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A clinical drug library screen identifies astemizole as an antimalarial agent

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The high cost and protracted time line of new drug discovery are major roadblocks to creating therapies for neglected diseases. To accelerate drug discovery we created a library of 2,687 existing drugs and screened for inhibitors of the human malaria parasite *Plasmodium falciparum*. The antihistamine astemizole and its principal human metabolite are promising new inhibitors of chloroquine-sensitive and multidrug-resistant parasites, and they show efficacy in two mouse models of malaria.

Only recently has a systematic high-throughput approach been used to screen existing drugs for previously unknown activities, and these screens have focused primarily on diseases with relatively low prevalence in the developing world[1]. Of the existing drug libraries reported, the largest contains less than 25% of the 3,400 drugs approved by the US Food and Drug Administration (FDA) and less than 10% of the approximately 11,500 drugs ever used in medicine (**Supplementary Fig. 1** online). We assembled a library of 1,937 FDA-approved drugs and 750 drugs that were either approved for use abroad or undergoing phase 2 clinical trials, and we screened this collection, called the Johns Hopkins Clinical Compound Library (JHCCL), for inhibition of *P. falciparum* growth (**Supplementary Methods** online). A preliminary screen using a concentration of 10 μM revealed 189 existing drugs, distributed across many drug classes, that resulted in >50% inhibition (**Fig. 1a** and **Supplementary Fig. 2** online). After eliminating topical drugs, known antimalarials, cytotoxic drugs and compounds previously reported to inhibit the malaria parasite, we determined half-maximal inhibitory concentration (IC_{50}) values for the 87 remaining drugs (**Supplementary Table 1** online). Some inhibitors, such as pyrvinium pamoate, have no absorption with oral dosing. Other weak *P. falciparum* inhibitors that we identified, such as paroxetine, could be improved upon by screening related analogs that have not been developed to phase 2 drug trials as antidepressants. The unique ability of individual drugs within a class to inhibit *P. falciparum* supports building a comprehensive library of existing drugs rather than selecting representative members of each mechanistic class.

One of the more promising drugs identified is the nonsedating antihistamine astemizole (**1**; **Fig. 1b**), which inhibits (at submicromolar concentrations) the proliferation of three *P. falciparum* parasite strains that differ in chloroquine sensitivity (**Table 1**). After oral ingestion in humans, astemizole is rapidly converted primarily to desmethylastemizole (**2**), which is ten-fold more abundant in plasma than astemizole and has a half-life of 7–9 d (ref. 2). Notably, desmethylastemizole had an IC_{50} of approximately 100 nM and was 2- to 12-fold more potent than astemizole in inhibiting *P. falciparum*, whereas the minor metabolite norastemizole (**3**) weakly inhibited the parasite. Astemizole and desmethylastemizole showed only an additive effect in combination with chloroquine (**4**), quinidine (**5**) and artemisinin (**6**) on the 3D7 and Dd2 *P. falciparum* strains (data not shown). During intraerythrocytic infection, *P. falciparum* parasites crystallize heme released from hemoglobin catabolism within the food vacuole, and quinoline antimalarials such as chloroquine inhibit this reaction[3]. Astemizole and desmethylastemizole (like the quinoline antimalarials) inhibit heme crystallization, concentrate within the *P. falciparum* food vacuole and co-purify with hemozoin in chloroquine-sensitive and multidrug-resistant parasites (**Supplementary Fig. 3** online).

To determine whether astemizole has *in vivo* antimalarial activity, we tested it in two mouse models using the 4-d parasite suppression test. We used intraperitoneal dosing of desmethylastemizole in mice because in humans this is the principal active metabolite, and the parent compound has an oral bioavailability of 95% (ref. 2). Vehicle-treated mice that were infected with the lethal, chloroquine-sensitive *P. vinckei* strain developed parasitemias of approximately 64% on day 5 (**Fig. 1c**). In contrast, mice treated with astemizole at 30 mg m^{-2} d^{-1} or desmethylastemizole at 15 mg m^{-2} d^{-1} had an 80% or 81% reduction in parasitemia, respectively. Mice infected with chloroquine-resistant *P. yoelii*, then treated with astemizole or desmethylastemizole at 15 mg m^{-2} d^{-1}, showed a 44% or 40% reduction in parasitemia, respectively (**Fig. 1d**). Recrudescence occurred for both strains if we stopped treatment after 4 d at these doses, although high doses of astemizole (300 mg m^{-2}) delivered *per os* for 4 d cured infection. Doses as high as 18.6 mg m^{-2} have been used in humans to treat seasonal allergic rhinitis[4].

Astemizole was introduced in 1983 under the brand name Hismanal as a nonsedating selective H_1-histamine receptor antagonist for treating allergic rhinitis and was sold in 106 countries and also over the counter[2]. The use patent for astemizole has expired. Although astemizole was voluntarily withdrawn in 1999 from the United States and Europe after decreased sales due to warnings about its safety and to the availability of antihistamines with fewer side effects[5], it is currently sold in generic form in over 30 countries, including Cambodia, Thailand and Vietnam, which are malaria endemic (Dr. Reddy's Laboratories, personal communication). Astemizole and desmethylastemizole potently inhibit the ether-a-gogo (HERG)

