The heat shock protein 90 of *Plasmodium falciparum* and antimalarial activity of its inhibitor, geldanamycin

Rajinder Kumar, Alla Musiyenko and Sailen Barik*

Address: Department of Biochemistry and Molecular Biology (MSB 2370), University of South Alabama, College of Medicine, 307 University Blvd., Mobile, AL 36688-0002, USA

Email: Rajinder Kumar - rkumar@usouthal.edu; Alla Musiyenko - musiyenkoalla@hotmail.com; Sailen Barik* - sbarik@jaguar1.usouthal.edu

* Corresponding author

**Abstract**

**Background:** The naturally occurring benzoquinone ansamycin compound, geldanamycin (GA), is a specific inhibitor of heat shock protein 90 (Hsp90) and is a potential anticancer agent. Since *Plasmodium falciparum* has been reported to have an Hsp90 ortholog, we tested the possibility that GA might inhibit it and thereby display antiparasitic activity.

**Results:** We provide direct recombinant DNA evidence for the Hsp90 protein of *Plasmodium falciparum*, the causative agent of fatal malaria. While the mRNA of Hsp90 was mainly expressed in ring and trophozoite stages, the protein was found in all stages, although schizonts contained relatively lower amounts. *In vitro* the parasitic Hsp90 exhibited an ATP-binding activity that could be specifically inhibited by GA. *Plasmodium* growth in human erythrocyte culture was strongly inhibited by GA with an IC50 of 20 nM, compared to the IC50 of 15 nM for chloroquine (CQ) under identical conditions. When used in combination, the two drugs acted synergistically. GA was equally effective against CQ-sensitive and CQ-resistant strains (3D7 and W2, respectively) and on all erythrocytic stages of the parasite.

**Conclusions:** Together, these results suggest that an active and essential Hsp90 chaperone cycle exists in *Plasmodium* and that the ansamycin antibiotics will be an important tool to dissect its role in the parasite. Additionally, the favorable pharmacology of GA, reported in human trials, makes it a promising antimalarial drug.

**Background**

As the causative agent of malaria, *Plasmodium* sp. claims between one and two million human lives annually worldwide. *Plasmodium falciparum* is particularly lethal and causes cerebral malaria [1]. A major area in malaria research is, therefore, focused on finding a potent and reliable anti-parasitic drug that would inhibit *Plasmodium* infection and growth. In nearly all the malaria-endemic populations, *Plasmodium* has developed resistance against the hallmark drug chloroquine and its derivatives [2–4]. It is thus appreciated that the new generation of drugs should use a rational strategy based on the structure and function of essential parasitic molecules. With this goal we have concentrated on understanding the signaling pathways of *P. falciparum* with special emphasis on protein phosphorylation. We and others have recently shown that *P. falciparum* contains a PP5 protein phosphatase containing a tetratricopeptide (TPR) domain [5,6]. We also showed that PP5 interacts with a 90 kDa protein of the parasite that is antigenically similar to mammalian heat shock protein 90 (Hsp90) [5]. Because of the enormous importance of PP5 and Hsp90 in cellular
physiology and signaling [7–9], further studies of both *Plasmodium* proteins were warranted.

Hsp90 is the most abundant chaperone in cells and plays an essential role in the folding, and hence functioning, of a large number of proteins, especially those participating in cell cycle regulation and signal transduction [8,9]. The list of the "client" proteins of Hsp90 is impressively long, and includes protein kinases such as Raf, Src, Lck, Wee1, MEK, Cdk4, Src, and CK2, and transcription factors such as steroid receptors and p53 [8,9]. Because of this, Hsp90 has been used as a drug target in basic as well as clinical applications [10–15]. Recent studies have revealed a number of structural and functional aspects of Hsp90 that include the N-terminal ATP-binding domain and a sophisticated ATP-dependent conformational change in the protein [16–19]. At least two natural antibiotics – geldanamycin (GA) and radicicol – have been experimentally demonstrated to compete with ATP for binding to the N-terminal domain [16–20]. GA, in particular, is considered a highly specific inhibitor of Hsp90 and its derivative, 17-(allylamino)-17-Demethoxygeldanamycin (17AAG), is in Phase I trials as an anticancer therapy candidate [16–20]. Geldanamycin is considered a highly specific inhibitor of Hsp90 and its derivative, 17-(allylamino)-17-Demethoxygeldanamycin (17AAG), is in Phase I trials as an antitumor agent [16–19]. Inhibition of Hsp90 by these antibiotics and others abolish Hsp90-dependent folding of immature client proteins and direct them to ubiquitin-mediated proteolytic degradation [21,22]. The gene and cDNA sequence of PfHsp90 have been characterized, and the deduced protein sequence revealed its obvious similarity to Hsp90 from other species and its high conservation among *Plasmodium* isolates [23,24]. The cDNA sequence was considered to correspond to this protein since a monoclonal antibody that reacted with the 90 kDa antigen was used to screen the cDNA library. Furthermore, the same antibody reacted with a 90 kDa *Plasmodium* protein that bound to ATP-agarose [23,25]. Sera of humans, mice, and squirrel monkeys, exposed to *Plasmodium*, contained abundant amounts of antibody reactive to the 90 kDa protein [25–27], suggesting that it may have a major antigenic role in malaria.

