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14. ABSTRACT
In malaria, drug resistance is a major treat to disease control efforts. Unfortunately, there is a significant knowledge gap in our understanding of the molecular mechanisms behind these phenomena. The current report provides an update in our efforts to explored the role of GRP78, a protein chaperone from the stress response, in arteminisin resistant parasites. The GRP78 expression at the mRNA and protein levels was evaluated in artemisinin sensitive and resistant Plasmodium falciparum strains. The results indicated a low base line GRP78 protein levels in the drug resistant parasites, and these levels did not increase significantly upon artemisinin exposure in any of the strains evaluated. A role for the chaperone in the recovery period after drug exposure will be investigated during the last period of this award. Additionally, P. falciparum GRP78 was expressed and purified from a heterologous system. The recombinant protein was used to characterize the binding of GRP78 inhibitors identified in the literature. The inhibitors were also evaluated in the growth inhibition activity against P. falciparum. This information identified inhibitors that preferentially affected PfGRP78, and the selected compounds will be used in the last period of the grant to evaluate the chaperone role upon artemsisin exposure.

15. SUBJECT TERMS
Malaria, artemisisin, drug resistance, chaperones, GRP78, stress response.

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1. INTRODUCTION:

One third of the world population lives in zones at risk of contracting malaria, a treatable vector borne infectious disease. The current recommended standard treatments are artemisinin combination therapies (ACTs), which are very effective against *Plasmodium falciparum*, the parasite responsible for over 90% of malaria related deaths. Unfortunately, reports of artemisinin resistant *P. falciparum* strains from Southeast Asia raise the spectrum of treatment failure and increase mortality. Our project goal is to determine the role of GRP78 (Glucose-regulated Protein 78kDa) in artemisinin resistance *P. falciparum* parasites. Research on artemisinin resistant parasites has revealed a significant increase in the expression of genes associated with the stress response including GRP78. We proposed that *P. falciparum* artemisinin resistant parasites upregulate their stress pathway compared with sensitive ones. Therefore, inhibition of GRP78 could restore parasite sensitivity to artemisinin. To accomplish our goals, we have been developing the following objectives: 1) characterization of GRP78 expression in artemisinin resistant *P. falciparum* parasites; and 2) determine if inhibition of GRP78 in artemisinin resistant *P. falciparum* parasites reverts resistance.

2. KEYWORDS:

Malaria, Plasmodium, falciparum, artemisinin, drug resistance, stress response, chaperone, GRP78, UPR, inhibitors.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Identify the expression and activity levels of GRP78 in artemisinin sensitive and resistant *P. falciparum* parasites		
Major Task 1: Characterize expression levels of GRP78 in different strains of *P. falciparum*	**Months**	**COMPLETION**
Subtask 1: Examine GRP78 mRNA levels in *Plasmodium* parasites using RT-qPCR Parasite strains used: 3D7, W2, MRA-1236, MRA-1239 and MRA-1241 [MR4]	1-3	100%
Subtask 2: Examine GRP78 expression levels in *Plasmodium* parasites by western blot. Parasite strains used: 3D7, W2, MRA-1236, MRA-1239 and MRA-1241 [MR4]	1-3	100%
Milestone(s) Achieved: determination of GRP78 expression in various Plasmodium *strains sensitive and resistant to artemisinin.*	3	**Achieved**
Major Task 2: Characterize expression levels of GRP78 in different strains of *P. falciparum* upon exposure to artemisinin	**Months**	
Subtask 1: Examine GRP78 mRNA levels in *Plasmodium* parasites exposed to artemisinin using RT-qPCR Parasite strains used: 3D7, W2, MRA-1236, MRA-1239 and MRA-1241 [MR4]	3-9	100%
Subtask 2: Examine GRP78 expression levels in *Plasmodium* parasites exposed to artemisinin by western blot. Parasite strains used: 3D7, W2, MRA-1236, MRA-1239 and	3-9	100%