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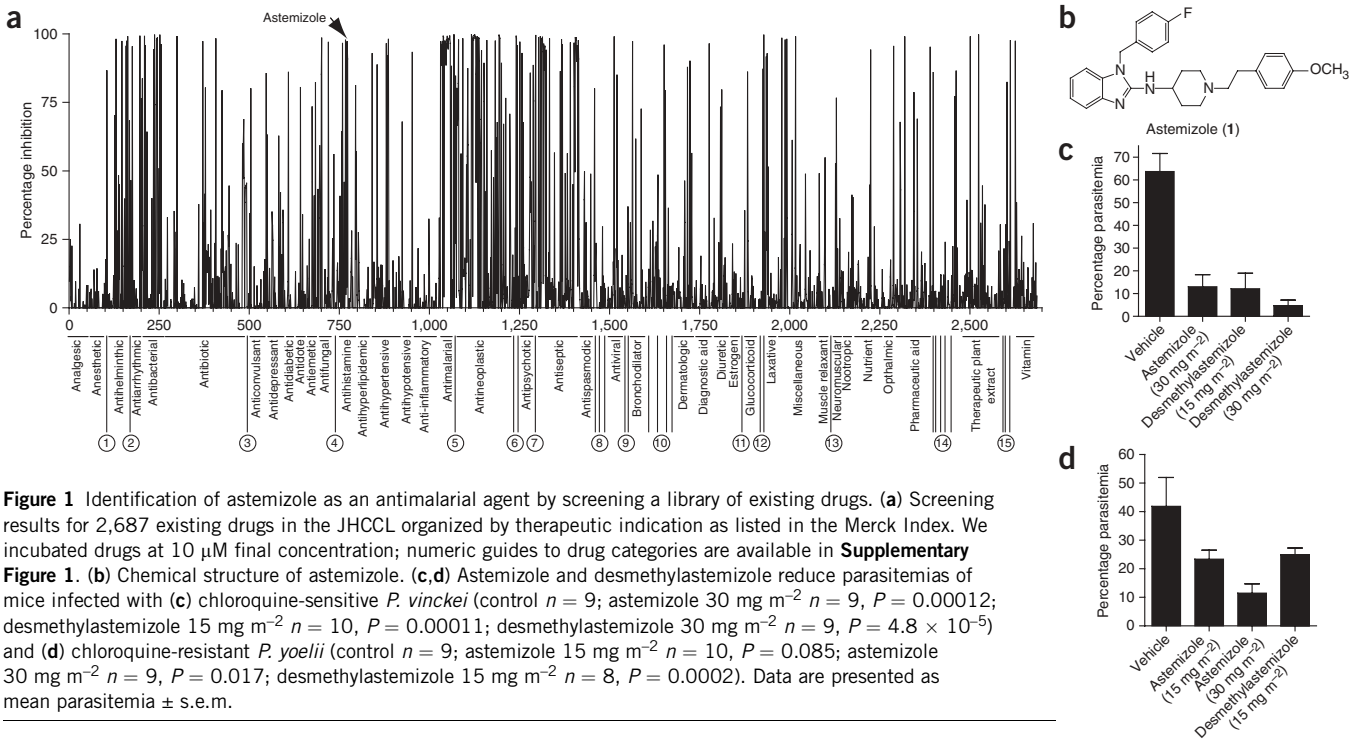

Figure 1 Identification of astemizole as an antimalarial agent by screening a library of existing drugs. (**a**) Screening results for 2,687 existing drugs in the JHCCL organized by therapeutic indication as listed in the Merck Index. We incubated drugs at 10 μM final concentration; numeric guides to drug categories are available in **Supplementary Figure 1**. (**b**) Chemical structure of astemizole. (**c**,**d**) Astemizole and desmethylastemizole reduce parasitemias of mice infected with (**c**) chloroquine-sensitive *P. vinckei* (control $n = 9$; astemizole 30 mg m^{-2} $n = 9$, $P = 0.00012$; desmethylastemizole 15 mg m^{-2} $n = 10$, $P = 0.00011$; desmethylastemizole 30 mg m^{-2} $n = 9$, $P = 4.8 \times 10^{-5}$) and (**d**) chloroquine-resistant *P. yoelii* (control $n = 9$; astemizole 15 mg m^{-2} $n = 10$, $P = 0.085$; astemizole 30 mg m^{-2} $n = 9$, $P = 0.017$; desmethylastemizole 15 mg m^{-2} $n = 8$, $P = 0.0002$). Data are presented as mean parasitemia ± s.e.m.

potassium channel at nanomolar concentrations[6]. Life-threatening cardiac arrhythmias can occur after astemizole overdose or when it is taken with drugs that block its metabolism via cytochrome P450 3A5 (CYP 3A4)[7]. Surveillance data from 17 countries over a decade revealed one cardiac rate or rhythm disorder per 8 million doses of astemizole and less than one cardiac fatality per 100 million doses[8]. Considerations relating to potential astemizole side effects in the treatment of malaria include the following: (i) antimalarial use is likely to be acute, in contrast to chronic administration as an antihistamine; (ii) malaria patients in resource-poor settings may be less likely to take interacting medications than were patients treated with astemizole in the past; and (iii) established quinoline antimalarials that less potently inhibit the HERG channel also have known cardiotoxicity[9]. Hundreds of astemizole analogs have been synthesized, and re-examination of this pharmacophore class may improve antimalarial activity and reduce HERG-related and other side effects[10].

Given the economic challenges of *de novo* drug development for neglected diseases, screening existing drugs for new activities may be helpful. Even though many leads lack the potency to immediately enter the clinic or have unacceptable toxicity, the pharmacophore we have identified is a starting point for further development. Currently, the JHCCL is undergoing expansion to include every available drug ever used in the clinic via phase 2 clinical trials or approval by the FDA or its foreign counterparts. When complete, the JHCCL will be available to any researcher interested in screening for existing drugs that may be useful as economically viable new therapies for diseases of the developing world.