Based on the foregoing, we conjectured that PfHsp90 might play a critical role in parasitic signaling and cell division, and by corollary, GA might inhibit *P. falciparum* growth. In this communication, we show that this is true and present detailed studies of the effect of GA on *P. falciparum* replication and morphology. Our evidence suggests that GA inhibits the ATP-binding activity of PfHsp90. This is likely to inhibit the Hsp90 chaperone cycle, thus providing a working hypothesis for the antiparasitic activity of GA.

**Materials and Methods**

**Antibodies, reagents, parasite culture, and drug treatment**

Monoclonal mouse antibody against Achlya Hsp90 was purchased from Sigma, and was found to react with PfHsp90 [5]. Antibody to the T7-tag peptide was from Novagen (EMD Biosciences, Inc., Madison, WI, USA). Chloroquine (CQ) was purchased from Sigma, and [8–3H]-hypoxanthine, from Perkin Elmer. Geldanamycin was provided by NCI, and its stock solution and appropriate dilutions were made in DMSO.

*P. falciparum* (3D7 or W2) was grown on A-positive human erythrocytes at 5% hematocrit in the presence of homologous serum as described earlier [28]. The parasite morphology and stage-specific development were evaluated by microscopic inspection of Giemsa-stained thin smears [29]. To determine parasitemia, about 500 erythrocytes were examined and the number of infected erythrocytes was reported as percentage of the total. Stage-specific development was assessed by examining a minimum of 400 parasitized cells on each smear for differential counting of rings, trophozoites, schizonts, and pyknotic forms whose exact morphology could not be established. The fraction of each group was calculated as a percentage of the total number of parasitized cells.

When needed, the cultures were synchronized to ring stage by D-sorbitol treatment as described earlier [28,30]. Four hours post-sorbitol treatment was taken as 0 h, and synchronized parasites were collected (or treated with drug) at various times afterwards, as described in the respective Figure legends. The different morphogenetic stages were timed as follows: early trophozoite (20 h); late trophozoite (26 h); early schizont (32 h); late schizont (40 h); ring (48 h). Synchrony persisted well through two cycles; the purity of individual stages was confirmed to be greater than 95% by light microscopic examination of the Giemsa-stained thin smears of the cultures [29].

Asynchronous or ring-stage synchronized culture at 4 h after sorbitol treatment was seeded in 12-well culture plates. When needed, the cultures were treated with the drugs (or DMSO control for GA and sterile de-ionized water control for CQ) for different time intervals as indicated in the figure legends. The [3H]-hypoxanthine incorporation was carried out as described earlier [31] with minor modifications. All incorporations were measured in triplicate, and the average presented.

