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High-Throughput *Plasmodium falciparum* Growth Assay for Malaria Drug Discovery[▽]

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New therapeutic agents for the treatment of malaria, the world's most deadly parasitic disease, are urgently needed. Malaria afflicts 300 to 500 million people and results in 1 to 2 million deaths annually, and more than 85% of all malaria-related mortality involves young children and pregnant women in sub-Saharan Africa. The emergence of multidrug-resistant parasites, especially in *Plasmodium falciparum*, has eroded the efficacy of almost all currently available therapeutic agents. The discovery of new drugs, including drugs with novel cellular targets, could be accelerated with a whole-organism high-throughput screen (HTS) of structurally diverse small-molecule libraries. The standard whole-organism screen is based on incorporation of [^{3}H]hypoxanthine and has liabilities, such as limited throughput, high cost, multiple labor-intensive steps, and disposal of radioactive waste. Recently, screens have been reported that do not use radioactive incorporation, but their reporter signal is not robust enough for HTS. We report a *P. falciparum* growth assay that is technically simple, robust, and compatible with the automation necessary for HTS. The assay monitors DNA content by addition of the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) as a reporter of blood-stage parasite growth. This DAPI *P. falciparum* growth assay was used to measure the 50% inhibitory concentrations (IC_{50}s) of a diverse set of known antimalarials. The resultant IC_{50}s compared favorably with those obtained in the [^{3}H]hypoxanthine incorporation assay. Over 79,000 small molecules have been tested for antiplasmodial activity using the DAPI *P. falciparum* growth assay, and 181 small molecules were identified as highly active against multidrug-resistant parasites.

Malaria has afflicted humans since the introduction of agriculture some 10,000 years ago, and inherited diseases, such as sickle cell anemia and the thalassemias, testify to its profound selection signature on the human genome (4). Malaria continues to be a major public health problem in large regions of the world, especially the developing world. The vast majority of malaria deaths are caused by *Plasmodium falciparum*, and young children and pregnant mothers in sub-Saharan Africa are the primary victims. Because most cases of malaria occur in medically underserved areas, completely accurate figures for the disease's impact are difficult to obtain, but by any measure, malaria is a pernicious disease. Some insight into its impact can be gained by using disability-adjusted life years, which measure the disease's toll on both longevity and quality of life, and the World Health Organization (WHO) estimates the malaria burden at 42 million disability-adjusted life years (20a).

Success in preventing and treating malaria varies. Only 50 years ago, WHO and others were optimistic that malaria could be eradicated through a combination of effective therapeutic agents to treat the existing disease, insecticides to eliminate its mosquito vector, and various public health measures for prevention. Optimism, not the disease, disappeared in the face of a perfect storm of factors: the disengagement of developed countries from regions where malaria is endemic, brought about by the independence of former colonies; the cessation of armed conflicts by developed countries in regions where malaria is endemic; the collapse of public health institutions in many developing nations; the lack of financial incentives for pharmaceutical companies to develop antimalarial agents; and the spread of resistance in the parasite (especially to chloroquine) and the mosquito vector (especially to dichlorodiphenyltrichloroethane). This combination of factors has led to the resurgence and spread of malaria in large parts of the developing world.

Resistance to the most commonly used and approved antimalarial drugs has been reported throughout Africa and Asia, and new drugs are needed. The range of current antimalarials is narrow. There are only four classes of compounds: those based on quinine (chloroquine, mefloquine, amodiaquine, and halofantrine) or other aminoquinolines (primaquine and tafenoquine), the antifolate compounds (pyramethamine, proguanil, cyocloguanil, dapsome, and sulfadoxine), the artemisinin derivatives (artesunate, artemether/arteether, Co-artem, and others), and, most recently, the hydroxynapthoquinone atovaquone. This lack of structural diversity means that previously developed therapeutic alternatives, really modifications of the same basic molecular templates, might prime new drug candidates for the rapid emergence of resistance.

Current antimalarials also have a limited range of cellular targets: only four to six processes in these sophisticated eukaryotic organisms with complex life cycles. Most drugs target either metabolism or the detoxification of hemoglobin, the parasite's food source. Drugs that target hemoglobin detoxification include the 4-aminoquinolines (chloroquine and amodiaquine) and the amino alcohols (quinine, mefloquine, halofantrine, and lumefantrine). The parasite's folate metabolism

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is targeted by the antifolates (sulfadoxine-pyrimethamine and dapsone), and its mitochondria are targeted by atovaquone (6). In addition to these targets, artemisinin, or more likely an artemisinin-derived molecule, probably targets the sarco- and/or endoplasmic reticulum Ca^{2+} ATPase (11). The *P. falciparum* genome has been sequenced, revealing over 5,500 genes of *P. falciparum* that surely contain a large, though admittedly unknown, number of targets that are suitable for small-molecule intervention (9). The discovery of new targets, especially those that can be affected by small molecules, can be most easily approached with a high-throughput screening (HTS) campaign with structurally diverse small-molecule libraries.

Conducting a HTS campaign to identify small molecules that might lead to drug candidates obviously requires a robust HTS, and such a screen does not currently exist. Previously, the only means of detecting parasite growth was microscopic examination of blood smears using a modified Wright-Giemsa stain or the [^{3}H]hypoxanthine incorporation assay (7). While the latter method could in principle be automated, it is not well suited for HTS, as it requires radioactive materials that pose safety and disposal problems and has multiple steps that are technically demanding. Recently, new nonradioactive screens have emerged, using DNA stains as a reporter to measure parasite growth (1, 5, 16, 17). The use of DNA stains to detect parasite DNA has greatly aided the ease of drug susceptibility testing. However, these methods are not sufficiently robust and are therefore not amenable to HTS of large compound libraries.

