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The adaptive proline response in *P. falciparum* is independent of *Pf*elK1 and eIF2 α signaling

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Abstract

We have previously identified the cytoplasmic prolyl tRNA synthetase in *Plasmodium falciparum* as the functional target of the natural product febrifugine and its synthetic analogue halofuginone (HFG), one of the most potent antimalarials discovered to date. However, our studies also discovered that short-term treatment of asexual blood stage *P. falciparum* with HFG analogues causes a 20-fold increase in intracellular proline, termed Adaptive Proline Response (APR), which renders parasites tolerant to HFG. This novel resistance phenotype lacks an apparent genetic basis but remains stable after drug withdrawal. Based on our findings that HFG treatment induces eIF2 α phosphorylation, a sensitive marker and mediator of cellular stress, we here investigate if eIF2 α -signaling is functionally linked to the APR. In our comparative studies using a parasite line lacking *Pf*elK1, the *Plasmodium* orthologue of the eIF2 α -kinase GCN2 that mediates amino acid deprivation sensing, we show that HFG activity and the APR are independent from *Pf*elK1 and eIF2 α signaling.

Graphical Abstract

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Author Contributions

LF conducted the HFG activity and induction assays, characterized the induced parasites, and wrote the manuscript. AD & CBC carried out the mass spectrometric experiments and analyzed the data. KS, SEB & SAS validated the antibodies and optimized the immunoblotting experiments. JDH & AKL participated in conception of the study and helped to draft the manuscript. DFW & RM conceived of the study, participated in its design and coordination and wrote the manuscript. All authors read and approved the final manuscript.

The authors declare no competing financial interests.

Keywords

malaria; Plasmodium; drug resistance; halofuginone; stress response

With 212 million infections and 429,000 deaths annually, malaria is a parasitic disease of global importance¹. Most of the mortality occurs in children under the age of 5, primarily in sub-Saharan Africa^{2,3}. Increased global vector control measures, together with access to artemisinin combination therapies (ACTs), have markedly reduced malaria-related illness and death in endemic areas⁴. However, emerging resistance to ACTs now threatens global malaria control and elimination efforts^{5,6}. A better understanding of the molecular mechanisms of parasite adaptation to drug regimens is needed to maintain the efficacy of current and future medicines in the global arsenal for malaria control. New antimalarial drugs aimed at previously unexploited parasite targets are needed, and there is a concerted effort in the scientific community to discover and develop novel therapies⁷. One such group of new targets is the family of amino acyl tRNA synthetases, from which several have been identified in *P. falciparum* as targets of potential new lead compounds for drug development^{8–11}.

Our previous studies identified the cytoplasmic prolyl tRNA synthetase in *Plasmodium falciparum* (PfPRS) as the long-sought biochemical target of halofuginone (HFG) and the natural product febrifugine, the active ingredient of the Chinese medicinal plant *Dichroa febrifuga*, which has been used for millennia as a traditional remedy for malaria^{12,13}. During the course of this work we analyzed the temporal evolution of HFG resistance. While long-term selection under increased drug-pressure yielded parasites with point mutations in a single codon in PfPRS that were functionally confirmed to confer high-level resistance to HFG (200 to 300-fold) and related analogues¹⁴, we made the surprising observation that the bulk parasite culture developed tolerance to HFG (10 to 20-fold increase in EC₅₀) within as

little as five generations (10 days)¹⁵. Sequencing of these “phenotypically resistant” parasites did not identify any consistent single nucleotide polymorphisms (SNPs) or amplification of the target *Pf*PRS gene. Notably, the phenotype was retained even when parasites were propagated in drug-free media for more than 50 generations¹⁵. Comprehensive metabolic profiling revealed that the genetically wildtype, but phenotypically resistant parasites, henceforth referred to as HFG-tolerant, have increased levels of free intraparasitic proline (20–30 fold), which is competitive with HFG. Specific changes in amino acid metabolism and homeostasis in response to specific drug exposure constitute an unprecedented and previously unrecognized mechanism of drug tolerance and resistance evolution in *Plasmodium*.

Prior work in other systems has demonstrated that the modulation of proline homeostasis was associated with cellular defense and stress response mechanisms^{16,17}. We have previously shown that treatment of *P. falciparum* with HFG results in the phosphorylation of the eukaryotic initiation factor 2- α (eIF2 α). In eukaryotes, eIF2 α phosphorylation functions as the central mediator of the Integrated Stress Response (ISR) following cellular insult or amino acid deprivation^{14,18}. Insufficient supply of amino acids, or aaRS inhibition, results in accumulation of uncharged tRNAs that bind and activate the eIF2 α kinase GCN2. This process triggers the Amino Acid Response (AAR), a specific component of the ISR, to restore cellular homeostasis.

