Identification of an inhibitory pocket in falcilysin bound by chloroquine provides a new avenue for malaria drug development

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Abstract

Despite their widespread use, our understanding of how malaria drugs work remains limited. This includes chloroquine (CQ), the most successful antimalarial ever deployed. Here, we used MS-CETSA and dose-response transcriptional profiling to identify possible protein targets and mechanism of action (MOA) of CQ, as well as MK-4815, a malaria drug candidate with a proposed MOA similar to CQ. Both compounds bind falcilysin (FLN) and hemoglobin digestion was the key biological pathway affected, with distinct MOA profiles between CQ-sensitive and CQ-resistant parasites. We showed that CQ and MK-4815 inhibit FLN proteolytic activity, and using X-ray crystallography, that they occupy a hydrophobic pocket situated within the large peptide substrate binding cavity of FLN. Studies using transgenic parasite line suggest the potential role of FLN in the CQ and MK-4815 MOA. Altogether, our data reveal a druggable pocket in the FLN substrate binding cavity that can be explored in future antimalarial development efforts.

Introduction

Malaria remains a major public health problem with > 200 million cases and 400,000 deaths in 2018 ¹. Declining efficacy for artemisinin-based combination therapy as the frontline treatment for malaria, is particularly worrisome, given the limited number of clinically approved treatments and widespread emerging resistance ²⁻⁵. The majority of antimalarial drugs used in the past and in present were derived from phenotypic-based screenings followed by empirical scaffold improvement ^{6,7}. As a result, knowledge of targets and associated mode of action (MOA) remains fragmentary for most antimalarial drugs. A prime example of this is chloroquine (CQ) that represents by far the most successful antimalarial in history, having saved millions of lives and prevented hundreds of millions of malaria cases ⁸. Initially synthesized in 1934, it was not until the 1940s when CQ was deployed worldwide for prophylaxis and treatment of malaria immediately after World War II ⁹. Unfortunately, reports of CQ resistance in *P. falciparum* began cropping up in the 1950s across different continents and by the late 1980s CQ resistance has spread to nearly all endemic regions in Asia, Africa, and South America, leading to the discontinuation of CQ use for the treatment of falciparum malaria ¹⁰. Although CQ is no longer used for malaria treatment, its efficacy, safety profile, and cost-effectiveness remain the gold standard for any novel antimalarial ⁸.

Extensive research over the last four decades links CQ antimalarial activity with its ability to disrupt hemoglobin degradation, a key biological process carried out by the malaria parasites during their intraerythrocytic developmental cycle (IDC). *Plasmodium falciparum*, the deadliest species of human malaria parasites, digests up to 65% of the host-cell hemoglobin through a cascade of proteolytic cleavages occurring within the parasite digestive vacuole (DV) ¹¹. This releases amino acids for parasite protein synthesis but also free heme which is toxic to the parasite through oxygen radicals generated by free heme ¹²⁻¹⁷. As a protective mechanism, *P. falciparum* converts the liberated heme into an inert crystalline form (hemozoin) via lipid ¹⁸⁻²⁰ and protein factors such as histidine-rich protein 2 ²¹ and

heme detoxification protein^{22–25}. CQ is a diprotic weak base, which, in its unprotonated form, is able to traverse the parasitized erythrocyte membrane traveling along the pH gradient to accumulate in the DV²⁶. There, it exerts its main activity through (i) interference with hemozoin formation by direct binding to crystals^{27,28}, (ii) formation of a complex with heme and (iii) inhibition of hemoglobin binding to falcipain 2, which in turn affects heme formation²². Indeed, reduced accumulation in the DV is the main cause of CQ resistance, a consequence of mutations to the *pfcrt* gene (*P. falciparum* Chloroquine Resistance Transporter protein), thus facilitating CQ efflux out of the DV^{29,30}.

Given its success, numerous efforts have been conducted to develop and/or screen for compounds that mimic CQ antimalarial activity, focusing predominantly on the quinoline scaffold as the key chemical moiety. The best examples include amodiaquine and mefloquine (MFQ) that were deployed for clinical use in the 1970s²⁶. More recently, the experimental compound MK-4815, a novel chemotype discovered from the MSD chemical collection, was shown to be effective against both CQ-sensitive (CQS) and CQ-resistant (CQR) parasites and highly efficacious in an animal malaria model³¹. MK-4815 appears to share a CQ-like MOA based on the observation that MK-4815 accumulates to low mM concentration in trophozoite/schizont-infected erythrocytes and interferes with heme polymerization³¹. Altogether, hemoglobin degradation disruption remains of high interest for the development of novel antimalarial compounds and one that would benefit from a deeper understanding of underlying molecular processes. Identifying proteins essential for hemoglobin digestion is likely to provide suitable drug targets for novel or improved antimalarials. Here, we used the mass-spectrometry based cellular thermal shift assay (MS-CETSA)^{32,33} to identify possible protein targets of CQ and MK-4815. CETSA methodology is based on the observation that drug-ligand binding typically results in protein stabilization and ultimately leads to a shift in protein melting temperature. Coupled with mass spectrometry, this methodology allows monitoring of the entire proteome simultaneously for changes in protein thermal stability under drug treatment. Here, we demonstrate that both drugs bind directly to a hydrophobic pocket and inhibit the function of a DV residing metalloprotease, falcilysin (FLN). It has been previously shown that FLN is essential for the survival of the parasites and plays roles in multiple organellar compartments, including late stage degradation of host cell hemoglobin in the DV and processing of proteins imported into the parasite apicoplast^{34,35}. Overall, our work introduces a ‘druggable’ pocket in FLN that can be explored further for future antimalarial development.

Results

MS-CETSA identifies falcilysin (FLN) as a protein target of CQ and MK-4815

At the onset of our study, we wished to utilize the mass-spectrometry based cellular thermal shift assay (MS-CETSA) to identify protein targets of CQ and MK-4815, applying a similar strategy as previously described for identifying targets for quinine and mefloquine^{32,33,36}. Specifically, we applied the Iso-Thermal Dose Response (ITDR) implementation of MS-CETSA that monitors protein thermal stability changes for increasing drug concentrations that cover both *in vitro* IC₅₀s and clinically relevant

concentrations of CQ (i.e., ranging from 1 nM to 10 μ M). As the anti-malarial activity of CQ requires cellular accumulation in the DV, we conducted ITDR with live *P. falciparum*-infected erythrocytes. Using the CQ sensitive (CQS) 3D7 strain of *P. falciparum*, we identified a total of nine proteins: six at ITDR 51°C (n=802 proteins) and three at ITDR 57°C (n=694 proteins), that exhibited a thermal stability shift upon CQ exposure (**Figure 1A and Table S1**). From the nine identified proteins, falcilysin (FLN; *PF3D7_1360800*) exhibited the most significant shift characterized by the largest increase in abundance (Δ AUC) following well-structured sigmoidal profile (R^2 value) ³². In contrast, CQ failed to induce significant thermal shift of any protein in the CQ resistant (CQR) Dd2 strain of *P. falciparum* (**Figure 1B**). The Dd2 strain is known to possess up to 10-fold higher EC_{50} for CQ compared to 3D7 ^{29,37}. The lack of response of the CQR Dd2 strain indicates that identified proteins in the 3D7 are likely to represent components of the CQ MOA. Next, we carried out ITDR using MK-4815 to examine expected overlaps with the putative CQ MOA. MK-4815 induced significant thermal shifts of eight proteins (n=509 total identified proteins at ITDR 51°C and n=501 at ITDR 57°C) (**Figure 1C and Table S1**) and three proteins (n=748 total identified proteins at ITDR 51°C and n=516 total identified proteins at ITDR 57°C) (**Figure 1D and Table S1**) in 3D7 and Dd2 strains, respectively. These proteins did not overlap with those identified in the CQ CETSA with the notable exception of FLN, which also exhibited the strongest stabilization amongst the MK4815-induced thermal shifts.

A closer look at FLN thermal stabilization profile showed that CQ induced thermal stabilization in FLN with a minimal dose threshold (MDT; minimum concentration required to induce thermal stabilization) of 13.6 nM in the CQS 3D7 strain, in range with its commonly reported *in vitro* IC_{50} (**Figure 1E**). In Dd2, CQ showed low levels of FLN stabilization at substantially higher MDT of 259.4 nM that is similar to its *in vitro* IC_{50} (**Figure 1F**). In contrast, MK-4815 exerted an equally strong effect on FLN in 3D7 and Dd2 strains, with MDTs of 113 nM and 139.4 nM, respectively (**Figures 1G and 1H**), which also corresponds to their *in vitro* IC_{50} s. This suggests that MK-4815 works equally well in both CQS and CQR strains, as previously suggested ³⁸. Taken together, FLN stabilization profiles from both CQS and CQR strains suggest that FLN engagement is related to CQ MOA.

Next, we conducted ITDR experiments using *P. falciparum* total protein lysate, in which altered thermal stability is more likely to originate from a direct drug interactions³⁹. In the CQ- and MK-4815-treated protein lysates from 3D7 and Dd2 strains, ITDR analysis detected thermal shift of FLN along with small unique sets of other *P. falciparum* proteins (**Figure S1A and Table S1**). Notably, in CQ-treated lysates, FLN thermal stabilization was only observed at concentration ~500-1,000 fold higher as compared to intact cell samples in both 3D7 (MDT of 56.1 μ M) and Dd2 (MDT of 29.2 μ M) (**Figures 1E and 1J**). The substantial difference in FLN thermal stabilization between lysate and intact cell suggests that drug accumulation inside of the parasite is the pre-requisite for protein engagement. This also indicates that in cell lysates, FLN interacts with CQ with the same affinity; regardless of the CQ sensitivity status. In contrast, FLN engages MK-4815 with similar affinities in both intact cell (**Figures 1G and 1H**) and lysate samples (**Figures 1K and 1L**), suggesting that drug accumulation might be less relevant for MK-4815 binding to FLN. All findings above were also validated with western blot analysis, showing that FLN

thermal stability was observed across different antimalarials, strains, and cell preparation types (**Fig. S1B**).

Taken together, these data support the notion that FLN is a direct protein binding partner for both CQ and MK-4815. Moreover, FLN is also a possible binding partner of mefloquine (MFQ), which was determined by its thermal stabilization in intact-cells, in our previous study³³. FLN is a crucial *Plasmodium* metalloprotease that contributes to hemoglobin degradation inside the DV^{35,40}. Next, we explored FLN interactions with CQ, MK4815 and MFQ using a recombinant FLN protein.