As part of a larger effort to develop antimalarial agents, we set about developing a HTS based on DNA staining with blood-stage *P. falciparum* that would identify inhibitory small molecules from structurally diverse small-molecule libraries. A whole-organism method allows all relevant blood-stage targets to be screened simultaneously and ensures that screening positives have at least minimal levels of desirable pharmacokinetic properties, such as cell permeability and activity in a cellular context. Such a screen can also be readily expanded to other parasite strains to study resistance profiles or to include variables, such as culture time, that would distinguish fast- from slow-acting compounds. The choice of a 4′,6-diamidino-2-phenylindole (DAPI) DNA stain led to a robust and reproducible reporter of parasite growth that responded to chemically and mechanistically diverse known antimalarial inhibitors. In addition, it is well suited for HTS performed using a 384-well microtiter plate, as demonstrated by its performance in a screen of approximately 79,000 compounds.

MATERIALS AND METHODS

Cultivation of *P. falciparum*. *P. falciparum* strains 3D7, HB3, and Dd2 were obtained from the Research and Reference Reagent Resource Center (MR4) (Manassas, VA). The three strains were maintained in vitro by a modification of the method of Trager and Jensen (18). Cultures were maintained in fresh type O-positive (O^+) human erythrocytes (Interstate Blood Bank, Memphis, TN) suspended at 4% hematocrit in complete medium containing 50 ml human O^+ serum (Interstate Blood Bank, Memphis, TN), 2.5 g albumax II (Gibco-BRL Laboratories, Gaithersburg, MD), 0.5 ml gentamicin (Sigma Aldrich Chemical Company, St. Louis, MO), 5.94 g HEPES (Sigma), 2.01 g sodium bicarbonate (Sigma), 0.050 g hypoxanthine (Sigma), and 10.44 g RPMI 1640 (JRH biosciences, Lenexa, KA) per liter at pH 6.74. Cultures were grown in 75-cm^2 flasks (BD Falcon, Franklin Lakes, NJ) at a volume of 25 ml and were flushed for 45 s with a standard gas environment of 5% CO_2, 1% O_2, and 94% N_2 and incubated at 37°C. A culture at approximately 1% parasitemia at ring stage was synchronized with 5% sorbitol (Sigma), as previously described (10), and was followed by a subsequent synchronization 7 to 8 h later. Forty-eight hours later, the percent parasitemia was determined as 5% parasitemia in ring stage using light microscopy by counting a minimum of 500 erythrocytes in a Giemsa-stained thin blood smear. Cultures were diluted in complete medium, and type O^+ human erythrocytes were suspended at 4% hematocrit to 100 ml into 225-cm^2 flasks (BD Falcon). The cultures were maintained by replacing the complete medium daily and were diluted in fresh complete medium at 4% hematocrit when they reached 5% parasitemia.

DAPI *P. falciparum* growth assay HTS design. To perform the DAPI *P. falciparum* growth assay, 30 μl of complete medium was dispensed into 384-well black opaque tissue culture treated microtiter plates (Corning, Corning, NY) using a Matrix WellMate liquid dispenser (Matrix, Hudson, NH). Subsequently, single chemical compounds were dispensed into the microtiter plates at the Institute of Chemistry and Cell Biology Longwood (ICCB-L) screening facility at Harvard Medical School. ICCB-L stock compounds were stored in 384-well microtiter plates at a minimal compound concentration of 10 mM in dimethyl sulfoxide (DMSO) and were dispensed using a Seiko compound transfer robot equipped with a 100-nl 384-pin head array (V&P Scientific, San Diego, CA). Next, 10 μl of 1.0% parasitized red blood cells (P-RBCs) (ring stage) and 3% hematocrit (type O^+ human RBCs) in complete medium were dispensed into the microtiter plates using a Matrix WellMate liquid dispenser at speed setting 2. The P-RBCs were continuously resuspended and dispensed in 30-ml intervals to ensure a uniform distribution of parasites into the microtiter plates. The plates were then incubated in a CO_2 1000 incubator (Forma Scientific, Marietta, OH) with a controlled gas environment of 5% CO_2 and 4% O_2 at 37°C for 72 h. After 72 h, the percent parasitemia was determined as 7% parasitemia in trophozoite stage by light microscopy, counting a minimum of 500 erythrocytes from a Giemsa-stained thin blood smear. Following incubation, the plates were carefully removed from the incubator to prevent the P-RBC pellet from being disturbed. The complete medium was gently aspirated from each well using a 24-channel wand with the pin height adjusted above the P-RBC pellet (V&P Scientific, San Diego, CA). If the microtiter plates could not be removed without disturbing the P-RBC pellet, the microtiter plates were centrifuged at 4,000 rpm for 4 min prior to aspiration. Next, 30 μl of a fluorochrome mixture was dispensed into each well using a Matrix WellMate liquid dispenser at speed setting 1. The fluorochrome mixture contained 20 mM Tris-HCl (pH 7.5) (Sigma), 5 mM EDTA (disodium salt) (Sigma), 0.0008% Saponin (Sigma), 0.001% Trition X-100 (Sigma), and a 1:100,000 final dilution of DAPI (5 mg/ml) (Molecular Probes, Inc. Eugene, OR). The microtiter plates were then incubated in the dark for 30 min and centrifuged at 4,000 rpm for 10 min. Next, the excess fluorochrome mixture was aspirated using a 24-channel wand, and 30 μl of 1× phosphate-buffered saline (PBS) (pH 7.4) was dispensed into each well using a Matrix WellMate liquid dispenser at speed setting 1. The microtiter plates were read using an EnVision plate reader (excitation and emission [ex/em] wavelengths, 355/460 nm; Perkin Elmer, Wellesley, MA). Screening was performed in duplicate on *P. falciparum* 3D7. For each assay, a screening-positive (chloroquine serial dilution) and -negative (infected erythrocytes with no drug added) and a background count (noninfected erythrocytes) were performed in replicate. The percentage of parasite growth in the test wells was calculated by the formula [test well $-$ $CQ(LD_{90})$/Avg_+ $-$ $CQ(LD_{90})$] $\times$ 100, in which $CQ(LD_{90})$ is the parasite nuclei detected in the presence of chloroquine at a lethal-dose concentration of 90% (LD_{90}) and Avg_+ is the mean of the negative control wells (infected erythrocytes with no drug added). A screening positive was arbitrarily determined to be any compound that suppressed parasite growth by 90% compared to the no-drug-added control well. To perform the assay for imaging-based analysis, an alternative procedure was followed. Prior to assay setup, plates were coated with CellTak (BD Biosciences, Franklin, NJ) or concanavalin A (Sigma). Following the growth incubation period, a 4.0% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) solution was added to each well, and the plates were incubated for 30 m. The plates were then centrifuged at 4,000 rpm for 10 min, excess fixative was aspirated using a 24-channel wand, and the plates were washed once with 1× PBS (Gibco-BRL). The plates were then centrifuged at 4,000 rpm for 10 min and aspirated prior to addition of the fluorochrome mixture. Following the 30-min incubation in the dark, the plates were washed once with 1× PBS as described above. As an alternative to coating the microtiter plates, following the growth incubation period, the medium was aspirated using a 24-channel wand, and a 4.0% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and 0.0075% glutaraldehyde (Fluka, Switzerland) solution was added to each well and incubated for 30 min. Final microtiter plate preparation was carried out as defined above for the plate-coating technique. Parasite DNA was detected using an Applied Precision cellWoRx imaging station (Applied Precision, Issaquah,