**Cloning and expression of recombinant PfHsp90 cDNA**

The cDNA of *P. falciparum* Hsp90 (PfHsp90) was first amplified by reverse transcriptase-PCR using the following primers based on the published sequence [23,24]:

- For primer: ATGTCAACGGAAACATCCGCAATTTAAC (sense), and
- Rev primer: TTAGTCAACTTCTCCATTTTAGAATCG (antisense) (the
Figure 1

(A) Native and recombinant expression of PfHsp90: E. coli BL21(DE3) containing pMICO plasmid [32] and either the pET23a-PfHsp90 clone (lane C) or just the pET-23a vector (no insert) (lane V) were induced with IPTG, analyzed in SDS-PAGE, followed by staining. Proteins from duplicate gels were probed in immunoblot with antibody against T7-tag or Hsp90 (Sigma), as described under Materials and Methods. In the far right panel, 50 µg of total parasitic protein was similarly analyzed, and probed with the same anti-Hsp90 antibody. The roughly 92 k His-tagged recombinant and 90 k parasitic Hsp90 bands are indicated by arrowheads (left and right, respectively); Mr values (in thousands) of protein markers are shown on the left. (B) Stage-specific expression of Hsp90 mRNA in the parasite: Parasites were synchronized by sorbitol treatment and harvested at time points corresponding to the following stages: 20 h (early trophozoite), 26 h (late trophozoite), 32 h (early schizont), 40 h (late schizont), 48 h (ring, from the second cycle of synchrony). The upper panel shows a Northern of the total parasitic RNA probed with 32P-labeled PCR product corresponding to nucleotide 450–990 of Hsp90 ORF. The 2.9 kb Hsp90 mRNA is so indicated. The lower panel is an ethidium bromide stained picture of the parental gel, showing the ribosomal RNAs and size markers (lane M). (C) Agreement of Northern data with microarray analysis [34]. The intensities of the PfHsp90 mRNA bands (panel B) were quantified by densitometry, normalized against rRNA, and plotted as green bars of relative height. The bars were superimposed on the DeRisi microarray data for Hsp90 mRNA throughout the intraerythrocytic cycle [34]. (D) Stage-specific expression of the Hsp90 protein: Immunoblot of 30 µg of total protein from the same parasitic stages as in panel (B). The Immunoblot for PfARP protein, which is constitutively expressed in all stages [35], serves as control for sample loading.
start codon ATG and the antisense stop codon TTA are underlined). The 2238 bp long product was further amplified with similar primers that additionally contained Bam HI and Xho I restriction sites, respectively, and the resultant final product cloned in the corresponding sites of pET-23a (Novagen), such that the recombinant protein would have a T7-tag at the N-terminus. The clone was confirmed by dideoxy sequencing using the ABI Big Dye terminator technology and introduced into E. coli BL21(DE3) containing the pMICO plasmid [32]. The resultant strain, upon induction with IPTG, produced the expected 92,000 Mr recombinant protein (Fig. 1).

**Interaction of PfHsp90 with ATP and GA**

Hsp90-binding to ATP-Sepharose column (Upstate Biotechnology, Inc., Lake Placid, NY) was tested essentially as described [19,23]. Saponin-purified parasite pellets were resuspended in an appropriate volume of buffer A (50 mM Tris-Cl, pH 7.8, 50 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 15 mM Na2MoO4, 2 mM MgCl2, 5 mM MnCl2), and lysed by pipetting and sonication. The lysate was clarified by centrifugation at 15,000 × g for 20 min in a refrigerated centrifuge. Each reaction sample was prepared by mixing 80 µg (by protein assay) of the *P. falciparum* lysate with 25 µl of ATP-Sepharose in a total volume of 250 µl. As and where mentioned (Fig. 2), additions or subtractions (e.g., GA, nucleotides etc.) were made to the *Plasmodium* extract prior to its addition to the resin. After incubation at 30 °C for 20 min (with mixing on a rotating shaker to prevent the settling of the resin), the resins were washed 4 times with ice-cold buffer A, and the bound Hsp90 was extracted by boiling in 1× Laemmli sample buffer [33] and analyzed by SDS-PAGE followed by immunoblot (Western) using the Super Signal Ultra chemiluminescence procedure (Pierce, Rockford, IL) as described [5]. In the control experiment, ovalbumin (20 µg), which has no ATP-binding activity, was used instead of the *Plasmodium* lysate.