Here we investigate if the drug-induced alteration in proline homeostasis, which we termed Adaptive Proline Response (APR), is mechanistically linked to GCN2-eIF2 α -signaling and an AAR-like stress response pathway. To test our hypothesis, we analyzed the APR in cells defective in the ability to phosphorylate eIF2 α . The eukaryotic initiation factor kinase-1 (eIK1) has previously been identified as the GCN2 homolog in *P. falciparum* and shown to be required for eIF2 α phosphorylation in response to amino acid starvation.¹⁹ Using a stable *Pf*eIK1 knockout parasite line (*Pf*eIK1(–)), which is competent for asexual growth¹⁹, we sought to determine if eIF2 α phosphorylation in response to HFG is mediated by *Pf*eIK1 and functionally required for downstream induction of the APR. First, we compared *Pf*eIK1(–) parasites to the wildtype line (*Pf*eIK1(+)) for differential sensitivity to HFG. We evaluated dose-dependent HFG-sensitivity of both lines in a standard growth assay²⁰ and determined the effective concentration that gave a half-maximal response (EC₅₀). There was no statistically significant difference in response to HFG between the *Pf*eIK1(+) and *Pf*eIK1(–) parasite lines (EC₅₀ = 1.4nM $\pm$ 0.38 (n=5) and 1.0nM $\pm$ 0.31 (n=4), respectively, p= 0.24) (Figure 1A). This finding indicates that the parasitocidal activity of HFG is not mediated by *Pf*eIK1.

To test our premise that *Pf*eIK1 is required for HFG-induced eIF2 α phosphorylation, we measured levels of phospho-eIF2 α in *Pf*eIK1(–) and *Pf*eIK1(+) parasites following HFG exposure and compared this response to control amino acid deprived or untreated parasites. The parasites were treated with 100 $\times$ EC₅₀ HFG or control conditions and probed for phospho-eIF2 α by Western blot analysis. In contrast to the wildtype strain, which consistent with our previous findings exhibited strong induction of eIF2 α phosphorylation, *Pf*eIK1(–) parasites were unable to modulate the phosphorylation state of eIF2 α in response to HFG treatment or control amino acid starvation conditions (Figure 1B). At the same time, we did

not observe changes in total eIF2 α protein under all tested conditions. This result demonstrates that HFG-induced eIF2 α phosphorylation is mediated by *Pf*IK1 and not by an independent eIF2 α kinase.

We next sought to investigate the requirement of *Pf*IK1-mediated signaling for the HFG-tolerant APR. Both *Pf*IK1(+) and *Pf*IK1(–) parasites were exposed to 4 $\times$ EC₅₀ (2.8nM) HFG for three generations and allowed to recrudesce in drug-free media (approximately 6–10 days). The sensitivity of the HFG-tolerant parasites was then assessed in a dose-response assay. Consistent with our previous findings in a Dd2 background, *Pf*IK1(+) induced parasites were ~17-fold less sensitive to HFG (EC₅₀ = 23.1nM $\pm$ 6.5 (n=4), p = 0.0079) (Figure S1)¹⁵. Notably, *Pf*IK1(–) induced parasites also displayed a comparable reduction (~18-fold) in HFG sensitivity (EC₅₀ = 17.8nM $\pm$ 5.8 (n=5), p=0.0286), suggesting that *Pf*IK1 is not required for the rapid acquisition of phenotypic tolerance to HFG (Figure 2A). This decrease in potency was specific to HFG and was not observed in response to other antimalarial drugs such as chloroquine and artesunate (Table S1).

We analyzed the *Pf*IK1(–) induced parasites in order to rule out reversion to the wildtype. As observed for the uninduced parasites, we found that total eIF2 α levels were unchanged and eIF2 α phosphorylation remained ablated in HFG-tolerant *Pf*IK1(–) parasites following HFG exposure and amino acid starvation. HFG-tolerant *Pf*IK1(+) parasites were competent for eIF2 α phosphorylation under the same conditions (Figure 2B). We also confirmed that *Pf*IK1(–) induced parasites retained the genetic knockout by PCR analysis of the *Pf*IK1 locus as described (Figure S2)¹⁹. To assess whether there was a genetic determinant of the observed resistance phenotype we sequenced the *Pf*PRS locus and measured *Pf*PRS copy number in the induced cell lines. Neither parasite line exhibited point mutations or gene amplification, indicating that variation at the *Pf*PRS locus was not responsible for the decreased HFG sensitivity (Figure S3). Taken together, these observations indicate that *Pf*IK1 is not required for the acquisition of rapid tolerance to HFG.