CQ, MFQ and MK-4815 bind to FLN and inhibit its proteolytic activity

First, we conducted isothermal titration calorimetry (ITC) measurements with recombinant FLN (**Figures 2A and S2A**). The ITC results showed a 1:1 interaction and K_d values of 1.79 μM ($\pm 0.306 \mu\text{M}$) for MK-4815, 116 μM ($\pm 18.8 \mu\text{M}$) for CQ, and 26.7 μM ($\pm 8.6 \mu\text{M}$) for MFQ, which agree with thermal stabilization values (i.e., EC_{50}) obtained from ITDR experiments (**Table S1**). Enthalpic contributions dominate the MK-4815–FLN interaction (with $\Delta H = -4.43 \pm 0.117 \text{ kcal/mol}$ and $-T\Delta S = -3.42 \text{ kcal/mol}$), whilst entropic contributions are proportionally higher for the CQ-FLN interaction (with $\Delta H = -2.37 \pm 0.251 \text{ kcal/mol}$ and $-T\Delta S = -3.00 \text{ kcal/mol}$). This agrees with the observation of a direct hydrogen bond established by MK-4815 with FLN (see below). As a negative control we tested artemether, an endoperoxide antimalarial with a chemical structure clearly distinct from the structures of either CQ, MFQ, or MK-4815, and found that artemether does not interact with FLN (**Figure S2C**).

Given the ability of CQ, MFQ, and MK-4815 to bind FLN *in vitro* and in intact live cells, we next tested whether binding led to inhibition of FLN protease activity using a ten amino-acid peptide substrate derived from the hemoglobin sequence⁴⁰. Indeed, all three compounds inhibit FLN proteolytic activity with IC_{50} values of 1.85 μM , 56.94 μM and 73.36 μM at pH 7.2 for MK-4815, CQ and MFQ, respectively (**Figure 2B**). At pH 5.2 resembling acidic environment found in the DV lumen, MK-4815 and CQ inhibited FLN with lower efficiency and IC_{50} values 206.7 μM and 20.51 μM while MFQ IC_{50} value dropped slightly to 51.7 μM . These measurements fall within the range of EC_{50} values obtained from lysate ITDR (**Table S1**), showing that FLN activity is inhibited at compound concentrations expected in DV at therapeutic doses. A Lineweaver-Burke plot shows that FLN inhibition by CQ and MK-4815 is noncompetitive (**Figure 2C**) with a decrease in the V_{max} of the reaction whilst the K_m value is not affected. This mode of inhibition is consistent with the observation that the binding pocket shared between the three inhibitors is at a distance of 14 Å from the active site (see below).

Crystal structures of CQ, MK-4815 and MFQ bound to FLN

We obtained co-crystal structures of FLN bound to CQ, MFQ and MK-4815 at 1.82, 1.55 and 1.9 Å resolution, respectively (**Table S2**). In the closed conformation of FLN captured, the large reaction chamber of the active site occupies a volume of 10,886 Å³ which is fully enclosed by the N and C terminal halves of the protein. The M16 protease active site contains a catalytic Zinc ion tetrahedrally coordinated by His129 and His 133, from the inverted 129-HXXEH-133 motif, Glu243 and a water molecule (**Figure 3A**). For all three compounds, clear residual electron density at a distance of ~14 Å away from the metalloprotease active site was present in a hydrophobic pocket lined by residues Phe82, Phe527, Ile 544 and Phe545 (**Figures 3C-3E**). The chloride moiety of CQ points towards this pocket and establishes a halogen bond with the phenyl ring of Phe545, a residue strictly conserved in other *Plasmodium* FLN proteins such as FLN from *P. vivax* and *P. malariae* (**Figures 3C and S3**). Parameters defining this interaction fall within the range of tabulated halogen-pi electron (C-Cl—π) interactions⁴¹ : (i) the distance between the Cl atom and the centroid of the Phe545 phenyl ring is 4.1 Å, (ii) the angle between the CQ and F545 aromatic rings is 69.9° and (iii) the distance between the two rings centroids is 7.1 Å. Thus, this halogen-pi electron interaction plays a key role to orient and stabilize CQ inside the FLN catalytic chamber (**Figure 3C**). Several aliphatic and aromatic residues Phe82, Ile513, Leu514, Ala517, Phe527 and Ile544 further contribute to CQ-FLN binding via Van der Waals and hydrophobic interactions (**Figure 3C**). MFQ, which binds to the same pocket as CQ establishes hydrophobic interactions between its six fluorine atoms and aromatic residues of the pocket including Phe545, Phe82 and Phe527 (note that these residues also make contact with CQ), while Phe44, Leu417, Ile510, Ile513, Leu514, Ala517 and Ile544 establish additional hydrophobic interactions not seen in the FLN-CQ complex (**Figure 3D**). A water molecule coordinated by Asp451 makes a bridge with the hydroxyl moiety of MFQ. In contrast, MK-4815 does not make direct interaction with the cluster of Phe residues (Phe527, Phe544 and Phe545), but rather establishes hydrophobic interactions with Leu417, Ile510 and Ile513, Leu514 and Ala517 via its two tri-methyl groups on its “hydrophobic side” (**Figure 3E**). On its other more “polar side”, its hydroxyl group hydrogen bonds with Glu530 and the amine group of MK-4815 hydrogen bonds with Asp451. When Glu530 was mutated to alanine (FLN-E530A mutant), the mutated recombinant FLN-E530A protein showed ~40 fold weaker binding to MK-4815 with a K_d of 78 μM as measured by ITC (**Figure S2C**). Consistently, the IC₅₀ value of MK-4815 for the FLN-E530A mutant increases to 9 μM (**Figure S2C**).

Next we modelled the structure of FLN in complex with a hemoglobin peptide substrate based on the experimental structure of human presequence protease PreP bound to an amyloid peptide substrate (PDB access code: 4NGE). PreP has an overall 23% amino acid sequence identity with FLN but a closely related 3D structure with a r.m.s.d. of 1.9 Å over 742 Cα atoms following superposition with FLN. This model indicates possible steric hindrance between the drug and peptide substrates longer than 15 amino acids (**Figure 3B**). Hydrophobic residues of a peptide substrate were seen to be anchored precisely in the cavity where CQ is bound, and this cavity was named “hydrophobic exosite”⁴². Therefore, inhibitors binding at this position could reduce the affinity of longer peptide substrates, impairing FLN enzymatic activity. Although in available crystal structures of M16C metalloprotease (PDB codes: 2FGE, 4NGE, 4RPU)⁴², only the closed conformations of M16 metalloproteases are represented, a transition between closed and open conformation in solution must occur to allow substrate entry, while the closed conformation is

conducive to peptide cleavage and at the end of the cycle, FLN must reopen to release cleaved peptides. This cycle between open and closed forms was shown experimentally using SAXS for PreP⁴². Thus, in addition to direct competition with peptide substrate binding, one possibility accounting for the observed enzymatic inhibition is that upon CQ or MK-4815 binding, the conformation of FLN becomes locked in a closed form.

CQ, MK-4815, and MFQ all interfere with hemoglobin digestion

Next, we carried out dose-response transcriptional profiling (DRTP) to analyze transcriptional responses of *P. falciparum* to CQ, MK-4815, and MFQ. The main rationale was to gain a better understanding of the cytotoxic activity induced by these three drugs and the parasite's response. Briefly, genome-wide transcriptional patterns were monitored in parasites treated for 6 hours with a range of drug concentrations as previously described⁴³. Overall, we identified 151, 597, and 200 genes upregulated and 106, 293, and 58 genes downregulated in the CQS 3D7 strain upon treatment with CQ, MK-4815, and MFQ, respectively (**Figure 4A and Supplementary data 1**). In the CQR Dd2 strain, 6 and 178 genes are upregulated and 11 and 293 genes were downregulated upon treatment with CQ and MK-4815, respectively (**Figure 4A and Supplementary data 1**). Consistent with the CETSA result, CQ failed to induce such response in the strain Dd2, which reflects its CQR status⁴⁴. This indicates that the CQ resistance mechanism prevents most if not all cytotoxic activities of the drug within the parasite cell. In the CQS 3D7 strain, the vast majority of the transcriptional changes coincided with the range of effective *in vitro* IC₅₀ values which likely reflects their nature of direct transcriptional responses (**Figures 4A and S5A**). Crucially, primaquine, an unrelated antimalarial with different MOA, induced highly distinct transcriptional response that also coincided with its IC₅₀ value. (**Figure S5B and Supplementary data 1**). This confirms the robustness of the DRTP method and indicates that the transcriptional changes were induced by compound-specific actions. In light of this, functional analyses of the differentially expressed genes revealed similarities in *P. falciparum* responses to CQ, MK-4815, and MFQ, that included genes involved in rRNA processing, protein folding, ribosomal biogenesis commonly to GTPase signaling, phosphate ion transport and translational initiation (**Figures 4B-4C, Supplementary data 2**). Crucially, hemoglobin digestion was overrepresented amongst the genes upregulated in CQ and MK-4815 treated 3D7 CQS parasites (**Figure 4C**). A closer inspection revealed that CQ caused upregulation in almost all genes involved in the main hemoglobin proteolytic cascade, including falcipains, plasmepsins, aminopeptidases, and heme detoxification protein (HDP) (**Figure 4D**). Some of these genes were also upregulated in MK-4815- and MFQ-treated 3D7 CQS and MK4815 Dd2 CQR parasites, suggesting a degree of similarity in their MOA. Conversely, hemoglobin digestion genes were largely unaffected in the CQ-treated Dd2 CQR strain which is consistent with the lack of effect produced by the drug in these parasites. In addition to the genes directly involved in the hemoglobin digestion process, two DV transmembrane transporters involved in CQ and MFQ resistance, *pfcr1* and *pfmdr1*, exhibited similar transcriptional response (**Figure 4D**). Upregulation of *pfcr1* and *pfmdr1* in MK-4815 treated parasites is

particularly interesting and suggests a tight link between these two transmembrane transporters with hemoglobin digestion.

