

Genetic mapping of targets mediating differential chemical phenotypes in *Plasmodium falciparum*

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Studies of gene function and molecular mechanisms in *Plasmodium falciparum* are hampered by difficulties in characterizing and measuring phenotypic differences between individual parasites. We screened seven parasite lines for differences in responses to 1,279 bioactive chemicals. Hundreds of compounds were active in inhibiting parasite growth; 607 differential chemical phenotypes, defined as pairwise IC₅₀ differences of fivefold or more between parasite lines, were cataloged. We mapped major determinants for three differential chemical phenotypes between the parents of a genetic cross, and we identified target genes by fine mapping and testing the responses of parasites in which candidate genes were genetically replaced with mutant alleles. Differential sensitivity to dihydroergotamine methanesulfonate (1**), a serotonin receptor antagonist, was mapped to a gene encoding the homolog of human P-glycoprotein (PfPgh-1). This study identifies new leads for antimalarial drugs and demonstrates the utility of a high-throughput chemical genomic strategy for studying malaria traits.**

Malaria is a serious public health burden that causes an estimated 1–2 million deaths and 300–500 million infections each year¹. There is no effective vaccine available, and parasites resistant to almost all antimalarial drugs currently in use have been reported, including parasites with reduced sensitivity to derivatives of the traditional Chinese medicine Qinghaosu (artemisinin, **2**)². Development of new drugs and a better understanding of the targets of antimalarial drugs and drug resistance are urgently needed.

Phenotypic characterization of human malaria parasites is limited because pathogenic stages live within red blood cells (RBCs), and laboratory models for *in vivo* investigations are often unsatisfactory. Changes in response to antimalarial drugs, differences in growth rate and variations in virulence are among the few phenotypes typically accessible³. When phenotypes are available, genetic mapping is a powerful tool for assigning them to particular determinants, and various high-throughput genotyping methods, including microarrays for detecting single nucleotide polymorphisms (SNPs) and microsatellites, have been developed for studies of *Plasmodium falciparum* traits⁴. However, there are ~5,400 predicted genes in the parasite's genome, and the function of the majority of these genes remains unknown⁵. Characterizing phenotypic differences in malaria parasites and identifying the genes affecting the differences may provide important information for investigating gene function.

A challenge in understanding drug action and mechanisms of drug resistance is to identify the relevant molecular target. One useful strategy is to synthesize an active compound and use it to affinity purify the protein targets to which the compound binds⁶. This approach, however, generally requires compounds that have high affinity for their

targets. Another strategy uses genetic mapping to link chromosomal loci that affect parasite responses to compounds, allowing molecular targets to be identified after fine mapping and functional characterizations of candidate genes. In addition to discovering potential new antimalarial compounds, these strategies can detect and define differential chemical phenotypes (DCPs) that show distinct signature responses to compounds among a variety of parasite isolates.

Here we demonstrate a strategy for identifying targets of chemical compounds in malaria parasites by integrating quantitative high-throughput screening (qHTS) with genetic mapping (**Fig. 1**). We tested seven *P. falciparum* lines, including parents of three genetic crosses^{7–9}, for their responses to 1,279 bioactive compounds and identified candidate genes for three DCPs using progeny from a genetic cross and genetic transfection methods of allelic replacement. These results show that differential responses of small molecules between parasite lines can be reliable phenotypes for exploring molecular mechanisms of pharmacologic interest. This study also provides an approach for investigating drug action and resistance mechanisms in diseases other than malaria.

RESULTS

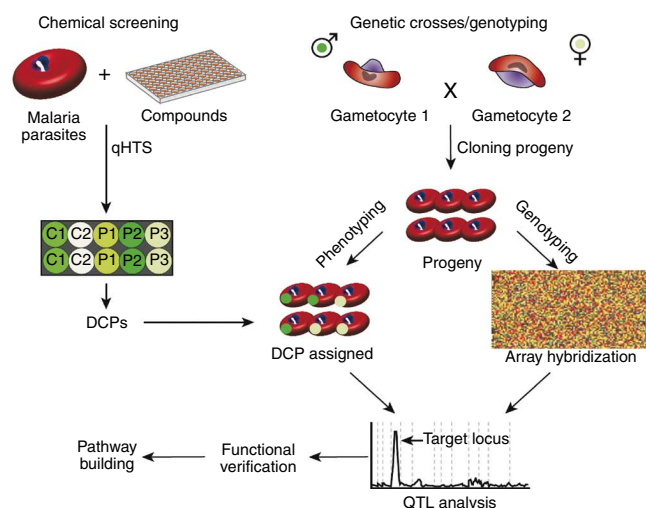
qHTS for inhibitors of *P. falciparum* proliferation

We tested *P. falciparum* proliferation within infected erythrocytes against the LOPAC¹²⁸⁰ collection of known bioactives (Sigma-Aldrich) by a qHTS¹⁰ of a SYBR DNA binding assay¹¹. Parasite proliferation was measured after 72 h of incubation (corresponding to 1.5 generations of intra-erythrocytic parasite growth), with each compound tested at seven or eight fivefold dilutions beginning at 29 μM. Two independent

