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A Repurposing Strategy Identifies Novel Synergistic Inhibitors of *Plasmodium falciparum* Heat Shock Protein 90

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Malaria is responsible for 3 million deaths annually. Antimalarial drug resistance is widespread, and few novel, well-defined targets exist. A robotic high throughput screen (HTS) was performed using 4000 small molecules from a natural compound (Spectrum), pharmacologically active (Lopac), and Food and Drug Administration (FDA) approved drug library (Prestwick) for competitive inhibition of the ATP-binding (GHKL) domain of *Plasmodium falciparum* (Pf) Hsp90, a highly conserved chaperone. Hits were further screened for specificity based on differential inhibition of PfHsp90 in comparison to human (Hs) Hsp90. PfHsp90-specific inhibitors showed 50% inhibitory concentrations (IC_{50}) in the nanomolar range when tested using a cell-based antimalarial validation assay. Three hits, identified as selective PfHsp90 inhibitors in the HTS, also demonstrated synergistic activity in the presence of the known antimalarial drug chloroquine. These data support PfHsp90 as a specific antimalarial target with potential for synergy with known antimalarials.

Introduction

The protozoan parasite *Plasmodium falciparum* is responsible for the most severe form of human malaria and causes great economic burden[1] leading to at least 3 million deaths per year,[2] particularly in many developing countries where failure to eradicate the *Anopheline* mosquito vector leads to occasional epidemics.[3] The emergence of resistance to the most commonly used antimalarial drugs and the lack of an effective vaccine drive an urgent need to develop novel drugs. Failure to delineate the mechanism of action of many current antimalarials has compounded the problem.[4]

Heat shock proteins (HSP[a]) are a class of highly conserved molecular chaperones that facilitate protein folding. One of the best-studied members of the HSP family is Hsp90, which is important for normal growth and development in eukaryotes.[5−7] In higher eukaryotes, cytosolic Hsp90 exists in the form of a multichaperone complex, and together with Hsp70 and Hsp60, helps newly synthesized proteins to fold and to modulate the activities of transcription factors and protein kinases.[5−7] Hsp90 serves as a buffer for other proteins by preventing cellular toxicity caused by mis-folded and aggregated proteins in response to stress. A number of independent studies have demonstrated that Hsp90 is essential in eukaryotes and that inhibition of Hsp90 activity by small molecules results effectively in lethality.[3,8−10] The N-terminal domain of Hsp90 has a conserved ATP-binding domain essential for chaperone function.[11] Human and yeast Hsp90 are similar in tertiary structure at the N-terminal ATP-binding domain. Binding of ATP to this domain can be competitively inhibited by geldanamycin (GA), a benzoquinone ansamycin drug shown to complex with the ATP-binding domain of yeast Hsp90.[12−14] However, GA has proven to be too toxic for human application.[15,16]

P. falciparum heat shock protein 90 (PfHsp90 or PfHsp86 PF07_0029) is essential for the development of the parasite during the intraerythrocytic cycle.[8,10,17] Significant similarity exists at the NH_2-terminal nucleotide-binding domain (also known as the GHKL domain) in the central acidic hinge region as well as at the COOH-terminal substrate-binding domain between the eukaryotic Hsp90 and the PfHsp90. Because of the presence of an EEVD motif at the COOH terminus, PfHsp90 has been localized in the cytosol.[3,8,18] PfHsp90 complexes play an important role in the parasite life cycle. Inhibition of PfHsp90 function using GA arrests parasite development between ring and trophozoite stage during the intraerythrocytic cycle.[8] Analysis of PfHsp90 function in the parasite has revealed its essential role in the regulation of parasite development following heat-shock stress.[19] Cowen and colleagues have shown that Hsp90 inhibitors synergize with known antifungals to which the microbe would otherwise be resistant.[20,21] PfHsp90 may play a role in the development of drug resistance in the malarial parasite because of its interaction with the chloroquine resistance-associated protein Cg4.[18] Hence, PfHsp90 has the potential not only to serve as a drug target but also to circumvent drug resistance to

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[a]Abbreviations: HTS, high throughput screen; GHKL, heat shock protein 90 ATP-binding domain; PfHsp90, *Plasmodium falciparum* heat shock protein 90; HsHsp90, *Homo sapiens* heat shock protein 90; IC_{50}, 50% inhibitory concentrations; HSP, heat shock protein; Hsp90, heat shock protein 90; GA, geldanamycin; FDA, Food and Drug Administration; FIC, fractional inhibitory concentration; PBS, phosphate buffered saline solution; bis-ANS, 4,4′-dianalino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt; APPA, (±)-2-amino-3-phosphonopropionic acid.