To test the overlap in their anti-malarial activity, we used isobologram analysis to uncover the synergy or antagonism between CQ and MK-4815, indicative of a common target. In addition we use o-phenanthroline (PHEN), a known zinc chelator and FLN inhibitor⁴⁵ to test its involvement in the shared MOA. Essentially, CQ was observed to show antagonism with MK-4815 and PHEN in 3D7, with a mean FIC index of 1.67 and 1.36, respectively (Fig. 4E). This is consistent with competition for a shared binding site within the FLN peptide binding cavity and points to some similarity in their respective MOA. In contrast, in Dd2 strain, combination of CQ/MK-4815 showed an additive effect (mean fractional inhibitory concentration; FIC = 1.04) and a less pronounced antagonism with a CQ-PHEN combination (mean FIC = 1.21) (**Figure 4E**). Combination of MK-4815/PHEN in both 3D7 and Dd2 strains was found to be additive, with a FIC index of 1.01 and 1.08, respectively (**Figure 4E**). Taken together, the transcriptional responses and the isobologram analyses are in a good agreement with a model in which inhibition of FLN by CQ, MK-4815 play a role in the drugs MOA via disruption of hemoglobin degradation.

Conditional knockdown of FLN affects CQ *in vitro* drug sensitivity

To gain further insight into the biological importance of FLN binding and inhibition by CQ- and MK-4815 for *P. falciparum* survival, we generated both an episomal overexpression and conditional knock down FLN transgenic parasite lines. These two approaches are the most permissible for studying the biological role of FLN, given its essentiality that precludes complete gene disruption⁴⁵. For FLN overexpression study, we generated a transgenic *P. falciparum* line using the 3D7 CQS strain. For that full length FLN (excluding stop codon) was fused to Hemagglutinin (HA) tag at the C-terminus and cloned into pBcamR_3xHA transfection vector, allowing adjustable expression via increased copy number (**Figure S6A**)⁴³. Crucially, we observed a pronounced toxic effect caused by FLN overexpression represented by severely distorted parasite morphology characterized by malformed trophozoites and schizonts between these two lines upon recovery, especially at schizont stage (**Figure 5A**) and 3-fold reduction in number of newly formed merozoites (**Figure 5B**). These morphological changes were observed throughout the transgenic parasite growth starting at the parasite recovery, approximately 23 and 30 days after transfection and initial application of Blasticidin S drug pressure. The reduction in merozoite formation is likely responsible for the severe growth impedence of the FLN overexpression line, keeping the overall parasitemia below 1% through the entire transgenic experiment (**Figure 5C**). In addition to the growth inhibition the FLN overexpression cell line contained dramatically increased proportion of sexual stages (Gametocytes) (**Figure 5A**), a phenomenon commonly associated with stress⁴⁶. Taken together, these observations complement the current view of FLN being highly essential for *P. falciparum* asexual stage growth and development showing that not only its presence, but also a precise expression level is crucial.

In the absence of the possibility of complete gene disruption, next we generated a *P. falciparum* CQS 3D7 transgenic line with a chromosomal *fln* gene copy fused with the HA tag and *Eschericia coli* dihydrofolate reductase destabilizing domain (DDD) (**Figure S6B**). The *E. coli* DDD approach has been used to successfully partially disrupt gene function by protein destabilization in the absence of the DDD ligand: trimethoprim (TMP)⁴⁷⁻⁴⁹. Indeed, upon validation by PCR and western blot (**Figures S6C and S6D**) we wished to use the transgenic cell line to test the effect of FLN protein level depletion/reduction on both growth as well as drug sensitivity profiles. Overall, we achieved reductions of the FLN protein levels by 83.6% that was observed immediately following the TMP withdrawal within the first IDC and as such reduced FLN levels were maintained at ~15-20% of its original level for at least three subsequent IDCs (**Figures 5D and 5E**). These reduced FLN expression level, however, had no effect on the parasite growth with no significant differences in the multiplication rates between parasites grown in the presence or absence of TMP (**Figure S6F**). Moreover, in the absence of TMP, the parasite morphology remained normal (**Figure S6E**). This indicates that the DDD-TMP-based approach can achieve only partial FLN depletion (up to 85%) with a minimal effect on the parasite growth and morphology; hence the parasite can survive with ~15% of the original FLN level. Crucially these partial depletions lead to increased susceptibility of the transgenic parasites to both FLN interacting drugs, CQ and MK-4815 (**Figure 5F**). Specifically, we observed subtle but statistically significant decreases of 50% inhibitory concentrations (IC₅₀) for both drugs measured by *in vitro* drug sensitivity assays initiated at two IDC developable stages; ring and trophozoite. Upon withdrawal of TMP, in rings, the average IC₅₀ dropped from of 12.9 nM to 11.3 nM (pValue ~0.019) for CQ and 243.2 nM vs 219.8 nM (pValue ~0.049) for MK-4815. Similar drops were observed in the trophozoite-based assay from 22.3 nM to 19.3 nM (pValue ~0.016) for CQ and 234.1 nM to 203.5 nM (pValue ~0.047) for MK-4815. no statistically significant difference was observed in similar assays conducted with a functionally unrelated antimalaria drug, dihydroartemisinin. Taken together, our data suggest that binding of CQ and MK-4815 to the inhibitory pocket of FLN contributes significantly to both drugs MOA and as such this binding site represents a potential druggable domain hat can be explored in future drug design efforts.

Discussion

Traditionally, the development of antimalarials relies heavily on phenotypic-based screening, in which compounds are screened based on their inhibitory potential. While this approach has led to the discovery of the most important antimalarials in history such as the aminoquinolines (e.g., chloroquine, mefloquine, and piperazine) and artemisinins (e.g., dihydroartemisinin, artesunate, and artemether), it is becoming more evident that new classes of antimalarials are urgently needed, given that parasites have developed resistance to most of these compounds (reviewed in ref 50). Knowledge of drug target(s) is imperative for the development of effective and safe antimalarials. Without knowing which part of the drug interacts with its target, chemical changes needed to improve the compound efficacy and safety are done in a trial-error manner^{51,52}. Here, our MS-CETSA based screening approach successfully identified a number of potential drug binding partners of CQ and MK-4815 in *P. falciparum*, with FLN exerting the strongest thermal stabilization. Follow-up biochemical and structural validation experiments demonstrate that FLN

is indeed one common protein binding partner of CQ, MK-4815, and MFQ and these compounds can inhibit its enzymatic activity at various levels. Interestingly, all drugs bind to the same hydrophobic binding pocket, albeit via different binding interactions. The main site for FLN biological activity is believed to be the DV, where FLN participates in hemoglobin degradation via its metalloprotease activity^{34,35,40,53}. The DV is also the main site of the CQ cytotoxic activity mediated by its interference with hemozoin formation that leads to increased levels of free heme⁵⁴, causing oxidative stress and subsequent parasite death. CQ must accumulate in the DV to reach mM concentrations which are achieved even in the presence of low μM external drug concentrations⁵⁵. Here, we uncovered several biological features that are consistent with a model in which this CQ-FLN interaction contributes to the drugs' MOA. First, CQ binds to FLN with $K_d=115\ \mu\text{M}$ and IC_{50} values of $56.94\ \mu\text{M}$ and $206.7\ \mu\text{M}$ at pH 7.5 and 5.2, respectively. These values are relevant to expected accumulations levels of CQ in the acidic environment of the DV essential for its activity⁵⁵⁻⁵⁸. Second, PfCRT-mediated efflux is known to lower CQ accumulation in the DV to a level at which the drug is no longer clinically creating CQR *P. falciparum* strains⁵⁹⁻⁶¹. This is consistent with the lack of FLN-CQ engagement that we observed in the studied CQR Dd2 strain in which CQ accumulation in the DV is thought to be reduced by 3-10-fold^{58,61-63}. Third, our transcriptomics experiment showed upregulation of genes involved in hemoglobin degradation which is currently the main functional assignment of FLN^{26,53,57,62,64-67}. Finally, here we showed that conditional knockdowns of FLN cause subtle but statistically significant levels of hypersensitivities of *P. falciparum* CQS strain to both FLN-binding drugs, CQ and MK-4815. Taken together, our data suggest that FLN and its inhibitory binding pocket contributes to the CQ and MK4815 MOAs. In future studies it will be interesting to assess precisely to which level FLN-CQ binding contributes to the overall CQ MOA that is known to be intrinsically complex involving multiple molecular factors (see above). Nevertheless, results from this study indicate that the inhibitory CQ binding site on FLN could constitute a promising target for novel antimalaria drugs in the future and put FLN on the list of potential antimalarial drug targets for development programs that are currently ongoing around the world^{51,52,68-70}.

Similar to CQ, our data showed that binding and inhibition of FLN enzymatic activity by MK-4815 and MFQ also requires relatively high concentration of these compounds, exceeding their respective *in vitro* IC_{50} values. This suggests that a degree of accumulation is also needed for the latter two compounds. At the moment, the extent of drug accumulation of MFQ and MK-4815 in live parasites is relatively unknown. In the case of MK-4815, the compound has been shown to selectively accumulate in infected red blood cells, although its exact localization site is yet to be determined³⁸. For MFQ, many studies have shown that the main MOA and targets are likely to be outside the DV and MFQ transport into DV through PfMDR1 is, in fact, a method to confer partial resistance^{29,71-73}. Given that FLN stabilization by MFQ requires drug concentration far exceeding its *in vitro* IC_{50} (MDT of $1\ \mu\text{M}$ in intact cell experiment³³ vs *in vitro* IC_{50} of $<50\ \text{nM}$), it is likely that (i) FLN-mediated inhibition plays at best a minor role in MFQ MOA and (ii) MFQ binds to cytosolic FLN rather than DV-based FLN.