WA) as a detector. The data were analyzed using MetaXpress imaging software (Molecular Devices Corporation, Sunnyvale, CA).

Assessment of the percentage of *P. falciparum*-infected erythrocytes with fluorescence intensity. To perform the assay for assessment of the percentage of *P. falciparum*-infected erythrocytes with fluorescence intensity, synchronized ring-stage parasites (0.5% parasitemia and 2% hematocrit) were serially diluted 1:1 in noninfected erythrocytes and complete medium containing 2% hematocrit in 96-well black mictrotiter plates, yielding a final parasitemia range of 0 to 7% and a final hematocrit of 2% in a final assay volume of 230 μl. The microtiter plates were then incubated in a chamber with the standard gas environment at 37°C for 72 h. The assay was performed in triplicate, and parasite growth was detected in parallel by using the [^{3}H]hypoxanthine incorporation, DAPI fluorimetry-based, and DAPI imaging-based assays. To perform the [^{3}H]hypoxanthine incorporation assay, after a 24-h incubation period, 1 μCi (10 μl of 0.1 μCi/μl) [^{3}H]hypoxanthine was added to each well. The microtiter plates were then returned to the gas-controlled chamber and incubated for an additional 48 h. Following incubation, the plates were stored at −80°C, thawed, and read as described below for the drug susceptibility test. To perform the DAPI *P. falciparum* growth assay, after the 72-h incubation period, the fluorochrome mixture was added to each well and the plates were processed as described above for the DAPI *P. falciparum* growth assay HTS design.

Drug susceptibility test. Parasite growth was detected in parallel by our DAPI *P. falciparum* growth assay and by the traditional [^{3}H]hypoxanthine incorporation assay as previously described by Desjardins and colleagues (7). To perform the [^{3}H]hypoxanthine incorporation assay, compounds (chloroquine, atovaquone, quinine, and artemisinin; Sigma) were serially diluted 1:1 into hypoxanthine-free complete medium to a final volume of 20 μl (final drug concentration range, 300 nM to 0.1 nM). Synchronized ring-stage parasites in hypoxanthine-free complete medium (0.5% parasitemia and 2% hematocrit) were added to each well at a volume of 200 μl. The microtiter plates were then incubated in chamber in a standard gas environment at 37°C for 72 h. After the 24-h incubation period, 1 μCi (10 μl of 0.1 μCi/μl) [^{3}H]hypoxanthine (specific activity, 1.0 mCi/ml; American Radiolabeled Chemicals, Inc., St. Louis, MO) was added to each well. The microtiter plates were then returned to the gas-controlled chamber and incubated for an additional 48 h. Following the incubation period, the plates were stored at −80°C, thawed, and harvested with a Filtermate cell harvester (Packard Biosciences, Meriden, CT) onto fiberglass paper disks, washed with distilled water, and fixed with ethanol. Each disk was placed in unifilter 96-well white microplates with a bonded GF/C filter (Perkin Elmer Life and Analytical Science, Boston, MA) containing 2 ml of Microscint scintillation cocktail (Microscint-High Efficiency LSC-Cocktail; Perkin Elmer Life and Analytical Science) for 1 h. The plates were counted in a Top Count NXT microplate scintillation and luminescence counter (Packard Biosciences). The mean values for [^{3}H]hypoxanthine incorporation in parasitized control and nonparasitized control erythrocytes were calculated.

To perform the DAPI *P. falciparum*-growth assay, compounds (chloroquine, atovaquone, quinine and artemisinin; Sigma) were serially diluted 1:1 in complete medium to a final volume of 20 μl (final drug concentration range, 300 nM to 0.1 nM). Synchronized ring-stage parasites (0.5% parasitemia and 2% hematocrit) were added to each well at a volume of 200 μl. The microtiter plates were then incubated in a chamber with a standard gas environment at 37°C for 72 h. After the 72-h incubation period, the fluorochrome mixture was added to each well and the plates were processed as described above for the DAPI *P. falciparum*-growth assay HTS design. Analysis of the counts obtained by both assay methods was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). The counts were plotted against the logarithm of the drug concentration, and the curve was fitted by nonlinear regression using the formula sigmoidal dose-response (variable slope) to yield the dose-response curve and resultant inhibitory concentrations at 50% (IC_{50}s).

Quantitative assay evaluation and optimization. Three 96- and 384-well microtiter plates were filled with parasite-infected erythrocytes (positive control), and three plates were filled with noninfected erythrocytes (negative control). The Z′ value was calculated from the data collected by the formula $z' = 1 - (3SD_+ + 3SD_-)/|Ave_+ - Ave_-|$, in which SD_+ is the positive control standard deviation, SD_- is the negative control standard deviation, Ave_+ is the mean value of the positive control, and Ave_- is the mean value of the negative control.