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conventional antimalarials when used in combination through synergy. Indeed, GA synergizes with calcineurin and cyclosporine A when tested in tandem for antimalarial activity.[10] Furthermore, the parasite interaction network exhibits functional differences with that of *Saccharomyces cerevisiae* and the human host,[22] highlighting the potential of this protein target for the design of specific antimalarial inhibitors.

Taken together, the following key factors suggest that PfHsp90 represents a promising target: (1) the antimalarial activity demonstration by known cross-species Hsp90 inhibitors such as GA; (2) the essential and multifaceted function chaperone function of Hsp90; (3) potential cross-talk with pathways involved in drug resistance; (4) unique structural features in the ATP-binding domain between human and PfHsp90; (5) difference between the human and *P. falciparum* Hsp90 interactome.[18]

In this study, three libraries consisting of natural compounds, FDA-approved drugs, and pharmacologically active compounds consisting of approximately 4000 small molecules were screened using a robotic, protein-based HTS. Malaria-specific hits were validated using a standard cell-based antimalarial assay. Our findings suggest that PfHsp90-specific inhibitors can be identified using this repurposing strategy and demonstrate synergistic activity with known antimalarials.

Results

Sequencing of the PfHsp90 ATP-Binding Domain from *P. falciparum* Malaria Patient Isolates. Fifty patient isolates were selected from returning travelers to diverse geographical locations for sequencing of the PfHsp90 ATP-binding domain. Three substitutions were identified at residue position 41 (Ile41Thr) and three substitutions at position 106 (Ser106Leu) (Figure 1a). Homology modeling of the PfHsp90 ATP-binding domain using the template of the human (Hs) Hsp90 ATP-binding domain (PDB code 2FWZ) revealed that Ile41 is outside the ATP-binding domain and Ser106 is located at the back of the helix facing away from the binding pocket (Figure 1b). Leucine substitution of this residue is not expected to affect substrate binding (Figure 1c).

Robotic High Throughput Screen with Recombinant PfHsp90 ATP-Binding Domain. The initial screen was based on competitive inhibition of bis-ANS binding with 4000 compounds consisting of natural compounds [Spectrum], FDA approved drugs [Prestwick], and pharmacologically active compounds [Lopac]. Forty-six compounds were identified that caused a reduction of ≥70% in fluorescence, suggesting competitive inhibition of PfHsp90 ATP-binding (see Supporting Information, Table S1). This threshold was set based on PfHsp90 inhibition by radicicol, a well-known cross-species Hsp90 inhibitor. The 46 small molecules were also screened for competitive inhibition of ATP-binding by the homologous region of HsHsp90 in order to identify compounds that cause selective inhibition of PfHsp90. Differential binding, defined as reduction of ≥70% in fluorescence for PfHsp90 but no significant reduction for HsHsp90, was observed for three compounds: (±)-2-amino-3-phosphonopropionic acid (**1**, Pubchem ID 44291306) (APPA) from LOPAC, harmine (harmaline) (**2**, Pubchem ID 5280953), and acrisorcin (**3**, Pubchem ID 24144) from SPECTRUM (Figure 2). Of note, no inhibition of fluorescence was observed in the presence of vehicle (DMSO) alone.

Compounds 1, 2, and 3 Show Antimalarial Potency in *P. falciparum* Drug Resistant Strains. The cell-based antimalarial assay relies on fluorescence staining of parasitized erythrocytes.[23] The three small molecules that showed PfHsp90-specific activity (**1**, **2**, **3**) demonstrated 50% inhibitory concentrations (IC_{50}) in the nanomolar range (Figure 3 and Table 1). These compounds also showed similar potency against the chloroquine-resistant strain W2 and the multidrug resistant clinical isolate 208432.

