Multi-omics based identification of specific biochemical changes associated with PfKelch13-mutant artemisinin resistant Plasmodium falciparum

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Key words: (3-10 key words) malaria, Plasmodium falciparum, artemisinin resistance, PfKelch13, proteomics, metabolomics, peptidomics

Running head: Biochemical impact of PfKelch13 mutations

Word count (text): 3499
Word count (abstract): 174
Figures: 5
Tables: 2
References: 49

Summary

Multi-omics analyses of PfKelch13-mutant artemisinin resistant and sensitive Plasmodium falciparum revealed artemisinin resistance to be associated with a two-fold decrease in PfKelch13 protein abundance, decreased haemoglobin digestion and increased glutathione production.
Abstract

Background. The emergence of artemisinin resistance in the malaria parasite, *Plasmodium falciparum*, poses a major threat to the control and elimination of malaria. Certain point mutations in the propeller domain of *PfKelch13* are associated with resistance, but *PfKelch13* mutations do not always result in clinical resistance. The underlying mechanisms associated with artemisinin resistance are poorly understood and the impact of *PfKelch13* mutations on cellular biochemistry is not defined.

Methods. This study aimed to identify global biochemical differences between *PfKelch13*-mutant artemisinin-resistant and -sensitive strains of *P. falciparum* by combining LC-MS-based proteomics, peptidomics and metabolomics.

Results. Proteomics analysis found both *PfKelch13* mutations examined to be specifically associated with decreased abundance of *PfKelch13* protein. Metabolomics analysis demonstrated accumulation of glutathione and its precursor, gamma-glutamylcysteine, and significant depletion of one other putative metabolite in resistant strains. Peptidomics analysis revealed lower abundance of several endogenous peptides derived from haemoglobin (HBα and HBβ) in the artemisinin resistant strains.

Conclusion. *PfKelch13* mutations associated with artemisinin resistance lead to decreased abundance of *PfKelch13* protein, decreased haemoglobin digestion and enhanced glutathione production.
Introduction

Malaria is a major global health problem, with over 200 million cases of malaria and an estimated 429,000 deaths in 2015 [1]. *Plasmodium falciparum* causes the most severe form of malaria and is responsible for the highest incidence, especially in the WHO African Region where 90% of malaria cases and 92% of deaths due to malaria occur [1].

Artemisinin-based combination therapy (ACT) has been the recommended treatment for *P. falciparum* malaria since 2005, and has contributed to a significant reduction in malaria incidence and mortality [2]. However, the prevalence of ACT failure is increasing, especially in South-East Asia. Resistance to ACT was first reported in Western Cambodia and has now spread across the Greater Mekong Subregion, an area known historically for the emergence of resistance against previous first-line antimalarials [3]. The spread of chloroquine resistance from South-East Asia to Africa had significant implications for malaria control, and the emergence of artemisinin resistance in Africa could have devastating consequences.

Clinically, artemisinin resistance is defined by slow parasite clearance in *P. falciparum*-infected patients post-treatment [3] and is associated with an increased survival of ring-stage parasites after short-term exposure to artemisinin *in vitro* [4, 5]. Recently, point mutations in the propeller domain of the PfKelch13 gene (PF3D7_1343700) were shown to be associated with resistance to artemisinin *in vitro* and *in vivo* in South-East Asian *P. falciparum* strains [6-8]. To date, a total of 186 different PfKelch13 alleles have been reported in Asia [9], but only a small subset of these mutants have been shown to be associated with artemisinin resistance [7, 8, 10-12], with four of these mutations (Y493H, R539T, I543T and C580Y) further confirmed *in vitro* [6, 8, 13]. Furthermore, mutations in the PfKelch13 gene have not yet been identified to be associated with artemisinin resistance in African *P. falciparum* strains [14-19].