Table 1 Astemizole inhibition of three *P. falciparum* strains of different chloroquine sensitivity

Plasmodium falciparum strain	Astemizole IC_{50} (nM)	Norastemizole IC_{50} (nM)	Desmethylastemizole IC_{50} (nM)	Chloroquine IC_{50} (nM)
3D7	227 ± 6.4	4,477 ± 15	117 ± 1.4	31.8 ± 3.5
Dd2	457 ± 12.3	3,590 ± 16	106.2 ± 10.3	79.3 ± 6.8
ItG	734 ± 2.2	2,230 ± 934	56.8 ± 27	107.3 ± 13.8

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

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AUTHOR CONTRIBUTIONS
C.C., J.L. and D.S. contributed to library design, construction and screening as well as manuscript preparation. X.C. assisted with the mouse malaria model. L.S. performed the *P. falciparum in vitro* screen.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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SUPPLEMENTARY FIGURE 1. Comparison of The Johns Hopkins Clinical Compound Library with other libraries of existing drugs. (a) There are approximately 11,500 existing drugs known to medicine, as indicated by FDA approval or the presence of a US Adopted Name, an International Non-proprietary Name, a Japanese Adopted Name, a British Adopted Name, or other national registry designation [1]. These drug names were entered into a database and cross-referenced with library content lists. (**b)** A list of FDA-approved drugs was obtained by Freedom of Information Act requests and cross-referenced as above.

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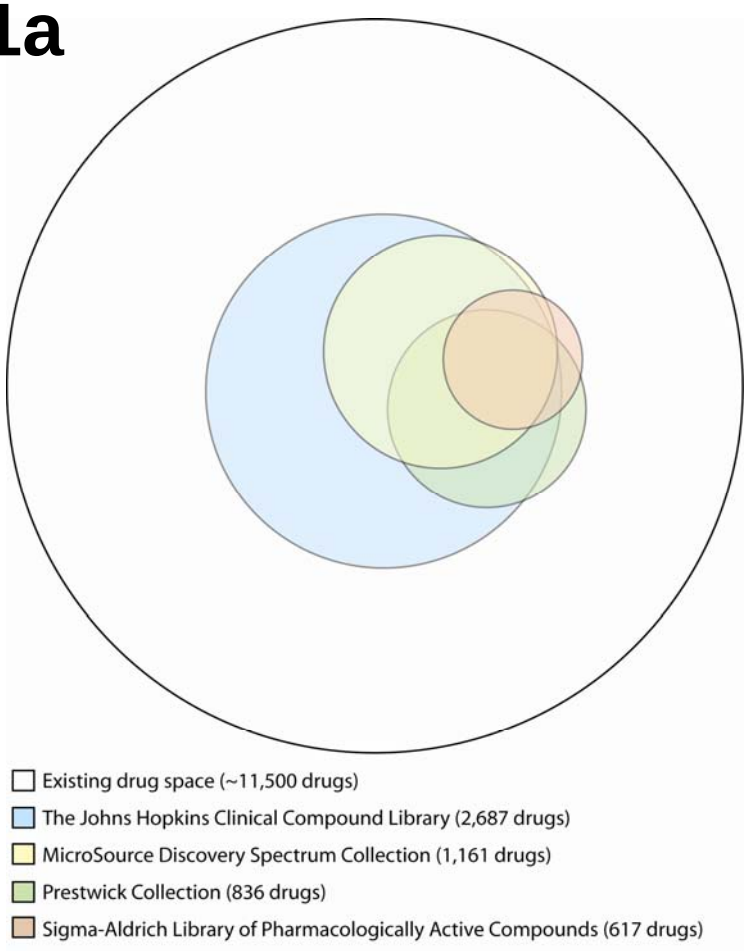
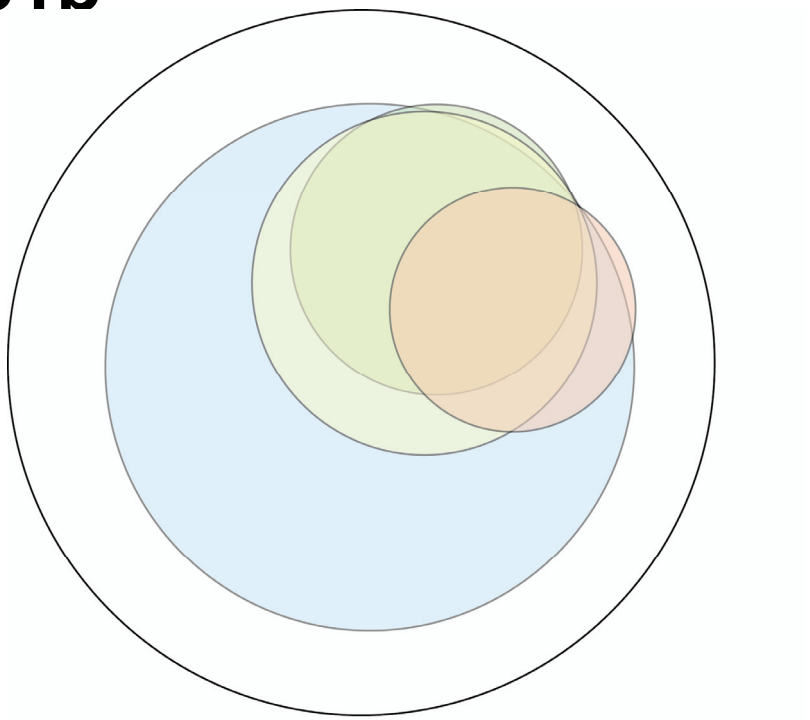
Figure S1a