How inhibition of FLN proteolytic activity translates into the parasite's death, remains to be fully investigated. In addition to its generally accepted MOA in the inhibition of heme detoxification and hemozoin formation, several observations also link CQ MOA with the hemoglobin degradation process. CQ treatment leads to marked accumulation of undigested hemoglobin levels⁶⁴, long-chain hemoglobin-derived peptides⁷⁴, and di-/tri-peptides⁷⁵, indicating disruption in the hemoglobin digestion cascade. In the DV, FLN is also likely to be present in a hemozoin formation complex along with HDP, falcipains, and plasmepsins, although the role of FLN in this complex is unknown²². It is plausible that in the DV, CQ exerts its action by inhibiting both FLN enzymatic activity and hemozoin formation/heme detoxification. Although the sequence of events leading to this phenomenon is unclear, it is likely that these processes are directly correlated to each other. Apart from the DV, FLN has been shown to accumulate in the apicoplast, in addition to a minor pool in the mitochondrion, where in the apicoplast it functions to cleave free signal transit peptide which otherwise could insert into membrane lipid bilayers and disrupt organelle function⁴⁵. The apicoplast has garnered attention as a drug target in recent years following the discovery of its essential function in providing the parasites with a number of important cofactors (reviewed in ref⁷⁶). At this point it is not known whether the binding of these compounds to apicoplast-based FLN will contribute to their respective cytotoxicity, but considering that apicoplasts are not similarly acidic as DV, it is less likely that these compounds are able to accumulate to concentrations required to inhibit apicoplast-based FLN (at least for CQ). It is also highly likely the cytotoxic effect of CQ as well as MFQ and MK-4815 is exerted via additional targets, some of which were identified in our CETSA survey (**Figures 1 and S1; Table S1**). This is consistent with the emerging view of the current antimalarial chemotherapeutic agents having pleiotropic biological effects. For instance, tRNA ligase, which we identified in our CQ MS-CETSA datasets, was shown to be inhibited in the presence of excess free heme as a consequence of CQ treatment⁷⁷, although at this stage, whether the observed thermal stabilization was due to direct binding or downstream effect remains to be determined. A complete study of the possible interplay between identified targets and CQ or MK-4815 MOA is beyond the scope of this study. However, we believe the present data show that further studies are warranted.

In recent years, MS-CETSA has garnered attention as a powerful tool to study drug target engagement. The CETSA methodology and its variants were originally developed to assist in anti-cancer drug target discovery efforts^{39,78}; however, the use of this methodology has now been expanded to study drug target engagement in human pathogens, such as *E. coli*⁷⁹, toxoplasma⁸⁰, and *P. falciparum*^{32,33,81,82}. In our previous work, we demonstrated the value of CETSA for antimalarial drug deconvolution by identifying PfPNP as a direct molecular target of QN but also of MFQ³³. In conclusion, we identified FLN as one binding partner of CQ, MK-4815 and MFQ and discussed potential implications for their respective MOAs. We propose that the hydrophobic pocket we uncovered in the FLN binding cavity constitutes a promising antimalarial drug target and that future SAR studies based on the compound-bound FLN structures presented in this work are warranted.

Materials And Methods

Parasite culture

P. falciparum 3D7 and Dd2 cultures were maintained in Malaria Culture Media (MCM) containing RPMI-1640 medium (Gibco) supplemented with 0.25% Albumax II (Gibco), 0.1 mM hypoxanthine (Sigma), 0.2% sodium bicarbonate (Sigma), and 10 mg/L gentamycin. Human blood group O were used for parasite culture and cultures were maintained at 37°C with 5% CO₂, 3% O₂, and 92% N₂. Culture media were replenished daily with freshly packed RBC added to the culture when necessary.

Sample preparation for ITDR MS-CETSA and LC/MS analysis

Samples for intact-cell and lysate ITDR MS-CETSA and LC/MS analysis were prepared as described in Dziekan *et al*^{32,33}. For both intact-cell and lysate MS-CETSA, synchronized mid-trophozoite stage (28±4 HPI) culture at 10% parasitaemia and 2% hematocrit was used. In case of intact-cell MS-CETSA, 1 mL of packed blood was loaded on MACS CS column (Milty Biotech), washed, and eluted. Enriched infected RBC (iRBC) (>80% parasitaemia) were then incubated for 1 hour in MCM at 37°C with agitation for recovery and equilibration. The iRBC were then divided into 10 aliquots (75-90 million cells in 6 mL MCM for each aliquot) and treated with 4-fold dilution series of CQ (Sigma, 10 µM top concentration), MK (100 µM top concentration), or an equivalent volume of their respective solvent at 0.1% concentration, and incubated for 1 hour at 37°C with agitation. Cells were pelleted by centrifugation (2,500 rpm, 5 minutes), washed with PBS, resuspended in 150 µL PBS per aliquot and transferred in 50 µL aliquots to three 96-well plates, each representing different heat challenge temperature (37°C, 51°C, or 58°C). Samples were subjected to thermal challenge for 3 minutes, followed by 3 minutes cooling at 4°C. After heat treatment, 50 µL of lysis buffer (50 mM HEPES pH 7.5, 5 mM beta-glycerophosphate, 0.1 mM Na₃VO₄, 10 mM MgCl₂, 2 mM TCEP, and cocktail EDTA-free protease inhibitors (Sigma)) were added to each well. Samples were then subjected to 3 times of freeze/thaw cycle, followed by mechanical shearing and centrifugation (20,000g, 20 minutes, 4°C) to isolate the soluble protein fraction. Protein quantification was then performed with bicinchonidic assay (BCA) protein assay kit (Pierce). In case of lysate MS-CETSA, the parasite culture was pelleted and incubated with 10x volume of 0.1% saponin in PBS (pH 7.2) for 5 minutes. Following centrifugation (2,500g, 5 minutes) the supernatant containing RBC cytosol was removed, whilst intact parasite pellet was washed 3 times with 50 mL of ice-cold PBS. Parasite was then resuspended in 1 mL of lysis buffer and subjected to 3 times of freeze/thaw cycle, followed by mechanical shearing. Parasite lysate was then centrifuged (20,000g, 20 min, 4°C) and supernatant containing soluble parasite proteins was isolated. Protein concentration was quantified with BCA protein assay kit. Ten aliquots of 20 µg of proteins were added to 4-fold dilution series of CQ (top concentration 500 µM), MK (top concentration 250 µM), or MFQ (Sigma, top concentration 1 mM), incubated at room temperature for 3 minutes, and heated at 37°C, 51°C, or 57°C for 3 minutes followed by 3 minutes cooling at 4°C. The post heating lysates were centrifuged at 20,000g for 20 minutes at 4°C and the supernatant was transferred to a fresh Eppendorf tube for further processing.

Peptide preparation and labeling

Following quantification of lysate and intact-cell samples, the volume equivalent to 20 µg total protein in post heating treatment was aliquoted and incubated with denaturation and reduction buffer containing 100 mM triethylammonium bicarbonate (TEAB, pH 8.5), 20 mM tris(2-carboxyethyl)phosphine) (TCEP, pH 7.0), and 1% (w/v) Rapigest (Waters) at 55°C for 20 minutes, followed by alkylation with 55 mM chloroacetamide (CAA) at room temperature for 30 minutes. Samples were then sequentially digested with Lys-C (0.23 AU LysC/µg of protein, Wako) for 3 hours, followed by trypsin (0.05 µg trypsin/µg of protein) for 18 hours at 37°C. Enzyme activation and Rapigest degradation was performed by incubation with 1% trifluoroacetic acid (TFA, Sigma) for 45 minutes at 37°C and the sample supernatant containing peptides was collected by centrifugation at 20,000g for 10 minutes. The samples were then dried in a centrifugal vacuum evaporator and resolubilized with 200 mM TEAB to 1 µg/µl concentration. Peptides were labeled with TMT10plex Isobaric Label Reagent Set (Thermo Fisher Scientific) according to manufacturer's protocol. Briefly, 4 µg of the digested protein was labeled for at least 1 hour at a condition of pH>6 and then quenched with 1 M Tris (pH 7.4). The labeled samples were subsequently pooled and desalted using a Oasis HLB column (Waters), followed by vacuum drying. Samples were resuspended in 5% (v/v) acetonitrile, 5% (v/v) ammonia and separated using high pH reverse phase Zorbax 300 extend C-18 4.6 mm x 250 mm (Agilent) column and liquid chromatography AKTA Micro system (GE Healthcare) was used for offline sample pre-fractionation into 96 fractions. The fractions were concatenated into 20 fractions and dried with a centrifugal vacuum concentrator.

LC/MS analysis

The dried peptide sample fractions were resuspended in 1% acetonitrile, 0.5% (v/v) acetic acid, and 0.06% TFA in water prior to analysis on LC/MS. Online chromatography was performed using the reverse phase liquid chromatography Dionex 3000 UHPLC system coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Each fraction was separated on a 50 cm x 75 µm Easy-Spray analytical column (Thermo Fisher Scientific) in 70 minutes gradient of programmed mixture of mobile phase A (0.1% formic acid) and mobile phase B (99.9% acetonitrile, 0.1% formic acid) to the following gradient over time: 1-55 min (2-25%), 55-57 min (25-50%), 57-58 min (50-85%), 58-63 min (85%), 63-70 min (2%). MS data were acquired using data-dependent acquisition (DDA) with full scan MS spectra acquired in the range of 350-1550 m/z at a resolution of 70,000 and AGC target of 3e6; Top12 MS² 35,000 and AGC target of 1e5, and 1e5 isolation window at 1.2 m/z.

Protein identification and quantification

Proteome Discoverer 2.1 software (Thermo Fisher Scientific) was used to perform protein identification and quantification based in the Xcalibur raw files. Raw spectra was submitted for database searching with Sequest HT (Thermo Scientific) search engine using combined Sanger Institute *P. falciparum* 3D7 or Dd2 Coding Sequences database and Uniprot database for human proteins. MS precursor mass tolerance was set at 30 ppm, fragment mass tolerance set at 0.06 Da and maximum missed cleavage sites of 3. Dynamic modifications include oxidation (M), deamidated (NQ), acetylation (N-terminal protein) while static modification includes carbamidomethyl (C). Forward/decoy searches were used for false discovery rate (FDR) estimation. Peptide and peptide spectrum matches (PSMs) with high=FDR 1% and medium=FDR 5% levels were accepted.

Quantitative MS data analysis and MS-CETSA data processing

For downstream analysis, only unique peptides were used for quantification of associated protein abundance and only proteins quantified by at least 3 PSMs were used. Relative abundances of proteins under different compound concentrations were normalized against untreated control and fed into “mineCETSA” R package (available at <https://github.com/nkdailingyun/mineCETSA>) for data extraction, clean-up, normalization, curve fitting, hit selection, and plotting. The ITDR hit selection considers the global responses from the measured protein population and applies two thresholds: a Median Absolute Deviation scheme where the threshold was set at median $\pm 3 \times \text{MAD}$ and the R^2 value of curve fitting (indicating goodness of fit) for the selection of the most prominent hits.