The use of PfKelch13 as a genetic molecular marker for artemisinin resistance is time-consuming and not always predictive of treatment outcome [9]. Additionally, labour intensive *in vitro* assays are required for confirmation of resistance [19, 20]. An alternative set of biomarkers of artemisinin resistance would be valuable for epidemiological monitoring, and potentially for point-of-care testing to assist with drug selection.

The genetic mutations responsible for artemisinin resistance likely mediate specific biochemical functions that help to define the resistance phenotype [21]. In this study, differences in the biochemical phenotype of three...
pairs of PfKelch13-mutant parasites and relevant controls were characterised by analysing their global proteome, metabolome and peptidome.

This integrative multi-omics approach identified putative biomarkers that were consistently associated with artemisinin resistant PfKelch13 mutants, including the PfKelch13 protein, glutathione-related metabolites and endogenous peptides derived from haemoglobin.
Methods

*P. falciparum* parasite culture

All *P. falciparum* strains were cultured as previously described [22] and all analyses were performed within six weeks of the parasites being thawed. Parasites were tightly synchronised by double treatment with sorbitol and when required, magnetic column purification was performed to obtain highly-enriched trophozoite stage infected red blood cells (iRBCs) as described previously [23, 24]. The artemisinin resistant and sensitive *P. falciparum* isolates used in this study included two common PfKelch13 mutants and the PfKelch13 wild-type on an isogenic background [13], and two field strains [25] (Table 1). Global proteomic analyses were performed on ring and trophozoite stage parasites, but global metabolomics and peptidomics analyses were focussed only on trophozoite stage parasites.

Global proteomic studies

Proteomics sample preparation

Intracellular parasites were purified from iRBCs using 0.1% saponin as described previously [26]. Parasite pellets were solubilised with lysis buffer (100 mM HEPES, 1% sodium deoxycholate (SDC), pH 8.1) supplemented with phosphatase and protease inhibitors for 5 min at 95 °C. Proteins were reduced, alkylated and then precipitated with trichloroacetic acid (TCA). The supernatants were kept for peptidomics sample preparation. The pellets were resuspended in 1 ml of lysis buffer and then 500-800 µg of total protein (accurately determined using Pierce BCA protein assay kit (Thermo Scientific™ Pierce™)) was incubated overnight with trypsin (Promega) (1:50). On the following day, dimethyl duplex labelling was performed [27]. After labelling, protein samples from the resistant and sensitive lines were mixed and the peptide mixture was fractionated using a disposable Strong Cationic exchange solid phase extraction cartridge (Agilent Bond Elut). Eluates (fractions) and flowthroughs collected were subjected to desalting using in-house generated StageTips as described previously [28]. The fractions were then dried and resuspended in 20 µL of 2% (v/v) acetonitrile (ACN) and 0.1% (v/v) formic acid (FA) for LC-MS/MS analysis.

Proteomics LC-MS/MS analysis and data processing

LC-MS/MS was performed using an Ultimate U3000 Nano LC system (Dionex) and Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (ThermoFisher®). Samples were loaded at a high flow rate onto a
reversed-phase trap column (100 μm x 2 cm) Acclaim PepMap media (Dionex) in 0.1 % (v/v) FA in water. Peptides were eluted from the trap column at a flow rate of 0.3 μl/min through a reversed-phase capillary column (75 μm x 15 cm) (LC Packings, Dionex) into the nanospray ion source of the mass spectrometer (MS). For proteomics analysis, the HPLC gradient was set to 115 min using a gradient that reached 30% ACN after 55 min, then 45% after 85 min, 55% after 90 min and 90% after 100 min. The MS was operated in a data-dependent mode with 2 microscan FTMS scan events at 70,000 resolution (MS) over the m/z range of 375-1800 Da in positive ion mode, and up to 20 data-dependent HCD MS/MS scans. Identification and quantification of proteins was performed using MaxQuant proteomics software [29]. Statistical analysis for the determination of differential proteins used parametric and non-parametric hypothesis testing, and a linear model approach. Log-transformed fold-difference for paired artemisinin resistant and sensitive samples and one sample t-test were calculated in MS-Excel to test the mean of combined experiment groups against the known mean (μ = 0) [30]. Bonferroni correction was applied to the significance threshold to adjust for multiple testing. Rank Product analysis, a non-parametric permutation test, was conducted on the Log-transformed fold-difference for paired artemisinin resistant and sensitive samples using the RankProd R package [31]. The significance of each protein was assessed by the p-value and false discovery rate (FDR). Limma analysis, a linear model designed to assess differential expression, was conducted using the limma R package [32]. The significance of each protein was assessed by the p-value and adjusted p-value (adjusted for multiple testing) calculated with the empirical Bayesian framework applied to the linear model.