		Overlapping drugs (% overlap)			
	Drugs in library (% Existing drugs)	JHCCL	Spectrum	Prestwick	LOPAC
The Johns Hopkins Clinical Compound Library (JHCCL)	2,687 (23%)	-	-	-	-
MicroSource Spectrum Collection	1,161 (10%)	1,120 (96%)	-	-	-
Prestwick Collection	836 (7%)	752 (90%)	635 (55%)	-	-
Sigma-Aldrich Library of Pharmacologically Active Compounds (LOPAC)	617 (5%)	546 (88%)	411 (35%)	332 (40%)	-

Figure S1b



		Overlapping FDA-approved drugs (% overlap)			
	FDA-approved Drugs in library (% Existing drugs)	JHCCL	Spectrum	Prestwick	LOPAC
The Johns Hopkins Clinical Compound Library (JHCCL)	1937 (56%)	-	-	-	-
MicroSource Spectrum Collection	821 (24%)	801 (98%)	-	-	-
Prestwick Collection	590 (17%)	560 (95%)	470 (80%)	-	-
Sigma-Aldrich Library of Pharmacologically Active Compounds (LOPAC)	418 (12%)	411 (98%)	257 (65%)	324 (78%)	-

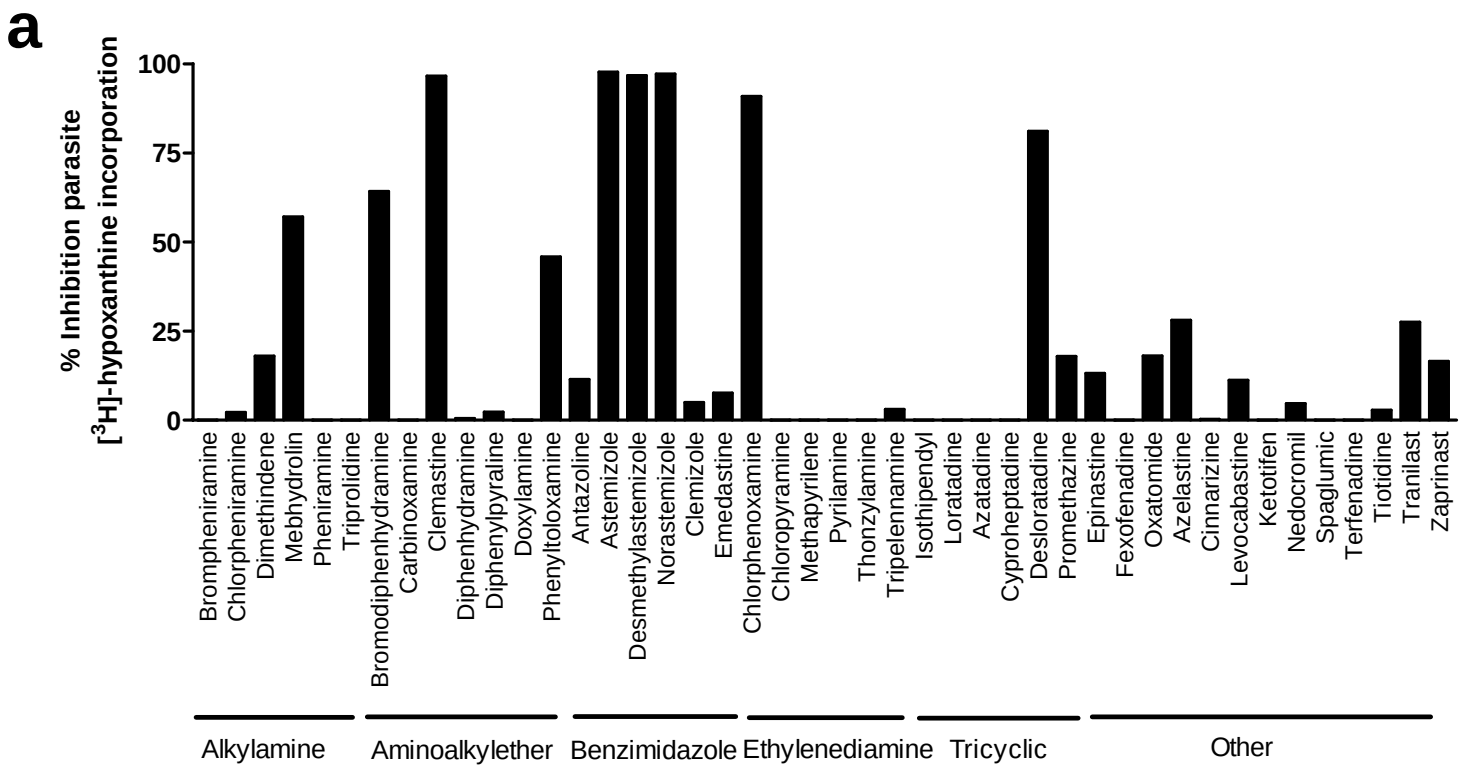
SUPPLEMENTARY MATERIALS: Guide to numeric indications in manuscript Figure 1