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Figure 1 A chemical genomic strategy for studying gene function in malaria parasites. qHTS of parasites against compounds in titration-response fashion identifies a large number of DCPs. Target genes associated with these DCPs can be identified using quantitative trait loci analysis after genotyping progeny from genetic crosses or field isolates. Gene functions can be deduced from classes of compounds that target specific biologic pathways. The green circles represent differential parasite responses to chemicals. C1 and C2 represent negative and positive controls, and P1–P3 represent responses from three parasites. Gametocytes are the sexual stage of the malaria parasite that can be cultured *in vitro*, and a genetic cross is started by feeding a mixture of gametocytes from two different parasites to mosquitoes.



screens of each parasite line performed well, showing 0.7 or higher average Z' factor and eightfold or higher signal-to-background ratio (**Supplementary Table 1**). The potencies of known antimalarial agents had values similar to those determined by the assay in 96-well plate format. Titrations of two control inhibitors, **2** and mefloquine (**3**), were present on every plate and showed expected half-maximal inhibitory concentration (IC_{50}) values (**Supplementary Table 1**). The antimalarial agents chloroquine (**4**) and quinine (**5**) were present in the collection, and IC_{50} values determined from the qHTS for **5** were similar, but the measurements for **4** were 15- to 20-fold higher than those from 96-well plate tests (**Supplementary Table 1**) because of lower solubility of **4** in DMSO (data not shown). Although the determined potencies of **4** were lower, the potencies in *P. falciparum* lines sensitive to **4** (HB3, 3D7 and D10) were clearly different from the potencies in resistant lines (Dd2, GB4, W2 and 7G8). The consensus IC_{50} and activity values for each of the 1,279 compounds in each of the seven *P. falciparum* lines are listed in **Supplementary Table 2**, and screening data are deposited in PubChem (AID 1828). Comparison of the replicate runs for each parasite line indicated excellent agreement of curve class assignment and IC_{50} determination. About 80% of actives (compounds associated with class 1.1, 1.2 and 2.1 curves¹⁰; see Methods for curve class definitions) identified in one replicate were active in the second replicate for all lines except Dd2, where 55% were active in both replicates. Of the actives that did not repeat, almost all showed inconclusive activity (class 2.2 and 3) in the other replicate, with few or none scoring as inactive (**Supplementary Table 3**). The potencies of compounds scored as active or inconclusive in both replicates correlated well (**Supplementary Fig. 1**), indicating good repeatability in determining IC_{50} values between replicates.

Discovery of potential antimalarial compounds

Screens of the seven parasite lines revealed a large number of consensus actives (active in both replicates or active in one replicate and inconclusive in the other), all of which inhibited parasite growth. Among the 1,279 compounds tested, about 20% to 30% were active in most lines—except in W2, where 40% of the compounds were active, and in D10 and Dd2, where 19% and 15% of the compounds were active, respectively (**Supplementary Table 3**). Of the hundreds of inhibitors identified for each line, about 50% or more showed IC_{50} values between 1 and 10 μ M, and 6% to 14% had IC_{50} values less than 1 μ M (**Supplementary Table 4**). There were 155 compounds that inhibited growth in all seven lines tested (**Supplementary Table 5**). The potency distribution of these pan inhibitors indicated differences in sensitivity between the lines; W2 was most sensitive, with 32% of the compounds having IC_{50} values of 1 μ M or less, whereas Dd2 was least sensitive, with 7% of the compounds having IC_{50} values below 1 μ M. We identified 25 potent compounds that inhibited proliferation in all parasite lines at lower than 2 μ M IC_{50} (**Supplementary Table 6**). Some of these compounds are well-known antimalarial

drugs (**5** and quinacrine (**6**)), whereas others—such as hexahydro-sila-difenidol hydrochloride (**7**), dequalinium dichloride (**8**), paclitaxel (**9**) and BW 284c51 (**10**)—are not compounds used for malaria prophylaxis or treatment.

Identification of a large number of DCPs

One of our study objectives was to identify and profile differences in parasite responses to different chemicals. These differences can be considered phenotypes that derive from underlying variations in each parasite genotype. Hierarchical clustering of activity category (**Fig. 2a**) and IC_{50} values (**Fig. 2b**) of actives showed clear differences in the parasite responses to many compounds. We therefore compared compounds between each pair of parasite lines that showed a fivefold or greater difference in IC_{50} (see Methods for specific criteria). Applying these criteria, we identified 149 compounds (**Supplementary Table 7**) that produced 607 potential DCPs between the seven lines (**Table 1**). Notably, the three chloroquine-sensitive (CQS) parasite pairs had the smallest differences in response to the chemicals: seven DCPs for HB3 and 3D7, two for HB3 and D10, and seven for 3D7 and D10. There were 58 DCPs between the parents of the three genetic crosses, with seven between 3D7 and HB3, 23 between GB4 and 7G8, and 28 between Dd2 and HB3 (**Table 1**). These DCPs can be used to identify responsible loci or genes via linkage mapping of recombinant progeny from the crosses.

