentrations of most of our target-based hits required to inhibit enzyme activity are higher than the concentrations needed to inhibit *P. falciparum* growth. There are a number of possible explanations for this cellular and enzymatic discrepancy. First, some of the targets inhibited by our antimalarial compounds may not be the only, or even the primary, target of the compound; inhibition of more than one protein (and/or non-protein targets) might contribute to cellular activity [52]. Second, it is possible that the mechanism by which a compound is affecting parasitic activity is completely unrelated to the biochemical activity reported here. Third, EC₅₀ values would be lower relative to biochemical IC₅₀ values if the test compound accumulates to higher concentrations inside the parasite. Lastly, comparing enzyme activity with antimalarial activity may not be particularly helpful, given unavoidable differences between intracellular and *in vitro* conditions (e.g. substrate concentrations), unless one is comparing structure activity relationships (SAR) among a set of chemically related analogs and relative enzyme and antimalarial inhibition. Derivation of the pharmacophore and correlation between enzyme-based and parasite-based inhibition is needed to identify meaningful SAR correlations and link target hits to the observed cell-based phenotype. Our highest priority will be to follow up on enzyme inhibitors with IC₅₀'s < EC₅₀'s, but investigations of other associations of high-value compounds and targets may also prove worthwhile to chemically validate targets.

Three targets (dUTPase, GDH and FPPS) lacked any hits among the compounds tested. It is possible that these enzymes are not essential to the parasite under our assay conditions. Alternatively, the broader library from which the antimalarial compounds were selected might have been lacking in chemical composition capable of inhibiting these enzymes. Regarding the latter possibility, these enzymes are predicted to have moderate to high druggability according to *in silico* methods (see Table 1). Our approach thus offered a test of these *in silico* predictions and suggested that they might benefit from further refinement.

To test whether inhibition of a particular biochemical target is causal for antimalarial activity, several approaches are possible. These include: (a) allowing cultures to evolve resistance to the compound and determining whether mutations occur in the gene for the presumed target [53]; (b) studying compounds chemically related to the original hit and looking for a correlation between IC₅₀'s against the presumed target and EC₅₀'s against the parasite [54]; (c) determining whether overexpression of the putative target overcomes the inhibition observed in the presence of the compound [55] (although genetic manipulation of *Plasmodium* can be challenging); (d) detecting a buildup of the presumed target's substrate or a depletion of its product in extracts of compound-treated cells [56–58], and/or determining whether parasite growth can be restored via supplementation of the media with the missing product [59]; and (e) using compounds as affinity probes to isolate target proteins from cell lysates [60].

Despite the extensive screening work reported here, the mechanisms of action of our 5,655 antimalarial compounds generally remain elusive. However, modifications of the present approach might lead to greater success in identifying mechanisms of action. For example, studying compounds with EC₅₀'s >1.25 μM might enable discovery of those with weak activity against the parasite but with EC₅₀/IC₅₀ data that strongly point to a particular target. Furthermore, considering that roughly 2 to 5% of an organism's genes may have legitimate potential as drug targets [61], the number of proteins tested here was relatively small due to the labor-intensive requirement of optimizing a separate biochemical assay for each protein. A generic method for assessing compound-protein binding, such as thermal melting [32] or Surface Plasmon Resonance [62], might allow more proteins to be studied simultaneously,

increasing the odds that some could be identified as the likely targets of antiparasitic compounds. Finally, it is possible that similar screening efforts might instead focus on finding new scaffolds for previously validated targets.

In conclusion, these data do not support our original proposition that target-based drug discovery can be accelerated by starting with cell-active compounds. While it is too early to say whether any of the enzyme-compound associations identified in these screens represent the compound's true mechanism of action in inhibiting parasite growth, these compounds nevertheless have utility in exploring the biology surrounding these possible targets. Such tool compounds can be employed in basic studies of enzyme function and pathway biology as well as starting points for structure-activity relationship (SAR) and selectivity studies. Screens of the kind performed here can thus yield valuable information even when they do not result in identification of biochemically potent lead compounds for drug development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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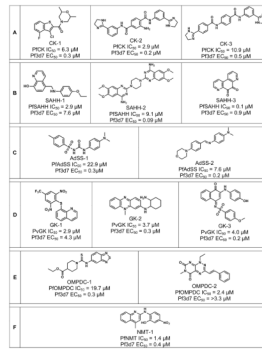


Figure 1. Chemically tractable inhibitors discovered by the biochemical screens. Abbreviations refer to the enzymes inhibited: (A) choline kinase (CK), (B) S-adenosylhomocysteine hydrolase (SAHH), (C) adenylosuccinate synthetase (AdSS), (D) guanylate kinase (GK), (E) OMP decarboxylase (OMPDC), (F) N-myristoyltransferase (NMT).