MRA-1241 [MR4]		
Milestone(s) Achieved: Characterization of GRP78 expression levels in artemisinin sensitive and resistant P. falciparum *strains after exposure to artemisinin.*	9	**Achieved**
Specific Aim 2: Determine if the inhibition of GRP78 will have an impact in artemisinin resistance parasites		
Major Task 1: Characterize the effects of GRP78 inhibition in *Plasmodium* growth and viability *in vitro*		
Subtask 1: Determine the IC_{50} of GRP78 inhibitors against artemisinin sensitive and resistant *P. falciparum* strains in an *in vitro* growth inhibition assay. Parasite strains used: 3D7, W2, MRA-1236 and MRA-1241 [MR4]	8-10	50%
Subtask 2: Examine GRP78 expression levels in *Plasmodium* parasites exposed to GRP78 inhibitors by western blot. Parasite strains used: 3D7, W2, MRA-1236 and MRA-1241 [MR4]	8-10	0%
Subtask 3: Determine the effect on parasite viability of exposing parasites cultures to artemisinin alone or in combination with non-lethal concentrations of GRP78 inhibitors. Parasite strains used: 3D7, W2, MRA-1236 and MRA-1241 [MR4]	12-18	0%
Milestone(s) Achieved: Determination of the growth inhibitory potential of GRP78 inhibitors against P. falciparum*, and its effects in combination with artemisinin.*	18	**Not achieved**
Major Task 2: Expression of recombinant GRP78 for binding studies		
Subtask 1: Generate bacterial expression plasmids for human and *P. falciparum* GRP78 nucleotide binding domains.	10-12	100%
Subtask 2: Expression and purification of recombinant GRP78 nucleotide binding domains.	10-12	100%
Subtask 3: Determination of affinity constants for GRP78 inhibitors used in 2.1.1 using recombinant GRP78 domains purified in 2.2.2	10-14	100%
Milestone(s) Achieved: Characterization of binding affinities between human and P. falciparum *GRP78 nucleotide binding domains and inhibitors.*	14	**Achieved**
Milestone(s) Achieved: Characterization of effects of GRP78 on artemisinin resistance; publication of 1-2 peer reviewed papers	18	**Not achieved**

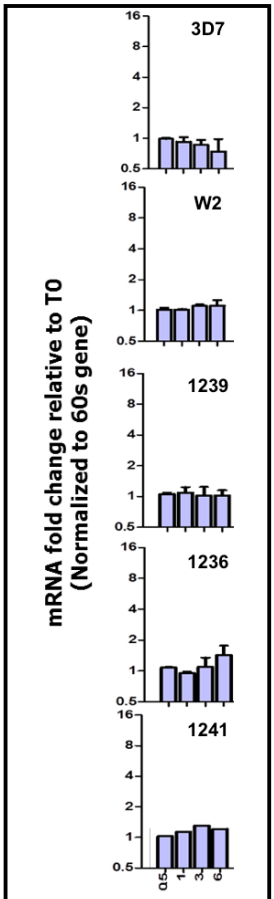

Figure 1. GRP78 mRNA level time-course in five *P. falciparum* strains with different levels of susceptibility to DHA. Gene expression normalized to

What was accomplished under these goals?

Aim 1: Identify the expression and activity levels of GRP78 in artemisinin sensitive and resistant *P. falciparum* parasites

Characterize DHA susceptibility of different *P. falciparum* strains

Parasites included in this study were chosen according their reported DHA susceptibility. To verify parasite maintained their differential susceptibility during the experimental conditions, we determined the survival rates for all strain after each assay (RSA assay). Survival rates were expressed as a percentage of viable parasites after 66 hours post drug removal compared with untreated controls (Table 1).

Characterize expression levels of GRP78 in different strains of *P. falciparum*

GRP78 mRNA levels in Plasmodium *parasites:* Gene expression of GRP78 chaperone was determined for ring stage parasites in a 6h time course. The mRNA levels were determined at 0.5, 1, 3 and 6 hours. These results provide the base line for the GRP78 expression of all *P. falciparum* strains used (Figure 1).

Table 1. RSA values of used *P. falciparum* strains.

Parasite Strain	RSA $^{0-4h}$ Survival rate* % (range)
3D7	2.76 (0.20 -7.25)
W2	2.79 (1.6 – 4.7)
1239	0.26 (0.10 – 0.53)
1236	12.91 (4.92 -18.64)
1241	32.04 (19.8 – 52.57)

GRP78 expression levels in Plasmodium *parasites by western blot:* Blot of parasites in basal conditions (ring-stage 0-4 hours) indicate differential expression of GRP78 between the five strains (Figure 2A). The cambodian strains showed less GRP78 compared to the reference strains 3D7 and W2. Between the samples, MRA-1236 had the lowest amount of GRP78. Our results did not suggest a correlation between basal expression of GRP78 and susceptibility to DHA. Because, the susceptible cambodian strain MRA-1239 showed similar GRP78 protein level to the less susceptible MRA-1241 (Figure 2B).