Compound libraries. The ICCB-L at Harvard Medical School and the Broad Chemical Biology (BCB) Program at the Broad Institute of Harvard and the Massachusetts Institute of Technology (MIT) have a collection of large libraries containing over 250,000 drug-like and natural-product-like small molecules. They are arrayed in 384-well microtiter plates as ~4 to 10 mM solutions in DMSO. These compounds include commercially available libraries, bioactive collections, natural products, and, at the BCB, products from diversity-oriented syntheses (2, 3). The purchased commercially available collections were obtained from ActiMol TimTec (8,518 compounds), Bionet (1,700 compounds), ChemBridge (10,560 compounds), ChemDiv (25,104), Enamine (6,004 compounds), I.F. Lab (6,835 compounds), Maybridge (12,215 compounds), Peakdale (352 compounds), and mixed commercial sources (599).

The Known Bioactives collection contains the National Institute of Neurological Disorders and Stroke (NINDS) library (1,040 compounds). This collection of characterized bioactive compounds was compiled by MicroSource Discovery Systems for the NINDS, the Huntington's Disease Society of America, the Amyotrophic Lateral Sclerosis Association, and the Hereditary Disease Foundation. The Specplus collection (960 compounds) contains pure natural products and their derivatives and includes simple and complex oxygen heterocycles, alkaloids, sequiterpenes, diterpenes, pentacyclic triterpenes, and sterols. The BIOMOL ICCB Known Bioactives collection (480 compounds) contains many compound classes, including ion channel blockers, G-protein coupled receptor ligands, second-messenger modulators, nuclear hormone receptor ligands, actin and tubulin ligands, kinase inhibitors, protease inhibitors, gene regulation agents, and lipid biosynthesis inhibitors, as well as other well-characterized compounds that perturb cellular pathways.

The natural-products collection contains partially purified extracts from endophytic fungi (fungi that live in higher plants) that grow in Costa Rica (2,902 extracts), as well as extracts from plants used in traditional Chinese medicine (2,025). Crude extracts from the plants and fungi were obtained and further fractionated to eliminate nuisance compounds, especially those of high molecular weight or high polarity.

RESULTS

Development of a DNA stain-based whole-organism *P. falciparum* HTS assay as a drug susceptibility test. We designed a DNA stain-based whole-organism assay as an HTS to identify new antimalarial agents, especially agents that work against new targets in the parasite. To identify an optimal reporter agent for the *P. falciparum* growth assay, we compared several DNA stains: DAPI, PicoGreen, and SYBR Green I (Molecular Probes, Inc. Eugene, OR). We observed that DAPI exhibited the most robust signal-to-noise ratio of 9:1, compared to observed signal-to-noise ratios of 3:1 for PicoGreen and SYBER Green I. To achieve this, using our DAPI *P. falciparum* growth assay (as described in Materials and Methods), we systematically determined the fluorescence signal output from heme, parasitized RBCs (heme minus parasitized RBCs and heme), and parasitized RBCs in the presence of known antimalarial chloroquine at the IC_{50} and IC_{100}. Although PicoGreen and SYBER Green I can be effective reporters of parasite growth, DAPI is the optimal reporter.

To perform the DAPI *P. falciparum* growth assay (Fig. 1), compounds and DMSO mock controls were added to microtiter plates containing complete medium. Next, synchronized ring-stage cultures were added to the microtiter plates in the presence and absence of compound, and the plates were incubated under a standard gas environment at 37°C for 72 h. Prior to incubation, the plates rested for 15 min to minimize the "edge effect" (13). Following the incubation period, DAPI DNA stain was added to detect the parasite DNA in infected erythrocytes. One significant note is the fact that the DAPI *P. falciparum* growth assay can be performed with two different detection methods. First, the assay can be performed using a traditional fluorescence plate reader as a detector to rapidly screen thousands of compounds per day in the primary DAPI *P. falciparum* growth assay HTS. Additionally, the DAPI *P. falciparum* growth assay can be performed using an automated microscope (here, we used an Applied Precision cellWoRx imaging station as a detector). The cellWoRx imaging station

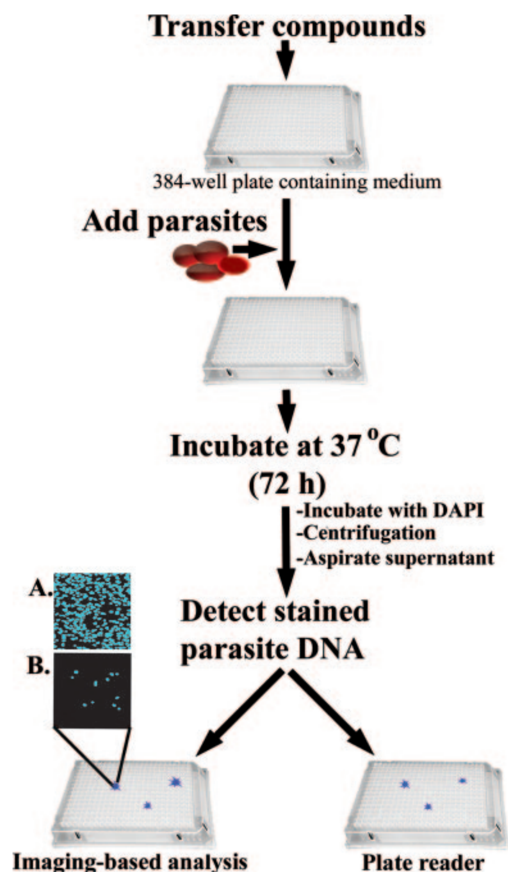

FIG. 1. DAPI *P. falciparum* growth assay design. Compounds and DMSO mock-treatment controls are added to 384-well microtiter plates containing complete medium. Next, synchronized ring-stage cultures of *P. falciparum* are added to the microtiter plates in the presence and absence of compound, and the plates are incubated under a standard gas environment at 37°C for 72 h. Following the incubation period, DAPI DNA stain is added to detect the parasite DNA in infected erythrocytes. Stained parasite DNA is detected using a fluorescence plate reader or an automated microscope, as described in Materials and Methods. (A) Imaging-based analysis image showing parasite growth for a control well with no drug added. (B) Image showing the effect of a small molecule on parasite growth, resulting in an LD_{90}.