**Results**

**Characterization of PfHsp90 cDNA and stage-specific expression of the protein**

To obtain direct evidence for the identity of PfHsp90 and to initiate studies of its biochemistry, we cloned its cDNA and expressed the recombinant protein in bacteria. Results (Fig. 1) show that the T7-tagged recombinant protein reacts with anti-T7 tag as well as human Hsp90 antibodies, thus confirming the cDNA sequence. The observed Mr of the protein (about 92 k) is in agreement with the predicted molecular weight of PfHsp90 (86.2 kDa) plus approximately 2 kDa for the T7-tag. The protein also has a predicted acidic pi of 4.94 [http://us.expasy.org/tools/pi_tool.html](http://us.expasy.org/tools/pi_tool.html), which should retard its mobility on SDS-PAGE. The stage-specific expression pattern of the Hsp90 mRNA (Fig. 1B) confirmed previous findings [23] that the mRNA is abundant in the ring and early trophozoite stages, but extremely low in schizont, indicating potential regulation at the level of transcription or mRNA stability. Our data closely matched the recent results of Bozdech et al [34] obtained by microarray analysis of the PfHsp90 transcript throughout the intraerythrocytic developmental cycle (Fig. 1C). Measurement of Hsp90 protein by immunoblot analysis (Fig. 1D) revealed
comparable amounts in all stages except the late schizont, where only low amounts are present. Comparing the RNA and protein levels we conjecture that Hsp90 is translated mainly in the ring and trophozoite stages; however, it is a relatively stable protein that continues to persist through much of the schizont stage, starting to disappear only in the late schizonts.

Interaction of PfHsp90 with ATP and GA
Crystalllographic as well as biochemical studies have documented that Hsp90 possesses an ATP-binding activity, and that GA, by virtue of its structural similarity to ATP competes for binding to the N-terminal ATP-binding pocket [8,16,17,19,20]. In vitro, purified Hsp90 was shown to bind to immobilized ATP linked to the matrix via the γ-phosphate, and this could be abolished by pre-incubation of the Hsp90 with GA [19]. Binding was also inhibited by free ATP and ADP, but not by GTP. Since the biochemistry of parasitic Hsp90 has not been studied, we carried out preliminary experiments in an attempt to understand GA action. As described under Discussion, p23 is a co-chaperone important to the function of Hsp90, and previous work showed that the formation of the p23-Hsp90 complex requires ATP and Mg²⁺ [36,37]. We have, therefore, adopted similar conditions for binding of native parasitic Hsp90 to ATP-Sepharose. As shown in Fig. 2, upon incubation of P. falciparum extracts with ATP-Sepharose, PfHsp90 was indeed detected in the bound material by immunoblot assay. In the control experiment, ovalbumin did not bind to the column [19], demonstrating specificity of binding (data not shown). The association of PfHsp90 was abolished in the presence of EDTA, confirming a role of divalent cations [23]. ATP and ADP also inhibited binding in a concentration-dependent manner whereas GTP failed to do so.

Antimalarial effect of GA is independent of CQ-resistance
The original antimalarial drug, chloroquine (CQ), serves as the hallmark against which new antimalarials are generally compared. In different CQ-sensitive strains, the IC₅₀ (i.e., concentration of the drug that causes 50% inhibition) has been reported to range from approximately 10 to 50 nM [38,39]. In our in vitro culture, when CQ and GA were compared against P. falciparum strain 3D7 under identical conditions, the IC₅₀ of CQ was about 12 nM, whereas that of GA was about 20 nM (Fig. 3). Thus, it appears that GA is about half as potent as CQ.

Since CQ-resistance is a major problem in malaria therapy, and multi-drug resistance is a common phenomenon in Plasmodium and other parasites [4,40], we tested if CQ-resistant parasites were simultaneously GA-resistant. Fig. 3 shows that GA is equally effective against the CQ-sensitive strain 3D7 and the CQ-resistant strain W2.

Geldanamycin and chloroquine are synergistic inhibitors of Plasmodium growth
In principle, two antimalarials can interact in a variety of ways: they may have no effect on each other, or they may facilitate or antagonize each other [4,38,39]. For example, the antimalarial effect of clotrimazole (IC₅₀ = 1 µM) is antagonized by CQ, but is synergistic to that of mefloquine [41]. Therefore, we tested how the antimalarial activity of GA interacts with that of CQ, a commonly used antimalarial. Various ratios of the two drugs were used over a range of concentrations spanning their individual IC₅₀ values, as described under Materials and Methods. The isobologram (Fig. 4) shows a concave curve, suggesting a synergistic interaction between CQ and GA in inhibiting parasite growth.