We then determined the concentration of free amino acids in both wildtype and *Pf*IK1(–) parasites using comprehensive LC-MS based metabolomic profiling. Of the 19 proteinogenic amino acids assayed from saponin-released purified parasites, only proline concentrations were highly increased in HFG-tolerant *Pf*IK1(+) and *Pf*IK1(–) parasites relative to their uninduced parental lines (Figure 3). These data are in agreement with our previous findings that the phenotypic resistance of HFG-tolerant parasites results from the modulation of intracellular proline levels and appears to be independent of their genetic background¹⁵. These results also establish that *Pf*IK1 is not required for the APR.

In this study, we have shown that *Pf*IK1(–) parasites are as susceptible as wildtype parasites to HFG, and that eIF2 α phosphorylation in response to HFG is mediated by *Pf*IK1. The observation that HFG exposure phenocopies amino acid starvation, suggests that *P. falciparum* parasites can also sense the deprivation of other amino acids besides isoleucine²¹. We further demonstrate that *Pf*IK1(–) parasites can develop tolerance to HFG after short-term treatment, and that this tolerance appears to be mediated by the upregulation of intracellular proline. Together, our data provide strong evidence that the APR is independent from an eIF2 α mediated stress-signaling cascade and that *Pf*IK1 is not

required for the modulation of proline homeostasis in response to HFG treatment, suggesting the existence of a previously unrecognized starvation sensing mechanism.

Future studies dissecting the mechanism underlying the APR will be a critical for the identification of the factors regulating proline homeostasis. Interrogation of the routes through which the parasites acquire proline (i.e. uptake, biosynthesis and/or hemoglobin digestion) will guide and inform those studies. In addition to illuminating the basis of this intriguing adaptive response, identifying the source of the increased proline is a potential vulnerability that could be exploited to develop drug combinations with *Pf*PRS inhibitors and could potentially forestall the emergence of drug resistance.

Methods

P. falciparum cell lines and culture conditions.

The 3D7 *Pf*IK1(+) and 3D7 *Pf*IK1(−) parasite lines were obtained from the Doerig lab²⁰ and maintained under standard conditions²². *Pf*IK1(−) parasites cultures were supplemented with 2.5µg/mL blasticidin²⁰. Synchronized cultures were obtained by sorbitol treatment, as previously reported²³. Cultures were monitored by Giemsa-stained thin smears and split as needed to maintain a parasitemia between 0.5–5%.

In vitro drug sensitivity and dose-response analysis.

Parasite growth was determined using a fluorescence assay based on the SYBR Green I method as reported previously^{20,24,25}. *P. falciparum* parasites were seeded in 384-well plates at 1% hematocrit and 1% starting parasitemia. Growth was assessed by SYBR Green staining of parasite DNA after 72-hour exposure to compound. All dose-response assays were carried out with 12-point dilutions in technical triplicate. Compounds were dispensed with an HP D300 Digital Dispenser (Hewlett Packard, Palo Alto, CA, USA). Fluorescence intensity measurements were performed on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA) and analyzed in GraphPad Prism version 7 (GraphPad Software, La Jolla, CA, USA) after background subtraction and normalization to control wells. EC₅₀ values were determined using a four-parameter nonlinear regression curve fit from at least three assays and are represented as mean ± standard deviation. Statistical significance was determined by the Mann Whitney test.

Western blot analysis.

Immunohistochemical analysis of phosphorylated eIF2α(Ser59) (PF3D7_0728000), equivalent to human eIF2α(Ser51), and total eIF2α was performed as previously reported¹⁴. In brief, asexual 3D7 parasites were exposed to either RPMI (negative control), amino acid free media or phosphate-buffered saline (PBS) (positive controls), or 100× EC₅₀ HFG for 4 hours. Protein lysates were prepared from saponin-released parasite pellets in a lysis buffer containing 1× Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) supplemented with 5% β-mercaptoethanol, 2% NP40 (G-Biosciences, St. Louis, USA), 1× Complete protease inhibitor cocktail (Roche Holdings AG, Basel, Switzerland), and 1× PhosStop phosphatase inhibitor cocktail (Roche Holdings AG, Basel, Switzerland). eIF2α pSer59 was detected with a custom polyclonal phospho-specific eIF2α Ab (rabbit) raised

against a synthetic peptide antigen sequence CGMILMSELpSKRRFR representing *P. falciparum* eIF2 α pSer59 (GenScript, Piscataway, NJ, USA). Total eIF2 α protein was detected using a polyclonal custom pAb (rabbit) raised against recombinantly expressed *P. falciparum* eIF2 α with C-terminal His6 tag (GenScript, Piscataway, NJ, USA). An anti-histone-H3 rabbit pAb was used as loading control (Abcam Cambridge, United Kingdom). Blots were then incubated with an anti-rabbit HRP-conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK), bands visualized by ECL detection reagent (Thermo Fisher Scientific, Waltham, MA, USA) and exposure to film (GE Healthcare, Buckinghamshire, UK).

