Biol. Chem., Vol. 392, pp. 431–438, May 2011 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2011.040

Screening for small molecule modulators of Hsp70 chaperone activity using protein aggregation suppression assays: inhibition of the plasmodial chaperone PfHsp70-1

Ingrid L. Cockburn[1], Eva-Rachele Pesce[1], Jude M. Pryzborski[2], Michael T. Davies-Coleman[3], Peter G.K. Clark[4], Robert A. Keyzers[3,4], Linda L. Stephens[1] and Gregory L. Blatch[1,*]

[1] Biomedical Biotechnology Research Unit, Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, Grahamstown 6139, South Africa
[2] Department of Parasitology, Faculty of Biology, Philipps University Marburg, D-35043 Marburg, Germany
[3] Department of Chemistry, Rhodes University, Grahamstown 6139, South Africa
[4] Centre for Biodiscovery, School of Chemical and Physical Sciences, Victoria University of Wellington, Wellington, New Zealand

*Corresponding author
e-mail: g.blatch@ru.ac.za

Abstract

Plasmodium falciparum heat shock protein 70 (PfHsp70-1) is thought to play an essential role in parasite survival and virulence in the human host, making it a potential antimalarial drug target. A malate dehydrogenase based aggregation suppression assay was adapted for the screening of small molecule modulators of Hsp70. A number of small molecules of natural (marine prenylated alkaloids and terrestrial plant naphthoquinones) and related synthetic origin were screened for their effects on the protein aggregation suppression activity of purified recombinant PfHsp70-1. Five compounds (malonganenone A-C, lapachol and bromo-β-lapachona) were found to inhibit the chaperone activity of PfHsp70-1 in a concentration dependent manner, with lapachol preferentially inhibiting PfHsp70-1 compared to another control Hsp70. Using growth inhibition assays on *P. falciparum* infected erythrocytes, all of the compounds, except for malonganenone B, were found to inhibit parasite growth with IC_{50} values in the low micromolar range. Overall, this study has identified two novel classes of small molecule inhibitors of PfHsp70-1, one representing a new class of antiplasmodial compounds (malonganenones). In addition to demonstrating the validity of PfHsp70-1 as a possible drug target, the compounds reported in this study will be potentially useful as molecular probes for fundamental studies on Hsp70 chaperone function.

Keywords: heat shock proteins; malaria; malonganenones; molecular chaperones; naphthoquinones; *Plasmodium falciparum*.

Introduction

Of the five *Plasmodium* species known to infect humans, *Plasmodium falciparum*, which can cause cerebral malaria, results in the highest number of deaths (Haldar and Mohandas, 2007) and thus is the focus of most malaria research. Although uncomplicated malaria is curable if treated early, current antimalarials are fast becoming ineffective due to increased drug resistance (Tuteja, 2007). This problem makes the search for effective new therapies and, in particular, novel drugs with new mechanisms of action and hence new targets, an extremely important and worthwhile research area.

Molecular chaperones, and more specifically heat shock proteins, are known to play an important role in the survival and virulence of many protozoan parasites, including *P. falciparum* (Neckers and Tatu, 2008). Heat shock proteins 70 kDa in size (Hsp70s) facilitate the correct folding and assembly of newly synthesized proteins, the refolding of incorrectly folded or aggregated proteins and the translocation of proteins across membranes (Mayer and Bukau, 2005). Structural features of Hsp70s include an approximately 45 kDa ATPase domain, an approximately 15 kDa peptide-binding domain and an approximately 10 kDa C-terminal domain ending in a conserved EEVD motif (Mayer and Bukau, 2005), which interacts with proteins containing a tetratricopeptide repeat domain (Blatch and Lässle, 1999; Scheufler et al., 2000). Heat shock proteins 40 kDa in size (Hsp40s), characterized by the presence of a conserved approximately 70 amino acid sequence termed the J-domain (Cheetham and Caplan, 1998), act as co-chaperones by interacting, via their J-domains, with the Hsp70 ATPase domain. Hsp40 co-chaperones can deliver peptide substrates to Hsp70 and stimulate the ATPase activity of Hsp70 (Cheetham et al., 1994).

The complex life cycle of *P. falciparum* is associated with many potential stresses to the parasite, including significant temperature increases due to febrile episodes in the human host (Acharya et al., 2007) and attack by host defense mechanisms (Sharma, 1992). The ability of the parasite to survive these stresses has been attributed to heat shock proteins (Kumar et al., 2003). In *P. falciparum*, as much as 2% of all genes encode molecular chaperones (Acharya et al., 2007), with six encoding Hsp70s (Shonhai et al., 2007) and at least 43 encoding Hsp40s (Botha et al., 2007). The major cytosolic plasmodial Hsp70, PfHsp70-1 (PF08_0054), has been shown to have chaperone activity using both ATPase and aggregation suppression assays (Matambo et al., 2004; Ramya et al., 2006; Shonhai et al., 2008; Misra and Ramachandran, 2009). Furthermore, PfHsp70-1 has been shown

to suppress the thermosensitivity of a mutant strain of *Escherichia coli* lacking DnaK (Shonhai et al., 2005). PfHsp70-1 is localized to the cytosol and the nucleus of the parasite (Kumar et al., 1991; Pesce et al., 2008) and is expressed at high levels in all the blood stages of the *P. falciparum* life cycle, with expression levels increasing after heat shock (Kumar et al., 1991). Taken together, these data suggest that PfHsp70-1 plays an important role in parasite viability in the human host, making it an attractive antimalarial drug target.