Western blotting

Western blots analysis was performed with either a) post-heating lysates generated from the corresponding lysate or intact-cell ITDR experiments (Fig. S8) or b) lysates generated from trophozoite of parasites cultured with or without TMP (Fig. 5D and Fig. S14D). For each well, 10 μg (for FLN-DD experiment) or 20 μg (for CETSA experiments) of protein lysates for each treatment condition was fractionated in 4-15% Mini-Protean TGX protein gels (Bio-Rad) and transferred to nitrocellulose membrane using Bio-Rad Trans-Blot Turbo Transfer System with “Turbo Mixed MW” settings (constant 2.5A for 7 minutes). Primary antibodies used are anti-FLN (rabbit) at 1:2000, anti-PfBIP (rabbit) at 1:5000, anti-HA at 1:5000 (rat), or anti-14-3-3 (mouse) in 5% nonfat milk in TBS-Tween (TBST). HRP-conjugated secondary antibodies (Biolegend) diluted at 1:2000 in 5% nonfat milk in TBST was used accordingly to their corresponding primary antibodies. Blot was developed with Clarity Western ECL system (Bio-Rad), and the image was generated and analyzed using LAS-3000 imager (Fujifilm)

Protein expression and purification

Falcilysin gene (UniProtKB - Q76NL8, gene ID: 814283) coding for residues 59-1193 was synthesized and cloned into pNIC28-Bsa4 downstream a 6xHis tag and TEV cleavage site (pNIC-FLN59-1193). BL21 (DE3) Rosetta T1R cells were transformed with pNIC-FLN59-1193 and grown at 37°C until OD600 reached 0.6. Protein expression was induced by addition of 0.5 mM IPTG for 16 hours at 16°C. The bacteria were harvested by centrifugation and cells were resuspended in 100 mM Na HEPES, 500 mM NaCl, 10 mM Imidazole, 10% (v/v) glycerol, 0.5 mM TCEP, pH 7.5, and lysed mechanically (LM20 microfluidizer). Lysate was cleared by centrifugation at 50,000 g for 1 h. Protein was purified by affinity chromatography using Ni-NTA beds (GE Healthcare) and eluted in 20 mM Na HEPES, 500 mM NaCl, 500 mM Imidazole, 10 % (v/v) glycerol, 0.5 mM TCEP, pH 7.5 before size exclusion chromatography using Hiload 16/600 Superdex 200 pg column (GE healthcare) equilibrated in 20 mM NA HEPES, 300 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP, pH 7.5. Protein purity was checked by SDS-PAGE, before protein was concentrated to 22 mg.ml⁻¹, flash frozen and stored at -80°C until use.

Crystallization, Data Collection and Processing

Crystallization conditions for falcilysin were screened with a protein concentration of

22 mg.ml⁻¹ using commercial kits (JCSG-plusTM & Morpheus from Molecular Dimensions; Index & PEG/Ion ScreenTM from Hampton Research) using a mosquito crystallization robot (TTP Labtech). Crystals were obtained after a few days. To obtain the structures of protein-inhibitor complexes, native FLN crystals were soaked with inhibitors at 10 mM concentration for approximately 1.5 h before being flash-frozen in liquid nitrogen. Detailed buffer composition for each structure are presented in **Table S2**. X-ray diffraction intensities were collected at the PXIII beamline (Swiss Light Source, Villigen), integrated and scaled using XDS⁸³. Structures were solved with molecular replacement as implemented in the program Phaser⁸⁴ using the *P. falciparum* falcilysin ligand-free structure (Goldberg et al, unpublished, PDB access code: 3S5M) as a search probe. Refinement and simulated annealing omit map calculations were done using the Phenix package. Manual model corrections were performed at the computer graphics using Coot⁸⁵. Data collection and refinement statistics are shown in **Table S2**. Structure similarity search was performed using ssm from EBI, (<https://www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmserver>)⁸⁶ and returned eight similar structures (r.m.s.d. of 1.78-2.17) all belonging to the M16C metalloprotease family. The volume of the catalytic cavity in the FLN protein was calculated using the 3V webserver⁸⁷. MAD scan was performed to confirm the presence of Zinc in FLN crystal (**Figure S4**). A dataset was collected at 1.278 Å near Zinc K edge and the anomalous signal was used to confirm the presence and the location of the active-site Zinc unambiguously (**Table S3**).

Isothermal titration microcalorimetry measurements

Isothermal titration calorimetry data were collected using MICROCAL PEAQ-ITC (Malvern) at 25 °C with 19 injections and 500 rpm stirring speed in buffer 20 mM Na-HEPES pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP, 1% DMSO. K_d of MK-4815 (1.79 μ M) was determined by titrating 200 μ L of 100 μ M FLN protein with 36.4 μ L of 1 mM MK-4815 (**Figure 2A**). Determination of K_d of CQ to FLN via direct ITC was impossible due to its low binding affinity and limited sensitivity of ITC equipment. Instead, K_d of CQ was estimated by displacement titration⁸⁸, titrating 200 μ L mixture of 100 μ M FLN protein and 200 μ M CQ with 36.4 μ L of 1 mM MK-4815, which yielded an apparent K_d of 5.5 μ M (Fig. 2B). The increase of apparent K_d for MK-4815 from 1.79 μ M to 5.5 μ M was caused by competition of CQ for the same binding site, from which a K_d of 116 μ M for CQ was estimated using competitive mode in Marven analysis software. Binding of MK-4815 to FLN was largely inhibited when 200 μ L mixture of 100 μ M FLN protein and 2 mM CQ was titrated with 36.4 μ L of 1 mM MK-4815 (**Figure S2A**).

Enzymatic assay

To determine IC_{50} values of the three inhibitors, FLN activity was monitored using substrate fluorescence quenching at pH 7.5 and pH 5.2 representing values encountered in the apicoplast/mitochondrion and DV respectively. The FLN protein at a concentration of 20 nM was incubated with 20 μ M of substrate peptide⁴⁰ (YNHHS⁻FFMEE where the arrow indicates the scissile peptide bond) in presence of various concentrations of inhibitors (ranging from 1 mM to 0.48 μ M for CQ and MFQ and from 100 μ M to 0.048 μ M for MK-4815). Measurements for each concentration were performed as triplicate. A TECAN 10M plate reader, operated at 336 nm and 495 nm wavelengths for excitation and emission respectively was used to follow FLN protease activity. The reaction was started with automated addition of the enzyme into a mixture of substrate and inhibitor, followed for a total duration of 1 min. The initial velocity was determined automatically using Magellan® software provided by the manufacturer. The V_{max} values obtained were plotted against the log of the inhibitor concentration in GraphPad Prism to obtain the IC_{50} values.

To assess the MOA of MK-4815 and CQ, Michaelis-Menten steady state kinetics parameters were measured in the presence of various inhibitor concentrations. A serial dilution of substrate starting from a maximum value of 20 μ M of substrate was incubated with three concentrations of inhibitor ranging from approximately 0.1 to 10 times (2.5 times for CQ due to its lower affinity) of their respective K_d values (0.1 μ M, 2 μ M and 10 μ M of MK-4815 and 1 μ M, 50 μ M and 250 μ M of CQ). The reaction was started with automated addition of the enzyme into a mixture comprising the substrate and inhibitor and followed for 1 min. The initial velocity was determined automatically using Magellan® software. The values of reciprocal velocity were plotted as a function of reciprocal concentration in a Lineweaver-Burk graph.

In vitro drug sensitivity assay and isobologram analysis

For isobologram analysis (**Figure 4E**), drug assays were performed in 100 μ L volume with mid-ring stage (12 ± 4 HPI) of 3D7 and Dd2 strains at 0.5-1% parasitaemia and 2% hematocrit in a 96-well format. For standard *in vitro* drug sensitivity assay (**Figures 5E and S5A**), ring stage (± 12 -16 HPI) was used. The top concentration of compounds used in was 3,200 nM CQ, 16,000 nM MK, 1,000 nM MFQ, and 100 μ M α -phenanthroline followed by 2-fold serial dilution and a drug-free control. For isobologram analysis, IC_{50} measurements were conducted for each drug alone and for combinations at fixed volumetric ratio (i.e., Drug A:Drug B ratio – 5:0, 4:1, 3:2, 2:3, 1:4, 0:5). For assays with 3D7-FLN DD, the parasites in -TMP group were cultured without TMP for at least 2 cycles prior to the commencement of assay to ensure sufficient depletion of FLN. For wash-off assays, parasites were incubated with the compounds for 6 hours, followed by 2x washes with MCM to remove the compounds completely from the MCM, followed by 66 hours incubation. A total of 72 hours incubation time was done for all assays. Following incubation, supernatant was removed and iRBCs were stained with 50 μ L of 8 μ M Hoechst 33342 in PBS (pH 7.2) for 15 minutes at 37°C, followed by addition of 200 μ L of PBS. Results readout was conducted with the LSR Fortessa X-20 Flow Cytometer (BD Biosciences) equipped with HTS sampler using UV laser (355 nm). Raw FCS data was gated and analyzed with FACS DIVA software. Linear regression analysis of dose-response curves and IC_{50} determination was subsequently done using GraphPad Prism 8 (Graphpad Software). Isobologram analysis was conducted according to Loewe additivity model, where the sum of fractional inhibitory concentrations (ΣFIC_{50}) for the two compounds in a combination is a measure of additivity, synergism, or antagonism. The combination with $\Sigma FIC_{50} > 1$ are considered antagonistic, < 1 synergistic, and equal to 1 is additive⁸⁹.

Drug treatment and RNA extraction for DRTP

For DRTP experiment, sorbitol-synchronized mid-trophozoite stage (28 ± 4 HPI) of 3D7 and Dd2 culture were used. Parasite culture was divided into 10 well in two 6-well plates with each well containing 6 mL of culture at 3% parasitaemia and 2% hematocrit and treated with 4-fold dilution series of CQ (10 μ M top concentration), MK (20 μ M top concentration), MFQ (10 μ M top concentration), primaquine (50 μ M), and an equivalent volume of DMSO at 0.1% concentration and incubated for 6 hours at 37°C with 5% CO₂, 3% O₂, and 92% N₂. Following incubation, the samples were harvested and centrifuged at 2,500 rpm for 5 minutes. After removal of supernatant, 1 mL of Trizol reagent (Invitrogen) was added to the iRBC pellet, followed by vigorous shaking and incubation at 37°C for 10 minutes. Following lysis, 200 μ L of chloroform was added and the mixture was incubated in ice for 5 minutes with periodical shaking. Samples were then centrifuged at 12,000g for 15 minutes at room temperature to separate the organic and aqueous phases. Five hundred μ L of the aqueous phase was collected and RNA was purified by using the Direct-zol-96 RNA kits (Zymo Research) as per manufacturer's protocol.

cDNA synthesis, SMART amplification, and microarray analysis

cDNA synthesis, Switching Mechanism at 5' End of RNA Template (SMART) amplification of double stranded cDNA, and microarray hybridization was performed as previously described by Bozdech *et al.*⁹⁰. Briefly, 500 ng of total RNA was used as the input for reverse transcription using SuperScript II (Invitrogen). The cDNA product was then amplified using SMART-PCR containing aminoallyl-dUTP and dNTPs. Four µg of SMART-amplified samples were then labelled with Cy5. On the other hand, reference cDNA generated from pooling together RNA from all IDC stages of 3D7 was labelled with Cy3. Equal amounts of Cy3- and Cy5-labelled products were then hybridized onto a microarray chip containing 10,265 probes covering ~5000 genes in the *P. falciparum* genome. Fluorescence intensity from labelled samples were scanned with Tecan Powerscanner open-format microarray scanner. Background subtraction, normalization, and filtering of raw signal intensities for each array were done using the R package 'limma'⁹¹.