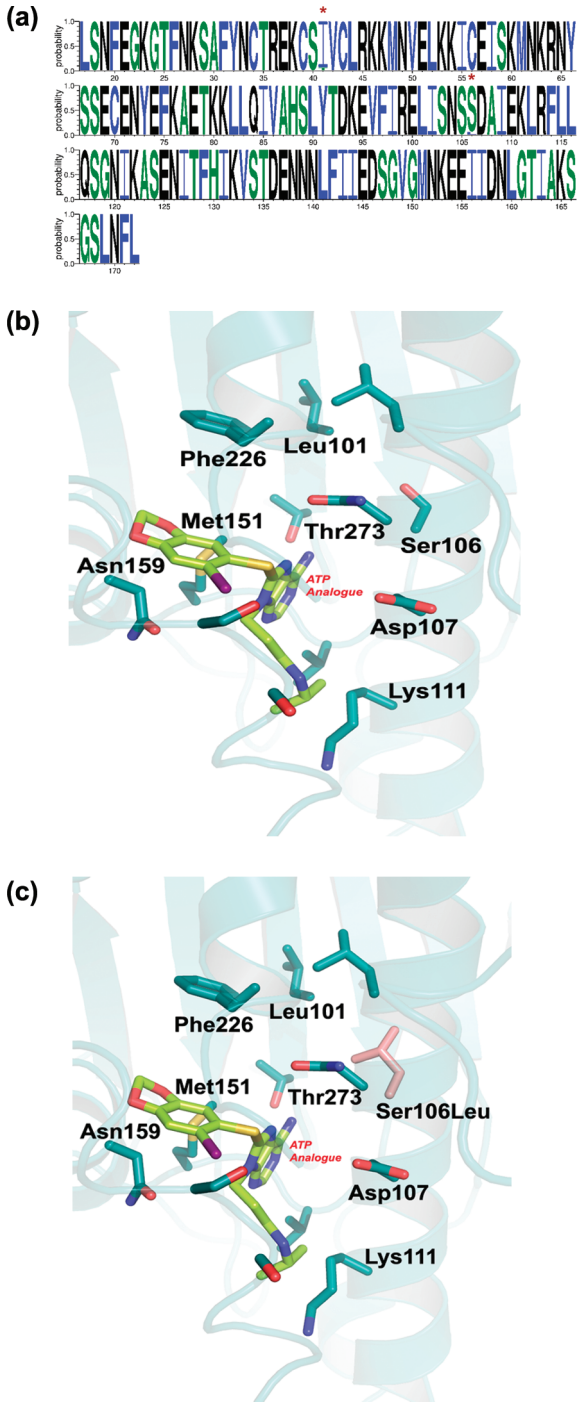

Figure 1. Sequencing of the Pfhsp90 ATP-binding domain from 50 globally distributed patient isolates. (a) WebLogo representation of the multiple sequence alignment. Asterisks indicate two low frequency mutations that were identified as Ile41Thr and Ser106Leu. The WebLogo representation was generated using WebLogo, version 3.[49] (b) Homology modeling of the domain revealed that Ile41 is outside the ATP-binding domain. (c) Substitution of Ser106 by leucine does not affect substrate binding in the ATP-binding domain. The homology model diagrams were generated using PyMol and labeled in Adobe Photoshop.