GA-mediated inhibition is rapid
To determine if the GA effect occurs in the same stage or if it is delayed, the morphological parasitemia (defined under Materials and Methods) was followed for short as well as long periods following GA addition to the culture. As shown in Fig. 5, the inhibitory effect on the rate of increase of parasitemia was immediately discernible starting at a GA concentration between 10–20 nM, which approximated the IC₅₀ value determined by DNA synthesis inhibition assay (i.e., hypoxanthine incorporation). At
GA concentrations of four times the IC$_{50}$ (80 nM) and higher, parasitemia never increased with time, and actually decreased below the initial number, suggesting that either the parasites or the infected RBCs were destroyed when the GA effect was severe. These results suggest that GA-mediated inhibition is manifested within a single cell cycle, and is thus, likely to be a relatively rapid effect.

**GA causes death and disintegration of all parasitic stages**

A series of experiments was next carried out to determine whether all the stages of the parasitic life cycle were directly affected by GA. A range of GA concentrations (10 to 100 nM) were first tested on *P. falciparum*-infected RBC, and growth of the parasite was measured by staining. In an asynchronous culture that contained the three major *Plasmodium* life stages (ring, trophozoite, and schizont), all became morphologically abnormal (Fig. 6) and later disappeared from the culture, confirming the results from Fig. 5.

To directly examine how GA affects the progression of the parasitic life cycle, a synchronous culture starting with early ring stage was exposed to a range of GA concentrations (spanning both sides of the IC50) and parasites were checked at 24 h and 48 h. As shown in Fig. 7, all rings in the control untreated (0 nM GA) culture advanced to trophozoite in 24 h, as expected [34]. In contrast, with increasing GA concentrations, progressively more and more parasites failed to make it to trophozoite, and instead shrank to pyknotic masses. After 48 h exposure to GA, some of these trophozoites made it to ring in the second cycle, but the remainder turned to pyknotic masses (Fig. 7). At GA concentrations of 40 nM and higher essentially all rings turned pyknotic after 48 h. Taken together, these results show that GA inhibits all intraerythrocytic...
Discussion

Our in vitro results presented here demonstrate that GA can function as an effective antimalarial, at least in erythrocytic culture, and that it is effective against chloroquine-resistant strains as well. The impressive reduction (5–10 fold) of IC50 of both drugs when used together (Fig. 4) suggests that GA can be used in combination with CQ. As mentioned before, GA and its derivatives are already FDA-approved for Phase I clinical trials in cancer patients, particularly those with advanced solid malignancies of the breast [11–15]. When treated with 17-(allylamino)-17-demethoxygeldanamycin (17AAG), derivative of GA, breast cancer cells were arrested in G1, underwent subsequent mammary differentiation, and then apoptosis. Interestingly, despite the spectrum of important proteins that are degraded in response to these drugs, they showed antitumor activity in animals at doses that are not particularly toxic. In human cancer patients, micromolar peak concentrations were achieved without significant toxicity, which suggested a favorable pharmacology [12–14]. Thus, it appears that GA and 17AAG are worth testing in animal and human Plasmodium infections.

It is important to note that pairs of antimalarial drugs interact with various degrees of cooperativity or antagonism which must be experimentally determined. For example, while mefloquine and clotrimazole are synergistic, CQ and clotrimazole are antagonistic [41]. The demonstration that CQ and GA act synergistically (Fig. 4) makes antimalarial therapy with a combination of these two drugs a viable option. It is also gratifying to find that development of CQ-resistance did not simultaneously lead to a resistance to GA (Fig. 3). Together, these results reinforce the facts that the CQ-resistant strains do not cause an efflux of GA and that the target of GA and CQ are indeed different, i.e., while GA most likely inhibits Hsp90, the principle target of CQ is the parasitic digestive vacuole and requires PfCRT, a membrane transporter [40].