**Global metabolomics studies**

*Metabolomics Sample preparation*

*P. falciparum* parasites were synchronised using sorbitol 24 hr before parasite harvest for metabolomics experiments. A control culture containing only uninfected RBCs was also analysed. Metabolites were extracted from magnetically enriched parasites (5 x 10⁷) at 60-70% parasitaemia using cold methanol as previously described [33]. Insoluble precipitates were removed by centrifugation and 100 μL of metabolite extract was transferred to glass LC-MS vials and stored at -20 °C until analysis. An aliquot (20 μL) of each sample was combined to generate a pooled biological sample for quality control (QC) procedures.
Metabolomics LC-MS analysis and data processing

Hydrophilic interaction liquid chromatography (ZIC-pHILIC, Merck) coupled to high resolution-mass spectrometry (Q Exactive™, ThermoFisher®) was used to analyse metabolomics samples as before [34]. Approximately 250 metabolite standards were analysed immediately preceding the batch run to determine accurate retention times to facilitate metabolite identification. Additional retention times for metabolites lacking authentic standards were predicted computationally as previously described [35]. Identification and quantification of metabolites was performed using the IDEOM workflow [36] and peak areas for significant metabolites were confirmed by manual integration of raw LC-MS data with TraceFinder™ (ThermoFisher®).

LC-MS peaks heights, representing metabolite abundances, were normalised according to the median identified peak for each sample. Univariate statistical analysis was done using IDEOM and Welch’s t-test [36]. Multivariate statistical analysis utilised partial least squares - discriminant analysis (PLS-DA) and was applied to log-transformed and auto-scaled data using the web-based analytical tool, MetaboAnalyst [37].

Global peptidomics studies

Peptidomics sample preparation

Supernatants collected post TCA precipitation from proteomics sample preparations were subjected to centrifugal filtration (10 kDa cut-off, Amicon Ultra). The flow-through was collected and an equal amount of ethyl acetate was added to remove residual SDC. Equal quantities of total peptides (50-70 µg by BCA) were then used for peptidomics analyses. Peptide samples were subjected to desalting [28], and were then dried and resuspended in 20 µL of 2% (v/v) ACN and 0.1% (v/v) FA for LC-MS/MS analysis.

Peptidomics LC-MS/MS analysis and data processing

LC-MS/MS was performed using the same methodology as the proteomics LC-MS/MS analysis (described above and in supplementary methods) with minor modifications. Mass identification with +1 and up to +8 charge was also enabled.

Peptide identification was conducted with de novo sequencing assisted database search using PEAKS DB software [38]. The identified peptide sequences from the Homo sapiens and P. falciparum proteome databases that were detected in multiple samples were shortlisted. The mass to charge ratio and retention time of shortlisted peptides were imported into TraceFinder™ (ThermoFisher®) and the peak intensity was obtained by
manually adjusting the integration and accounting for retention time drift when required. Log-transformed fold-difference and Welch’s t-test were calculated in MS-Excel for paired artemisinin resistant and sensitive samples.
Results

This study combined metabolomics, peptidomics and proteomics (Figure 1) to identify novel potential biomarkers for PfKelch13 mutant *P. falciparum* isolates associated with artemisinin resistance. Resistant and sensitive *P. falciparum* parasite isolates (Table 1) were synchronised and grown *in vitro* to the equivalent stage of the intra-erythrocytic cycle (rings: 6-12 hours post invasion (hrs p.i), trophozoites: 24-30 hrs p.i), followed by parallel extractions for metabolomics, peptidomics and proteomics analysis by LC-MS (Figure 1).