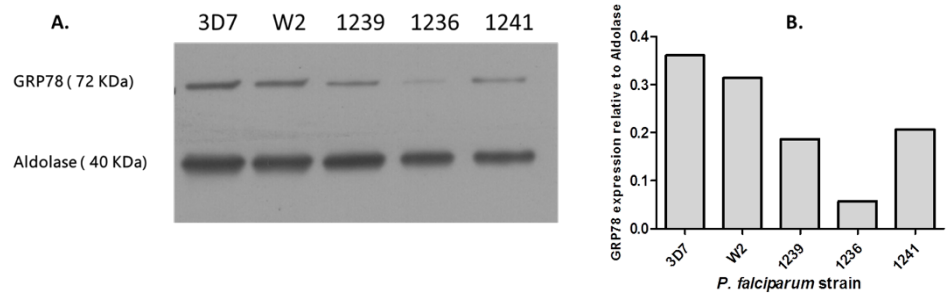

Figure 2. Basal GRP78 expression in five *P. falciparum* strains. (**A**) Western blot of parasites synchronized at ring stage 0-4 hours and stained with antibodies against PfGRP78 and PfAldolase. (**B**) Band intensities were estimated with the Image Studio™ Lite software and expressed as the ratio between GRP78 and Aldolase.

Characterize expression levels of GRP78 in different strains of *P. falciparum* upon exposure to artemisinin

GRP78 mRNA levels in Plasmodium *parasites exposed to artemisinin:* RT-qPCR was used to evaluate mRNA levels of GRP78 in *Plasmodium* parasite cultures exposed to 100nM DHA. The treated parasites did not alter the chaperone transcript levels in a statistically significant manner during the DHA exposition time. In fact, the mRNA levels showed similar changes as a reference gene seryl-tRNA synthetase (Figure 3).

GRP78 expression levels in Plasmodium *parasites exposed to DHA:* During the monitored period, there was no significant changes in the quantity of GRP78 protein between DHA exposed and control parasite cultures (Figure 4).

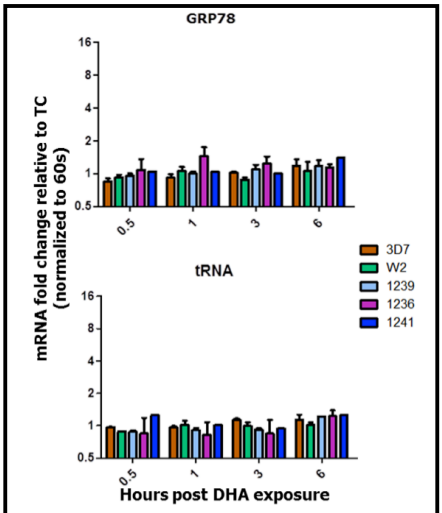

Figure 3. GRP78 chaperone expression between *P. falciparum* strains upon DHA exposure. The mRNA levels GRP78 measured during treatment with DHA. Values were normalized to 60S ribosomal subunit protein and reported relative to respective control parasites at each time point.

In conclusion, the results suggest the response mechanism in tolerant parasites strains is not associated with change the GRP78 chaperone at least during the 6 hours of the DHA treatment. We could not rule out an important role for the chaperone yet, because the differences between strains could be associated with the recovery phase once DHA is removed after 6 hours of treatment.

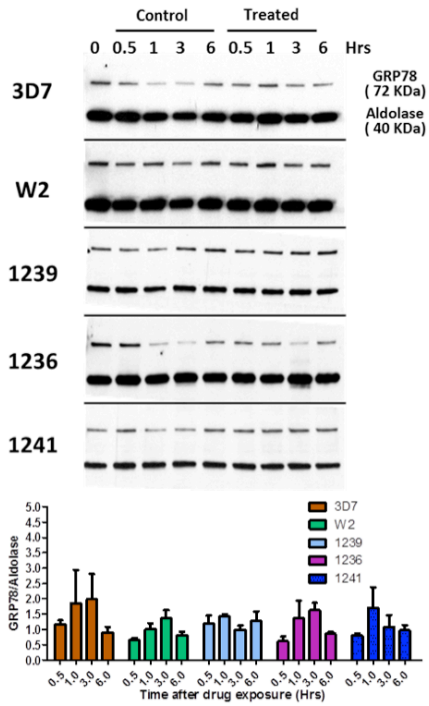

Figure 4. Time course of GRP78 protein levels in *P. falciparum* strains upon DHA exposure. (*top*) Representative western-blots of PfGRP78/PfAldolase levels from control and DHA exposed parasite cultures at different time points. (*bottom*) Protein levels are shown as the band intensity chaperone/Aldolase ratio, and normalized to expression control parasite cultures. Results are presented as mean +/- SEM of three biological replicates

Materials and methods:

Parasites cultures: Two *P. falciparum* reference strains sensitive to DHA (3D7 and W2) and three parasite lines with different levels of susceptibility to DHA (isolated and culture adapted from Cambodian patients) were used to evaluate the chaperone expression under basal condition and during DHA exposure. The Cambodian parasites coded as MRA-1239, MRA-1236 and MRA-1241 were obtained from the Malaria Research and Reagent Resource Repository (MR4) - BEI Resources. The blood-stage asexual parasites were maintained in continuous culture under the standard conditions previously described in this document. Parasites were synchronized to obtain rings between 0 and 4 hours post erythrocyte invasion using the methodology de-

scribed by [1]. Cultures with parasitaemia higher than 5% were exposed to cycles of synchronization with sorbitol at 5 and 15 hours after the transition between schizont and rings. One cycle before the experiment, schizonts were harvested using Accudenz. A parasite suspension at hematocrit of ~2% was underlid with Accudenz solution 60% in PBS 1x and centrifuged 20 minutes at 200g with acceleration 5/deceleration 4 in Sorvall ST 8 centrifuge (Thermofisher). The parasites were harvested from the parasitized cell layer containing schizonts. Recovery parasites were washed with RPMI and returned to culture. Parasites were monitored until they contained again a significant fraction of bursting schizonts and the rings (newly invaded RBCs) begin to appear. After 2-3 more hours of incubation, the cultures were treated with sorbitol 5% to remove schizont remainder and let rings between 0 and 4 hours.

Drug exposure: A 52 ml suspension of ring-stage parasites (0-4 h post-invasion of RBCs) at 4% parasitaemia and 1.5% of hematocrit were split in 2 50ml tubes (22 ml each one) and 1 15ml tube (5ml). The parasites in the 50ml tubes were exposed to DHA 100nM or DMSO 0.014%. The parasite exposed were split in 4 culture dishes of 5 ml each one. The remainder from each tube was used to check final parasitaemia, hematocrit and conduct a Ring Survival Assay modified. The 5ml suspension in the 15ml tube corresponds to the control time cero and was processed immediately for protein and RNA extraction. Culture dishes contained control (DMSO) and treated (DHA) parasites were incubated at 37˚C in 5% O_2 and 5% CO_2 atmosphere. One of each control and treated group were harvest at time 0.5, 1, 3 and 6 hours post exposure. Samples were processed immediately they were harvested. The parasites were centrifuged 5 min at 5000g and washed twice with PBS 1x 5000g for 1 min at 4˚C. Pellet were resuspended with 1ml of 0.02% saponin-PBS solution (Sigma, Aldrich) and centrifuged 10 min 11000g at 4˚C. Parasite pellet was washed twice with PBS 1x 11000g 10 min at 4˚C. The pellet was resuspended with 100µl of PBS and 30ul were separated and mixed with 70µl of Laemlii lysis buffer (BioRad) and 7µl of BME (Thermofisher) and heated at 100C for 10 min and stored at -20C until use for western blot. The remainder 70µl were mix with 200µl of lysis buffer (NucleoSpin® RNA Plus, Macherey-Nagel GmbH & Co.) and keep at -80˚C until mRNA extraction was conducted.

Ring-stage Survival Assay (RSA): We used the RSA in this study to verify the differential levels of DHA susceptibility in the parasites strains evaluated. This method allows detect difference in the susceptibility to DHA that are not capture in the regular growth inhibition assay. Additionally, this method is well correlated with the in vivo response to DHA. A 200µl aliquots of the parasite exposed to DMSO 0.014% and DHA 100nM (control and treated respectively) were incubated for 6 hours in a 96 wells plate at 37˚C in 5% O_2 and 5% CO_2 atmosphere. After 6 hours of incubation, the media was removed and the parasites were washed twice with RPMI, resuspended in 200µl of complete media and returned to incubation for an additional 66 hours. Culture from each well was transferred into a 1.5ml microcentrifuge tube and centrifuged 5min at 800g. Approximately 2-5µl of the pellet was used to make a thin blood smear from control and treated samples. Smears were fixed with methanol and stained with Giemsa 10%. Parasitaemia of viable parasite was determined at 100x magnification under immersion oil in a total of 5,000 RBCs for both samples. The percentage of survival was calculated dividing the parasitaemia in the treated sample by the parasitaemia in the control sample.

Quantitative reverse transcriptase PCR (qRT-PCR): RNA from controls and treated samples from each time point were extracted using the NucleoSpin® RNA Plus Kit (Machery-Nagel, Germany). RNA was quantified using NanoDrop Spectrophotometer (A_{260}) and treated with DNAse I according to the protocol supplied by the manufacturer (Ambion). DNAse I was inactivated at 75˚C for 10 min after the addition of EDTA at final concentration of 5mM. RNA was

[1] Stanley C Xie et al., "Optimal Assay Design for Determining the in Vitro Sensitivity of Ring Stage Plasmodium Falciparum to Artemisinins.," *International Journal for Parasitology* 44, no. 12 (October 15, 2014): 893–99, doi:10.1016/j.ijpara.2014.07.008.