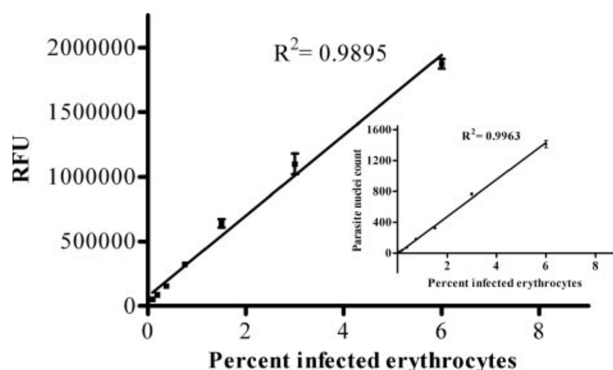

FIG. 2. Assessment of the percentage of *P. falciparum*-infected erythrocytes with fluorescence intensity, using the fluorimetry-based DAPI *P. falciparum* growth assay. A serial twofold dilution of synchronized ring-stage culture (6% parasitemia) of *P. falciparum* strain 3D7 was performed, as described in Materials and Methods. The inset shows the assessment of the percentage of *P. falciparum*-infected erythrocytes with fluorescence intensity, using the imaging-based DAPI *P. falciparum* growth assay.

automatically tracks, focuses, and captures fluorescent images of the cells within each well across an entire plate. The data can then be analyzed using imaging software. To circumvent the problem of defining a field of focus in a nonadherent culture system, plates can be precoated or a fixative solution can be added prior to staining, causing the cells to adhere to the surface of the microtiter plate, as described in Materials and Methods. The DAPI *P. falciparum* growth assay ensures that detection of stained parasite DNA by the DAPI reporter is dependent on parasite replication (see below).

The relationship between the percentage of parasite-infected erythrocytes and the fluorescence intensity is linear. To test the sensitivity of the DAPI *P. falciparum* growth assay and to ensure that the detection of stained parasite DNA was dependent on parasite viability, we compared the percentage of infected erythrocytes as determined by the [^{3}H]hypoxanthine incorporation, DAPI fluorimetry-based, and DAPI imaging-based assays. To achieve this, a serial dilution of synchronized ring-stage-infected erythrocytes was performed using 96-well microtiter plates, with duplicate microtiter plates used for each detection technique. The plates were then incubated in a chamber under the standard gas environment at 37°C for 72 h. After the first 24-h incubation, [^{3}H]hypoxanthine was added to two microtiter plates as described in Materials and Methods. Following an additional incubation period of 48 h, the fluorochrome mixture was added to the remaining four plates, and the percent parasite growth was detected as described in Materials and Methods. As shown in Fig. 2, there is a linear relationship between the percentage of infected red blood cells and the fluorescence signal. Monitoring parasite growth using the DAPI *P. falciparum* growth assay by fluorimetry-based detection or the [^{3}H]hypoxanthine incorporation assay showed comparable sensitivities, with a detection range of 0.25 to 6.0% of trophozoite-stage-infected erythrocytes with an observed r^2 of 0.989 and 0.971, respectively. However, the DAPI *P. falciparum* growth assay using imaging-based detection had a far superior detection sensitivity, with a detection range of 0.01 to 6.0% of trophozoite-stage-infected erythrocytes and an observed r^2 of 0.996 (Fig. 2).

Antimalarial inhibitors exhibit similar activities in the DAPI *P. falciparum* growth assay and the conventional [^{3}H]hypoxanthine incorporation assay. To demonstrate that the activities of antimalarials measured in the DAPI *P. falciparum* growth assay are consistent with activities measured in the conventional [^{3}H]hypoxanthine incorporation assay, IC_{50}s were determined for known antimalarials, chloroquine, atovaquone, quinine, and artemisinin, on the *P. falciparum* strain 3D7, a drug-sensitive parasite. To perform the assay, compounds were serially diluted 1:1 into complete medium (final drug concentration, 300 nM to 0.1 nM). Synchronized ring-stage parasites were added to each well. The microtiter plates were then incubated in a chamber with the standard gas environment at 37°C for 72 h. After the 24-h incubation period, [^{3}H]hypoxanthine was added to each well. The microtiter plates were then returned to the gas-controlled chamber and

TABLE 1. Comparison of IC_{50}s of known antimalarials by fluorescence and radioactive incorporation assays[a]

Known antimalarial	IC_{50} (nM)[b]		
	DAPI *P. falciparum* growth assay		[³H]hypoxanthine incorporation
	Plate reader	Imaging	
Chloroquine	12.63 ± 2.34	10.11 ± 1.54	12.07 ± 2.52
Atovaquone	0.66 ± 0.13	0.63 ± 0.10	0.61 ± 0.14
Quinine	66.52 ± 6.71	69.19 ± 9.13	62.77 ± 9.49
Artemisinin	10.78 ± 2.94	9.67 ± 2.81	10.11 ± 2.63

[a] Each comparative assay was repeated three times.
[b] Values are means ± standard errors of the mean for experiments run in triplicate.