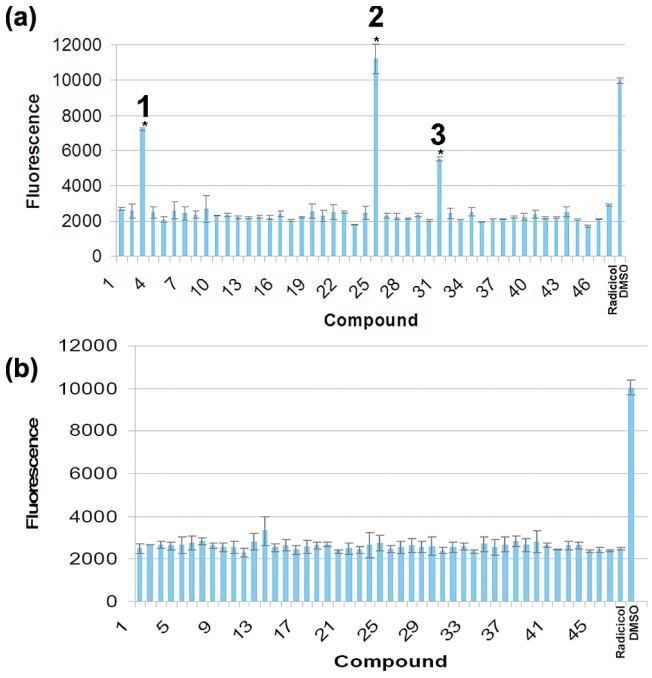

Figure 2. Competitive binding assay using the fluorescent ATP analogue bis-ANS for the identification of PfHsp90 specific inhibitors at a screening final concentration of 2.5 μM: (a) human (Hs) Hsp90 ATP-binding domain; (b) malaria (Pf) Hsp90 ATP-binding domain. The asterisk identifies compounds that did not inhibit ATP-binding with HsHsp90 ATP-binding domain. The error bars represent standard deviation of duplicate readings. Inhibition is defined as ≥70% reduction of fluorescence in this assay.

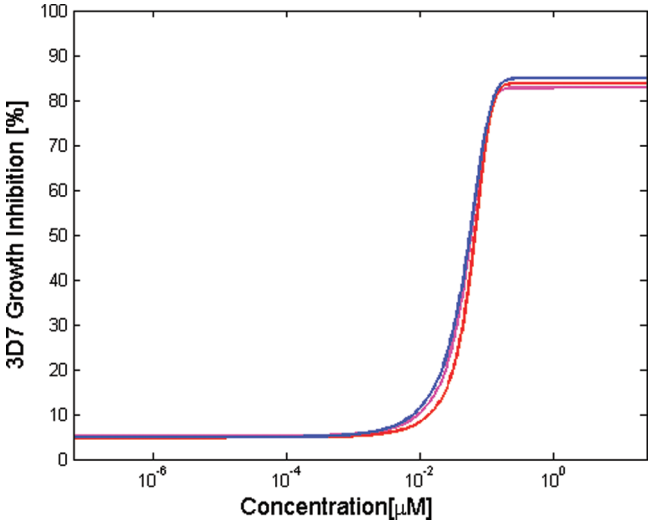

Figure 3. Antimalarial activity of **1** (blue), **2** (red), and **3** (pink) using a standard cell-based fluorescence assay. Representative IC_{50} curves are shown for the three specific inhibitors for laboratory strain 3D7. Error bars represent standard deviation of duplicate samples. Actual IC_{50} values are summarized in Table 1.

Compounds 1, 2, and 3 Act Synergistically with Chloroquine. The selective PfHsp90 hits were evaluated for synergy in combination with chloroquine. Inhibition of growth curves were generated for the synergistic combination of each drug as shown (Figure 4). Fractional inhibitory concentration (FIC) ratios were calculated as previously described.[24] **1**, **2**, and **3** exhibited FIC ratios of 0.11, 0.08, and 0.01, respectively, in combination with chloroquine. FIC ratios of <0.5 indicate synergistic activity.[24]

Table 1. Summary of Antimalarial Assay IC_{50} Values for the Inhibitors Identified in This Study When Compared to Known Antimalarials[a]

	IC_{50} (μM)		
compd	3D7	W2	208432
acrisorcin	0.0513 ± 0.0237	0.3124 ± 0.0156	0.0544 ± 0.0071
APPA	0.0603 ± 0.0145	0.0844 ± 0.0137	0.0471 ± 0.0069
harmine	0.0501 ± 0.0098	0.0280 ± 0.0054	0.1824 ± 0.0293
artemisinin	0.0061 ± 0.0014	0.0073 ± 0.0021	0.0683 ± 0.0091
mefloquine	0.0021 ± 0.0019	0.0032 ± 0.0011	0.0894 ± 0.0134
chloroquine	0.0046 ± 0.0015	0.2524 ± 0.0542	0.4514 ± 0.0463

[a] 3D7 is a fully susceptible laboratory strain. W2 is a chloroquine-resistant laboratory strain. 208432 is a clinical isolate from a patient with multidrug resistant malaria from West Africa.