Hsp70s have been implicated in a number of diseases and illnesses including cancer, viral infections and protein conformational diseases such as Parkinson's disease (Brodsky and Chiosis, 2006). For this reason, there are increasing numbers of studies aimed at identifying small molecule modulators of Hsp70, both inhibitors and activators, for use in the development of chemotherapeutic agents (Jinwal et al., 2009). Compared with Hsp90, inhibition or modulation of Hsp70s (specifically the ATPase activity) by small molecules is thought to be more challenging. This, as reviewed by Massey (2010), has been attributed to various structural and biochemical properties of the chaperones, including the more hydrophilic ATP binding site of Hsp70 compared to that of Hsp90 in addition to a very high affinity of Hsp70 for ATP and ADP, making inhibition by competitive binding more difficult than for Hsp90, which has a lower affinity for nucleotides.

A number of Hsp70 inhibitors have been identified, including adenosine analogs (ATP mimics), spergualins, pyrimidinones, fatty acids and peptides (Evans et al., 2010). ATP mimics of Hsp70 have been found to be relatively selective and have been shown to have cytotoxic effects on HCT116 (human colon cancer) cells, which in some cases was attributed to Hsp70 modulation by these compounds (Williamson et al., 2009). 15-Deoxyspergualin (DSG), an immunosuppressive drug belonging to the spergualins, has been found to modulate Hsp70 chaperone activity, including both ATPase and aggregation suppression activity (Brodsky, 1999; Ramya et al., 2006). Structural analogs of DSG, the pyrimidinone-peptoids, have also been tested as modulators of Hsp70 chaperone activity, resulting in the identification of inhibitors of either the Hsp40-stimulated ATPase activity of Hsp70 or the basal Hsp70 ATPase activity with certain inhibitors exhibiting Hsp70 inhibition that correlated with inhibition of breast cancer growth (Fewell et al., 2004; Wright et al., 2008). These pyrimidinones have proven useful in rational design and testing of novel Hsp70 inhibitors, in which, it was revealed that aromatic or hydrophobic groups were important features for inhibition of Hsp70 ATPase activity (Wisén et al., 2008). Recently, a pyrimidinone compound was found to act as an artificial co-chaperone to Hsp70, stimulating its chaperone activity and binding adjacent to the site on Hsp70 involved in interaction with the J-domain of Hsp40. When steric bulk was added to this compound, inhibition of Hsp70 chaperone activity was observed (Wisén et al., 2010). These findings provide useful information on the possible mechanism of modulation of Hsp70 by pyrimidinones and related compounds. Finally, a number of pyrimidinones were tested as potential inhibitors of the ATPase activity of PfHsp70-1 and for inhibitory effects on *P. falciparum* growth. Of the compounds screened, nine were found to have significant inhibitory effects on parasite growth, in addition to effects on the ATPase activity of PfHsp70-1 (Chiang et al., 2009).

In this study, two classes of compounds were screened for potential effects on PfHsp70-1 chaperone activity. The first class, the marine tetraprenylated alkaloids malonganenones A, B and C, are natural products extracted from *Leptogorgia gilchristi*, a Mozambique sea fan. These novel marine compounds were found to have moderate cytotoxicity towards a number of oesophageal cancer lines (Keyzers et al., 2006). The second class of compounds is the 1,4 naphthoquinones, specifically lapachol and its derivatives, of both natural and synthetic origin. Lapachol is a common naturally occurring naphthoquinone found in the heart-wood of various tree species belonging to the bigonaceous family, found in Brazil and other tropical regions of the western hemisphere (da Silva Júnior et al., 2009). Lapachol and its derivatives have been shown to have both anticancer (Bonifazi et al., 2010) and antiplasmodial activity (Pérez-Sacau et al., 2005). Both compound classes were selected for this study because of their established biological activity and the structural similarities that they share with ATP (purine analogs) and with known inhibitors of Hsp70 and Hsp90 chaperones, like geldanamycin (quinone analogs). The tetraprenylated alkaloid class of compounds screened in this study also contain peptoid components, which in addition to pyrimidinone groups have been found to be important for Hsp70 interaction and modulation (Wright et al., 2008). In this study we report the identification of two novel classes of small molecule inhibitors of PfHsp70-1, one representing a new class of antiplasmodial compounds.

Results

PfHsp70-1 protein aggregation suppression activity is inhibited by selected marine prenylated alkaloids and naphthoquinone compounds

PfHsp70-1, as previously reported (Shonhai et al., 2008), was found to be effective in suppressing the thermally induced aggregation of malate dehydrogenase (MDH), reducing the aggregation of MDH by more than half at substoichiometric levels (Figure 1). A non-chaperone protein (BSA) and the solvent (DMSO) used to dissolve the test compounds had no effect on the aggregation of MDH (Figure 1). Following the testing of our pure compound repository (Table 1), five compounds (malonganenones A-C, lapachol and bromo-β-lapachona) showed notable inhibition of the aggregation suppression activity of PfHsp70-1 (Figure 2). With the exception of bromo-β-lapachona, PfHsp70-1 aggregation suppression activity was almost completely inhibited (>90% MDH aggregation) by each of the five compounds at 300 μM. Furthermore, in most cases the inhibition of PfHsp70-1 occurred in a concentration dependent manner (Figure 2), indicative of a specific interaction. Control assays in which no MDH was present and no detectable aggregates were observed suggested that the reversal of the aggregation