Mapping molecular targets underlying selected DCPs

To confirm that genes underlying DCPs could be identified using genetic mapping, we examined **1**, trimethoprim (**11**) and triamterene (**12**) because they exhibited relatively large differences in IC_{50} between the parents of a GB4 \times 7G8 cross. **1** (**Fig. 3a**) is a competitive serotonin receptor antagonist and has been used to treat migraine headache^{12,13}. From the qHTS, **1** was ten times more potent in 7G8 (0.2 μ M) compared with GB4 (2 μ M). Follow-up tests in 96-well plates confirmed the IC_{50} difference between 7G8 (0.37 \pm 0.01 μ M) and GB4 (2.8 \pm 0.37 μ M; **Fig. 3b**). We then tested the **1** responses of 32 progeny from the cross and used the resulting IC_{50} values and genotypic data from 285 microsatellite markers⁹ to map genetic loci controlling the difference in parasite responses (**Fig. 3b**). Quantitative trait loci (QTL) analysis identified a major locus on chromosome 5 with a logarithm of the odds (LOD) score of 16.4 and another locus on chromosome 12 having a LOD score of 3.0 (**Fig. 3c**). A perfect genotype-phenotype association was found within a \sim 150-kb segment of chromosome 5 bounded by crossovers at marker B7M114 in progeny JB8 and marker

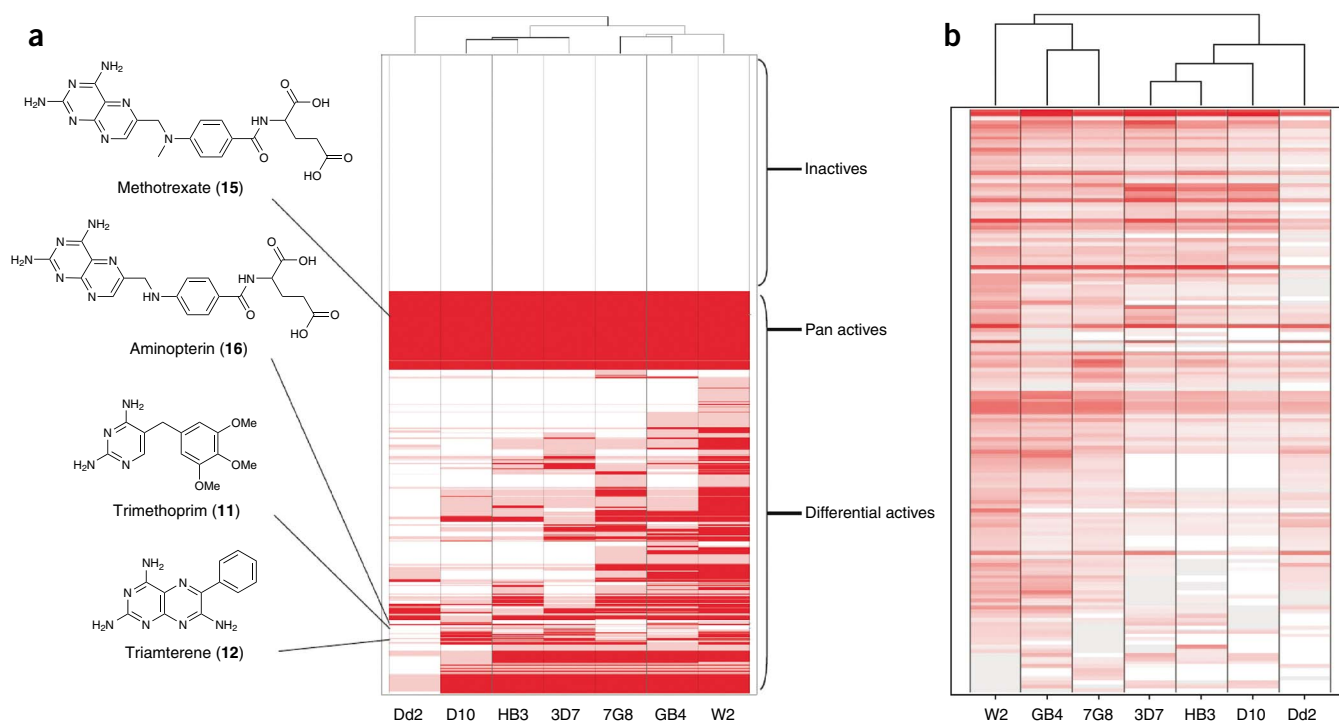


Figure 2 Hierarchical clustering of compound activities in seven *P. falciparum* lines. (a) Clustering of compounds based on activity category from parasite responses to the compounds. Each compound was scored as consensus active (class 1.1, 1.2 or 2.1 in one or both replicates; red), consensus inconclusive (class 2.2 or 3 in both replicates; pink) or consensus not active (class 4 in one or both replicates; white) for each parasite line. Structures of four known dihydrofolate reductase inhibitors and their positions in the clustering are shown. (b) Hierarchical clustering of IC₅₀ values of differentially active compounds in seven *P. falciparum* lines. IC₅₀ values of 149 compounds having fivefold or greater potency differences between two or more strains were clustered. IC₅₀ values ranged from 12 nM (dark red) to >29 μM (inactive; white). Gray indicates excluded IC₅₀ values.

B5M86 in progeny XE7, LA10 and WE2 (Fig. 3b). This 150-kb segment contained *pfmdr1*, the gene encoding a homolog of the human P-glycoprotein (PfPgh-1) involved in drug resistance, and 33 other predicted genes (Supplementary Table 8). The smaller peak on chromosome 12 suggests that a second gene might modulate the response to **1** (Fig. 3c).