Discussion

Multidrug resistance is an issue of great concern in malaria.[25−27] Recent reports demonstrate the emergence of artemisinin-resistant parasites in South East Asia, the last line of pharmacotherapy against this disease.[28−30] Hsp90 is a hub in several intracellular pathways required for cell survival under stress conditions such as heat shock. Experiments performed in yeast, fruit flies, plants, and animal systems support the idea that in addition to helping newly synthesized proteins to fold, Hsp90 also regulates cell cycle, development, potentiation of drug resistance, buffering of phenotypic variation, and evolution of morphological development.[6,20,31] In fungi, inhibitors of Hsp90 have been able to reverse resistance to existing antifungals.[20,21] Malaria PfHsp90 appears to be induced and translocated to the nucleus upon heat stress at 41 °C.[9] Indeed, heat shock during hallmark febrile episodes of malaria infection implies that heat shock stress may not only be clinically relevant but also essential to *P. falciparum* growth and survival.[18]

We demonstrate here that the PfHsp90 ATP-binding domain is highly conserved in clinical isolates from around the world. Four thousand compounds from three separate libraries were robotically screened, and 46 inhibitors of the PfHsp90 ATP-binding domain were identified. Three of these compounds, **1**, **2**, and **3**, specifically inhibit the PfHsp90 ATP-binding domain when compared to the HsHsp90 ATP-binding domain. **2** has been used to treat breast cancer cells[32] and has shown effectiveness and low toxicity in single therapy in mice against lung carcinoma[33] and depression.[34] **1** is a normal human metabolite found in diverse tissues, such as liver, intestine, and spleen. It has shown pharmacologic activity as a metabotropic glutamate receptor agonist. It is able to block the amyloid precursor protein release evoked by glutamate receptor stimulation in neurons of the cortex and hippocampus, a condition that is believed to produce nerve damage in Alzheimer's disease.[35,36] Compound **1** was tested in the Alzheimer's mouse model in which it showed effectivity at 60 nmol.[37] Compound **3** consists of a combination of 9-aminoacridine and 4-hexylresorcinol and has been used for over 40 years as an antifungal agent for the treatment of *tinea versicolor*, a skin infection that causes discolored patches of skin in humans.[38−40] Recently, the active component of **3**, 9-aminoacridine, has shown activity in malaria mouse models infected with the mouse pathogens *Plasmodium berghei* and *Plasmodium chabaudi*.[41] We have shown here that these compounds exhibit nanomolar range antimalarial potency against the drug sensitive strain 3D7 and, importantly, to the