Quantitative dimethyl-based proteomic analysis

Global proteome analysis was performed using reductive dimethyl labelling to identify quantitative differences in protein levels between artemisinin sensitive and resistant *P. falciparum* isolates. Two clonal artemisinin resistant isolates with defined PfKelch13 mutations (Cam3.II{R539T} and Cam3.II{C580V}) were compared to their isogenic PfKelch13 wild-type strain (Cam3.II{rev}) [13] and two independent field isolates from the Pailin region of Cambodia, a PfKelch13-mutant artemisinin resistant isolate (PL7) and PfKelch13 wild-type sensitive (PL2) isolate [25] were compared to each other (Table 1).

Saponin lysed ring-stage parasite proteins from Cam3.II{R539T} and Cam3.II{rev} were labelled for quantitative proteomics from three biological replicates. Ring stage parasites were chosen for initial proteomic analysis, as this is the stage where differential *in vitro* sensitivity to artemisinin has been reported [6-8]. A total of 920 proteins were identified with at least two unique peptides, with 432 proteins in common between the three replicates (Supplementary data file 1). Compared with the control, a significant reduction in the abundance of six proteins was observed (p≤0.05; log fold-change > 0.5), including the PfKelch13 protein itself (Figure 2), and only one protein, Pfl3_0341, a putative DNA-directed RNA polymerase 2, was significantly more abundant in the resistant strain (Supplementary data file 1). It was noted that the ring-stage proteomics data demonstrated relatively poor coverage and high variability, likely due to the challenges associated with purification of ring-stage parasites following saponin lysis. Whilst decreased ring-stage sensitivity to artemisinins is the accepted *in vitro* model for artemisinin resistance, decreased sensitivity has also been reported for trophozoite stages when treated for a short duration (< 2 h) [25], suggesting that the resistance mechanism is not stage-specific.

Therefore, further studies were performed on trophozoite-stage parasites, allowing improved reproducibility and purity from uninfected RBC contaminants.
For trophozoite stage parasites, a total of 2824 proteins were identified from three to four biological replicates of three pairs of parasite strains (Supplementary data file 1). Data from all eleven experiments were combined in order to detect significant proteins associated with all three resistant strains tested, and three types of statistical tests were applied to minimise the likelihood of false discoveries. As the rank product analysis performs poorly with data sets containing missing values, the data set was restricted to proteins detected in all eleven experiments, resulting in a set of 520 proteins for analysis. The limma analysis was also conducted on an expanded data set of 1133 proteins that were detected in at least one experiment for each of the three pairs of parasite strains and the same significant proteins were identified (data not shown). Only five proteins were apparently different (p-value < 0.05) according to each of the statistical tests applied (Table 2). However, when taking multiple testing corrections for all three tests into account (Bonferroni correction for t-test, FDR for rank product analysis and adjusted p-value for limma), only PfKelch13 abundance was found to be significantly different across all parasite strains (Table 2). PfKelch13 abundance was approximately two-fold lower in the resistant strain across all 14 individual experiments including ring and trophozoite-stage parasites (Figure 3A). The identification of PfKelch13 was confidently assigned based on the detection of 20 unique peptides, and manual analysis of the data confirmed lower abundance of peptides from both the N-terminal region and the C-terminal propeller domain in resistant (PfKelch13-mutant) compared to the sensitive (wild-type) strains (Figure 3B).