11 (Fig. 4a) is an antifolate that targets DHFR and is used mainly for prophylaxis and treatment of urinary tract infections¹⁴. In follow-up assays, the parasite clone 7G8 had a much higher IC₅₀ (37 ± 1.2 μM) for **11** than did GB4 (2.5 ± 0.2 μM). Analysis of responses of 32 GB4 × 7G8 progeny to **11** showed two groups of recombinants that were separated by a large difference in IC₅₀ as well as some variation in IC₅₀ within each group, particularly in the resistant group (Fig. 4b). QTL analysis identified a major peak on chromosome 4 with a LOD score of 15.6 and some minor peaks on chromosomes 6 and 11 with LOD scores lower than 1.5 (Fig. 4c). A perfect genotype-phenotype association was found at marker C4M69 within a segment

of chromosome 4 bounded by crossovers at marker C4M19 in progeny DAN and at marker C3M35 in progeny AUD and JH6 (Fig. 4b). The chromosome 4 segment spanned ~59 kb of DNA and contained ten genes (Supplementary Table 9), including the gene encoding *P. falciparum* dihydrofolate reductase (PfDHFR).

12 (Supplementary Fig. 2a) is a Na⁺ channel blocker¹⁵ with activity against edema from heart, kidney or liver disease. In assays with **12**, the 7G8 parasite had an IC₅₀ ~24 times higher than that of GB4 (Supplementary Fig. 2b). We tested 32 progeny from the 7G8 and GB4 cross with **12**, and we identified two groups that were separated by large differences in IC₅₀, with some variations in IC₅₀ within each group (Supplementary Fig. 2b), suggesting contributions from one major gene and several minor genes. QTL analysis of the genotypes and phenotypes from the progeny mapped a major determinant to a locus on chromosome 4 with an LOD score of 14.1 and to another locus on chromosome 12 with an LOD score of ~2.3. Like **11**, the response to **12** mapped to a ~59-kb locus defined by informative crossovers in

Table 1 Compounds with fivefold or greater differences in IC₅₀ values between parasite pairs

Parasite	3D7	7G8	D10	Dd2	GB4	HB3	W2
3D7	0						
7G8	23	0					
D10	7	34	0				
Dd2	33	21	31	0			
GB4	27	23	39	33	0		
HB3	7	18	2	28	26	0	
W2	39	21	74	53	16	52	0

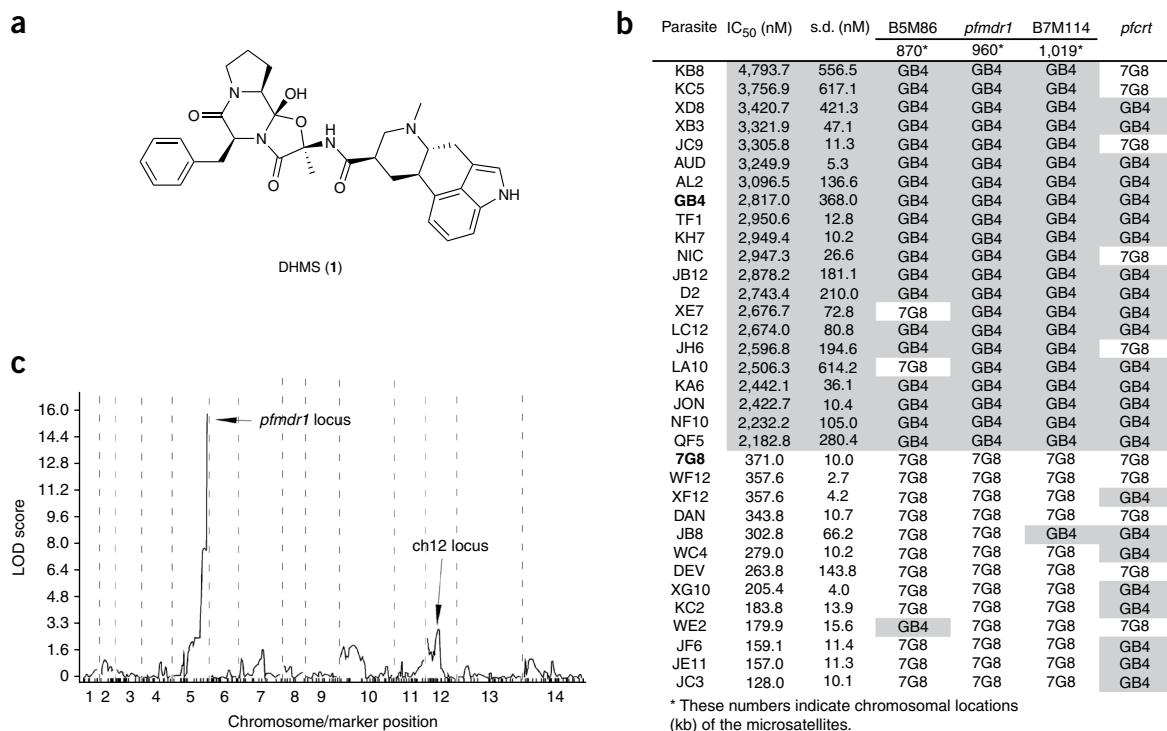


Figure 3 Mapping genetic loci contributing to IC₅₀ differences between 7G8 and GB4 in response to dihydroergotamine methanesulfonate (DHMS, **1**). (a) Chemical structure of **1**. (b) IC₅₀ values (mean and s.d.) of **1** and allelic designations of three microsatellite markers on chromosome 5 and one at the gene encoding the chloroquine-resistant transporter (*pfcr*) on chromosome 7 are shown for the parents and progeny of a GB4 × 7G8 cross. There is a perfect match of sensitivity to **1** and microsatellite polymorphism in *pfmdr1*. (c) Peaks of quantitative trait loci analysis linked to differential **1** responses are shown. Predicted genes within the chromosome 5 locus can be found in **Supplementary Table 8**.

the same three progeny between marker C4M19 (DAN) and marker C3M35 (AUD and JH6) (**Supplementary Fig. 2b**) containing *pfdhfr* and nine other genes (**Supplementary Table 9**). These results suggested that a single locus confers resistance to both **11** and **12**.