Table 1

Selection of promising drug targets for biochemical screens

Enzyme name (PlasmoDB ID)	Essentiality	Druggability score*	Genome-wide percentile ranking**
Adenylosuccinate synthase (PF13_0287)	Part of purine salvage pathway, which should be essential because <i>Plasmodium</i> cannot synthesize purines de novo [63].	0.8	99.5%
Choline kinase (PF14_0020)	Chemical validation of the Kennedy pathway for phosphatidylcholine synthesis is suggested by the work of Henri Vial [64].	0.6	97.9%
dUTPase (PF11_0282)	dUTPase is most likely essential because it prevents the buildup of dUTP and makes dUMP for dTTP synthesis [47].	0.6	99.8%
Farnesyl pyrophosphate synthase (PVX_092040)	Since protein farnesyltransferase is a valid drug target in <i>Plasmodium</i> [51], FPPS should be as well because it supplies the substrate for PFT. A recent paper reported an ability to predict cell-killing activity from bisphosphonates' inhibition of FPPS [54].	0.8	99.3%
Glutamate dehydrogenase (PF14_0164)	Thought to be a major source of NADPH for glutathione reductase, which is thought to be essential [65]. This is based in part on the fact that <i>Plasmodium</i> is "exposed to multiple oxidative stress due to its high metabolic rate, the degradation of heme and reactive oxygen species imposed by the host immune system" [66].	0.8	99.7%
Guanylate kinase (PVX_099895)	Based on metabolic maps, guanylate kinase appeared to be the only route by which <i>Plasmodium</i> can convert GMP to GDP or dGMP to dGDP. (A possible bypass is noted in the text.) Also, expression during liver stage [45] suggests importance in that stage.	0.2	98.4%
N-myristoyltransferase (PF14_0127)	As argued by Gelb et al. [49], "Genetic studies have shown that the NMT gene is essential for viability in a range of species, including <i>Drosophila melanogaster</i> , <i>S.</i>	0.8	98.4%

Enzyme name (PlasmoDB ID)	Essentiality	Druggability score*	Genome-wide percentile ranking**
	<i>cerevisiae</i> and <i>Candida albicans</i> and <i>Cryptococcus neoformans</i> .”		
OMP decarboxylase (PF10_0225)	Part of the de novo pyrimidine synthesis pathway, which should be essential in Plasmodium because it does not have a pyrimidine salvage pathway [63]. Also, chemical validation of the de novo pyrimidine pathway comes from studies of dihydroorotate dehydrogenase [46].	N/A	98.6%
S-adenosylhomocysteine hydrolase (PFE1050w)	Part of a methylation cycle whose blockage should be fatal [39].	0.8	99.9%

* Druggability scores were taken from TDRtargets.org. Scores range from 0.0 to 1.0, with 1.0 signifying maximum druggability and ~0.2 signifying an average score.

** TDRtargets.org was used to perform a multiparameter weighted search, with points awarded for various features considered desirable in a potential drug target. Percentiles were then computed from a rank-order list of all genes in the Plasmodium genome. Points were awarded as follows: 8 points to each gene for each life cycle stage (merozoite, early and late ring, early and late schizont, and early and late trophozoite) in which their expression ranks in the top quintile (80th to 100th percentile) for a given erythrocyte life cycle stage; 4 points to each gene for each life cycle stage in which expression was in the 60th to 80th percentile; 50 points to each gene with genetic validation (according to manual curation of the literature); 35 points to each gene associated with one or more publications (according to an automated analysis of PubMed); 50 points to each gene with one or more associated crystal structures; 30 points to each gene for which there is a 3D structural model (ModBase); 25 points to each gene lacking an ortholog in humans; 20 points to each gene lacking a predicted transmembrane domain; 20 points to each gene coding for a protein less than 100 kilodaltons; 100 points to each gene coding for an enzyme; 40 points to each gene whose orthologs are essential in at least one model organism; and 30 points to each gene for which there is an activity assay available.