stored at -80˚C until it was used. The GRP78 (PF3D7_0917000) gene expression was assessed. The seryl-tRNA synthetase (PF07-0073) and the 60S ribosomal subunit protein L18 (MAL13P1.209) were used as reference genes to normalize the chaperones levels. All samples were run in triplicate using One Step RT-qPCR (BioRad) according to the manufacturer's instructions. The following cycling conditions were used: 10 mins at 50˚C, 1 min at 95˚C for initial denaturation and enzyme activation, followed by 40 cycles of 95˚C for 10 sec, 58.4˚C for 30 secs, followed by a melting curve analysis beginning at 65˚C with a gradual increase in temperature of 0.5˚C/5s to 95˚C. The mean C_t and the relative expression ($\Delta\Delta C_t$ method) was determined using Bio-Rad CFX Manager 3.1 Software (Bio-Rad). Controls were analyzed relative to sample Time cero and treated samples were analyzed relative to controls at the same time point. Difference in the gene expression due to DHA exposure was evaluated using analysis of variance (ANOVA)

Quantification of GRP78 protein expression: The GRP78 protein levels were determined by western blot. 10 µl of sample from each time point were separated by SDS-PAGE 4-20% gradient gel (BioRad) run 10 min at 60V followed by 50 min 130V. Proteins were then transferred to PVDF membrane using Trans-Blot Turbo Transfer System (BioRad). After blocking overnight in nonfat dry milk 3% in TBS-T, the membrane was incubated with polyclonal sera specific for *P. falciparum* GRP78 (provided by Dr. Kumar) and anti-*P. falciparum* aldolase. Posteriorly, membrane was incubated with donkey anti-rabbit HRP-labeled antibodies. The blot was developed using Pierce ECL Western Blotting Substracte (ThermoFisher Scientific) and the chemiluminescent signal was capture with autoradiography film (Genesse Scientific). Western blots signal was analyzed using Image Studio Lite software (LI-COR Biosciencess). Signal was normalized using aldolase.

Aim 2: Determine if the inhibition of GRP78 will have an impact in artemisinin resistance parasites

Characterize the effects of GRP78 inhibition in *Plasmodium* growth and viability *in vitro*

A major goal of our study was to evaluate the drug target potential of *P. falciparum* GRP78, thus we evaluated the effect of the chaperon inhibition in the malaria parasite. Our approach took advantage of several GRP78 inhibitors previously characterized against cancer cell lines, which were commercially available[2]. These compounds were tested against two different parasite strains with opposite sensitivity towards the antimalarial drug chloroquine, 3D7 (sensitive) and W2 (resistant). *In vitro* cultures of these malaria parasites were exposed to a concentration series of each compound in a growth inhibition assay to determine its half maximal effective concentration (EC_{50}) (Table 2). The five compounds tested showed strong antiplasmodial activity

[2] Hyungseoph J Cho et al., "A Small Molecule That Binds to an ATPase Domain of Hsc70 Promotes Membrane Trafficking of Mutant Cystic Fibrosis Transmembrane Conductance Regulator.," *Journal of the American Chemical Society* 133, no. 50 (December 21, 2011): 20267–76, doi:10.1021/ja206762p; Jessica R Kirshner et al., "Elesclomol Induces Cancer Cell Apoptosis Through Oxidative Stress," *Molecular Cancer Therapeutics* 7, no. 8 (August 1, 2008): 2319–27, doi:10.1158/1535-7163.MCT-08-0298; Alba T Macias et al., "Adenosine-Derived Inhibitors of 78 kDa Glucose Regulated Protein (Grp78) ATPase: Insights Into Isoform Selectivity.," *Journal of Medicinal Chemistry* 54, no. 12 (June 23, 2011): 4034–41, doi:10.1021/jm101625x; A Matsumoto and P C Hanawalt, "Histone H3 and Heat Shock Protein GRP78 Are Selectively Cross-Linked to DNA by Photoactivated Gilvocarcin v in Human Fibroblasts.," *Cancer Research* 60, no. 14 (July 15, 2000): 3921–26; Aikaterini Rousaki et al., "Allosteric Drugs: the Interaction of Antitumor Compound MKT-077 with Human Hsp70 Chaperones.," *Journal of Molecular Biology* 411, no. 3 (August 19, 2011): 614–32, doi:10.1016/j.jmb.2011.06.003.

with MKT-077, Gilvocarcin A and Elesclomol having submicromolar EC_{50} values, which were in the same range as the antimalarial drug chloroquine. The two remaining compounds, Apoptozole and VER-155008, were less toxic against the malaria parasite with EC_{50}s in the micromolar range. However, these GRP78 inhibitors showed stronger activity against the chloroquine resistant *P. falciparum* strain W2.

TABLE 2. Growth inhibition effect of GRP78 inhibitors against malaria parasites *in vitro* (EC_{50} ± std. dev. in µM).