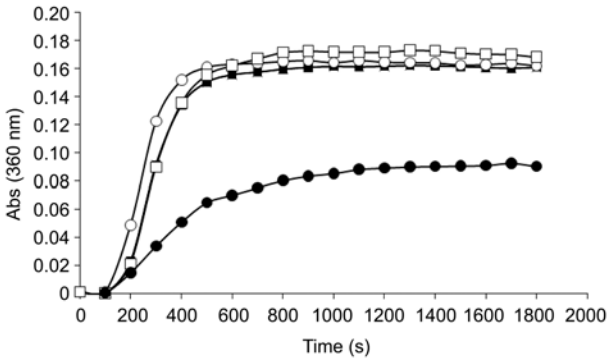


Figure 1 PfHsp70-1 suppresses the thermal aggregation of MDH. Progress curve showing MDH aggregation over time (0.72 μM, closed squares) and aggregation suppression by PfHsp70-1 (0.36 μM, closed circles). Controls performed show that the observed aggregation suppression is due to chaperone activity and not the presence of a second protein (BSA, 0.36 μM; open circles) and showing that DMSO, at the maximum concentration used (1%) in any subsequent inhibitor screening reactions, has no effect on MDH aggregation (open squares). These curves represent a typical set of control reactions using one batch of purified PfHsp70-1 and which was repeated at least twice on separate batches of purified protein.

suppression activity was not due to aggregates formed by either the compounds themselves or the compounds in combination with PfHsp70-1 or by PfHsp70-1 itself (data not shown). Additional control assays were conducted with MDH in the presence of each of the compounds, without chaperones. All of the compounds had no affect on MDH aggregation, except at the highest concentration (300 μM), where malonganenone A caused a slight enhancement of MDH aggregation (data not shown) and all the other compounds had minor effects on MDH aggregation (for example, lapachol, Figure 3). Overall these data suggest that marine prenylated alkaloids and naphthoquinones represent two novel classes of small molecule modulators of PfHsp70-1.

Lapachol differentially affects Hsp70s from different organisms

Recombinant human Hsp70 was found to be ineffective in MDH assays, as the chaperone itself aggregated at 48°C and therefore was unable to suppress the thermal aggregation of MDH at this temperature (data not shown). Thus, the compounds were tested for effects on *Medicago sativa* (alfalfa) Hsp70 as an alternative control Hsp70. Lapachol was shown to reproducibly inhibit PfHsp70-1 protein aggregation suppression activity in a concentration dependent manner but had little to no effect on the alfalfa Hsp70 activity (Figure 3). These data suggested that Hsp70 chaperones from different organisms, despite relatively high sequence similarities, can be differentially inhibited by selected small molecules.

Inhibitors of PfHsp70-1 also inhibit *P. falciparum* growth

The five compounds found to inhibit PfHsp70-1 *in vitro* chaperone activity were tested for their potential effects on *P. falciparum* growth in culture. All five compounds, except for malonganenone B, were able to inhibit parasite growth with IC_{50} values of <20 μM (Table 2). These IC_{50} values were found to be comparable to that of MAL3-39 (Table 2), an Hsp70 small molecule modulator previously shown to have inhibitory effects on *P. falciparum* growth (Chiang et al. 2009). Malonganenone A and malonganenone C, in particular, were found to have the highest inhibitory effects on parasite growth.

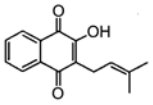
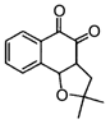
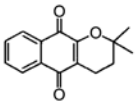
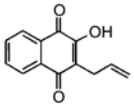
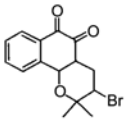
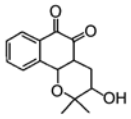
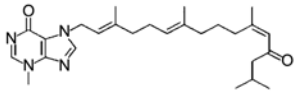
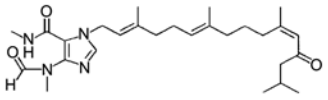
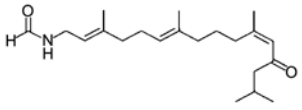
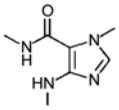
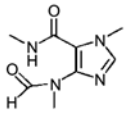
Discussion

To our knowledge, this is the first report of a screening for Hsp70 inhibitors using the MDH aggregation suppression assay. This study has resulted in the identification of two novel classes of small molecule inhibitors of PfHsp70-1, one representing a new class of antiplasmodial compounds (malonganenones). In addition to demonstrating the validity of PfHsp70-1 as a possible drug target, these compounds will be potentially useful as molecular probes for fundamental studies on Hsp70 chaperone function. The latter will be enhanced by the elucidation of the binding sites and mechanism of action for these classes of compounds. These compounds could be triggering the release of MDH from PfHsp70-1 simply by competitive binding to the substrate binding site. However, it is also possible that some of the compounds are associating with the ATP-binding site and causing an allosteric effect that lowers the affinity of PfHsp70-1 for MDH, as occurs on ATP binding (Shonhai et al., 2008).

In this study, relatively high compound concentrations were required for high levels of inhibition of PfHsp70-1 (100–300 μM). In a study by Chiang et al. (2009), similar concentrations of pyrimidinone compounds (300 μM) were required for inhibition of the ATPase activity of PfHsp70-1. However, these same compounds were shown to inhibit at relatively low concentrations (IC_{50}<2 μM) the growth of *P. falciparum* using parasite-infected erythrocyte cultures (Chiang et al., 2009).

The biological activities of lapachol and its derivatives have been well studied. Naphthoquinones have been found to be cytotoxic towards various human cancers including ovarian, breast, lung and cervical cancers lines (Bonifazi et al., 2010; da Silva Júnior et al., 2010), with many compounds displaying IC_{50} values of <5 μM. This group of compounds has also been shown to exhibit trypanocidal activity (Ferreira et al., 2006; Silva et al., 2006; da Silva Júnior et al., 2008) and antiplasmodial activity. A group of aminonaphthoquinones was tested in *P. falciparum* (clones W2 and D6) growth inhibition assays, with one of the aminoquinones displaying higher potency than chloroquine towards the W2 clone (Kapadia et al., 2001). A number of phenazine derivatives of lapachol were tested for inhibitory effects in both *P. falciparum* growth inhibition assays and *P. berghei* infectivity assays in mice, with certain compounds shown to have inhibitory effects on both systems (de Andrade-Neto et al.,

Table 1 Names, structures and numbers of naphthoquinones, prenylated alkaloids and derivatives.