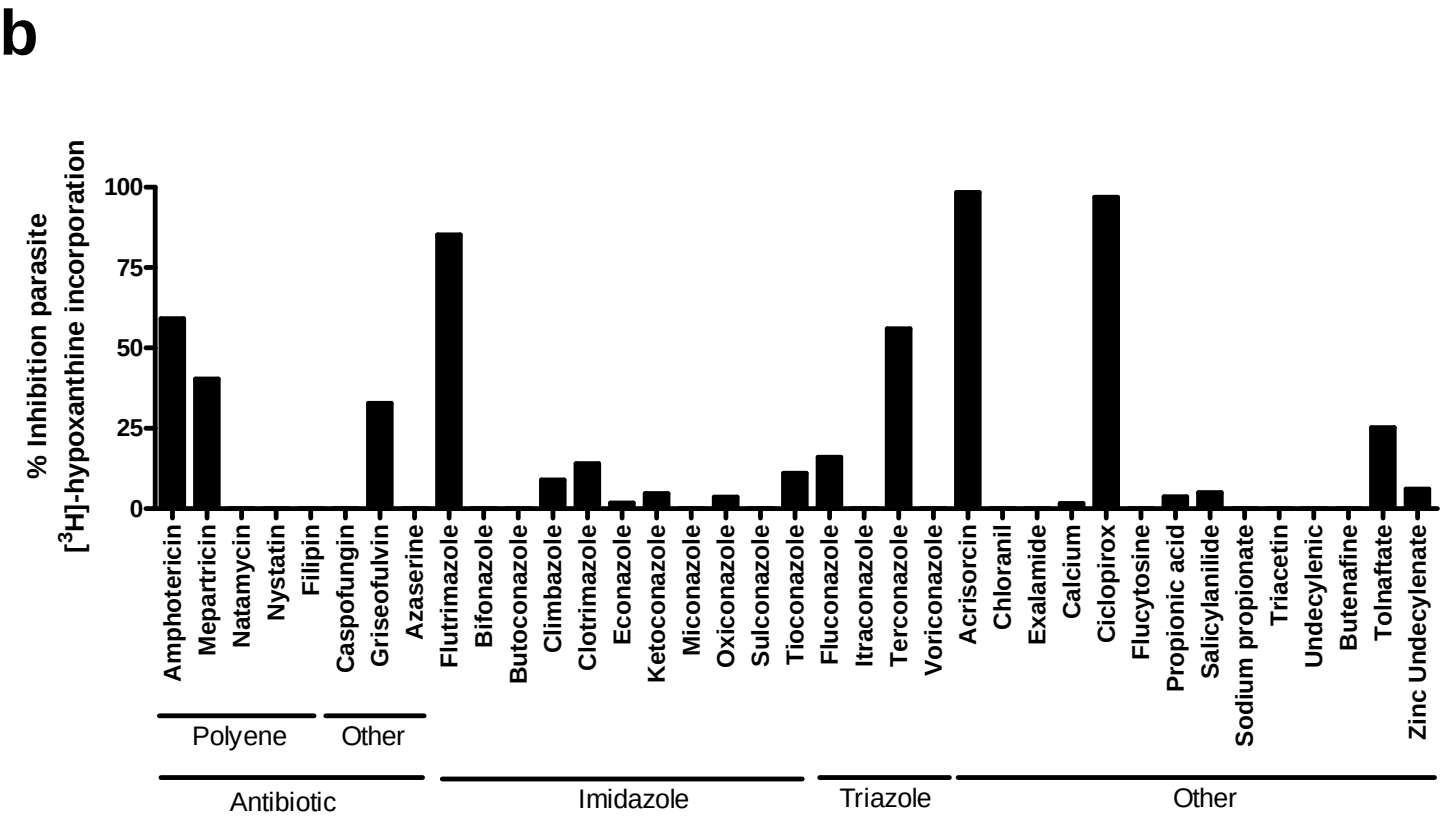
1: Antacid
2: Antianginal
3: Anticoagulant
4: Antiglaucoma
5: Antimigraine
6: Antiparkinsonian, antiprotozoal, respectively
7: Antirheumatic
8: Antithrombotic, antitussive, antiulcerative, respectively
9: Anxiolytic, bone resorption inhibitor, respectively
10: Cardiotonic, choleretic, cholinergic, decongestant, respectively
11: Expectorant
12: Hemostatic, immunosuppressant, respectively
13: Mydriatic
14: Pituitary, plasma volume expander, progestogen, sedative, steroid, respectively
15: Thyroid, tocolytic, vasodilator, respectively.

The miscellaneous category includes abortifacient, alcohol deterrent, anorexic, antiamebic, anticholelithogenic, antidiarrheal, antiflatulent, antmethemoglobinemic, antiobesity, antiurolithic, capillary protectant, contraceptive, ectoparasiticide, erectile dysfunction, gastroprokinetic, hemantic, hepatic protectant, immunomodulator, insecticide, mucolytic, oxytocic, prostaglandin, respiratory stimulant, sialagogue, surfactant, uricosuric, urologic, and vaccine, respectively.