Metabolomics analysis of artemisinin resistant parasites

Comparative untargeted metabolomics was used to investigate the impact of resistance-associated PfKelch13 mutations on the metabolome of two clonal resistant lines Cam3.II©539T and Cam3.II©580Y, relative to the sensitive line Cam3.II© (n = three biological replicates). Univariate statistical analysis of all identified metabolite features revealed few significant differences between the wild-type and both mutant lines. Supervised multivariate analysis (PLS-DA) revealed a combination of metabolic features in the second component (PC2) that were associated with resistance (Figure 4A). Detailed analysis of this model revealed four metabolites with VIP scores above 0.3, and subsequent manual integration of LC-MS data for these features confirmed that one metabolite was significantly depleted in the resistant lines, and three were more abundant (Figure 4B). Levels of glutathione, and its precursor gamma-glutamylcysteine, were 74% and 57%, higher in the resistant parasites than sensitive, respectively. Levels of NAD+ were only 20% higher in the resistant parasites than sensitive. The depleted metabolic feature in both resistant strains was putatively identified as L-
proline amide. This metabolite was detected in the uninfected RBCs, and is likely derived from the host cells (Supplementary data file 2).

**Peptidomics analysis of artemisinin resistant parasites**

Proteolysis is an important biochemical function in *P. falciparum*, and degradation of parasite (via the proteasome) and host (via the digestive vacuole) proteins has been proposed to influence the mechanism of action and/or resistance of artemisinin [39]. Most endogenous peptides liberated by protein digestion processes are not detected on the standard metabolomics and proteomics methods, necessitating the incorporation of a dedicated peptidomics analysis to investigate these proteolytic pathways. Peptidomics analysis identified 146 endogenous peptides that aligned with proteins from the *P. falciparum* (68 peptides) and *H. sapiens* (78 peptides) databases (Supplementary data file 3). Significant reductions in the abundance of 19 peptides were observed in artemisinin resistant lines compared to their appropriate controls (p≤0.05; log fold-change > 0.5), including eight peptides that originated from Hbβ, ten originated from Hba (Figure 5) and one originated from another *H. sapiens* protein, Uniprot ID number POU3F3 (Supplementary data file 3). No significant differences in the abundance of *P. falciparum*-derived peptides were observed.
Discussion

Integrative multi-omics approaches provide a comprehensive analysis of the abundance of cellular biochemicals, which can help in understanding the mechanism of artemisinin resistance. In this study, the proteomic, peptidomic and metabolic profiles of artemisinin resistant *P. falciparum* strains were investigated to reveal the biochemical impact of artemisinin resistance-associated *PfKelch13* mutations. The multi-omics analysis revealed that *PfKelch13* mutations do not have a widespread impact on the biochemistry of *P. falciparum* under standard *in vitro* culture conditions. The abundance of *PfKelch13* protein itself was found to be consistently lower in all the resistant lines compared to the sensitive strains. Endogenous peptides originating from haemoglobin, and one putative host-derived metabolite, were also decreased in abundance, while levels of glutathione and gamma-glutamylcysteine were higher in artemisinin resistant lines compared to the *PfKelch13* wild-type, artemisinin-sensitive, controls.

Artemisinin resistance is associated with point mutations on the *PfKelch13* allele. The exact function of *PfKelch13* is not yet known, however, the C-terminal propeller domain shows homology to the human Keap1 protein, whose main function is to respond to oxidative stress by regulating levels of the transcription factor Nrf2 [6, 40]. Interestingly, the global proteomics analysis of ring and trophozoite parasite stages in both artemisinin resistant laboratory-generated clonal lines and field isolates identified decreased abundance of *PfKelch13* protein as the only significant differentiating feature (Figures 2 and 3, Table 2). Whilst no Nrf2 homologue has been identified in *P. falciparum*, based on Keap1 function, it is plausible that lower levels of *PfKelch13* protein in artemisinin resistant parasites would decrease the Kelch-mediated negative regulation of the stress response, thereby making parasites more resilient to drug-induced oxidative stress. The observed increase in glutathione abundance supports this hypothesis. It has also been hypothesised that the *PfKelch13* protein is involved in the ubiquitination and proteasomal degradation of proteins within the endoplasmic reticulum and is involved in the correct folding of proteins within the parasite [39]. In this case it is anticipated that depletion of *PfKelch13* protein in artemisinin resistant parasites would be associated with a dysregulated unfolded protein response, which is consistent with the previously reported increase in mRNA expression of genes associated with unfolded protein response [41], although no difference in abundance of those proteins was observed in the untreated *in vitro* conditions described here (Supplementary data file 1).