	Plasmodium falciparum	
	3D7	W2
CQ	0.05 ± 0.011	0.23 ± 0.03
DHA	0.0004 ± 0.00007	0.0001 ± 0.00002
MKT-077	0.07 ± 0.02	0.07 ± 0.03
Apoptozole	6.8 ± 1.4	4.5 ± 0.4
VER-155008	82.1 ± 49.7	39.5 ± 0
Gilvocarcin	0.02 ± 0.004	0.08 ± 0.04
Elesclomol	0.05 ± 0.017	0.06 ± 0.01

Expression of recombinant GRP78 for binding studies

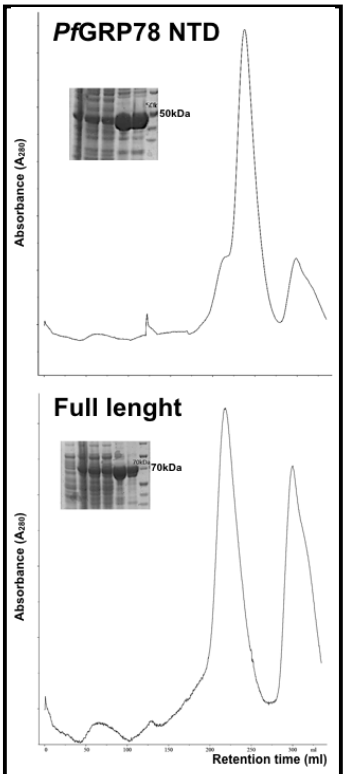

Figure 5. *Pf*GRP78 recombinant protein production. Absorbance traces of the last purification step a step of gel filtration. SDS-page gels representative of the purification protocol (last lane corresponds to the chromatography traces).

Two distinct recombinant *P. falciparum* GRP78 protein constructs were successfully expressed and purified from bacterial culture (Figure 5). The longest construct dubbed PfGRP78-FL, included the nucleotide and the substrate binding domains, residues S24 to K629. Two mutations were introduced in this construct T226A and 449-TYQDNQP-455 to VGG to mimic the ATP bound state. Because, it has been previously shown that these changes are essential to express the full-length protein[3]. The second construct encompassed the NBD (residues I26 to G404) and it will be referred as PfGRP78-NTD. The last protein construct was used for the inhibitor binding studies.

To quantify the binding affinity between PfGRP78-NTD and the selected compounds, dissociation constants (K_d) were determined by surface plasmon resonance (SPR). These results showed a large difference in the ADP and ATP binding affinities between *Plasmodium* and human GRP78 NBD proteins (Table 3). The malaria chaperone showed a 33- and 23-fold decrease in affinity towards ADP and ATP, compared to its human counterpart. The malaria parasite chaperone also showed low affinity for the inhibitor VER-155008. But, both *Plasmodium* and human GRP78 showed similar affinities for Apoptozole and MKT-077. Finally, two compounds were classified as non-binders, Gilvocarcin-A and Elesclomol (Table 3).

Three out of the five tested compounds against *P. falciparum* strains *in*

TABLE 3. Nucleotide and inhibitor binding affinities of *Plasmodium* and human GRP78 determined by SPR (K_d in µM).

	PfGRP78-NTD	huGRP78-NTD
ADP	16.2	0.49
ATP	333	14.0
Apoptozole	4.27	6.9
MKT-077	16.4	16
VER-155008	164	52.7

[3] Jiao Yang et al., "Close and Allosteric Opening of the Polypeptide-Binding Site in a Human Hsp70 Chaperone BiP.," *Structure* 23, no. 12 (December 1, 2015): 2191–2203, doi:10.1016/j.str.2015.10.012.

vitro showed also binding to PfGRP78-NTD. However, there was a very poor correlation between EC_{50} and K_d values for Apoptozole, MKT-077 and VER-155008. The discrepancies between the binding and the antiplasmodial activity showed stronger than expected activity for MKT-077 and VER-155008, while the EC_{50} value recorded for Apoptozole was clearly in line with its K_d. Finally, these three GRP78 inhibitors showed different growth inhibition effects on host cells (Table 2). VER-155008 selectivity index was 3.8 in favor of host cells, but Apoptozole and MKT-077 were 2.9 and 14 times more selective towards *P. falciparum*.