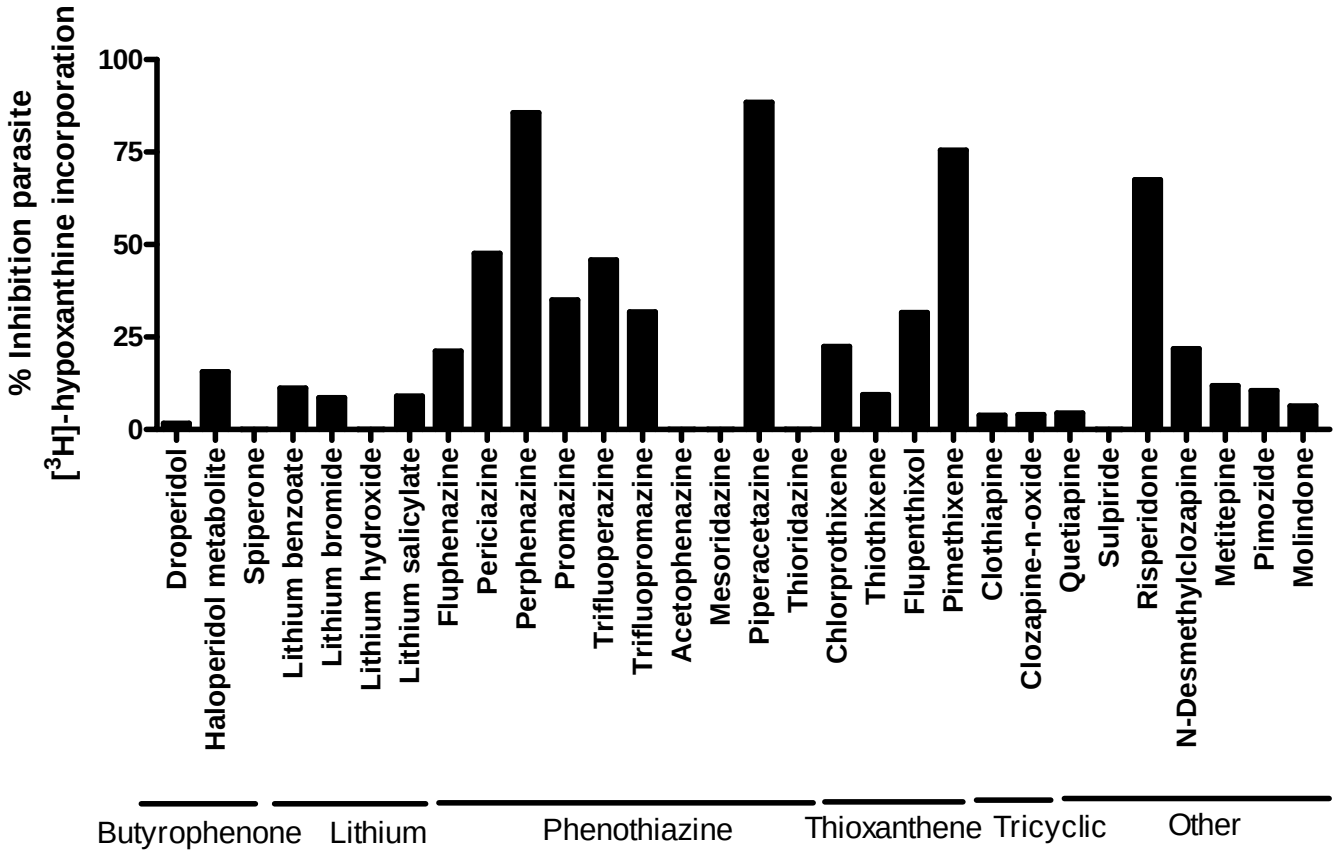
SUPPLEMENTARY FIGURE 2. Selected screening results from drug classes of interest. Drugs were incubated at a 10 μM final concentration with 3D7 *P. falciparum* for 96 h; antihistamines (**a**), antifungals (**b**), antipsychotics (**c**), antihypertensives (**d**), antidepressants (**e**).