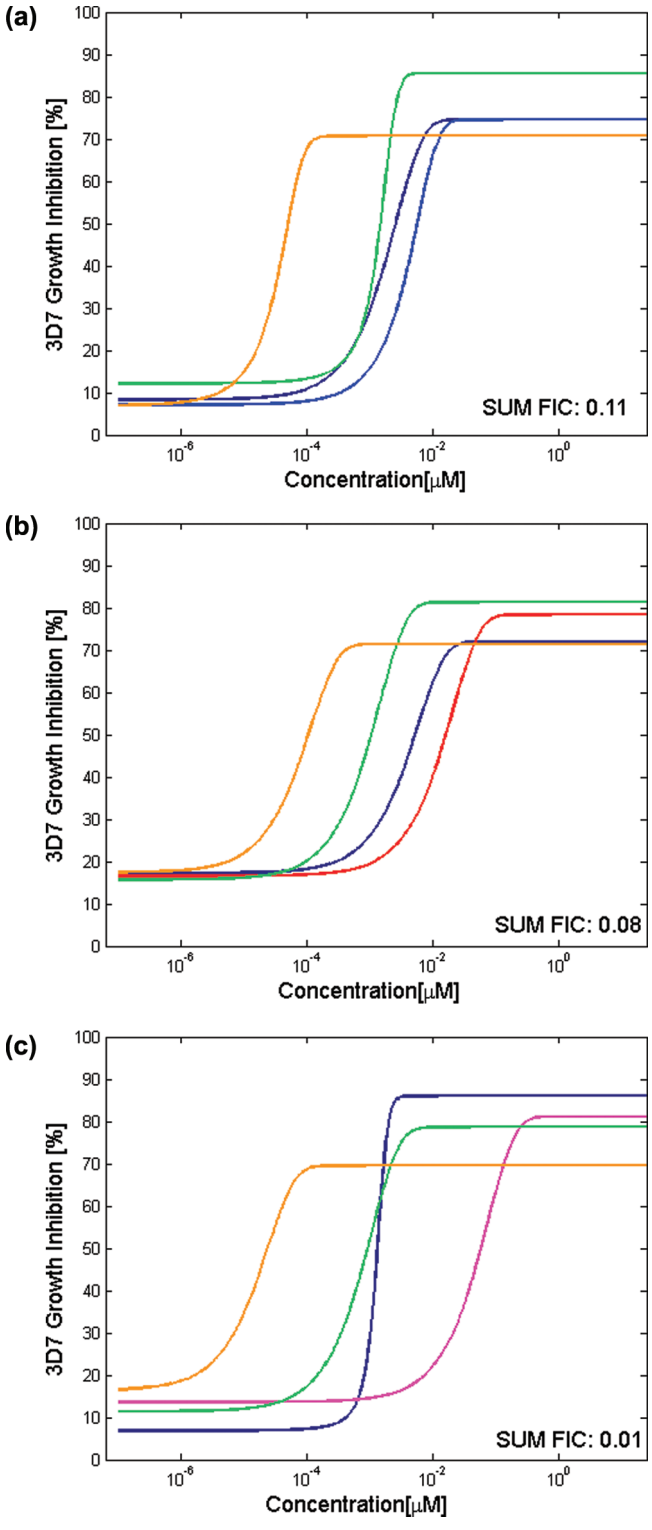

Figure 4. Synergistic activity of **1** (a), **2** (b), and **3** (c) in combination with the known antimalarial chloroquine for laboratory strain 3D7. The light (PfHsp90 inhibitor identified in this study) and dark (chloroquine) blue lines represent the IC_{50} curves of each antimalarial when tested alone. The green line depicts the IC_{50} curve for the PfHsp90 inhibitor in combination with 0.125 pM chloroquine. The orange line depicts the IC_{50} curve for chloroquine in combination with 1.25 pM novel PfHsp90 inhibitor. **1**, **2**, and **3** exhibited FIC ratios of 0.11, 0.08, and 0.01, respectively, in combination with chloroquine. FIC ratios of <0.5 indicate synergistic activity. A single representative experiment is shown.

drug resistant strain W2 and multidrug resistant clinical isolate 208432.

Our recombinant ATP-binding domain assay relies on competitive inhibition of an ATP analogue, suggesting that these compounds compete for the ATP-binding domain. While we have not fully elucidated the binding modes of these hits, the Hsp90 ATP-binding domain contains several basic and hydrophobic residues that are characteristic of protein binding pockets that bind structurally diverse compounds. The need for synergistic antimalarials is paramount, as the parasite inexorably develops resistance to single drug therapy.[42] Combination therapy has been widely used for the first line antimalarial artemisinin because single therapy with artemisinins has encountered drug resistance.[43,44,28,29] The three malaria-specific inhibitors identified in this study exhibited synergistic activity based on FIC with the drug combination IC_{50} in the picomolar range. The potential synergy of PfHsp90 inhibitors will serve as a strong basis for combination therapy in human disease. A potential limitation of this study is that our protein-based assay relied on competitive inhibition ATP-binding rather than inhibition of ATPase activity. Nevertheless, the significant antimalarial effect and the prior use of the candidates identified in this repurposing study for other human conditions taken together make these compounds attractive for further clinical development. Future efforts will focus on establishing the antimalarial acitivity, oral bioavailability, and toxicity in the *P. berghei* mouse model of malaria infection.