Whilst the link between *PfKelch13* mutations and artemisinin resistance is well-established, *PfKelch13* expression was not previously associated with artemisinin resistance [41], suggesting that the differential protein
abundance observed here may arise due to decreased translation, or enhanced degradation of the PfKelch13 protein, rather than transcriptional regulation. It has previously been demonstrated that artemisinin resistance in laboratory-generated PfKelch13C580T mutant parasites (NF54 strain) is associated with an increased abundance of *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K) and its lipid product phosphatidylinositol-3-phosphate (PI3P), likely due to a deficiency in PfKelch13-mediated ubiquitin-dependent degradation of PfPI3K [42]. This hypothesis is consistent with the decreased abundance of PfKelch13 observed here, albeit these studies are based on parasites with different genetic backgrounds. Unfortunately this untargeted metabolomics and proteomics approach was not capable of detecting PI3P and PfPI3K (Supplementary data files 1 and 2). Other published untargeted proteomics studies of blood stage *P. falciparum* have also failed to detect peptides from PfPI3K in the absence of phosphopeptide enrichment [43]. Nevertheless, this study was able to successfully demonstrate the specific impact of two common PfKelch13 mutations (C580Y and R539T) on PfKelch13 protein abundance and suggest that there is potential to develop a quantitative biomarker assay for artemisinin resistance that is independent of the specific PfKelch13 genotype. Further studies on diverse PfKelch13 genotypes are necessary to confirm the association between PfKelch13 protein abundance and clinical artemisinin resistance.

Whilst the PfKelch13 mutant parasites did not exhibit significant changes to the proteome (beyond PfKelch13) under these *in vitro* conditions, enhanced levels of glutathione and its precursor, gamma-glutamylcysteine, were observed in the metabolomics analysis. Glutathione is a co-factor required for detoxification enzymes such as glutathione peroxidase and glutathione S-transferase and its abundance is suggestive of the parasite’s ability to defend itself against additional oxidative stresses, such as that induced by artemisinin treatment [25, 44]. It has been proposed that artemisinin resistant parasites are able to better manage oxidative damage [39], and increased glutathione concentration been reported in rodent models of artemisinin-resistance [45, 46].

Our global peptidomics analysis identified a number of endogenous peptides originating from haemoglobin (alpha and beta subunits) that were decreased in abundance in the resistant lines (Figure 5), suggesting down-regulation of haemoglobin uptake or digestion. This decrease in haemoglobin catabolism could result in decreased production of haem, which is necessary to activate artemisinin in order to mediate parasite killing. Disrupted haemoglobin digestion and endocytosis has previously been demonstrated in artemisinin resistant rodent parasites [45, 47]. The decreased haemoglobin digestion could be associated with quiescence, or a higher proportion of ring stage parasites in the samples. However, the highly similar proteomics and metabolomics...
profiles observed here confirmed that all samples were correctly normalised and at the same lifecycle stage at the time of analysis, as stage-specific profiles would have been anticipated if significant differences in development were apparent [48]. Dysregulation in the abundance of haemoglobin-derived peptides in chloroquine-resistant *P. falciparum* lines has previously been reported [49]. However, chloroquine resistance was associated with peptide accumulation, and it is therefore unlikely that haemoglobin-derived peptides will provide a sensitive and selective biomarker in the context of multi-drug resistance.