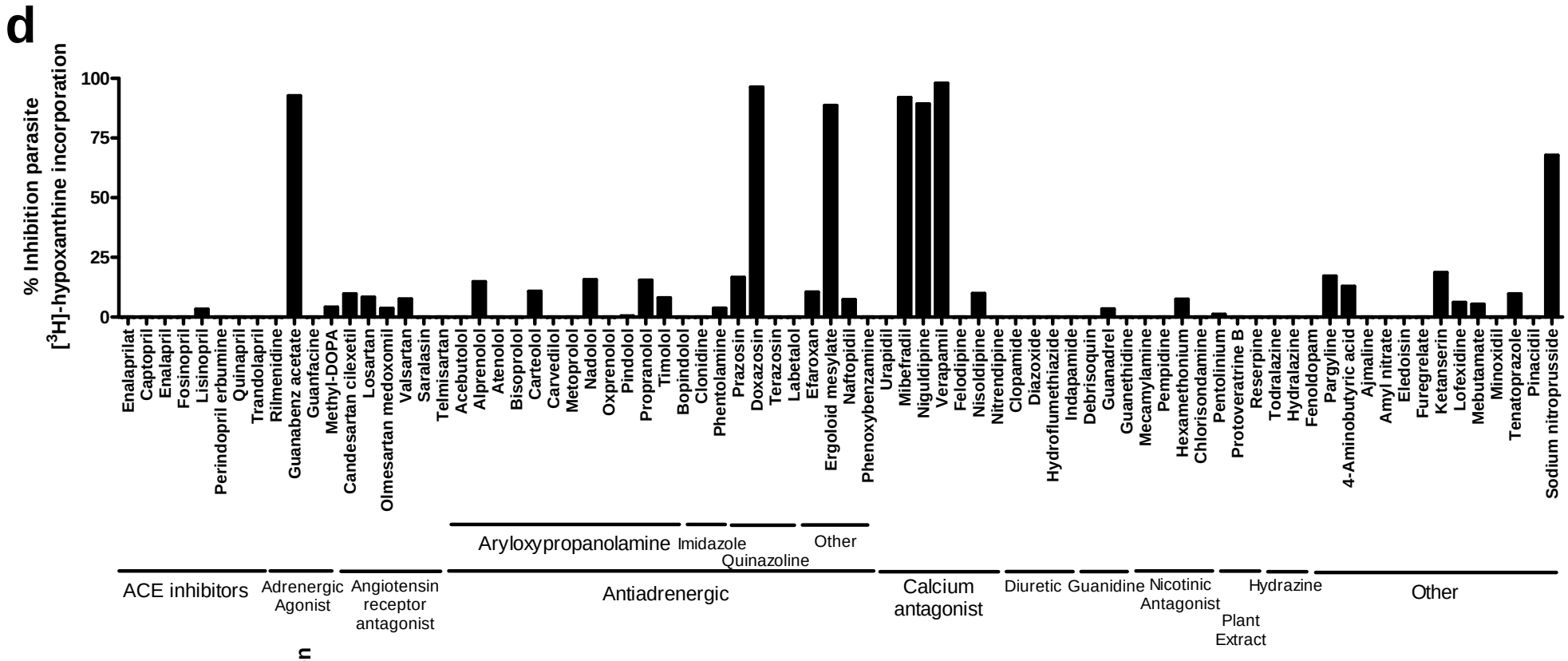
Figure S2


a
% Inhibition parasite [3H]-hypoxanthine incorporation
100
75
50
25
0
Brompheniramine
Chlorpheniramine
Dimethindene
Mebhydrolin
Pheniramine
Triprolidine
Bromodiphenhydramine
Carbinoxamine
Clemastine
Diphenhydramine
Diphenylpyraline
Doxylamine
Phenyltoloxamine
Antazoline
Astemizole
Desmethylastemizole
Norastemizole
Clemizole
Emedastine
Chlorphenoxamine
Chloropyramine
Methapyrilene
Pyrilamine
Thonzylamine
Tripelennamine
Isothipendyl
Loratadine
Azatadine
Cyproheptadine
Desloratadine
Promethazine
Epinastine
Fexofenadine
Oxatomide
Azelastine
Cinnarizine
Levocabastine
Ketotifen
Nedocromil
Spaglumic
Terfenadine
Tiotidine
Tranilast
Zaprinast
Alkylamine
Aminoalkylether
Benzimidazole
Ethylenediamine
Tricyclic
Other


b
% Inhibition parasite [3H]-hypoxanthine incorporation
100
75
50
25
0
Amphotericin
Mepartricin
Natamycin
Nystatin
Filipin
Caspofungin
Griseofulvin
Azaserine
Flutrimazole
Bifonazole
Butoconazole
Climbazole
Clotrimazole
Econazole
Ketoconazole
Miconazole
Oxiconazole
Sulconazole
Tioconazole
Fluconazole
Itraconazole
Terconazole
Voriconazole
Acrisorcin
Chloranil
Exalamide
Calcium
Ciclopirox
Flucytosine
Propionic acid
Salicylanilide
Sodium propionate
Triacetin
Undecylenic
Butenafine
Tolnaftate
Zinc Undecylenate
Polyene
Other
Antibiotic
Imidazole
Triazole
Other

C


% Inhibition parasite
[3H]-hypoxanthine incorporation
100
75
50
25
0
Droperidol
Haloperidol metabolite
Spiperone
Lithium benzoate
Lithium bromide
Lithium hydroxide
Lithium salicylate
Fluphenazine
Periciazine
Perphenazine
Promazine
Trifluoperazine
Trifluopromazine
Acetophenazine
Mesoridazine
Piperacetazine
Thioridazine
Chlorprothixene
Thiothixene
Flupenthixol
Pimethixene
Clothiapine
Clozapine-n-oxide
Quetiapine
Sulpiride
Risperidone
N-Desmethylclozapine
Metitepine
Pimozide
Molindone
Butyrophenone
Lithium
Phenothiazine
Thioxanthene
Tricyclic
Other


d
% Inhibition parasite
[3H]-hypoxanthine incorporation
100
75
50
25
0
Enalaprilat
Captopril
Enalapril
Fosinopril
Lisinopril
Perindopril erbumine
Quinapril
Trandolapril
Rilmenidine
Guanabenz acetate
Guanfacine
Methyl-DOPA
Candesartan cilexetil
Losartan
Olmesartan medoxomil
Valsartan
Saralasin
Telmisartan
Acebutolol
Alprenolol
Atenolol
Bisoprolol
Carteolol
Carvedilol
Metoprolol
Nadolol
Oxprenolol
Pindolol
Propranolol
Timolol
Bopindolol
Clonidine
Phentolamine
Prazosin
Doxazosin
Terazosin
Labetalol
Efaroxan
Ergoloid mesylate
Naftopidil
Phenoxybenzamine
Urapidil
Mibefradil
Niguldipine
Verapamil
Felodipine
Nisoldipine
Nitrendipine
Clopamide
Diazoxide
Hydroflumethiazide
Indapamide
Debrisoquin
Guanadrel
Guanethidine
Mecamylamine
Pempidine
Hexamethonium
Chlorisondamine
Pentolinium
Protoveratrine B
Reserpine
Todralazine
Hydralazine
Fenoldopam
Pargyline
4-Aminobutyric acid
Ajmaline
Amyl nitrate
Eledoisin
Furegrelate
Ketanserin
Lofexidine
Mebutamate
Minoxidil
Tenatoprazole
Pinacidil
Sodium nitroprusside
Aryloxypropanolamine
Imidazole
Quinazoline
Other
ACE inhibitors
Adrenergic Agonist
Angiotensin receptor antagonist
Antiadrenergic
Calcium antagonist
Diuretic
Guanidine
Nicotinic Antagonist
Plant Extract
Hydrazine
Other
e
% Inhibition parasite
[3H]-hypoxanthine incorporation
100
75
50
25
0
Citalopram
Escitalopram
Nomifensin
Paroxetine
Trazodone
Sertraline
Amitriptyline
Amoxapine
Clomipramine
Desipramine
Dibenzepine
Doxepin
Imipramine
Nortriptyline
Protriptyline
Trimipramine
Maprotiline
Mirtazapine
Iproniazid
Isocarboxazid
Nialamide
Selegiline
Alaproclate
Bupropion
Tryptophan
Fenclonine
Fluoxetine
Fluvoxamine
Hypericin
Minaprine
Nefazodone
Phenelzine
Quipazine
Rubidium
Tranylcypromine
Venlafaxine
Viloxazine
Cotinine
Bicyclic
Tricyclic
Tetracyclic
Other