Mutations in PfPgh-1 confer resistances to **1**

Among the genes in the chromosome 5 locus, *pfmdr1* was a primary candidate because it encodes an ABC transporter involved in drug resistance and because there are no known targets to **1** (such as 5-HT serotonin receptor or α -adrenergic receptor) in the locus^{12,13,16}. To investigate whether *pfmdr1* mediates differential susceptibility to **1**, we examined *pfmdr1* mutations and responses to **1** in parasite isolates from different regions of the world (**Supplementary Table 10**). There are five known nucleotide substitutions in the *pfmdr1* genes, resulting in codon polymorphisms for N86Y, Y184F, S1034C, N1042D and D1246Y (refs. 17,18). Examination of the *pfmdr1* haplotypes and **1** responses in the field isolates suggested that the N1042D substitution was associated with a greater than 50% reduction in IC₅₀ and that the S1034C substitution was associated with a further reduction of 50% or more (**Supplementary Table 10**). These mutations account for the majority of the difference between 7G8 (the New World *pfmdr1* haplotype) and GB4 (the Old World haplotype). The impacts of N86Y, Y184F and D1246Y on sensitivity to **1** were unclear, as parasites with the same substitutions had quite different IC₅₀ values in some cases (**Supplementary Table 10**). Field isolates usually display substantial genetic heterogeneity, and the differences in genetic background could contribute to the variations in IC₅₀ values observed.

To evaluate the influence of genetic background on the IC₅₀ measurements, we tested several parasite lines where the *pfmdr1*-coding regions were replaced with versions carrying different

polymorphisms by allelic exchange^{18,19}. Parasites with substitutions on the background of D10 (which has the same *pfmdr1* allele as GB4) and on the background of 7G8 have been reported¹⁸. Five parasites with different *pfmdr1* alleles on 7G8 or D10 genetic backgrounds were compared (**Supplementary Table 10**). Measurements of IC₅₀ values from three of the five parasites were consistent with the predicted drug-response phenotypes, whereas two parasites (D10^{7G8/1} and D10^{7G8/2}) yielded discrepant results. We therefore designed PCR primers to amplify specific sequences from the two parasites (**Supplementary Fig. 3**). Sequencing of the PCR products showed that the *pfmdr1* alleles in the two parasites were not the reported sequences; instead, the amino acids deduced from the DNA sequences matched the IC₅₀ values observed in our drug tests (NYCDY for D10^{7G8/1} and NYSND for D10^{7G8/2}; **Supplementary Table 10**). The apparent discrepancies between genotype and phenotype may have arisen from the mislabeling of parasites or cross-contamination during *in vitro* culture.

Additionally, we tested six transfected lines with *pfmdr1* alleles exchanged in two progeny (GC03 and 3BA6) of a Dd2 × HB3 cross¹⁹. Responses of these allelic-exchanged parasites to **1** showed that an amino acid change of N1042D reduced the **1** IC₅₀ by more than half, and changes of S1034C and D1246Y lowered it further to less than 300 nM (**Supplementary Table 10**). Although the F184Y substitution slightly reduced the IC₅₀ in D10 and 3D7 parasites (compared with C2A and 224), the same change in 7G8^{D10} and D10^{D10} had little effect, suggesting that the F184Y substitution had little influence on the response to **1**. Together, the results from field isolates and allelic-exchanged parasite lines demonstrate a strong association of S1034C and N1042D substitutions and resistance to **1**. Notably, verapamil, a compound that inhibits human P-glycoprotein and can reverse the