DRTP and functional pathway enrichment analysis

Selection of dose-dependent up- or downregulation was performed with the 'mineCETSA' package with modifications. Gene expression log₂ ratios of treated samples were first normalized and converted into fold change of gene expression against untreated samples for each drug. The fold-change expression data were then fed into 'mineCETSA' package for data normalization and fitting. Following curve fitting, dose-dependent up- and down-regulation were then selected using the following criteria: 1) at least 30% fold change increase compared to untreated control in the top 3 highest concentration, 2) goodness of sigmoidal curve fitting (R²) value greater than 0.8 in 2 biological replicates, and 3) unidirectionality of each gene response in 2 biological replicates. Genes showing dose-dependent expression changes were then subjected to functional pathway enrichment analysis to calculate functionally enriched pathways in a given dataset using the functional gene annotation from Gene Ontology (GO) and Malaria Parasite Metabolic Pathway (MPMP) databases as described previously⁹². Over-representation of pathways as compared to their respective frequency in the genome was calculated based on hypergeometric test ($p < 0.05$).

Plasmid construction and transfection

To generate plasmid for overexpression study, full length FLN open reading frame (omitting the stop codon) was PCR amplified from *P. falciparum* 3D7 genomic DNA with primers 5- T⁵ GCA C and 5- TGCTTAGCTAGCTTCTATTAATACCTTTTTA-3 and cloned into the NcoI/NheI sites of pBcamR_3xHA in-frame with the coding sequences of 3xHA to generate the plasmid pFLN OE. To generate a chromosomal FLN-3HA-DD fusion, a 670 bp fragment at the 3' end of the FLN open reading frame (omitting the stop codon) was PCR amplified from *P. falciparum* 3D7 genomic DNA with primers 5- TAAGCACTCGAGTAAAGTAAATGATCCAAC-3 and 5- TGCTTACCTAGGTTCTATTAATACCTTTTTA-3 and

cloned into the XhoI/AvrII sites of pFLN-HADB in-frame with the coding sequences of 3xHA and *E. coli* destabilization domain. One hundred micrograms each of the resulting plasmid and pHDFR were co-electroporated into ring stage parasites of Dd2 parasites as previously described⁹³. Plasmid-containing parasites were selected with 5 µM TMP (Sigma) and 2.5 µg/mL Blasticidin (Sigma), followed by limiting dilution of surviving transfectants. Clones were screened by PCR and one clone with validated integration was selected for further analysis, designated Dd2-FLN DD.

Data availability

The data that support the findings of this study are available from the corresponding author upon request

Declarations

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Author contributions

Z.B. and J.L. conceived and developed the idea for this article. G.W., J.M.D., J.Q.L., and A.E.S. wrote the manuscript and performed data analysis. G.W., J.M.D., J.Q.L., A.E.S., N.E.B.Z., J.B., R.T.J.K., S.T., K.D.G., and H.Y. carried out experimental work for the experiments presented. G.W., J.M.D., J.Q.L., A.E.S., A.P., D.O., N.P., R.M.S., and P.N. contributed to the development of experimental protocol and reagents in this article. All authors provided feedback and changes to the manuscript. G.W., J.M.D., and Z.B. brought together the text and finalized the manuscript.

References

- 1 World Health Organization. World Malaria Report 2020. (Geneva, Switzerland, 2020).
- 2 Ashley, E. A. *et al.* Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* **371**, 411-423, doi:10.1056/NEJMoa1314981 (2014).

- 3 Dondorp, A. M. *et al.* Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* **361**, 455-467, doi:10.1056/NEJMoa0808859 (2009).
- 4 van der Pluijm, R. W. *et al.* Determinants of dihydroartemisinin-piperaquine treatment failure in *Plasmodium falciparum* malaria in Cambodia, Thailand, and Vietnam: a prospective clinical, pharmacological, and genetic study. *Lancet Infect Dis* **19**, 952-961, doi:10.1016/s1473-3099(19)30391-3 (2019).
- 5 Phyo, A. P. *et al.* Declining Efficacy of Artemisinin Combination Therapy Against *P. Falciparum* Malaria on the Thai-Myanmar Border (2003-2013): The Role of Parasite Genetic Factors. *Clin Infect Dis* **63**, 784-791, doi:10.1093/cid/ciw388 (2016).
- 6 Guiguemde, W. A. *et al.* Global phenotypic screening for antimalarials. *Chemistry & biology* **19**, 116-129, doi:10.1016/j.chembiol.2012.01.004 (2012).
- 7 Cowell, A. N. & Winzeler, E. A. Advances in omics-based methods to identify novel targets for malaria and other parasitic protozoan infections. *Genome Med* **11**, 63, doi:10.1186/s13073-019-0673-3 (2019).
- 8 Wahlgren, M. & Perlmann, P. *Malaria: molecular and clinical aspects*. (CRC Press, 2003).
- 9 Krafts, K., Hempelmann, E. & Skórska-Stania, A. From methylene blue to chloroquine: a brief review of the development of an antimalarial therapy. *Parasitology research* **111**, 1-6, doi:10.1007/s00436-012-2886-x (2012).
- 10 Jensen, M. & Mehlhorn, H. Seventy-five years of Resochin in the fight against malaria. *Parasitology research* **105**, 609-627, doi:10.1007/s00436-009-1524-8 (2009).
- 11 Krugliak, M., Zhang, J. & Ginsburg, H. Intraerythrocytic *Plasmodium falciparum* utilizes only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. *Mol Biochem Parasitol* **119**, 249-256, doi:10.1016/s0166-6851(01)00427-3 (2002).
- 12 Aft, R. L. & Mueller, G. C. Hemin-mediated oxidative degradation of proteins. *J Biol Chem* **259**, 301-305 (1984).
- 13 Aft, R. L. & Mueller, G. C. Hemin-mediated DNA strand scission. *J Biol Chem* **258**, 12069-12072 (1983).
- 14 Chou, A. C. & Fitch, C. D. Mechanism of hemolysis induced by ferriprotoporphyrin IX. *J Clin Invest* **68**, 672-677, doi:10.1172/jci110302 (1981).
- 15 Klouche, K. *et al.* Mechanism of in vitro heme-induced LDL oxidation: effects of antioxidants. *Eur J Clin Invest* **34**, 619-625, doi:10.1111/j.1365-2362.2004.01395.x (2004).

- 16 Shinar, E. & Rachmilewitz, E. A. Oxidative denaturation of red blood cells in thalassemia. *Semin Hematol* **27**, 70-82 (1990).
- 17 Vincent, S. H. Oxidative effects of heme and porphyrins on proteins and lipids. *Semin Hematol* **26**, 105-113 (1989).
- 18 Pandey, A. V. *et al.* Hemozoin formation in malaria: a two-step process involving histidine-rich proteins and lipids. *Biochem Biophys Res Commun* **308**, 736-743, doi:10.1016/s0006-291x(03)01465-7 (2003).
- 19 Fitch, C. D., Cai, G. Z., Chen, Y. F. & Shoemaker, J. D. Involvement of lipids in ferriprotoporphyrin IX polymerization in malaria. *Biochim Biophys Acta* **1454**, 31-37, doi:10.1016/s0925-4439(99)00017-4 (1999).
- 20 Pisciotta, J. M. *et al.* The role of neutral lipid nanospheres in Plasmodium falciparum haem crystallization. *Biochem J* **402**, 197-204, doi:10.1042/bj20060986 (2007).
- 21 Sullivan, D. J., Jr., Gluzman, I. Y. & Goldberg, D. E. Plasmodium hemozoin formation mediated by histidine-rich proteins. *Science* **271**, 219-222, doi:10.1126/science.271.5246.219 (1996).
- 22 Chugh, M. *et al.* Protein complex directs hemoglobin-to-hemozoin formation in Plasmodium falciparum. *Proceedings of the National Academy of Sciences* **110**, 5392-5397, doi:10.1073/pnas.1218412110 (2013).
- 23 Jani, D. *et al.* HDP-a novel heme detoxification protein from the malaria parasite. *PLoS Pathog* **4**, e1000053, doi:10.1371/journal.ppat.1000053 (2008).
- 24 Nakatani, K., Ishikawa, H., Aono, S. & Mizutani, Y. Heme-binding properties of heme detoxification protein from Plasmodium falciparum. *Biochem Biophys Res Commun* **439**, 477-480, doi:10.1016/j.bbrc.2013.08.100 (2013).
- 25 Kapishnikov, S. *et al.* Unraveling heme detoxification in the malaria parasite by in situ correlative X-ray fluorescence microscopy and soft X-ray tomography. *Sci Rep* **7**, 7610, doi:10.1038/s41598-017-06650-w (2017).
- 26 Foley, M. & Tilley, L. Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. *Pharmacol Ther* **79**, 55-87, doi:10.1016/s0163-7258(98)00012-6 (1998).
- 27 Weissbuch, I. & Leiserowitz, L. Interplay between malaria, crystalline hemozoin formation, and antimalarial drug action and design. *Chem Rev* **108**, 4899-4914, doi:10.1021/cr078274t (2008).
- 28 Olafson, K. N., Ketchum, M. A., Rimer, J. D. & Vekilov, P. G. Mechanisms of hematin crystallization and inhibition by the antimalarial drug chloroquine. *Proc Natl Acad Sci U S A* **112**, 4946-4951, doi:10.1073/pnas.1501023112 (2015).