Materials and methods:

Growth Inhibition Assay: A synchronized *P. falciparum* culture, with over 80% ring forms, was used in the growth inhibition assay. Each strain was assayed at 0.5% parasitaemia and 1.5% hematocrit. A 190µL aliquot of parasite culture was dispensed per well to microplates that were pre-dispensed with the compounds at various dilutions. A 10µL aliquot of 11 dilutions per compound had been previously dispensed into a 96-well flat-bottom, in duplicate. Dilutions of VER-155008, Apoptozole and MKT-077 inhibitors and DHA were prepared in water with a final concentration of DMSO below 0.05% (v/v). DMSO at this concentration did not impact *P. falciparum* growth. Control parasites and parasites exposed to CQ dilutions did not contain any DMSO. The microplates were incubated at 37˚C in a controlled atmosphere (5% O_2, 5% CO_2 Nitrogen balanced) for 72 hours. After incubation, the plates were subject to a freeze-thaw cycle (-80˚C) and the parasite growth was quantified by the SYBR Green I method as previously described [4]. Briefly, 100µl of homogenized parasite culture was transferred into 96-well black plates. 100µl of SYBR Green lysis buffer (2x SYBR Green I, 100mM Tris-HCl pH 7.5, 10mM EDTA, 0.016% Saponin and 1.6% Triton X-100) were added to each well. The plates were incubated at room temperature in darkness for 1 hour. The fluorescence signal was read in a Synergy HT (Biotek Instruments, Inc., Winooski, VT) plate reader with $485_{Ex}/520_{Em}$ nm. The 50% inhibitory concentrations (IC_{50}) for each compound were calculated using the online program ICEstimator 1.2 (http://www.antimalarial-icestimator.net/). IC_{50} values were used in the analysis only if the parasite growth index, determined by the ratio of fluorescence units (FU) between the parasite grown without compound versus signal at maximum compound concentration, was equal to or higher than two. Growth inhibition experiments were conducted at least three times per compound. The IC_{50} differences for each inhibitor between the two *P. falciparum* strains were compared with a *t*-test. The statistical analysis was performed with the software Prism v5.01 (GraphPad Software, San Diego, CA).

Cloning of P. falciparum GRP78 and protein expression plasmids: The malaria GRP78 (PlasmoDB ID PF3D7_0917900) was cloned from *P. falciparum* 3D7 cDNA. The ATPase domain named PfGRP78-ATP was obtained by inserting a PCR-generated DNA fragment into vector pET28-MHL (*GenBank* accession EF456735) that included residues I26 to A404. The Y39F mutant PfGRP78-ATP was generated by site-directed mutagenesis of the wild-type construct. A PCR using following long primers that contained the mutant codon (forward – TTGTATTTCCAGGGCATTGAGGGACCCGTTATTGGTATTGACTTGGGTACCACTTTTAGTTG CGTTGGTG; and reverse - CAAGCTTCGTCATCAACCTAAAATAATACCTGCTTGGATAGCAG CACCATAAGCAACAGCTTCATCAGG) (Integrated DNA Technologies, USA. After PCR, methylated DNA template obtained from E. coli was digested by digestion with *DpnI*. DH5a (Thermo Fisher Scientific) competent cells were transformed and selected on Kanamycin/sucrose LB plates. Insert positive colonies were sent for sequencing validation. The modified full-length

[4] David J Bacon et al., "Comparison of a SYBR Green I-Based Assay with a Histidine-Rich Protein II Enzyme-Linked Immunosorbent Assay for in Vitro Antimalarial Drug Efficacy Testing and Application to Clinical Isolates.," *Antimicrobial Agents and Chemotherapy* 51, no. 4 (April 2007): 1172–78, doi:10.1128/AAC.01313-06.