The Hsp90 sequence is highly conserved throughout evolution. The sequence conservation is particularly notable in the approximately 220 residues at the N-terminus, which forms the ATP/GA-binding pocket, and the roughly 500 residue long C-terminus, which is mainly involved in interaction with other proteins such as the co-chaperones [8,9,16,17]. The sequence between these two domains is variable but generally consists of highly charged (acidic and basic) amino acid residues. Interestingly, most species studied to date contain at least two Hsp90 paralogs, and P. falciparum seems to be no exception. While the PfHsp90 presented in this paper is located on chromosome 7 (PlasmoDB ID: PF07_0029), P. falciparum has at least one other Hsp90 paralog, located on chromosome 12 (PlasmoDB ID: PFL1070c; manuscript in preparation). Fig. 8 shows a multiple alignment of the N-terminal ATP-binding domain of the two Plasmodium paralogs and their comparison with a representative mammalian Hsp90, namely the major human Hsp90 (P07900). The high sequence conservation was evident by the fact that the N-terminal domain of PfHsp90 (Fig. 8) shared a remarkably high 49% identity with the other paralog and 73% with the human ortholog. When the full-length sequences were compared, these numbers were 36% and 60% respectively. The in vitro results of ATP-PfHsp90 interaction (Fig. 2) and its inhibition by GA closely matched those of Grenert et al [19] who used human Hsp90β in similar studies. Successful competition for ATP-binding by GA (Fig. 2) suggests that as with mammalian Hsp90, the P. falciparum protein also has an ATP-binding pocket where both GA and ATP compete to bind. Mutagenesis and crystallographic studies have identified a number of amino acid residues in this pocket important for interaction with ATP and GA (16, 17). It is notable that all of these residues, as highlighted in Fig. 8, are conserved in the two Plasmodium Hsp90 sequences, suggesting that the second PfHsp90 may have similar ATP-binding activity that may also be inhibited by GA. As a corollary, the antimalarial
The effect of GA reported here could be due to the inhibition of either or both Plasmodium Hsp90 homologs. Clearly, identification of the exact target of GA in Plasmodium must await studies of expression of the other Hsp90 homologs of Plasmodium and determination of their relative GA sensitivity.

Besides GA, there are a number of other compounds that also inhibit Hsp90 and interfere with its chaperone function. These include members of the ansamycin antibiotic family, namely herbimycin, and macbeic I and II, which are structurally similar to GA and bind to the nucleotide-binding pocket of Hsp90, and coumarin-type antibiotics, exemplified by novobiocin, originally discovered as an inhibitor of bacterial DNA gyrase B [42–44]. Radicicol, a macrocyclic antifungal structurally unrelated to GA, also specifically binds to and inhibits Hsp90. As expected, many of these drugs inhibit the ATPase activity of the Hsp90 complex, and all of them promote proteolytic degradation of Hsp90 client proteins. Based on the high degree of sequence similarity between PfHsp90 and mammalian Hsp90, we predict that all of these compounds may also act as antimalarials.

There is now mounting evidence, albeit indirect, supporting the existence of a highly active protein chaperone system in Plasmodia. First, our results and previous publications have led to the characterization of a Plasmodium ortholog of Hsp90, an ATP-utilizing molecular chaperone conserved across evolution, and sequence analysis suggested that there might be others (Fig. 8). Consistent with its greater need in conditions that lead to protein misfolding, PfHsp90 transcript levels increased three to four fold when erythrocytic parasite culture was shifted from 37°C to 41°C [24].

Second, as mentioned before, a large variety of important cell cycle proteins, kinases, and transcription factors depend on Hsp90 for proper folding and stability [8,9]. Consistent with this task, Hsp90 is known to be essential in all eukaryotes. The mechanism of Hsp90 action is complex and many of its aspects are still being elucidated. In its chaperone cycle, Hsp90 forms transient complexes with a number of participating proteins that include Hsp70, Hip (Hsp70 interacting protein), Hop (p60), p23, and immunophilins [8,9]. The orthologs of all of these proteins also seem to exist in Plasmodium. Multiple Hsp70-like genes and a grp78 (glucose-regulated protein),
another stress protein of the same family, have been described in Plasmodium [45,46]. Like PfHsp90, these proteins are also highly immunogenic, and the transcription of these genes is elevated upon heat shock. A 58 kDa Plasmodium protein (called heat shock-related protein, Hrp) has been identified that has significant similarity to Hip and contains TPR motifs [47]. A homology search of the P. falciparum genome sequence also revealed sequences with significant homology to Hop (chr14_1.gen_156) and p23 (chr14_1.gen_248) [48], our unpublished data.