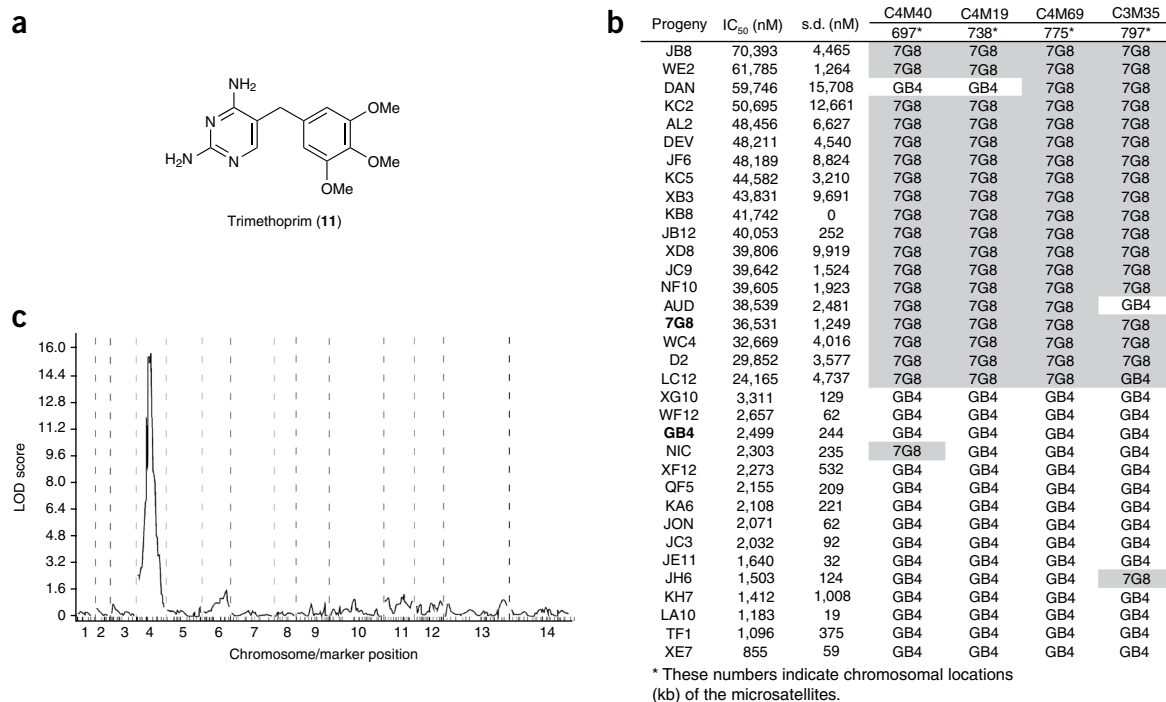


Figure 4 Identification of genetic loci linked to response to trimethoprim (**11**). **(a)** Chemical structure of **11**. **(b)** IC₅₀ values (mean and s.d.) of **11** and allelic designations for four microsatellite markers on chromosome 4 are shown for the parents and progeny of a GB4 × 7G8 cross. **(c)** Peaks of quantitative trait loci analysis linked to differential responses to **11** are shown. Predicted genes within the chromosome 4 locus can be found in **Supplementary Table 9**.

effects of many antimalarial and anticancer drugs¹⁹, did not have any effect on parasite response to **1** (**Supplementary Table 11**).

Polymorphisms in PfDHFR confer resistance to **11** and **12**

Variations in PfDHFR among the seven parasite lines support the association of differential parasite responses to **11** and **12**. Although the locus on chromosome 4 contained ten genes, we considered *pf dhfr* as the primary candidate for resistance to both **11** and **12** because **11** is a known DHFR inhibitor¹⁴ and inhibition of human leukocyte DHFR activity by **12** has also been reported²⁰. Many mutations in PfDHFR have been shown to contribute to parasite responses to antifolate drugs, particularly substitutions at amino acid positions 51, 59 and 108 (ref. 21). Sequencing of the *pf dhfr* coding region showed only two codon changes between 7G8 and GB4, with GB4 encoding Asn51 and Ser108 and 7G8 encoding Ile51 and Thr108 (**Supplementary Table 12**). We also determined seven known polymorphic sites of PfDHFR at codons 16, 50, 51, 59, 82, 108, 140 and 164 from the five other parasites but found alterations only at codons 51, 59 and 108 (**Supplementary Table 12**). Much like the pattern of response to pyrimethamine (**13**, a DHFR antagonist), parasites with substitutions at three positions in the gene had higher IC₅₀ values for **11** and **12** than parasites with substitutions at two positions in the gene, which in turn were more resistant than parasites having one substitution in the gene²². These results suggested that mutations in PfDHFR confer resistance to **11** and **12**.

To rule out the influence of genetic backgrounds on parasite response, we tested two 3D7 lines that were transformed to express integrated alleles of the gene encoding PfDHFR with a S108T substitution from HB3 or a triple N51I C59R S108T substitution from Dd2 (pDT-HB3 and pDT-Dd2; **Supplementary Table 12**)²³. As expected for parasites with integrated alleles, DNA sequencing confirmed the PfDHFR genotypes in the two parasites, which revealed mixed

serine and threonine codons at position 108 for pDT-HB3, and mixed codons at positions 51, 59 and 108 for pDT-Dd2 (**Supplementary Fig. 4** and **Supplementary Table 12**). The 3D7 parasite transfected with the HB3 PfDHFR allele had IC₅₀ values similar to those of HB3, and the parasite transfected with the Dd2 allele had even higher IC₅₀ values, although the IC₅₀ values were lower than those of the Dd2 parasite. These results demonstrated that introduction of a mutant PfDHFR allele into a sensitive parasite increases the IC₅₀ values to both drugs. Sequencing of DNA from progeny of the GB4 × 7G8 cross showed that *pf dhfr* alleles in these parasites also matched the phenotypes observed (data not shown). These results, obtained from parasite lines, transfected parasites and progeny, demonstrate that polymorphisms in PfDHFR indeed confer differential responses to **11** and **12**.

DISCUSSION

Progeny from genetic crosses, high-density microsatellite and SNP maps, and high-throughput genotyping methods^{4,24,25} provide a powerful platform to identify parasite genes involved in differential responses to bioactive compounds. As a proof of principle, we identified a large number of phenotypic variations (or DCPs) between individual parasites and linked two genes to parasite responses to three compounds using genetic mapping. Identification of many DCPs is consistent with a highly polymorphic *P. falciparum* genome^{26–28}. It may also be possible to map DCPs by genome-wide association studies using genetically heterogeneous parasite populations⁴. The DCPs identified here may lead to the identification of many target genes and to a better understanding of the molecular interactions within the parasite.