Common name	Structure	Number
Lapachol and synthetic derivates		
Lapachol		1
Nor-β-lapachona		2
α-Lapachona		3
c-Alil-lausona		4
Bromo-β-lapachona		5
Hydroxy-β-lapachona		6
Prenylated alkaloids and synthetic derivatives		
Malonganenone A		7
Malonganenone B		8
Malonganenone C		9
PGKC4_7C		10
PGKC4_4I		11

2004). In another study, lapachol and bromo-β-lapachona were found to inhibit the growth of *P. falciparum* F32 with IC_{50} values of 24.4 and 2.7 μM, respectively (Pérez-Sacau et al., 2005). These values were comparable to the IC_{50} values for parasite growth inhibition determined in this study using *P. falciparum* 3D7. When comparing malonganenones A-C, in terms of their performance in PfHsp70-1 *in vitro* chaperone assays vs. *P. falciparum* growth inhibition assays, malonganenones A and C exhibited greater inhibitory activity than malonganenone B in both assays. Similarly, two simplified synthetic analogs of malonganenone B (PGKC4_7C and PGKC4_4I) were inactive in our assays. Integrating all these data, it is tempting to speculate that Hsp70 is a potential cellular target of both the naphthoquinones and malonganenones. Interestingly, celastrol (a quinine), was found to activate the human heat shock response. This activation,

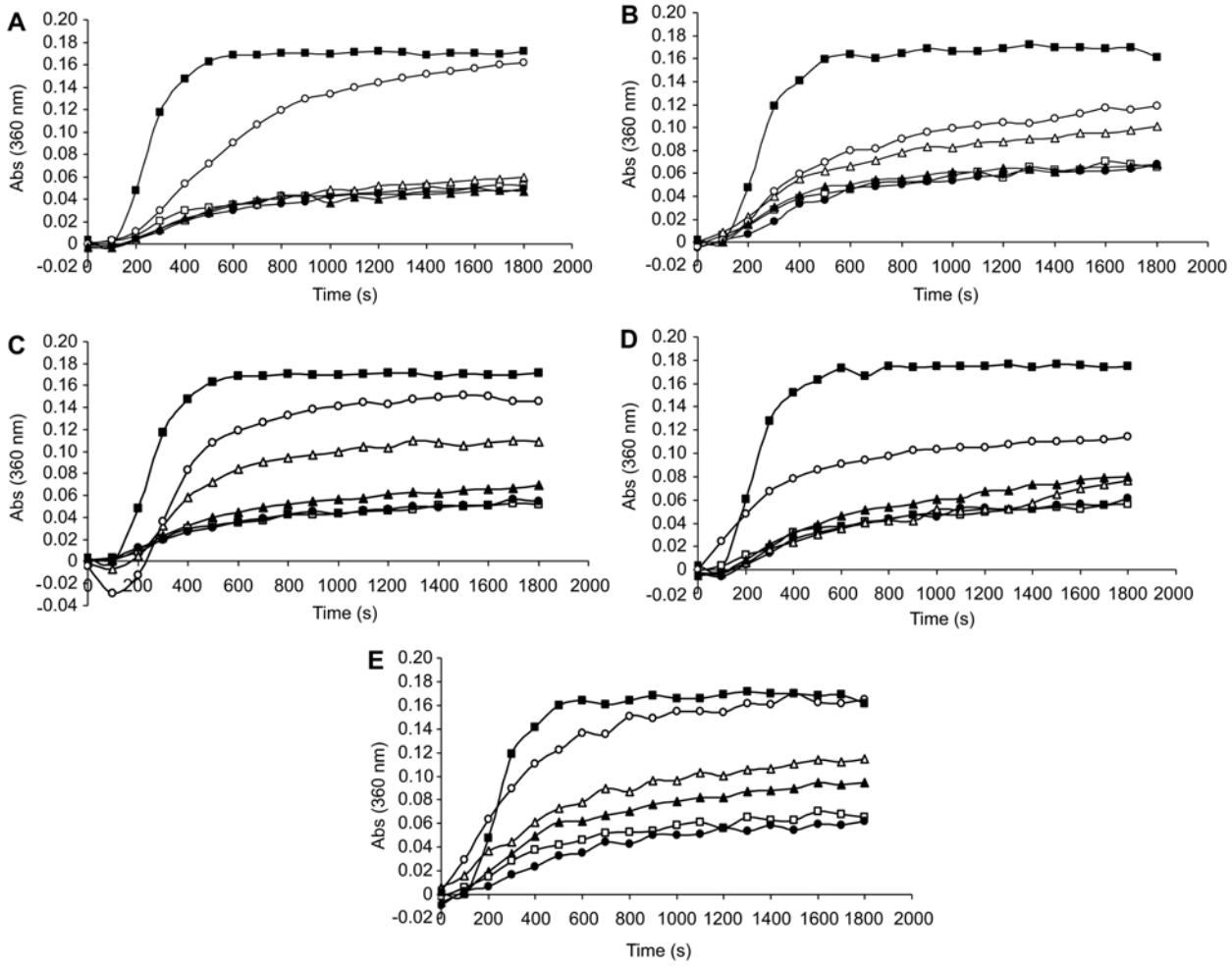


Figure 2 The protein aggregation suppression activity of PfHsp70-1 is inhibited in a concentration dependent manner by naphthoquinone and marine prenylated alkaloid compounds.
MDH (0.72 μM) aggregation at 48°C was monitored in the presence of PfHsp70-1 (0.36 μM) and various compounds (0, 1, 10, 100, 300 μM). Compounds found to inhibit PfHsp70-1 were lapachol (A), bromo-β-lapachona (B), malonganenone A (C), malonganenone B (D) and malonganenone C (E). For each set of compounds, progress curves were produced for: MDH only (closed squares); MDH and PfHsp70-1 (open squares); MDH, PfHsp70-1 and 1 μM compound (closed circles); MDH, PfHsp70-1 and 10 μM compound (closed triangles); MDH, PfHsp70-1 and 100 μM compound (open triangles); MDH, PfHsp70-1 and 300 μM compound (open circles). This set of figures represents a typical series of experiments on one batch of purified PfHsp70-1 in each case and each compound was tested on at least two separate batches of purified protein giving similar results.