- 29 Sidhu, A. B., Verdier-Pinard, D. & Fidock, D. A. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations. *Science* **298**, 210-213, doi:10.1126/science.1074045 (2002).
- 30 Ecker, A., Lehane, A. M., Clain, J. & Fidock, D. A. PfCRT and its role in antimalarial drug resistance. *Trends in parasitology* **28**, 504-514, doi:10.1016/j.pt.2012.08.002 (2012).
- 31 Powles, M. A. *et al.* MK-4815, a potential new oral agent for treatment of malaria. *Antimicrob Agents Chemother* **56**, 2414-2419, doi:10.1128/aac.05326-11 (2012).
- 32 Dziekan, J. M. *et al.* Cellular thermal shift assay for the identification of drug-target interactions in the *Plasmodium falciparum* proteome. *Nat Protoc*, doi:10.1038/s41596-020-0310-z (2020).
- 33 Dziekan, J. M. *et al.* Identifying purine nucleoside phosphorylase as the target of quinine using cellular thermal shift assay. *Sci Transl Med* **11**, doi:10.1126/scitranslmed.aau3174 (2019).
- 34 Eggleston, K. K., Duffin, K. L. & Goldberg, D. E. Identification and characterization of falcilysin, a metallopeptidase involved in hemoglobin catabolism within the malaria parasite *Plasmodium falciparum*. *The Journal of biological chemistry* **274**, 32411-32417, doi:10.1074/jbc.274.45.32411 (1999).
- 35 Murata, C. E. & Goldberg, D. E. *Plasmodium falciparum* falcilysin: a metalloprotease with dual specificity. *J Biol Chem* **278**, 38022-38028, doi:10.1074/jbc.M306842200 (2003).
- 36 Martinez Molina, D. *et al.* Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science* **341**, 84-87, doi:10.1126/science.1233606 (2013).
- 37 Wellems, T. E. *et al.* Chloroquine resistance not linked to *mdr*-like genes in a *Plasmodium falciparum* cross. *Nature* **345**, 253-255, doi:10.1038/345253a0 (1990).
- 38 Powles, M. A. *et al.* MK-4815, a potential new oral agent for treatment of malaria. *Antimicrobial agents and chemotherapy* **56**, 2414-2419, doi:10.1128/AAC.05326-11 (2012).
- 39 Dai, L. *et al.* Horizontal Cell Biology: Monitoring Global Changes of Protein Interaction States with the Proteome-Wide Cellular Thermal Shift Assay (CETSA). *Annu Rev Biochem*, doi:10.1146/annurev-biochem-062917-012837 (2019).
- 40 Murata, C. E. & Goldberg, D. E. *Plasmodium falciparum* falcilysin: an unprocessed food vacuole enzyme. *Mol Biochem Parasitol* **129**, 123-126, doi:10.1016/s0166-6851(03)00098-7 (2003).
- 41 Matter, H. *et al.* Evidence for C-Cl/C-Br...pi interactions as an important contribution to protein-ligand binding affinity. *Angewandte Chemie (International ed. in English)* **48**, 2911-2916, doi:10.1002/anie.200806219 (2009).

- 42 Johnson, K. A. *et al.* The closed structure of presequence protease PreP forms a unique 10,000 Angstroms³ chamber for proteolysis. *The EMBO journal* **25**, 1977-1986, doi:10.1038/sj.emboj.7601080 (2006).
- 43 Rocamora, F. *et al.* Oxidative stress and protein damage responses mediate artemisinin resistance in malaria parasites. *PLoS Pathog* **14**, e1006930, doi:10.1371/journal.ppat.1006930 (2018).
- 44 Reiling, S. J., Krohne, G., Friedrich, O., Geary, T. G. & Rohrbach, P. Chloroquine exposure triggers distinct cellular responses in sensitive versus resistant *Plasmodium falciparum* parasites. *Sci. Rep.* **8**, 11137, doi:10.1038/s41598-018-29422-6 (2018).
- 45 Ponpuak, M. *et al.* A role for falcilysin in transit peptide degradation in the *Plasmodium falciparum* apicoplast. *Mol. Microbiol.* **63**, 314-334, doi:10.1111/j.1365-2958.2006.05443.x (2007).
- 46 Josling, G. A., Williamson, K. C. & Llinás, M. Regulation of Sexual Commitment and Gametocytogenesis in Malaria Parasites. *Annu. Rev. Microbiol.* **72**, 501-519, doi:10.1146/annurev-micro-090817-062712 (2018).
- 47 Beck, J. R., Muralidharan, V., Oksman, A. & Goldberg, D. E. PTEX component HSP101 mediates export of diverse malaria effectors into host erythrocytes. *Nature* **511**, 592-595, doi:10.1038/nature13574 (2014).
- 48 Muralidharan, V., Oksman, A., Iwamoto, M., Wandless, T. J. & Goldberg, D. E. Asparagine repeat function in a *Plasmodium falciparum* protein assessed via a regulatable fluorescent affinity tag. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 4411-4416, doi:10.1073/pnas.1018449108 (2011).
- 49 Muralidharan, V., Oksman, A., Pal, P., Lindquist, S. & Goldberg, D. E. *Plasmodium falciparum* heat shock protein 110 stabilizes the asparagine repeat-rich parasite proteome during malarial fevers. *Nature communications* **3**, 1310, doi:10.1038/ncomms2306 (2012).
- 50 Wicht, K. J., Mok, S. & Fidock, D. A. Molecular Mechanisms of Drug Resistance in *Plasmodium falciparum* Malaria. *Annu. Rev. Microbiol.* **74**, 431-454, doi:10.1146/annurev-micro-020518-115546 (2020).
- 51 Forte, B. *et al.* Prioritization of Molecular Targets for Antimalarial Drug Discovery. *ACS infectious diseases* **7**, 2764-2776, doi:10.1021/acsinfecdis.1c00322 (2021).
- 52 Yang, T. *et al.* MalDA, Accelerating Malaria Drug Discovery. *Trends in parasitology* **37**, 493-507, doi:10.1016/j.pt.2021.01.009 (2021).
- 53 Goldberg, D. E. Hemoglobin Degradation in *Malaria: Drugs, Disease and Post-genomic Biology*. (eds R. W. Compans *et al.*) 275-291 (Springer Berlin Heidelberg, 2005).

- 54 Combrinck, J. M. *et al.* Insights into the role of heme in the mechanism of action of antimalarials. *ACS Chem. Biol.* **8**, 133-137, doi:10.1021/cb300454t (2013).
- 55 Geary, T. G., Jensen, J. B. & Ginsburg, H. Uptake of [3H]chloroquine by drug-sensitive and -resistant strains of the human malaria parasite *Plasmodium falciparum*. *Biochem Pharmacol* **35**, 3805-3812, doi:10.1016/0006-2952(86)90668-4 (1986).
- 56 Bray, P. G. *et al.* PfCRT and the trans-vacuolar proton electrochemical gradient: regulating the access of chloroquine to ferriprotoporphyrin IX. *Molecular microbiology* **62**, 238-251, doi:10.1111/j.1365-2958.2006.05368.x (2006).
- 57 Sullivan, D. J., Jr., Gluzman, I. Y., Russell, D. G. & Goldberg, D. E. On the molecular mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci U S A* **93**, 11865-11870, doi:10.1073/pnas.93.21.11865 (1996).
- 58 Bray, P. G., Mungthin, M., Ridley, R. G. & Ward, S. A. Access to hematin: the basis of chloroquine resistance. *Molecular pharmacology* **54**, 170-179, doi:10.1124/mol.54.1.170 (1998).
- 59 Fidock, D. A. *et al.* Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular cell* **6**, 861-871, doi:10.1016/s1097-2765(05)00077-8 (2000).
- 60 Sanchez, C. P. *et al.* Differences in trans-stimulated chloroquine efflux kinetics are linked to PfCRT in *Plasmodium falciparum*. *Mol Microbiol* **64**, 407-420, doi:10.1111/j.1365-2958.2007.05664.x (2007).
- 61 Sanchez, C. P., Stein, W. & Lanzer, M. Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in *Plasmodium falciparum*. *Biochemistry* **42**, 9383-9394, doi:10.1021/bi034269h (2003).
- 62 Fitch, C. D. Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs. *Life sciences* **74**, 1957-1972, doi:10.1016/j.lfs.2003.10.003 (2004).
- 63 Saliba, K. J., Folb, P. I. & Smith, P. J. Role for the *plasmodium falciparum* digestive vacuole in chloroquine resistance. *Biochemical pharmacology* **56**, 313-320, doi:10.1016/s0006-2952(98)00140-3 (1998).
- 64 Famin, O. & Ginsburg, H. Differential effects of 4-aminoquinoline-containing antimalarial drugs on hemoglobin digestion in *Plasmodium falciparum*-infected erythrocytes. *Biochem Pharmacol* **63**, 393-398 (2002).
- 65 Zhang, Y. Inhibition of hemoglobin degradation in *Plasmodium falciparum* by chloroquine and ammonium chloride. *Experimental parasitology* **64**, 322-327, doi:10.1016/0014-4894(87)90042-7 (1987).