METHODS

Library construction. 32,000 FDA drug approvals from 1938-2003 obtained by U.S. Freedom of Information Act requests were condensed to 3,400 unique drug formulations. 1,937 FDA approved drugs and 750 drugs that entered phase II clinical trials or are used abroad were purchased from Sigma, Spectrum Chemicals, MicroSource Discovery, MP Biomedicals, The Johns Hopkins Hospital pharmacy, Biomol, and Tocris. 10 mM stock solutions were made using DMSO, water, or ethanol as solvents. Drugs were arrayed in 96-well plates and screened at a final concentration of 10 μM.

Parasite culture and screening. Synchronized ring stage parasites from chloroquine-sensitive 3D7 or multidrug resistant Dd2 or ItG were cultured in RPMI 1640 medium with 10% human serum and incubated for either 48 or 96 h in the presence of drug and [^{3}H]-hypoxanthine[1, 2]. A 96 well plate with 0.2 mL of culture material per well at 0.2% parasitemia and 2-4% hematocrit, gives a radioactive incorporation signal of approximately 10,000 cpm at 48 h and 20,000 cpm at 96 h with background counts less than 500 cpm. Screening experiments were performed in duplicate and percent inhibition is reported as the average of two experiments.

Heme crystallization assays. Heme crystal was synthesized as previously described[3]. For high-throughput screening 2.5 nmol of heme crystal was used to seed a crystal extension reaction with 50 μM heme in 0.1 M ammonium acetate, pH 4.8 in a 0.1 mL half area 96 well plate, and incubated at 37°C for 16 h. This assay relies on the differential solubility of free versus crystalline heme in 0.15 M sodium bicarbonate to quantify the amount of crystal extension [3]. Compounds that showed 50% inhibition at 50 μM in this assay were selected for further characterization of heme crystallization as previously described[3]. For pH profile experiments the same buffers were used as previously[3]. IC_{50} values for heme crystallization and parasite

proliferation assays were determined using four-parameter logarithmic analysis with GraphPad Prism and are presented as mean ± s.e.m. for triplicate experiments.

Co-purification of [^{3}H]-astemizole with heme crystals or hemozoin. [^{3}H]-astemizole (27 Ci/mmol) was purchased from Vitrax (Placentia, CA) and various amounts were incubated in the heme crystal extension assay in triplicate with a 5 nmol heme crystal seed and 50 μM heme substrate in 0.5 mL 0.1 M ammonium acetate, pH 4.8 for 16 h at 37°C. The insoluble heme crystal produced was centrifuged at 15,000 × *g* for 10 min, resuspended by sonication in 0.1 mL 50 mM Tris HCl, pH 8.0, placed on top of 1 mL of 1.7 M sucrose, 50 mM Tris HCl, pH 8.0, and centrifuged at 200,000 × *g* for 15 min[4]. The heme crystal pellet was then decrystallized with 50 mM NaOH, 2% SDS and the radioactivity was quantified using a scintillation counter. For parasite hemozoin copurification experiments, duplicate 12 mL 3D7 or Dd2 cultures at 5% parasitemia and synchronized at ring stages were incubated with 1.5 μCi astemizole for 20 h. At the trophozoite stage erythrocytes were harvested, washed once, and hemozoin was extracted by hypotonic lysis and purified as described above[4].

Animal experiments. All animal experiments were performed on a protocol approved by The Johns Hopkins Animal Care and Use Committee in accordance with institutional standards. Male 5-6 week old 25 ± 2 g C57/BL6 mice were purchased from the National Cancer Institute. The *P. vinckei* Rhodain strain was obtained from ATCC; the *P. yoelii* 17X lethal strain was the gift of N. Kumar (JHMRI). Stock solutions of astemizole tartrate or acetate were used. Mice were given astemizole or equivalent volume of vehicle intraperitoneal or *per os*. 2 h post infection with 1×10^8 parasites intraperitoneal on day 0, and once daily for the indicated length of time[5]. Blood was taken from the tail vein on day five. Parasitemias were determined in a blinded fashion by counting four fields of approximately 200 erythrocytes per field, and *P*-values

comparing drug treated with control animals were determined using the two-tailed Student's T-test. Data are presented as mean parasitemia ± s.e.m. Mice that survived for 30 days post infection with complete disappearance of parasitemia and no recrudescence within the next 30 days were considered cured. Previous literature reports establish the minimum effective dose of chloroquine in murine *P. vinckei* as 7.5 mg/m^2/day and 150 mg/m^2/day in *P. yoelii*[6].

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