however, was caused by specific activation of heat shock transcription factor 1 and not a direct effect on chaperones (Westerheide et al., 2004).

The potential that these compounds might differentially inhibit different Hsp70s needs to be investigated further, particularly for the comparison of PfHsp70-1 and human Hsp70s, such as Hsc70, Hsp70, BiP and Grp75. Because human Hsp70 is not amenable to thermally based protein aggregation suppression assays, we intend to test the differential effects of these compounds using other chaperone assays such as the luciferase refolding assay, which has been successfully used by others (Yamamoto et al., 2010). All of these chaperone assays can be adapted to examine Hsp70 together with its co-chaperones and, therefore, it will also be possible to analyze the effect of these compounds on Hsp40-stimulated Hsp70 chaperone activity.

Materials and methods

Protein purification

The expression and purification of $(His)_6$-tagged PfHsp70-1 from *E. coli* XL1 Blue [pQE30-PfHsp70-1] cells was carried out as previously described (Matambo et al., 2004; Shonhai et al., 2008). Purified protein was dialyzed into the MDH assay buffer.

Source of compound library

Lapachol (compound 1, Table 1) and several synthetic derivatives of lapachol (compounds 2–5, Table 1) were kindly provided by Professor Antonio Ventura Pinto (Núcleo de Pesquisas em Produtos Naturais, UFRJ, 21944-971 Rio de Janeiro, RJ, Brazil).

The malonganenones were isolated from *Leptogorgia gilchristi*, collected at a depth of 20 m from a reef near Ponto Malongane,

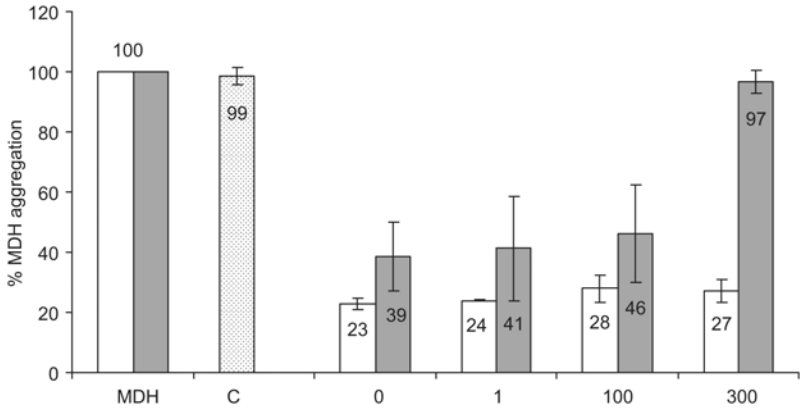


Figure 3 Lapachol differentially affects the aggregation suppression activities of *Medicago sativa* Hsp70 and PfHsp70-1.
Lapachol (0, 1, 100 and 300 μM) was tested for effects on the MDH (0.72 μM) aggregation suppression activities of PfHsp70-1 (0.36 μM) compared with *M. sativa* Hsp70 (0.36 μM). White bars, *M. sativa* Hsp70; grey bars, PfHsp70-1. The X-axis labels refer to MDH only (MDH) and lapachol concentrations in μM (0, 1, 100 and 300), in the presence of MDH and Hsp70. A control assay (shaded bar; labeled C) was conducted to assess the aggregation of MDH in the presence of lapachol (300 μM) without chaperone. The percentage MDH aggregation levels were calculated using the absorbance values after 1800 s, with the reaction of MDH alone taken as 100% MDH aggregation. For the compound testing, the standard deviation is shown and the numbers associated with the bars indicate the average percentage MDH aggregation.

Mozambique in 1995 with the aid of SCUBA. Freeze dried gorgonian (148 g) was exhaustively extracted with methanol that was removed under reduced pressure. The dried extract was partitioned between water (4.3 g) and ethyl acetate (3.1 g). A portion of the organic partition (0.9 g) was re-dissolved in methanol that was then defatted with hexanes, after which repeated bench-top chromatography using both normal- (DIOL) and reversed- (HP20) phase materials, followed by normal-phase HPLC (DIOL) resulted in the isolation of malonganenones A (29 mg; compound 7), B (14 mg; compound 8) and C (4 mg; compound 9). The purification and establishment of chemical structures of these compounds using mass spectrometry and nuclear magnetic resonance has been described (Keyzers et al., 2006, Table 1). Two synthetic analogs of malonganenone B were also prepared (compounds 10 and 11, Table 1). Briefly, caffeine (1.00 g) was refluxed in a mixture of 1 M $NaOH_{(aq)}$ (17 ml) and EtOH (8 ml) for one hour. Repeated recrystalization from EtOAc resulted in the purification of PGKC4_7C (0.11 g; 40% yield; compound 10). PGKC4_7C (0.28 g) was then refluxed in neat formic acid (14 ml) to provide the formate PGKC4_4I (compound 11) that was purified by recrystalization from toluene (0.20 g; 60% yield). The identity of both products was established from mass spectrometry and nuclear magnetic resonance data (data not shown).