Induction experiment.

HFG-tolerant parasites (on both *Pf*IK1(+) and *Pf*IK1(−) backgrounds) were generated in the same manner as previously reported¹⁵. Three independent 25mL cultures of mixed-stage 3D7 *Pf*IK1(+) and *Pf*IK1(−) parasites at 4% parasitemia were exposed to media containing HFG (4 $\times$ EC₅₀) for 144h (three intra-erythrocytic developmental cycles). The cultures were then maintained in compound-free complete RPMI growth medium with regular media exchange until healthy parasites reappeared (approximately 6–10 days).

DNA extraction, PCR, and amplicon sequencing.

Genomic DNA was extracted and purified from parasite pellets using a QiAmp DNA Blood Mini kit (Qiagen, Venlo, Limburg, the Netherlands) according to manufacturer's specifications.

Verification of the *Pf*IK1 locus was performed by diagnostic PCR using primers as previously described²⁰. PCRs were carried out in a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) with a 10 μ L total reaction volume. Reaction mixtures consisted of 1 $\times$ Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA), supplemented with 0.5 μ M both forward and reverse primers and 100ng genomic DNA. Samples were resolved by gel electrophoresis on a 1% agarose gel, visualized on an ultraviolet (UV) transilluminator, and product size determined by comparison to a Quick-Load 1kb Plus DNA Ladder (New England Biolabs, Ipswich, MA, USA).

The cPRS gene was amplified as previously described¹⁴. PCR products were then purified with DNA Clean & Concentrator columns per the manufacturer's directions (Zymo Research, Irving, CA, USA), and sequenced using BigDye termination chemistry on an ABI prism automated sequencer (Applied Biosystems, Foster City, CA, USA) from both the forward and reverse strands (Macrogen Inc., Seoul, South Korea). Raw sequence data were evaluated and aligned to the WT reference sequence. SNPs were identified using the ClustalW algorithm in MacVector (MacVector Inc., Cary, NC, USA).

Quantitative PCR - relative copy number analysis.

Quantitative PCR (qPCR) was performed on genomic DNA samples to determine the relative genomic copy number of the cPRS locus amplified using our previously published primers¹⁵. qPCR analysis was performed on an Agilent 7900HT Fast Real-Time System (Agilent Technologies, Santa Clara, CA, USA) using unlabeled primers and Power SYBR

Green master mix. cPRS copy number was determined using the Ct method included in SDS version 2.3.2 (Applied Biosystems User Bulletin 2) relative to two control loci, SRS and β -tubulin.

LC-MS analysis of amino acids and polar metabolites.

Highly synchronous (within 4 hours) early schizonts were magnetically purified with MACS CS columns (Miltneyi Biotec Inc., San Diego, CA, USA) and divided into two equal volumes: one for saponin lysis (0.015%) and one for whole iRBC extraction. Each sample was washed twice in PBS, and then suspended in 10 μ l PBS (Life Technologies, Carlsbad, CA, USA). Polar metabolites were extracted using nine volumes of 74.9:24.9:0.2 (v/v/v) acetonitrile/methanol/formic acid containing stable isotope-labeled internal standards (0.2ng/ μ l valine-d8 (Sigma Aldrich, St. Louis, MO, USA); and 0.2ng/ μ l phenylalanine-d8 (Cambridge Isotope Laboratories, Tewksbury, MA, USA) and stored at -80°C prior to the metabolite profiling assays.