TABLE 2. Comparison of IC_{50}s of known antimalarials against multidrug-resistant strains by the fluorimetry-based assay[a]

Known antimalarial	IC_{50} (nM)[b]		
	3D7	HB3	Dd2
Chloroquine	12.63 ± 2.34	9.47 ± 3.24	124.61 ± 71.13
Mefloquine	12.29 ± 2.01	9.63 ± 1.98	25.99 ± 7.78
Pyrimethamine	10.01 ± 2.13	49.88 ± 11.45	44.10 ± 7.45
Artemisinin	10.78 ± 2.01	9.70 ± 1.67	12.35 ± 1.46

[a] Each comparative assay was repeated three times.
[b] Values are means ± standard errors of the mean for experiments run in triplicate.

incubated for an additional 48 h. It is important to note that for the DAPI *P. falciparum* growth assay, the fluorochrome mixture was added at the end of the 72-h incubation period. Incorporated [³H]hypoxanthine and stained parasite DNA were detected using a plate reader, as described in Materials and Methods. As shown in Table 1, the IC_{50}s measured for the antimalarials tested in the DAPI *P. falciparum* growth assay by both fluorometric and imaging detection techniques were comparable to those values measured in the widely used [³H]hypoxanthine incorporation assay. The effect of a single freeze-thaw cycle on the sensitivity of the assay was not significant; the differences in the IC_{50}s were negligible.

The DAPI *P. falciparum* growth assay can be formatted as an HTS. To demonstrate that the DAPI *P. falciparum* growth assay is suitable for HTS, the assay was automated using standard laboratory automation instrumentation. Four known antimalarial compounds (chloroquine, mefloquine, pyrimethamine, and artemisinin) were distributed randomly in (12) 96-well black microtiter plates at each inhibitory concentration, IC_{50}, IC_{90}, or $2 \times IC_{90}$. The plates were then incubated under the standard gas environment at 37°C for a 72-h incubation period. Following the incubation period, the fluorochrome mixture was added and the stained parasite DNA was detected using an EnVision plate reader as described in Materials and Methods. To identify parasite growth, inhibition wells that exhibited a 50%, 90%, or ≥90% inhibition of stained parasite DNA detected compared to that in the control wells, to which no compound was added, were scored as screening positives, and the numbers of screening positives identified in the screen were then plotted relative to the number of total inhibitors present. The results showed that the DAPI *P. falciparum* growth assay identified 83% (10 of 12) of the compounds present at their respective IC_{50}s and 100% at both their respective IC_{90}s and $2 \times IC_{90}$s. These data demonstrate that the DAPI *P. falciparum* growth assay is sufficiently sensitive to reproducibly identify various classes of antimalarial compounds in an HTS microtiter plate and suggests a low rate of false negatives.

The DAPI *P. falciparum* growth assay was converted to a 384-well microtiter plate and was validated for screening. HTS demands a minimum of a 384-well microtiter plate. Miniaturization of the DAPI *P. falciparum* growth assay from a 96- to a 384-well microtiter plate would allow an increase in throughput and would greatly reduce the amount of compound and biomaterials. It was not obvious to us from the literature whether *P. falciparum* could be grown in 384-well microtiter plates. The challenge in developing a 384-well culturing system for *P. falciparum* was to identify a hematocrit concentration that would allow adequate parasite reinvasion. We observed that the standard 96-well microtiter plate growth conditions of 0.5% parasitemia and 2% hematocrit were inadequate because they did not support parasite growth and development. To develop a 384-well culturing system for *P. falciparum*, we determined the optimal parasitemia and hematocrit growth conditions by performing serial dilutions of each assay component. From our growth analysis, we determined that the optimal parameters for parasite growth in 384-well microtiter format were 0.22% parasites (ring stage) and 0.75% hematocrit final assay concentrations. To ensure that the parasites were uniformly and reproducibly dispensed at a starting concentration of 0.9% parasitemia and 3% hematocrit, the Z′ values were calculated from both 96-well and 384-well microtiter plates as described in Materials and Methods. The Z′ value reflects the dynamic range, as well as the variation, of the assay and is a useful tool for assay comparisons and assay quality determinations (21). Typically, a Z′ value of $0.9 > Z' > 0.7$ is considered to be favorable for HTS. The DAPI *P. falciparum* growth assay exhibited a signal-to-noise ratio of 6:1 and 9:1 and a Z′ value of 0.799 and 0.802 in the 96- and 384-well microtiter plates, respectively. For all assays, drug control plates were prepared in which chloroquine, pyrimethamine, mefloquine, and artemisinin were serially diluted, and their IC_{50}s were measured to monitor assay performance and to verify appropriate drug phenotypes of parasite strains (Table 2). The dose-response curves for chloroquine against 3D7 and the multidrug-resistant (MDR) strains HB3 and Dd2 are shown in Fig. 3. Collectively, these data demonstrate that the DAPI *P. falciparum* growth assay has similar screening parameters in the 96- and 384-well microtiter plates, and thus, that the DAPI *P. falciparum* growth assay is amenable to HTS in either microtiter plate format.

Preliminary studies of a DAPI DNA stain-based HTS of blood-stage *P. falciparum*-infected cells using ICCB-L libraries. As an initial pilot project, we screened a small subset of the ICCB-L small-molecule library collection using our DAPI *P. falciparum* growth assay to see how the library performed. To accomplish this, 2,500 individual compounds from the Known Bioactives library in the ICCB-L collection were screened for their antimalarial activities against *P. falciparum* strain 3D7.

From this initial screen of 2,500 small molecules, we identified 30 reproducible screening positives after retesting. Of these 30 screening positives, the identities of 20 inhibitory compounds were identified in ChemBank (a publicly accessible

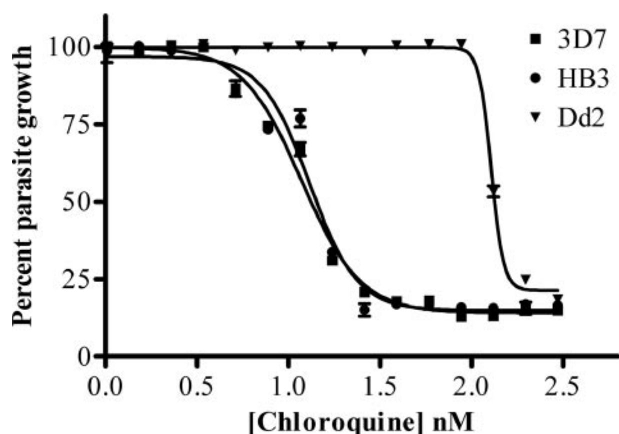

FIG. 3. Determination of the IC_{50}s for chloroquine against 3D7, HB3, and Dd2 by the DAPI *P. falciparum* fluorimetry-based growth assay in 384-well format. Synchronized ring-stage cultures of erythrocytes infected by *P. falciparum* strains 3D7 (■), HB3 (●), and Dd2 (▼) were incubated with different concentrations of chloroquine. Parasite growth was measured at 72 h as described in Materials and Methods. The IC_{50}s are reported in Table 2.