Several large-scale screens for antimalarial compounds have been described, most of which assayed compounds at one or two concentrations (typically 10 μM)^{11,29–33}. In contrast, we measured

parasite responses to each compound at concentrations across four orders of magnitude, allowing us to generate concentration-response curves and derive IC_{50} values for all tested compounds immediately (**Supplementary Table 2**). Many active compounds showed IC_{50} values less than 1 μM (**Supplementary Table 6**), some of which may be tested to treat malaria without extensive clinical trials because many of the compounds in the LOPAC collection have been used for treating human diseases. Our study also showed that a compound active against one parasite line was often not effective in killing other parasites with different genotypes.

The observation of more DCPs among parasites resistant to **4** suggests that mutations and compensatory changes selected by **4** may impact parasite responses to other chemicals. Similarly, Dd2 was derived from W2 after **3** selection³⁴, and Dd2 was less sensitive to many compounds than W2 (**Supplementary Table 3**). These observations suggest that following drug selection, parasites may become more resistant to multiple antimalarial drugs more quickly, consistent with the proposal of parasites showing the accelerated resistance to multiple drugs (ARMD) phenotype³⁵. Because Dd2 and W2 have a very similar genetic background but are known to differ in *pfmdr1* copy number, the variation in *pfmdr1* copy number may play a role in some differential responses.

Mutation and amplification of *pfmdr1* have been associated with drug responses in *P. falciparum*^{18,36,37}. There are some similarities in the associations of *pfmdr1* alleles to responses to **1** and antimalarial agents such as **2**, **3** and halofantrine (**14**). Amino acid changes in PfPgh-1 (from NFDY to NFSND; see **Supplementary Table 10** for more details) increase the IC_{50} values of **2**, **3** and **14** by twofold to sixfold and decrease the IC_{50} of **5** in some genetic backgrounds^{18,19}. Likewise, **1** showed a higher IC_{50} in parasites containing the NFSND haplotype. However, the changes in IC_{50} to **2**, **3** and **14** after allelic exchange might be parasite-specific because 7G8 and GB4 have very similar IC_{50} values to both **2** and **3** (**Supplementary Table 1**). Because PfPgh-1 is an ABC transporter homolog, mutations in this protein may affect its ability to transport **1** and other drugs.

Although **12** is a known Na^+ channel blocker¹⁵, our results showing that **12** acts on PfDHFR were consistent with observations that **12** interferes with folate metabolism *in vitro* and *in vivo*^{20,38,39}. Notably, the known chemical structures of DHFR inhibitors and epithelial sodium channel blockers share a similar amino-pyrimidine ring and tethered aryl group (**Supplementary Fig. 5**). It is possible that these structural features account for the inhibition of DHFR and some sodium channels, and these proteins may share similar binding pockets⁴⁰.

Our results showing that **1** interacts with or is transported by an ABC transporter instead of the expected 5-HT2B receptor highlight how such studies can identify ‘off target’ effects of bioactive molecules. The observation that mutations in an ABC transporter can alter sensitivity to **1** may provide important information for studies and treatment of migraine syndrome.

METHODS

Parasites and compounds. The *P. falciparum* lines used in this study have been described previously⁴¹, and their origins and responses to **2**, **3**, **4** and **5** are summarized in **Supplementary Table 1**. Parasites Dd2, HB3, GB4, 7G8 and 3D7 are parents of three separate genetic crosses^{7–9}. Dd2 is a clone derived from W2 that was originally isolated from Southeast Asia³⁴. Progeny of a genetic cross (GB4 \times 7G8) were described recently⁹. Transgenic parasites with the gene encoding a homolog of the human P-glycoprotein (PfPgh-1) replaced by mutant alleles were reported previously^{18,19}. The parasites described previously¹⁸ were obtained from MR4 (<http://www.mr4.org/>). Parasites transfected with mutant genes encoding PfDHFR were described previously²³. All parasites were cultured *in vitro* according to methods described⁴². Briefly, parasites were

maintained in RPMI 1640 medium containing 5% human O^+ erythrocytes (5% hematocrit), 0.5% (w/v) Albumax (GIBCO), 24 mM sodium bicarbonate and 10 $\mu g ml^{-1}$ gentamycin at 37 °C with 5% CO_2 , 5% O_2 and 90% N_2 .

The LOPAC¹²⁸⁰ collection (<http://www.sigmaaldrich.com/chemistry/drug-discovery/validation-libraries/lopac1280-navigator.html>) was purchased from Sigma-Aldrich, and the compounds were prepared and plated as described¹⁰. **1**, **2**, **3**, **4**, **5**, **11** and **12** were purchased from Sigma-Aldrich and had purities of 98% or better.