Profiles of amino acids were measured using LC-MS as described previously²⁶. Briefly, positive ionization, multiple reaction mode (MRM) data were acquired using a Q-Exactive Focus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Nexera X2 U-HPLC (Shimadzu Corporation, Kyoto, Japan). Cell extracts (10 μ l) were injected onto a 150 $\times$ 2.1mm Atlantis HILIC column (Waters, Milford, MA, USA). The column was eluted isocratically at a flow rate of 250 μ l/minute with 5% mobile phase A (10 mM ammonium formate and 0.1% formic acid in water) for 1 minute followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 minutes. The ion spray voltage was 3.5kV and the source temperature was 350 $^{\circ}\text{C}$. TraceFinder 3.3 software (Thermo Fisher Scientific, Waltham, MA, USA) was used for automated peak integration, and metabolite peaks were manually reviewed for quality of integration and compared against a known standard to confirm identity. Stable isotope-labeled internal standards valine-d8 and phenylalanine-d8 were used to eliminate samples with poor data quality. Metabolite peaks signals were total-signal normalized with all 119 metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HFG	halofuginone
APR	Adaptive Proline Response
eIF2α	eukaryotic Initiation Factor-2 α

eIK1 eukaryotic Initiation Factor Kinase-1

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Figure 1. *Pfk1K1* is not essential for the antiparasitic activity of HFG but is required for the HFG-dependent induction of eIF2 α phosphorylation.

A. HFG is equipotent in *Pfk1K1*(+) and *Pfk1K1*(-) parasites. HFG activity was determined using a 72hr SYBR Green dose-response assay (*Pfk1K1*(+) $EC_{50} = 1.4 \text{ nM} \pm 0.38$; *Pfk1K1*(-) $EC_{50} = 1.0 \text{ nM} \pm 0.31$). Values represent mean $EC_{50} \pm$ standard deviation of at least four independent assays. Dose-response curves from a single representative assay are shown.

B. HFG-induced phosphorylation of eIF2 α is mediated by *Pfk1K1*. Western blot analysis shows robust phosphorylation of eIF2 α in *Pfk1K1*(+) parasites in response to amino acid starvation (-AA RPMI) or HFG treatment (100 $\times$ EC_{50}). Control treatment with regular growth medium (RPMI) does not activate this stress-response. In contrast, *Pfk1K1*(-) parasites were unable to modulate the phosphorylation state of eIF2 α under identical conditions, indicating that *Pfk1K1* is required for the eIF2 α signaling observed. There was no measurable change in total eIF2 α or a histone H3 loading control in all parasite lines. Western blots are representative of four independent experiments.

Figure 2. *Pfk1* is not required for induced HFG-resistance.

Short-term HFG treatment induces rapid drug tolerance in *Pfk1*(+) and *Pfk1*(-) parasites. Mixed-stage *Pfk1*(+) and *Pfk1*(-) parasites were cultured in media containing $4 \times EC_{50}$ HFG for three intra-erythrocytic developmental cycles and allowed to recrudesce.

A. HFG treatment desensitizes *Pfk1*(-) parasites to HFG. HFG-tolerant *Pfk1*(-) parasites displayed a 20-fold shift in response to HFG relative to their untreated parental line (Parental *Pfk1*(-) $EC_{50} = 1.0 \text{ nM} \pm 0.31$, HFG-tolerant *Pfk1*(-) $EC_{50} = 17.8 \text{ nM} \pm 5.8$). Values represent mean $EC_{50} \pm$ standard deviation of at least four independent assays. Dose-response curves from a single representative assay are shown. A similar fold-change is observed in HFG-tolerant wildtype (*Pfk1*(+) parasites (see Supplemental Figure 2).

B. HFG induction of *Pfk1*(+) and *Pfk1*(-) parasites does not alter their eIF2α phosphorylation response. Western blot analysis of HFG-tolerant *Pfk1*(+) and *Pfk1*(-) parasites shows that eIF2α phosphorylation in response to amino acid starvation (-AA RPMI) or HFG ($100 \times EC_{50}$) remains consistent with parental untreated lines. Untreated *Pfk1*(+) and HFG-tolerant *Pfk1*(+) parasites show robust phosphorylation of eIF2α upon treatment with amino acid depleted media or HFG. HFG-tolerant *Pfk1*(-) parasites are unable to phosphorylate eIF2α under identical conditions just as their parental precursor. There was no measurable change in total eIF2α or histone H3 loading control in all parasite lines. Western blots are representative of three independent experiments.

Figure 3. HFG-induced modulation of proline homeostasis is independent of *Pf*eIK1.

Short-term HFG treatment increased intracellular proline levels in *Pf*eIK1(+) and *Pf*eIK1(-) parasites. Of the 19 proteinogenic amino acids assayed from saponin-released purified parasites, only proline concentrations were increased in HFG-tolerant *Pf*eIK1(+) and *Pf*eIK1(-) parasites. The fold-increase in amino acid concentration after HFG-induction is expressed as a ratio of induced parasite line measurement over untreated parental line measurement. Amino acid levels of saponin-released parasites were quantified from the normalized integrated peak intensity determined by LC-MS. Error bars denote standard deviation across 5 biological replicates.