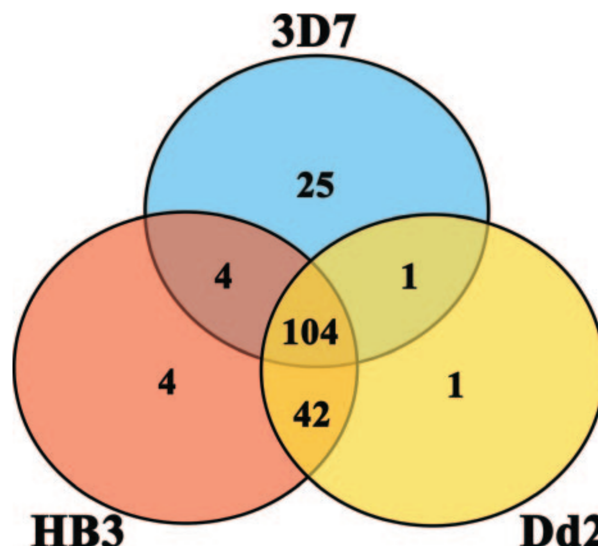

FIG. 4. Distribution of identified screening positives against *P. falciparum* strains 3D7, HB3, and Dd2. The Venn diagram shows the number of screening positives that were unique to each strain, and the overlapping regions illustrate the number of screening positives shared by each of the two strains and the number of screening positives shared by all three strains. A screening positive was defined as a compound that inhibited parasite growth to greater than 90% (LD_{90}) of that observed in the no-drug control well. A compound was identified to be selective for a specific strain or strains if it inhibited parasite growth at an LD_{90} for one strain and an LD_{10} for another strain(s). The HTS DAPI *P. falciparum* fluorimetry-based growth assay was performed in which ~20,000 small molecules at a drug concentration of 10 mM in DMSO were dispensed using a Visio compound transfer robot equipped with a 20-nl, 384-pin head array into 384-well microtiter plates containing 30 μl of medium. Synchronized ring-stage cultures of erythrocytes infected by *P. falciparum* strains 3D7, HB3, and Dd2 were dispensed in each well, and parasite growth was measured at 72 h as described in Materials and Methods.

database of biologically active small molecules created and maintained at the Broad Chemical Biology Program at the Broad Institute of Harvard and MIT [http://chembank.broad.Harvard.edu]) and determined to be similar to known antimalarial drugs, including chloroquine, phloretin, artemisinin, quinine analogues, atovaquone analogues, and iron chelators. Additionally, three inhibitory compounds were steroids and seven were flavonoids, and both steroids and flavonoids have been reported to have growth-inhibitory properties against *Plasmodium*.

The DAPI *P. falciparum* growth assay can be utilized for the execution of a large-scale HTS. To demonstrate the utility of the DAPI *P. falciparum* growth assay for the execution of a large-scale HTS, we screened approximately 79,000 individual compounds for their antimalarial activities. These compounds consisted of 71,887 compounds in the ICCB-L collection of purchased commercial products, 4,927 natural product extracts, and 2,480 compounds in the Known Bioactives library. The ICCB-L stock compound plates are stored at a drug concentration of 10 mM in DMSO and were dispensed using a Visio compound transfer robot equipped with a 100-nl 384-pin head array. To perform the assay, single chemical compounds were dispensed into 384-well black microtiter plates containing 30 μl of complete medium in the ICCB-L screening facility (Fig. 1). Screening was performed in duplicate on *P. falciparum* strain 3D7, using our DAPI *P. falciparum* growth assay. A screening positive was arbitrarily determined to be any compound that suppressed growth by 90%, as described in Materials and Methods. The final compound concentration in the assay was approximately 30 μM. A total of 1,000 screening positives were identified in this way. Compounds that were evaluated as not having drug-like properties as defined by the criteria of Lipinski et al. (12) or were identified as known antimalarials were removed, leaving 900 screening positives. This primary screen resulted in a hit rate of 1.36%, a high value that most likely reflects the 30 μM compound concentration that was used.

To identify the most potent compounds from the 900 screening positives identified in our primary HTS, a secondary dose-down screen was performed. To perform the secondary dose-down screen, we rescreened the 100 compound library plates identified to contain the 900 screening positives (19,968 total compounds) at ~5-fold-lower concentration than the primary screen. The compounds were dispensed using a Visio compound transfer robot equipped with a 20-nl 384-pin head array into 384-well microtiter plates containing 30 μl of complete medium, resulting in a final compound concentration of approximately 6 μM. Screening was performed in duplicate on *P. falciparum* strain 3D7 and MDR laboratory strains HB3 and Dd2 using the DAPI *P. falciparum* growth assay as described in Materials and Methods. For each assay, the drug phenotype for each MDR strain was verified by measuring the IC_{50} for chloroquine, pyrimethamine, mefloquine, and artemisinin. The MDR strain HB3 was originally isolated in Honduras and is sensitive to chloroquine and mefloquine but resistant to pyrimethamine. The strain Dd2 was originally isolated in Indochina and is resistant to chloroquine, mefloquine, and pyrimethamine. From this secondary screen of 19,968 small molecules, we identified 181 reproducible screening positives either to selectively inhibit each parasite strain or to inhibit multiple strains. A screening positive was identified

as a compound that inhibited parasite growth to greater than 90% (LD_{90}) of that observed in the no-drug control well. A compound was identified as selective for a specific strain or strains if it inhibited parasite growth at an LD_{90} for one strain and an LD_{10} for another strain(s). We observed several screening positives to be selective for 3D7 (25 compounds), HB3 (4 compounds), and Dd2 (1 compound). Additionally, several compounds were selective for multiple strains: 3D7, HB3, and Dd2 (104 compounds); 3D7 and HB3 (4 compounds); 3D7 and Dd2 (1 compound); and HB3 and Dd2 (42 compounds) (Fig. 4).