PfGRP78 (residues S24 – K629) sequence was commercially synthesized (Life Technologies Corporation, a Thermo Fisher Scientific). This construct contains two modifications T226A and $_{449}$TYQDNQP$_{455}$ to VGG) to prevent protein aggregation and ATP hydrolysis, as previously described for the human GRP78[5]. Protein expressing plasmids were sequenced validated.
Recombinant protein production and purification: PfGRP78 expression vectors were transformed into BL21(DE3) competent *E. coli*, these cells were used to start a 5mL LB 50 µg/ml kanamycin culture. After shaking at 220 RPM for 4 hours at 37˚C, 4.5 ml were inoculated into 100ml LB flasks and incubated overnight at 37˚C. The 100ml LB culture was transferred into 1L TB cultures and incubated until OD_{600} reached 2.0 to 3.0. Protein production was induced with 200µM IPTG and cell cultures were incubated overnight at 16˚C.Cells were harvested by centrifuging at 3,600 RPM for 20min at 4˚C, and the pellets were frozen at -80˚C until purification. All the PfGRP78 protein constructs were purified using the same protocol. A frozen pellet was thawed and re-suspended in 150ml of buffer containing 20mM Tris-HCl pH 7.5, 200mM NaCl, 1mM 2-βME (Buffer A) with additional 0.1% of IGEPAL, 1mM benzamidine, and 1mM PMSF. Resuspended cells were disrupted by French Pressure under high pressure (22000 psi). Then the lysate was centrifuged at 15000 RPM for 20min at 4°C and the supernatant loaded onto superloop of the fast protein liquid chromatography (FPLC) system for a tandem chromatographic purification, metal affinity and size exclusion chromatography. After the sample was completely loaded onto a Nickel column (NiNTA Quiagen, USA), two wash steps using 2% and 8% of buffer B, Buffer B contained buffer A plus 250mM imidazole. An initial elution fraction was obtained with 20% buffer B, and a second fraction was eluted with 100% buffer B. Both elution samples were collected and named “20W” and “100E”. After metal affinity chromatography, the fractions “20W” and “100E” were individually loaded onto a size exclusion column, HiLoad 26/600 Superdex 200pg (GE Healthcare Life Sciences, USA). 5 ml fractions were collected based on UV signal. The purified protein fractions were evaluated by SDS-Page electrophoresis, and fractions pooled accordingly. Protein concentration was determined by A_{280} spectrophotometric read (Nanodrop 2000, Thermo Fisher Scientific, USA). His-tag purification tag was cleaved with TEV protease using a ratio of 1:50 after a 4°C overnight incubation, followed by Nickel column to remove uncut proteins. For co-crystallization, aliquots of TEV-cleaved PfGRP78 proteins were mixed with 10X fold molar excess of either nucleotides (ADP or ATP) or inhibitors and incubated overnight at 4°C. The protein–nucleotide/inhibitor complexes were concentrated to no less than 30 mg/ml.
Biacore Analysis: SPR measurements were performed on Biacore T200 instrument (GE Healthcare) at 25˚C. Purified *Pf*GRP78-FL, PfGRP78-ATP wild-type and mutant, and human GRP78-ATP were immobilized on a CM5 sensorchip using NHS/EDC coupling following the manufacturer protocol to a level of <10,000 RUs, a reference surfaces without immobilized proteins served as a control for nonspecific binding and refractive index changes. Seven different concentrations of the ligands between 0.4nM to 1mM, were injected in triplicate over the sensor chip at 30µL/min in random order. The running buffer was 10mM HEPES, pH 7.4, 150mM NaCl, 0.005% P20, 1% DMSO and 2mM $MgCl_2$. Buffer alone injections were used as blanks. The Biacore responses recorded during the 30 seconds of the injection were used to estimate the association constant (k_{on}) and the 300 seconds after the injection were used to estimate the dissociation constant (k_{off}). All dissociation and kinetic constants were estimated using double subtracted sensorgrams using BiaEvaluation Software (GE Healthcare). All the reported parasite dissociation constants (K_d) were estimated with affinity analysis, and all the reported human K_ds were calculated from kinetic constants.

[5] Yang et al., “Close and Allosteric Opening of the Polypeptide-Binding Site in a Human Hsp70 Chaperone BiP..”

What opportunities for training and professional development has the project provided?
Two graduate students (one master and one doctoral) have been trained as pas of the project. The master student completed his program requirements and successfully obtained his MSc. from Tulane University, School of Medicine. The doctoral student, Ms. Claribel Murillo has been trained as part of this project and some of the results will be part of her dissertation.

How were the results disseminated to communities of interest?
Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
For the last reporting period, our efforts will be concentrated to complete the major task of the second aim. It includes the characterization of the effects of GRP78 inhibition in *Plasmodium* growth and viability *in vitro.* Additionally, we will be preparing manuscripts to report our findings and also attending the Military Health System Research Symposium (MHSRS).

4. IMPACT:
What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. CHANGES/PROBLEMS:
Nothing to Report.

6. PRODUCTS:
Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Juan C Pizarro
Project Role	Principal investigator
Researcher Identifier (e.g.ORCID ID):	orcid.org/0000-0003-0643-518X
Nearest person month worked:	4
Contribution to Project:	Research design, training, supervision, data analysis and reporting

Name:	Hee-Won Park
Project Role	Co-investigator
Researcher Identifier (e.g.ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Research design, supervision protein production.

Name:	Nirbhay Kumar
Project Role	Co-investigator
Researcher Identifier (e.g.ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Experimental design and supervision.

Name:	Tetyana Antoshchenko
Project Role	Research technician
Researcher Identifier (e.g.ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Protein production and purification

Name:	Claribel Murillo-Solano
Project Role	Graduate student
Researcher Identifier (e.g.ORCID ID):	N/A
Nearest person month worked:	9
Contribution to Project:	Parasite cultures, RNA extraction, RT-qPCR, western-blot and growth inhibition assays.

Name:	Yun Chen
Project Role	Master student
Researcher Identifier (e.g.ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Protein purification and binding studies
Funding Support:	Tulane University

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS
Nothing to Report.

9. APPENDICES:
Nothing to include.