Table 2

Screening reactions and detection strategies

Enzyme name (EC number)	Chemical reactions	Detection strategy	Control wells*
Adenylosuccinate synthetase (6.3.4.4)	Primary: GTP + IMP + L-aspartate → GDP + P _i + adenylosuccinate	Detect GDP using Cisbio Transcreener ADP kit.	Hadacidin [42]
Choline kinase (2.7.1.32)	Primary: ATP + choline → ADP + phosphocholine	Detect ADP using Cisbio Transcreener ADP kit.	No choline
dUTPase (3.6.1.23)	Primary: dUTP + H ₂ O → dUMP + PP_i	Detect PP _i with Lonza PPiLight kit.	CAS: 14270-73-6,84472-83-3
Farnesyl pyrophosphate synthase (2.5.1.-)	Primary: geranyl diphosphate + isopentenyl diphosphate → farnesyl diphosphate + PP_i	Detect PP _i with Lonza PPiLight kit.	compound 11 from [67]
Glutamate dehydrogenase (1.4.1.4)	Primary: L-glutamate + H ₂ O + NADP ⁺ → 2-oxoglutarate + NH ₃ + NADPH + H ⁺	Detect NADPH fluorescence.	No glutamate
Guanylate kinase (2.7.4.8)	Primary: ATP + GMP → ADP + GDP	Detect ADP and GDP using Cisbio Transcreener ADP kit.	Iodoacetamide
N-myristoyltransferase (2.3.1.97)	Primary: myristoyl-CoA + glycylpeptide → CoA + N-myristoylglycylpeptide	Detect CoA's free -SH groups with ThioGlo.	aurintricarboxylate
OMP decarboxylase (4.1.1.23)	Primary: OMP → UMP + ATP CMP kinase: UMP + ATP → UDP + ADP	Detect ADP using Cisbio Transcreener ADP kit.	No OMP
S-adenosylhomocysteine hydrolase (3.3.1.1)	Primary: S-adenosylhomocysteine + H ₂ O → adenosine + homocysteine Adenosine deaminase: adenosine → inosine + NH ₃	Detect homocysteine's free -SH groups with ThioGlo.	No S-adenosylhomocysteine

* Control wells either contained a known inhibitor of the enzyme or lacked a substrate so as to mimic the condition of complete inhibition of the enzyme.

Table 3

Results of biochemical screens

Enzyme	Z' score	Hit rate*	# Hits	Reconfirmed (powders)	Reconfirmation Enzyme (IC ₅₀ μM)	3d7 <i>faiciparum</i> (EC ₅₀ μM)
Pf Choline Kinase	0.802	1.2%	69	9 of 23	2.9 – 19.0	0.04 – 0.7
Pf dUTPase	0.466	3.1%	95	0 specific to dUTPase	n/a	n/a
Pf OMP Decarboxylase	0.751	0.5%	26	4 of 11	2.4 – 58.0	0.093 – 6.0
Pf S-adenosyl-Homocysteine Hydrolase	0.400	0.7%	38	7 of 19	0.1 – 9.1	0.9 – 7.6
Pf Glutamate Dehydrogenase	0.783	2.9%	164	0 of 78	n/a	n/a
Pv Guanylate Kinase	0.571	0.3%	17	4 of 7	2.9 – 4.0	0.092 – 3.4
Pf Adenylosuccinate Synthase	0.667	0.2%	9	5 of 6	1.7 – 22.9	0.2 – 1.6
Pf N-Myristoyl Transferase	0.640	0.2%	6	5 of 5	1.4 – 8.6	0.04 – 2.6
Pf Farnesyl Pyrophosphate Synthase	0.667	0.2%	6	0 specific to FPPS	n/a	n/a

* Determined based on size of compound collection screened. See Methods for details.