In vitro drug assays. The SYBR Green viability assay (**Supplementary Table 13**) was adapted from methods described previously^{11,32}. Briefly, 3 μl of culture medium was dispensed into 1,536-well black clear-bottom plates (Aurora Biotechnologies) using a Multidrop Combi (Thermo Fisher Scientific Inc.). Then, 23 μl of compounds in DMSO were added by a pin tool (Kalypsys), and 5 μl of *P. falciparum*-infected RBCs (0.3% parasitemia, 2.5% hematocrit final concentration) were added. The plates were incubated at 37 °C in a humidified incubator in 5% CO_2 for 72 h, and 2 μl of lysis buffer (20 mM Tris-HCl, 10 mM EDTA, 0.16% saponin (w/v), 1.6% Triton-X (v/v), 10 \times SYBR Green I supplied as 10,000 \times final concentration by Invitrogen) were added to each well. The plates were mixed for 25 s with gentle shaking and incubated overnight at 22–24 °C in the dark. The following morning, fluorescence intensity at 485 (14) nm excitation and 535 (25) nm emission wavelengths was measured on an EnVision (Perkin Elmer) plate reader. The compound library was screened against each line at eight (seven for Dd2) fivefold dilutions beginning at 29 μM . Antimalarial drugs **2** and **3** and DMSO were included as positive and negative controls for each plate, respectively.

Follow-up SYBR Green assays in 96-well plate format were performed as described⁴³. Briefly, 150 μl synchronized parasites diluted to 1% parasitemia with 1% hematocrit were mixed with 50 μl medium containing compound. Compound stocks (10 mM) were dissolved in ethanol or DMSO and tested at 11 twofold dilutions in triplicate. The beginning concentration of each compound was adjusted depending on IC_{50} values from the initial qHTS. The plates were incubated at 37 °C under 5% CO_2 , 5% O_2 and 90% N_2 for 72 h, incubated for another 30 min after addition of 50 μl lysis buffer, and read in a FLUOstar OPTIMA reader (BMG Labtech). Data were analyzed using software for 96-well assays at the US National Institute of Allergy and Infectious Diseases Bioinformatic Resource website (<http://exon.niaid.nih.gov/drap>).

Data analysis. Analysis of compound concentration-response data from the qHTS was performed as previously described¹⁰. Plate reads were first normalized relative to the control inhibitor (0.29 μM **2**) and vehicle (DMSO) wells present on each plate and then corrected by an algorithm using vehicle-only control plates at the beginning and end of the compound plate stack. Concentration-response data for each compound were curve-fitted and categorized into four major classes as described¹⁰ using custom software (<http://ncgc.nih.gov/pub/openhts/curvefit/>). Briefly, compounds in class 1 produce a complete response curve containing two asymptotes, while compounds in class 2 have incomplete response curves containing one asymptote. Compounds with class 1 and 2 curves are further divided based on efficacy, with subclasses 1.1 and 2.1 having 80% or greater efficacy and subclasses 1.2 and 2.2 having less than 80% efficacy. Compounds with class 3 curves show activity only at the highest concentration or are poorly fit class 1 or 2 curves with r^2 less than 0.9. Compounds with class 4 curves are inactive, and either they do not have a curve fit or the curve fit is below three s.d. of the mean basal activity (about 30% efficacy).

Compound activities were categorized as active (class 1.1, 1.2 or 2.1), inconclusive (class 2.2 or 3) or inactive (class 4). Consensus activity between two replicates was scored as active (class 1.1, 1.2 or 2.1 in both replicates or class 1.1, 1.2 or 2.1 in one replicate and class 2.2 or 3 in the other), inconclusive (class 2.2 or 3 in both replicates) or inactive (class 4 in one or both replicates). For determining the potencies of consensus actives (**Supplementary Tables 4–6**), the IC_{50} values of both replicates were averaged. Differentially active compounds were defined as having fivefold or greater IC_{50} differences between pairs of parasite lines, with IC_{50} values determined as follows: if active in both replicates or active in one replicate and inconclusive in the other replicate, curve fits with P values of 0.05 or less were used or averaged. If inconclusive with curve fit P values of 0.05 or less for both replicates, IC_{50} values were averaged. If inactive in both replicates, the IC_{50} value was set to 29 μM , the highest tested

concentration. If inconclusive with a curve fit P value greater than 0.05 or inactive in one replicate, the IC_{50} value was not used. Unsupervised hierarchical clustering of compound consensus activities and parasite lines was performed using Spotfire DecisionSite (TIBCO Software Inc.).

DNA sequencing, genotyping and QTL analysis. To verify nucleotide substitutions in the genes encoding PfPfgH-1 and PfDHFR, primers specific to the genes were commercially synthesized to amplify DNA sequences with known polymorphic sites (**Supplementary Fig. 3**). PCR products were sequenced directly without cloning into a plasmid vector as described⁴⁴. Microsatellite genotypes for the parents and progeny of the genetic GB4 × 7G8 cross have been reported^{9,24}. QTL analysis was carried out using J/qtl⁴⁵.

Accession codes. PubChem: Screening data have been deposited with accession code AID 1828.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

J.Y. performed drug assay qHTS, parasite culture and data analysis; R.L.J. performed assay optimization, qHTS and data analysis and helped write the manuscript; J.W. performed qHTS; R.H. performed data analysis; H.J. performed drug assays; K.H. performed progeny cloning and helped write the manuscript; D.A.F. transfected parasites and helped write the manuscript; T.E.W. produced progeny and helped write the manuscript; C.P.A. and J.L. planned the project and helped write the manuscript; X.-z.S. conceived the project, analyzed data and helped write the manuscript.

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