Experimental Section

Sequencing of the PfHsp90 ATP-Binding Domain. Genomic DNA was extracted from *P. falciparum* infected patient whole blood using the QiaAMP DNA Mini Kit (QIAGEN). The PfHsp90 ATP-binding domain was amplified using the primers Fwd 5′-GAAATGCTCCACACAATTAA-3′ and Rev 5′-CACCAAATTGTCCGATAATA-3′. DNA sequencing of this amplicon was performed using a standard capillary gene sequencer (3130xl genetic analyzer, Applied Biosystems) with the same primers. Homology modeling of the domain was done using Phyre[45] (protein fold recognition server), and the structure alignment and substitution modeling were achieved using the Ccp4 suite of programs.[46]

***P. falciparum* Culture Methods.** *P. falciparum* strains 3D7 and W2 and clinical isolate 208432 were grown in RPMI 1640 medium supplemented with 0.25% Albumax II, 2 g/L sodium bicarbonate, 0.1 mm hypoxanthine, 25 mm HEPES (pH 7.5), 50 g/L gentamycin, and human erythrocytes at 37 °C, 5% O_2, and 6% CO_2.

Antimalarial Drug Screening Cell Assay. Growth inhibition of *P. falciparum* cultures was quantified using a flow cytometric assay.[23] The cultures were synchronized to 0.8% rings and 0.5% hematocrit and cultured in the presence of each of the inhibitors. After a 48 h growth period in the presence or absence of inhibitor, cultures were stained for 1 h at room temperature (RT) with 1× SYBR Green in phosphate buffered saline (PBS) solution. Samples were analyzed with a Cytomics FC500 MPL flow cytometer, Beckman Coulter. Uninfected erythrocytes background autofluorescence was minimal. Parasite growth in each sample was determined relative to infected erythrocytes without drug treatment (DMSO vehicle alone). Chloroquine, mefloquine, and artemisinin were used as standardization controls with each assay run. FIC ratios were calculated as previously described.[24]

Cloning and Protein Purification. A *P. falciparum* Hsp90 GHKL domain construct was amplified from genomic DNA harvested from the intraerythrocytic life cycle of *P. falciparum* strain 3D7, which was cloned into pET28b, expressed in Bl21

(DE3) CodonPlus cells grown in terrific broth, and induced with 0.4 mM IPTG overnight at 24 °C. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM HEPES, pH 7.5, 10% glycerol, 20 mM imidazole, 500 mM NaCl, 0.5% NP-40) and supplemented with 1× bacterial protease inhibitor cocktail (Sigma). Cells were lysed by sonication. The cell debris were removed by centrifugation, and the protein was purified using Ni-NTA resin (QIAGEN). TEV protease was added at a ratio of 1:50 TEV protease in dialysis buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl2, and 0.01 mM bis-ANS) and incubated overnight at 4 °C. This mixture was washed over a nickel column to remove the TEV protease and cleaved 6x His tags were from the purified protein. The proteins were concentrated to ∼10 mg/mL. The same conditions were used for the expression and purification of the HsHsp90 ATP-binding domain. The clone was kindly provided by Dr. Daniel Gewirth (Hauptman-Woodward Medical Research Institute).

4,4′-Dianalino-1,1′-binaphthyl-5,5′-disulfonic Acid Dipotassium Salt (Bis-ANS) Binding Assay with PfHsp90 ATP-Binding Domain. By use of a previously established technique, the fluorescent probe 4,4′-dianalino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt (bis-ANS, Sigma-Aldrich) was used to demonstrate nucleotide binding to the GHKL domain of PfHsp90.[47] Recombinant purified protein (final protein concentration 1 μM) was preincubated for 45 min at 37 °C with no drug or in the presence of drug to a final concentration of 100 nM (Spectrum and Lopac libraries) and 50 nM (Prestwick library). bis-ANS was then added to a final concentration of 5 μM in binding buffer (20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl) in a final volume of 20 μL and incubated at 37 °C for 30 min. Fluorescence emission data were collected on a EnVision fluorescent monochromator spectrophotometer (Perkin-Elmer Life Sciences). Excitation wavelength for bis-ANS was set at 372 nm, and emission was captured at 490 nm.

Purity. All chemical compounds had ≥99% purity by high performance liquid chromatography (HPLC).

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Supporting Information Available: Table S1 listing compounds that competitively inhibit malaria PfHsp90 ATP-binding. This material is available free of charge via the Internet at http://pubs.acs.org.

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