The Plasmodium Hop and Hsp70 proteins also contain TPR motifs, and Hsp90 is known to have a propensity to interact with TPR-domain proteins [8,9]. We have already shown that PfHsp90 binds to the TPR-phosphatase, PIP5P [5]. Taken together, these findings not only suggest the existence of an active chaperone pathway in Plasmodium, but also point to the possibility that many members of the pathway are regulated by reversible phosphorylation. Clearly, further characterization of this process will lead to important directions in the regulation of parasitic gene expression.

Folding and stability of proteins are intimately connected: the vast majority of improperly folded proteins are generally ligated to ubiquitin and degraded by the proteasome machinery [49]. Thus, by blocking Hsp90 function, GA promotes the degradation of the proteins that depend on Hsp90 for optimal folding [21,22,44]. Recent reports have, in fact, described a proteasome S4 ATPase homolog [50] and ubiquitin in P. falciparum [51] and, interestingly, polyubiquitin expression was regulated during parasite development and by heat shock. It is thus tempting to speculate that Plasmodium has an ATP-ubiquitin-proteasome pathway that is functionally similar to higher eukaryotes and mediates non-lysosomal degradation of cytosolic proteins of the parasite. Our preliminary results (not shown) indicated that a specific subset of P. falciparum proteins is indeed degraded in GA-treated parasites,

Figure 8
Hsp90 sequence similarity. Only the N-terminal domains of three Hsp90 sequences are shown: PfHsp90 (the P. falciparum Hsp90 studied in this paper), PfHsp90-2 (another potential Hsp90 paralog in P. falciparum), and a major Homo sapiens (human) Hsp90 (HsHsp90). The total amino acid residues of the full-length sequences are 745, 821, and 732 respectively. Residues known to be important for ATP and GA binding are colored red. Details are in the Results.
suggesting that this may make additional contributions to the antimalarial mechanism of GA.

After our manuscript was submitted, a recent publication by Banumathy et al [52] came to our attention that described inhibition of *P. falciparum* by GA, thus vindicating the basic conclusion of our paper. Unfortunately, however, use of different procedures and lack of appropriate controls made evaluation of some of their results difficult. For example, these authors reported an IC50 (termed LD50) of 200 nM for GA against erythrocytic cultures of *P. falciparum* 3D7, which is ten-fold higher than our IC50 value of 20 nM. We believe the difference is due to the fact that Banumathy et al exposed the parasite culture to GA for 24 hr only, whereas we exposed for 48 hr. Initially, we used a 24 hr exposure protocol, and also obtained an IC50 value of 200 nM; however, under the same conditions (i.e., 24 hr exposure), CQ exhibited an IC50 of 250 nM, which is more than ten times the accepted value in literature [38,39]. Thus, we optimized the drug exposure time to 48 hr, which resulted in the IC50 values of 20 nM and 15 nM, respectively for GA and CQ (Fig. 3). Banumathy et al did not use any known antimalarial drug as positive control. These authors also performed density gradient and pull-down experiments to determine the binding partners of PfHsp90; however, lack of quantitation and specific detection of the associated proteins left their identities uncertain. Based solely on GA’s ability to inhibit Plasmodium growth, Banumathy et al concluded that Hsp90 is essential in the parasitic life cycle. We believe the conclusion is premature as it is still unknown whether Hsp90 is the exclusive parasitic target of GA. Isolation of spontaneous GA-resistant mutants of *P. falciparum* and mapping of the mutations to specific PfHsp90 gene(s), currently in progress in our laboratory, should shed light on the relative roles and essentiality of Hsp90 in the parasite.

Conclusion
The heat shock protein 90 (Hsp90) of *P. falciparum* is highly similar to its orthologs in other species in both sequence and biochemical activities that are relevant to its chaperone function. The inhibitory effect of geldanamycin on the ATP-binding activity of *Plasmodium* Hsp90 offers a potential biochemical mechanism for the antiparasitic effect of geldanamycin. Further characterization of the parasitic Hsp90 chaperone pathway and its client proteins may provide important targets for novel antiparasitic drugs. The relatively low IC50 (20 nM) of GA against *Plasmodium* and the substantially higher concentration (in the micromolar range) achieved in human serum without overt toxicity make it a potential candidate for further development as an antimalarial.

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