DISCUSSION

A DAPI stain-based *P. falciparum* whole-organism growth assay was developed that is technically simple, robust, flexible in microtiter plate format and incubation time, and compatible with the automation necessary for HTS. The DAPI *P. falciparum* growth assay responded to several chemically and mechanistically diverse antimalarial inhibitors and compared positively to the [^{3}H]hypoxanthine incorporation assay. The utility of the DAPI *P. falciparum* growth assay for large-scale (>50,000 compounds) HTS in 384-well microtiter plate format was demonstrated by screening approximately 79,000 individual compounds for their antimalarial activities.

In addition to HTS, the DAPI *P. falciparum* growth assay can be used to monitor the emergence of drug resistance in the developing world. Since the assay can be performed in 96-well microtiter plates, laboratories without access to automated equipment or HTS facilities can use the assay for a multitude of applications to measure parasite growth. In addition to being nonradioactive, the assay is inexpensive, and microtiter plates can be stored indefinitely and can be read on a basic-model plate reader. This is of great importance for field-based research occurring in developing countries that cannot perform radioactive experiments or whose funds are too limited to support antibody-based growth assays.

The DAPI *P. falciparum* growth assay design uses the DAPI DNA stain as a reporter for parasite growth. We have determined that the DAPI DNA stain has a signal-to-noise ratio superior to those of other DNA stains. The differences observed in the signal intensity likely originate in the autofluorescence of heme. The low signal-to-noise ratio observed with fluorescein isothiocyanate-based DNA stains can be explained by their ex/em wavelengths of 485/530 nm, coinciding with the ex/em wavelengths of heme/hematin (450/500 nm). DAPI's ex/em wavelengths of 355/460 nm lie almost entirely outside of the heme wavelengths, which would account for its more robust signal.

The DAPI *P. falciparum* growth assay has flexibility in its detection method. First, the assay can be performed using a traditional fluorescence plate reader as a detector to rapidly screen thousands of compounds per day in a HTS. Additionally, the DAPI *P. falciparum* growth assay can be performed using an imaging detector (CellWorX imaging station). An imaging-based DAPI *P. falciparum* growth assay is desirable, because it allows individual parasites to be visualized rather than relying on uniform well readout. Screening by fluorescence imaging of cells has been shown to be significantly more sensitive and reliable than fluorescence plate readers because imaging measures the biological variability of individual cells within a well, not a single intensity per well (14). For example, we are able to detect as little as one individual parasite in our imaging-based DAPI *P. falciparum* growth assay compared to a uniform well readout of 0.25% parasites observed in the DAPI *P. falciparum* growth assay and [^{3}H]hypoxanthine assay—significantly greater sensitivity and reliability.

The DAPI *P. falciparum* growth assay can be readily expanded to other parasite strains to study resistance profiles or to include variables such as culture time that would distinguish fast- from slow-acting compounds. We have demonstrated the utility of exploring resistance profiles by performing a HTS of structurally diverse small molecules against several *P. falciparum* strains (3D7, HB3, and Dd2) that have unique drug phenotypes. Using our DAPI *P. falciparum* growth assay, we identified screening positives that are either unique to each strain, shared by each of the two strains, or shared by all three strains.

The genetic cross of MDR strains HB3 and Dd2 has provided a rich source of biological tools to identify the small-molecule target or loci. The progeny of this cross have been maintained and used repeatedly to genetically map drug resistance determinants (20). Quantitative trait locus mapping of the progeny has identified multiple transporters associated with responses to chloroquine and quinine and has identified multiple genes that contribute to the evolution of quinine resistance (8, 15). Thus, combining the resources of HTS, *Plasmodium* genomics, and quantitative trait locus mapping will provide insight into the molecular fingerprint for each small molecule tested within a biological system. Ultimately, these data support the need to integrate field analysis of MDR parasites with standard and innovative laboratory methods to expand our knowledge of the molecular mechanisms of drug resistance and to advance therapeutic-drug development.

The whole-organism assay offers possibilities not available to target-based assays. First, whole-organism-based assays select compounds that are able to penetrate cells and reach both host and human intracellular targets, and compounds that lack these essential drug-like properties are eliminated. Second, compounds identified in whole-organism assays are active in the context of the cell environment; their activity validates their (unknown) target(s). In contrast, a small molecule identified in a target-based screen might prove to be ineffective due to compensatory or redundant pathways in the whole organism. Finally, whole-organism assays can identify compounds that hit multiple synergistic targets, i.e., compounds that might be weakly active in a target-based assay but potent in a whole-organism-based assay. Of course, whole-organism assays have the liability of requiring target identification. However, it is important to note that target identification is not absolutely necessary for the successful discovery and development of a new malaria drug. For example, atovaquone was originally thought to target dihydroorate dehydrogenase, but it actually targets a cytochrome (19), and the target of artemisinin is still not completely clear (11). However, target identification is an important step in any comprehensive development of antimalarial genomic studies.

This new DAPI *P. falciparum* growth HTS assay is highly robust, reproducible, technically simple, and suitable for automation, the five hallmarks of a successful high-throughput screen. Our current screening capacity for one person in a

384-well microtiter plate is approximately 34,000 compounds/week or approximately 94 library plates in duplicate; illustrating the robustness and ease of the assay. A phased screening of the entire 250,000 diverse small molecules in the BCB collection is in progress. It is our hope that this DAPI *P. falciparum* growth HTS will greatly facilitate the screening of large chemical libraries against *P. falciparum* to aid in the identification of new antimalarial agents, especially agents that work against new targets in *P. falciparum*.

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