Table 2 IC_{50} values of test compounds on *P. falciparum* 3D7 parasite growth.

Compound	IC_{50} (μM)
Lapachol	18.67±2.27
Bromo-β-lapachona	17.29±4.44
Malonganenone A	0.81±0.24
Malonganenone B	>50
Malonganenone C	5.20±2.49
MAL3-39	29.58±16.03
DMSO	ND[a]

[a]ND (not determined): the DMSO IC_{50} value was not determined due to the extremely low cytotoxicity of the solvent.

Protein aggregation suppression assays

MDH-based protein aggregation suppression assays using PfHsp70-1 were performed according to previously described methods (Boshoff et al., 2008; Shonhai et al., 2008). Briefly, the aggregation of MDH in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 48°C was monitored using a Helios Alpha DB spectrophotometer with a Peltier-controlled cell, measuring light scatter due to protein aggregates at 360 nm over a 30 min period. As shown previously (Shonhai et al., 2008), the aggregation suppression activity of PfHsp70-1 was found to be ATP dependent, with ATP lowering the affinity of PfHsp70-1 for MDH (data not shown). The MDH assay was adapted for use as a screening tool for potential modulators of PfHsp70-1 chaperone activity, specifically the ability to suppress the thermal aggregation of a substrate protein. MDH (pig heart, Roche, Mannheim, Germany) was used at a concentration of 0.72 μM and recombinant PfHsp70-1 and *M. sativa* Hsp70 (purchased from Alfa Biogene International, Germany) were used at a concentration of 0.36 μM to give a level of aggregation suppression which would allow detection of both stimulation and inhibition by the various test compounds. Before screening, a spectral scan of each compound was performed at a range of 200–900 nm, to ensure that the compounds had no effect on absorbance at or around 360 nm. None of the compounds showed any significant absorbance above 300 nm (data not shown). The compounds (marine prenylated alkaloids and naphthoquinones) were dissolved in DMSO and screened at a final concentration of 300 μM in the assay. The compounds were briefly incubated with the chaperone prior to addition of MDH. Only those compounds that showed an effect on the aggregation suppression activity of PfHsp70-1 activity at 300 μM were further assayed at a range of concentrations (0.36, 1, 10, 100, 200 and 300 μM) on at least two independently purified batches of PfHsp70-1. DMSO was shown to have no effect on PfHsp70-1 activity in the assay in a reaction in which DMSO was included at 1% (the maximum final concentration in inhibition experiments). Control reactions without MDH were carried out with DMSO alone, the test compounds alone, the chaperones alone and the chaperones with each compound in addition to DMSO. These were conducted

to ensure that any reversal of aggregation suppression activity in a test reaction was not due to aggregates formed by either the compounds themselves or the compounds in combination with the chaperone or by the chaperone itself (data not shown). In addition, each compound was tested at 300 μM with MDH alone (no chaperone) to assess the effect of the compounds on MDH aggregation.

Growth inhibition assays

Plasmodium falciparum 3D7 parasites were cultured according to Trager and Jensen (1976). Growth inhibition assays were conducted on *P. falciparum* 3D7-infected erythrocytes using the LDH method and a starting parasitaemia of 0.1% (Makler et al., 1993). A 'no compound' control was included on each plate and was taken as the '100% growth' value for that plate. Each assay plate also included cyclohexamide treated cultures to account for any initial or background LDH activity. These values were subtracted from all other LDH activities. The reported IC_{50} values and the accompanying variation represent the 95% confidence interval of IC_{50} values as calculated using GraphPad Prism 4 (San Diego, CA, USA) software.

Acknowledgements

This research was funded by a Deutsche Forschungsgemeinschaft (DFG) German-African Cooperation Project in Infectology grant (DFG [Ref: LI 402/12-0]). In addition, funding from the National Research Foundation (South Africa) and the Department of Environmental Affairs through the SEACHANGE program is gratefully acknowledged. ILC was awarded Rhodes University and Deutscher Akademischer Austausch Dienst (DAAD) Masters bursaries, ERP is a Claude Leon Foundation Postdoctoral Fellow, LLS was awarded a South African Malaria Initiative (SAMI) Postdoctoral Fellowship and PGKC holds VUW Masters and Woolf-Fisher Scholarships. The authors would like to thank Dr. Stefan Baumeister (Philipps University Marburg, Germany) for his help with the GraphPad software.

References

Acharya, P., Kumar, R., and Tatu, U. (2007). Chaperoning a cellular upheaval in malaria: heat shock proteins in *Plasmodium falciparum*. Mol. Biochem. Parasitol. *153*, 85–94.

Blatch, G.L. and Lässle, M. (1999). The tetracopeptide repeat: a structural motif mediating protein-protein interactions. BioEssays *21*, 932–939.

Bonifazi, E.L., Ríos-Luci, C., León, L.G., Burton, G., Padrón, J.M., and Misico, R.I. (2010). Antiproliferative activity of synthetic naphthoquinones related to lapachol. First synthesis of 5-hydroxylapachol. Bioorg. Med. Chem. *18*, 2621–2630.

Boshoff, A., Stephens, L.L., and Blatch, G.L. (2008). The *Agrobacterium tumefaciens* DnaK: ATPase cycle, oligomeric state and chaperone properties. Int. J. Biochem. Cell Biol. *40*, 804–812.

Botha, M., Pesce, E.R., and Blatch, G.L. (2007). The Hsp40 proteins of *Plasmodium falciparum* and other apicomplexa: regulating chaperone power in the parasite and the host. Int. J. Biochem. Cell Biol. *39*, 1781–1803.