In conclusion, through a combined proteomics, metabolomics and peptidomics analysis, the present study revealed the effect of *PfKelch13* mutations on protein expression, metabolic pathways and endogenous peptide levels in artemisinin-resistant lines compared to their sensitive (*PfKelch13* wild-type) controls. The functional consequences of *PfKelch13* mutations were observed in the form of decreased haemoglobin digestion and increased glutathione production. The abundance of *PfKelch13* protein was two-fold lower in artemisinin resistant *PfKelch13* mutants, and reproducible perturbations in the levels of other proteins were not observed. Further validation in field isolates is necessary to determine the potential of *PfKelch13* to be developed as a quantitative biomarker for rapid diagnostic testing and monitoring resistance.

**Acknowledgements**

The Australian Red Cross Blood Service in Melbourne is gratefully acknowledged for providing human erythrocytes used for *in vitro* cultivation of *P. falciparum* parasites. We thank Prof. David Fidock for the genetically modified Cambodian isolates and Prof. Leann Tilley for field-derived Pailin isolates. Carson Yuan assisted with the data analysis workflow for peptidomics analysis.

**Authorship Contributions**

GS performed experiments; GS, AS, and AR analysed results and made figures; GS and DJC designed the research; GS, AS, and DJC wrote the manuscript; DJC directed the overall research program.

**Conflicts of interest**

All authors declare no competing financial interests.

**Funding**

DJC is funded by a National Health and Medical Research Council Career Development Fellowship (APP1088855)
Figure Legends

Figure 1. Flow chart of the integrative multi-omics approach (metabolomics, proteomics and peptidomics) used for the analysis of P. falciparum infected red blood cells (iRBCs). For metabolomics (indicated in black boxes) equal number of 5x 10^7 cells were used for metabolite extractions, samples were analysed using LC-MS and data was processed using IDEOM. For peptidomics (indicated in light grey boxes and arrows) an equal amount of peptides (50-70 µg) was used for analysis using LC-MS/MS and data was processed and quantitatively analysed using PEAKS. For proteomics (indicated in grey boxes) equal amount of total protein (500-800 µg) was labelled using reductive dimethyl labelling for quantitative analysis. Samples from artemisinin resistant and sensitive iRBCs were then analysed using LC-MS/MS and the data was processed and analysed using Maxquant.

Figure 2. Volcano plot of ring-stage protein abundance in Cam3.II^{R539T} compared to Cam3.II^{rev}. Log_2 fold changes (x-axis) and T-test p-values (y-axis) of all proteins in ring stage parasites (6-12 hour post invasion). Protein names are labelled next to significantly-different proteins.

Figure 3. PfKelch13 is consistently and significantly down-regulated in artemisinin resistant lines compared to their relevant controls. (A) Relative abundance (Mean ± standard deviation) of PfKelch13 in three different artemisinin resistant lines compared to their appropriate controls (see Table 1). (B) Representative raw MS spectra of tryptic peptides from a 1:1 mixture of Cam3.II^{R539T}/PL7 (heavy labelled) and control and Cam3.II^{rev}/PL2 (light labelled) extracts. The tryptic peptides (190-FSTVNVNDTYEK-202, 623-SSGAAFNQIYVGGIDNEHNLDSVEQYQPFNKR-659, 717-FGHSVLIANI-726), belonging to N- and C-terminal regions of PfKelch13, show approximately two-fold lower relative abundance for the heavy-labelled peptides compared to light-labelled peptides.

Figure 4. Differentially abundant metabolites in artemisinin resistant (Cam3.II^{R539T} and Cam3.II^{C580Y}) versus artemisinin sensitive (Cam3.II^{rev}) lines. (A) Multivariate analysis (PLS-DA) of all putative metabolite features detected in Cam3.II^{R539T}, Cam3.II^{C580Y} and Cam3.II^{rev}. Sample projection (3 groups) onto the first PLS-DA discriminant plane of Cam3.II^{rev} and Cam3.II^{R539T}, Cam3.II^{C580Y}. (B) Relative abundance of the four most
differentially abundant metabolites from iRBCs in two artemisinin resistant (Cam3.II^{R539T}, Cam3.II^{C580Y}) lines compared to the control (Cam3.II^{rev}). Data are presented as mean ± standard deviation (n=3).