Brodsky, J. (1999). Selectivity of the molecular chaperone-specific immunosuppressive agent 15-deoxyspergualin: modulation of Hsc70 ATPase activity without compromising DnaJ chaperone interactions. Biochem. Pharmacol. *57*, 877–880.

Brodsky, J.L. and Chiosis, G. (2006). Hsp70 molecular chaperones: emerging roles in human disease and identification of small molecule modulators. Curr. Top. Med. Chem. *6*, 1215–1225.

Cheetham, M.E. and Caplan, A.J. (1998). Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. Cell Stress Chaperones *3*, 28–36.

Cheetham, M.E., Jackson, P., and Anderton, B.H. (1994). Regulation of 70 kDa heat-shock protein ATPase activity and substrate binding by human DnaJ-like proteins, HSJ1a and HSJ1b. Eur. J. Biochem. *226*, 99–107.

Chiang, A.N., Valderramos, J.C., Balachandran, R., Chovatiya, R.J., Mead, B.P., Scneider, C., Bell, S.L., Klein, M.G., Huryn, D.M., Chen, X.S., et al. (2009). Select pyrimidinones inhibit the propagation of the malarial parasite, *Plasmodium falciparum*. Bioorg. Med. Chem. *17*, 1527–1533.

da Silva Júnior, E.N., de Souza, M.C.B.V., Fernandes, M.C., Menna-Barreto, R.F.S., Pinto, M.C.F.R., Lopes, F.A., de Simone, C.A., Andrade, C.K.Z., Pinto, A.V., Ferreira, V.F., et al. (2008). Synthesis and anti-*Trypanosoma cruzi* activity of derivatives from nor-lapachones and lapachones. Bioorg. Med. Chem. *16*, 5030–5038.

da Silva Júnior, E.N., Pinto, M.C.F.R., de Moura, K.C.G., de Simone, C.A., Nascimento, C.J., Andrade, C.K.Z., and Pinto, A.V. (2009). Hooker's 'lapachol peroxide' revisited. Tetrahedron Lett. *50*, 1575–1577.

da Silva Júnior, E.N., de Deus, C.F., Cavalcanti, B.F., Pessoa, C., Costa-Lotufo, L.V., Montenegro, R.C., de Moraes, M.O., Pinto, M.C.F.R., de Simone, C.A., Ferreira, V.F., et al. (2010). 3-Arylamino and 3-alkoxy-nor-β-lapachone derivatives: synthesis and cytotoxicity against cancer cell lines. J. Med. Chem. *53*, 504–508.

de Andrade-Neto, V.F., Goulart, M.O.F., da Silva Filho J.F., da Silva, M.J., Pinto, M.C.F.R., Pinto, A.V., Zalis, M.G., Carvalho, L.H., and Krettli, A.U. (2004). Antimalarial activity of phenazines from lapachol, β-lapachone and its derivatives against *Plasmodium falciparum in vitro* and *Plasmodium berghei in vivo*. Bioorg. Med. Chem. Lett. *14*, 1145–1149.

Evans, C.G., Chang, L., and Gestwicki, J.E. (2010). Heat shock protein 70 (Hsp70) as an emerging drug target. J. Med. Chem. *53*, 4585–4602.

Ferreira, V.F., Jorqueira, A., Souza, A.M.T., da Silva, M.N., de Souza, M.C.B.V., Gouvêa, R.M., Rodrigues, C.R., Pinto, A.V., Castro, H.C., Santos, D.O., et al. (2006). Trypanocidal agents with low cytotoxicity to mammalian cell line: a comparison of the theoretical and biological features of lapachone derivatives. Bioorg. Med. Chem. *14*, 5459–5466.

Fewell, S.W., Smith, C.M., Lyon, M.A., Dumitrescu, T.P., Wipf, P., Day, B.W., and Brodsky, J.L. (2004). Small molecule modulators of endogenous and co-chaperone-stimulated Hsp70 ATPase activity. J. Biol. Chem. *279*, 51131–51140.

Haldar, K. and Mohandas, N. (2007). Erythrocyte remodeling by malaria parasites. Curr. Opin. Hematol. *14*, 203–209.

Jinwal, U.K., Miyata, Y., Koren, J., Jones, J.R., Trotter, J.H., Chang, L., O'Leary, J., Morgan, D., Lee, D.C., Shults, C.L., et al. (2009). Chemical manipulation of Hsp70 ATPase activity regulates tau stability. J. Neurosci. *29*, 12079–12088.

Kapadia, G.J., Azuine, M.A., Balasubramanian, V., and Sridhar, R. (2001) Aminonaphthoquinones – a novel class of compounds with potent antimalarial activity against *Plasmodium falciparum*. Pharmacol. Res. *43*, 363–367.

Keyzers, R.A., Gray, C.A., Schleyer, M.H., Whibley, C.E., Hendricks D.T., and Davies-Coleman, M.T. (2006). Malonganenones A–C, novel tetraprenylated alkaloids from the Mozambique gorgonian *Leptogorgia gilchristi*. Tetrahedron. *62*, 2200–2206.

Kumar, N., Koski, G., Masakadu, H., Masamichi, A., and Hong, Z. (1991). Induction and localization of *Plasmodium falciparum* stress proteins related to the heat shock protein 70 family. Mol. Biochem. Parasitol. *48*, 47–58.

Kumar, R., Musiyenko, A., and Barik, S. (2003). The heat shock protein 90 of *Plasmodium falciparum* and antimalarial activity of its inhibitor, geldanamycin. Malar. J. *2*, 30.

Makler, M.T., Ries, J.M., Williams, J.A., Bancroft, J.E., Piper, R.C., Gibbins, B.L., and Hinrichs, D.J. (1993). Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. Am. J. Trop. Med. Hyg. *48*, 739–741.