Figure 5. Differentially abundant endogenous peptides originating from Hb in artemisinin resistant vs artemisinin sensitive lines. (A and C) Sequence coverage of Hbα and Hbβ from peptidomics analysis of resistant lines compared to sensitive. The black bars represent endogenous peptides identified that were not significantly different in abundance. The red bars represent endogenous peptides identified that were significantly decreased in abundance in the resistant lines compared to sensitive, statistical analysis is shown in supplementary data file 3. (B and D) Chromatograms from all detected Hbα and Hbβ peptides in Cam3.II^{C580Y} versus Cam3.II^{rev}, PL7 versus PL2, and Cam3.II^{R539T} versus Cam3.II^{rev}. The chromatograms demonstrated an overview of the decrease in abundance of peptides identified from Hb in the resistant lines (above axis) compared to the sensitive (below axis).
Table 1. *P. falciparum* strains used in this study. Parasite strains, their response to artemisinin determined using *in vitro* ring-stage assay 0 to 3 hours/3 hours (RSA$_{0-3h}$), *PfKelch13* genotype, identifying description and geographical source are listed.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Response to artemisinin (700nM)</th>
<th><em>PfKelch13</em> Genotype</th>
<th>Identifying description</th>
<th>Geographical source</th>
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<td>Cam3.II$^{ov}$</td>
<td>0.7% survival using an <em>in vitro</em> RSA$_{0-3h}$</td>
<td>Wild-type</td>
<td>Cam3.II$^{ov}$, isogenic derivative of Cam3.II$^{R539T}$</td>
<td>Pursat province, Cambodia [13]</td>
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<td>Cam3.II$^{R539T}$</td>
<td>49% survival using an <em>in vitro</em> RSA$_{0-3h}$</td>
<td>R539T</td>
<td>Cam3.II$^{R539T}$, parent of Cam3.II$^{R539T}$ and Cam3.II$^{C580Y}$</td>
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<tr>
<td>Cam3.II$^{C580Y}$</td>
<td>24% survival using an <em>in vitro</em> RSA$_{0-3h}$</td>
<td>C580Y</td>
<td>Cam3.II$^{C580Y}$, isogenic derivative of Cam3.II$^{R539T}$</td>
<td></td>
</tr>
<tr>
<td>PL2</td>
<td>5% minimum viability using an <em>in vitro</em> RSA$_{3h}$</td>
<td>Wild-type</td>
<td>Pailin- PL2, field isolate (genetically distinct from PL7)</td>
<td>Western Cambodia [25]</td>
</tr>
<tr>
<td>PL7</td>
<td>20% minimum viability using an <em>in vitro</em> RSA$_{3h}$</td>
<td>R539T</td>
<td>Pailin- PL7, field isolate (genetically distinct from PL2)</td>
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Table 2. List of *P. falciparum* proteins differentially regulated in expression in trophozoite stage parasites of artemisinin resistant (Cam3.II^{R539T}, Cam3.II^{C580Y}, PL7) compared to artemisinin sensitive (Cam3.II^{rev} and PL2). Mean relative abundance ± standard deviation, Student’s t-test p-value, Rank Products p-value and false discovery rate, limma p-value and adjusted limma p-value are shown, n= (11). PfKelch13 is the only protein significantly down-regulated in artemisinin resistant lines according to all three analyses after adjustment for multiple testing (p < 0.05). *Less than Bonferroni-corrected p-value threshold (α = 4.03 x 10⁻⁵).

<table>
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<th>Protein</th>
<th>N</th>
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<th>Limma p-value</th>
<th>Rank product p-value</th>
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<td>PfKelch13 protein, putative</td>
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<td>0.63±0.09</td>
<td>2.63 x 10⁻⁶*</td>
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References