Massey, A.J. (2010). ATPases as drug targets: insights from heat shock proteins 70 and 90. J. Med. Chem. *53*, 7280–7286.

Matambo, T.S., Odunuga, O.O., Boshoff, A., and Blatch, G.L. (2004). Overproduction, purification, and characterization of the *Plasmodium falciparum* heat shock protein 70. Protein Expr. Purif. *33*, 214–222.

Mayer, M.P. and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol. Life Sci. *62*, 670–684.

Misra, G. and Ramachandran, R. (2009). Hsp70-1 from *Plasmodium falciparum:* protein stability, domain analysis and chaperone activity. Biophys. Chem. *142*, 55–64.

Neckers, L. and Tatu, U. (2008). Molecular chaperones in pathogen virulence: emerging new targets for therapy. Cell Host Microbe. *4*, 519–527.

Pérez-Sacau, E., Estévez-Braun, A., Ravelo, A.G., Yapu, D.G., and Turba, A.G. (2005). Antiplasmodial activity of naphthoquinones related to lapachol and β-lapachone. Chem. Biodivers. *2*, 264–274.

Pesce, E.-R., Acharya, P., Tatu, U., Nicoll, W.S., Shonhai, A., Hoppe, H.C., and Blatch, G.L. (2008). The *Plasmodium falciparum* heat shock protein 40, Pfj4, associates with heat shock protein 70 and shows similar heat induction and localization patterns. Int. J. Biochem. Cell Biol. *40*, 2914–2926.

Ramya T., Surolia, N., and Surolia, A. (2006). 15-Deoxyspergualin modulates *Plasmodium falciparum* heat shock protein function. Biochem. Biophys. Res. Commun. *348*, 585–592.

Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F.U., and Moarefi, I. (2000). Structure of TPR domain–peptide complexes: critical elements in the assembly of the Hsp70–Hsp90 multichaperone machine. Cell *101*, 199–210.

Sharma, Y.D. (1992). Structure and possible function of heat-shock proteins in *Falciparum* malaria. Comp. Biochem. Physiol. *102B*, 437–444.

Shonhai, A., Boshoff, A., and Blatch, G.L. (2005). *Plasmodium falciparum* heat shock protein 70 is able to suppress the thermosensitivity of an *Escherichia coli* DnaK mutant strain. Mol. Genet. Genomics. *274*, 70–78.

Shonhai, A., Boshoff, A., and Blatch, G.L. (2007). The structural and functional diversity of Hsp70 proteins from *Plasmodium falciparum*. Protein Sci. *16*, 1803–1818.

Shonhai, A., Botha, M., De Beer, T.J., Boshoff, A., and Blatch, G.L. (2008). Structure-function study of a *Plasmodium falciparum* Hsp70 using three dimensional modelling and *in vitro* analyses. Protein Pept. Lett. *15*, 1117–1125.

Silva, R.S.F., Costa, E.M., Trindade, U.L.T., Teixeira, D.V., Pinto, M.C.F.R., Santos, G.L., Malta, V.R.S., de Simone, C.A., Pinto, A.V., and de Castro, S.L. (2006). Synthesis of naphthofuranquinones with activity against *Trypanosoma cruzi*. Eur. J. Med. Chem. *41*, 526–530.

Trager, W. and Jensen, J.B. (1976). Human malaria parasites in continuous culture. Science *193*, 673–675.

Tuteja, R. (2007). Malaria – an overview. FEBS J. *274*, 4670–4679.

Westerheide, S.D., Bosman, J.D., Mbadugha, B.N., Kawahara, T.L., Matsumoto, G., Kim, S., Gu, W., Devlin, J.P., Silverman, R.B., and Morimoto R.I. (2004). Celastrols as inducers of the heat shock response and cytoprotection. J. Biol. Chem. *279*, 56053–56060.

Williamson, D.S., Borgognoni, J., Clay, A., Daniels, Z., Dokurno, P., Drysdale, M.J., Foloppe, N., Francis, G.L., Graham, C.J., Howes, R., et al. (2009). Novel adenosine-derived inhibitors of 70 kDa heat shock protein, discovered through structure-based design. J. Med. Chem. *52*, 1510–1513.

Wisén, S., Androsavich, J., Evans, C.G., Chang, L., and Gestwicki, J.E. (2008). Chemical modulators of heat shock protein 70 (Hsp70) by sequential, microwave-accelerated reactions on solid phase. Bioorgan. Med. Chem. *18*, 60–65.

Wisén, S., Bertelsen, E.B., Thompson, A.D., Patury, S., Ung, P.M.-U., Chang, L., Evans, C.G., Walter, G.M., Wipf, P., Carlson, H.A., et al. (2010). Binding of a small molecule at a protein-protein interface regulates the chaperone activity of Hsp70-Hsp40. ACS Chem. Biol. *5*, 611–622.

Wright, C.M., Chovatiya, R.J., Jameson, N.E., Turner, D.M., Zhu, G., Werner, S., Huryn, D.M., Pipas, J.M., Day, B.W., Wipf, P., et al. (2008). Pyrimidinone-peptoid hybrid molecules with distinct effects on molecular chaperone function and cell proliferation. Bioorg. Med. Chem. *16*, 3291–3301.

Yamamoto, S., Nakano, S., Owari, K., Fuziwara, K., Ogawa, N., Otaka, M., Tamaki, K., Watanabe, S., Komatsuda, A., Wakui, H., et al. (2010). Gentamicin inhibits HSP70-assisted protein folding by interfering with substrate recognition. FEBS Lett. *584*, 645–651.

Received November 8, 2010; accepted December 17, 2010