- 66 Fitch, C. D. Involvement of heme in the antimalarial action of chloroquine. *Transactions of the American Clinical and Climatological Association* **109**, 97-105; discussion 105-106 (1998).
- 67 Zarchin, S., Krugliak, M. & Ginsburg, H. Digestion of the host erythrocyte by malaria parasites is the primary target for quinoline-containing antimalarials. *Biochem Pharmacol* **35**, 2435-2442 (1986).
- 68 Burrows, J. N. *et al.* New developments in anti-malarial target candidate and product profiles. *Malar. J.* **16**, 26, doi:10.1186/s12936-016-1675-x (2017).
- 69 Hooft van Huijsduijnen, R. & Wells, T. N. The antimalarial pipeline. *Curr Opin Pharmacol* **42**, 1-6, doi:10.1016/j.coph.2018.05.006 (2018).
- 70 Wells, T. N., Hooft van Huijsduijnen, R. & Van Voorhis, W. C. Malaria medicines: a glass half full? *Nat Rev Drug Discov* **14**, 424-442, doi:10.1038/nrd4573 (2015).
- 71 Rohrbach, P. *et al.* Genetic linkage of pfmdr1 with food vacuolar solute import in Plasmodium falciparum. *EMBO J.* **25**, 3000-3011, doi:10.1038/sj.emboj.7601203 (2006).
- 72 Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K. & Cowman, A. F. Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. *Nature* **403**, 906-909, doi:10.1038/35002615 (2000).
- 73 Sanchez, C. P., Rotmann, A., Stein, W. D. & Lanzer, M. Polymorphisms within PfMDR1 alter the substrate specificity for anti-malarial drugs in Plasmodium falciparum. *Mol. Microbiol.* **70**, 786-798, doi:10.1111/j.1365-2958.2008.06413.x (2008).
- 74 Birrell, G. W. *et al.* Multi-omic Characterization of the Mode of Action of a Potent New Antimalarial Compound, JPC-3210, Against Plasmodium falciparum. *Molecular & cellular proteomics : MCP* **19**, 308-325, doi:10.1074/mcp.RA119.001797 (2020).
- 75 Creek, D. J. *et al.* Metabolomics-Based Screening of the Malaria Box Reveals both Novel and Established Mechanisms of Action. *Antimicrob Agents Chemother* **60**, 6650-6663, doi:10.1128/aac.01226-16 (2016).
- 76 McFadden, G. I. & Yeh, E. The apicoplast: now you see it, now you don't. *International journal for parasitology* **47**, 137-144, doi:10.1016/j.ijpara.2016.08.005 (2017).
- 77 Jain, V., Yogavel, M. & Sharma, A. Dimerization of Arginyl-tRNA Synthetase by Free Heme Drives Its Inactivation in Plasmodium falciparum. *Structure* **24**, 1476-1487, doi:10.1016/j.str.2016.06.018 (2016).
- 78 Martinez Molina, D. & Nordlund, P. The Cellular Thermal Shift Assay: A Novel Biophysical Assay for In Situ Drug Target Engagement and Mechanistic Biomarker Studies. *Annu Rev Pharmacol Toxicol* **56**, 141-161, doi:10.1146/annurev-pharmtox-010715-103715 (2016).

- 79 Mateus, A. *et al.* Thermal proteome profiling in bacteria: probing protein state in vivo. *Mol Syst Biol* **14**, e8242, doi:10.15252/msb.20188242 (2018).
- 80 Herneisen, A. L. *et al.* Identifying the Target of an Antiparasitic Compound in Toxoplasma Using Thermal Proteome Profiling. *ACS Chemical Biology* **15**, 1801-1807, doi:10.1021/acschembio.0c00369 (2020).
- 81 Favuzza, P. *et al.* Dual Plasmepsin-Targeting Antimalarial Agents Disrupt Multiple Stages of the Malaria Parasite Life Cycle. *Cell host & microbe* **27**, 642-658.e612, doi:10.1016/j.chom.2020.02.005 (2020).
- 82 Lu, K.-Y. *et al.* Plasmodium chaperonin TRiC/CCT identified as a target of the antihistamine clemastine using parallel chemoproteomic strategy. *Proceedings of the National Academy of Sciences* **117**, 5810-5817, doi:10.1073/pnas.1913525117 (2020).
- 83 Kabsch, W. XDS. *Acta crystallographica. Section D, Biological crystallography* **66**, 125-132, doi:10.1107/s0907444909047337 (2010).
- 84 McCoy, A. J. *et al.* Phaser crystallographic software. *Journal of applied crystallography* **40**, 658-674, doi:10.1107/s0021889807021206 (2007).
- 85 Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta crystallographica. Section D, Biological crystallography* **60**, 2126-2132, doi:10.1107/s0907444904019158 (2004).
- 86 Krissinel, E. & Henrick, K. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta crystallographica. Section D, Biological crystallography* **60**, 2256-2268, doi:10.1107/s0907444904026460 (2004).
- 87 Voss, N. R. & Gerstein, M. 3V: cavity, channel and cleft volume calculator and extractor. *Nucleic acids research* **38**, W555-562, doi:10.1093/nar/gkq395 (2010).
- 88 Sigurskjold, B. W. Exact analysis of competition ligand binding by displacement isothermal titration calorimetry. *Analytical biochemistry* **277**, 260-266, doi:10.1006/abio.1999.4402 (2000).
- 89 Foucquier, J. & Guedj, M. Analysis of drug combinations: current methodological landscape. *Pharmacol Res Perspect* **3**, e00149, doi:10.1002/prp2.149 (2015).
- 90 Bozdech, Z., Mok, S. & Gupta, A. P. DNA microarray-based genome-wide analyses of Plasmodium parasites. *Methods Mol Biol* **923**, 189-211, doi:10.1007/978-1-62703-026-7_13 (2013).
- 91 Smyth, G. K. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**, Article3, doi:10.2202/1544-6115.1027 (2004).

92 Gupta, A. P. *et al.* Histone 4 lysine 8 acetylation regulates proliferation and host-pathogen interaction in *Plasmodium falciparum*. *Epigenetics Chromatin* **10**, 40, doi:10.1186/s13072-017-0147-z (2017).

93 Wu, Y., Sifri, C. D., Lei, H. H., Su, X. Z. & Wellems, T. E. Transfection of *Plasmodium falciparum* within human red blood cells. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 973-977, doi:10.1073/pnas.92.4.973 (1995).

Figures

Figure 1

Protein with stability shifts for CQ and MK in 3D7 and Dd2. (A-D) Whole proteome analysis in intact-cell ITDR experiments under 0-10 μM of CQ in 3D7 and Dd2 strains (CQS and CQR strains, panel A and B, respectively) and under 0-100 μM of MK-4815 in 3D7 and Dd2 strains (panel C and D, respectively). Thermal challenges were done at 51°C (circles) or 57°C (triangles). Distribution of protein stabilization is plotted as a function of R² value (goodness of curve fit) against ΔAUC (area under the curve of heat-challenged sample normalized against non-denaturing 37°C control) for all proteins detected in the

assay. Three times of median absolute deviation (MAD) of Δ AUC in each dataset ($\text{MAD} \times 3$) and $R^2 = 0.8$ cutoffs are indicated on the graph. Proteins with significant thermal stabilization are labeled. **(E-H)** Thermal stabilization profile of FLN identified in **(A-D)**. Minimal dose threshold (MDT) stated are from ITDR 57°C, where FLN exhibited the strongest thermal stabilization. Stabilization under thermal challenges [51°C (blue) or 57°C (red)] is plotted relative to no-drug control with non-denaturing control (37°C) plotted in black. **(I-L)** Thermal stabilization profile of FLN obtained from in lysate experiments (Figure S4-S7). Minimal dose threshold (MDT) stated are from ITDR 57°C, where FLN exhibited the strongest thermal stabilization. Stabilization under thermal challenges [51°C (blue) or 57°C (red)] is plotted relative to no-drug control with non-denaturing control (37°C) plotted in black. Data are derived from 2 independent experiments, with the exception of **(A)** and **(E)** where 3 independent experiments were conducted.

Figure 2

FLN enzymatic inhibition by CQ, MK-4815, and MFQ. (A) FLN binding thermodynamics to MK-4815, CQ and MFQ measured by isothermal titration calorimetry. The top panels show heat differences upon injection of ligand and lower panels show integrated heats of injection and the best fits (solid lines) to a single-site binding model. The fitted K_d and thermodynamic values are shown in the Table. (B) FLN protease activity inhibition by MK-4815 (purple squares), CQ (blue points) and MFQ (red squares) at

pH=7.5 and pH=5.2. Protease activities were measured in triplicates and normalized for each inhibitor molecule. Squares denote mean values and error bars denote standard deviations. **(C)** Double reciprocal (Lineweaver-Burk) plot of FLN enzymatic activity in presence of 0.1 μM (pink triangles), 2 μM (purple squares) and 10 μM (blue points) of MK-4815. Each point represents the mean value of measurements performed in triplicate. Error bars which represent standard deviations are too small to be visible.

Figure 3

Crystal structures of FLN bound to inhibitors. (A) Sliced view of the inner FLN cavity in complex with CQ (yellow sticks). The molecular surface of FLN is shown, colored according to domain 1, residues 1-337, blue, domain 2, residues 338-587, cyan, domain 3, residues 658-990, orange, domain 4, residues 991-1193, red. Linker domain (residues 588-657) connecting domains 2 and 3, is colored in green. Active site residues H₁₂₉, H₁₃₃ and E₂₄₃ of domain 2 are shown as sticks and colored in cyan. The catalytic zinc ion is shown as a grey sphere. The inhibitors binding pocket (boxed) is situated at a distance of about 14 Å from the catalytic zinc. A model of the complex with the peptide substrate (pink sticks) was obtained by superimposing the hPrep structure (PDB access code: 4NGE) onto the FLN structure (this work). Of note, the inhibitors binding site overlaps with part of the hPrep binding (named hPrep substrate exosite, with sticks colored in purple). (B) Close-up view of the FLN inner substrate binding chamber. FLN is shown as ribbons. An anomalous map collected at the zinc absorption edge contoured at 5 σ is shown as a purple mesh. The modeled substrate (pink sticks) inferred from superimposition with hPrep, forms an antiparallel β -sheet with strand β 5 of FLN. Crystal structures of inhibitors (shown as sticks) bound to FLN (grey molecular surface): CQ (C, colored yellow), MFQ (D, magenta) and MK-4815 (E). Binding pocket residues are shown as sticks and colored in grey. For each bound compound, the corresponding simulated annealing Fo-Fc omit map (where the compound was removed during the calculation) contoured at a level of 3 σ is shown as a green mesh. To confirm the position of the Cl atom of CQ, the Fo-Fc omit map for CQ (obtained following simulated annealing) contoured at 5 σ is also shown colored in purple. The distance between the Chloride moiety of CQ and the center of the F545 ring is displayed. 2D interactions diagram for each inhibitor are shown below (calculated with the Schrödinger Maestro Suite).

Figure 4

Characterization of antimalarials MOA with DRTP and isobologram analysis. (A) Heatmaps of gene that were up- (red) or down- (green) regulated in a dose dependent manner upon treatment with stated compounds for 6 hours. Numbers in red or green represent numbers of genes that were up- or downregulated, respectively. Blue lines on the triangle scale represents the 6 hours drug assay IC_{50} s of the compound. (B) Venn diagram of genes similarly up- (top) or down- (bottom) regulated between

antimalarials. **(C)** Significantly over-represented pathways ($-\log$ hypergeometric $p > 1.3$) based on genes affected in (A). Overrepresentation of up- and down-regulated pathways are shown in red and green, respectively. Functional gene/pathway annotations listed are based on Gene Ontology Biological Processes (GO-BP) database, with the exception of hemoglobin digestion (Malaria Parasite Metabolic Pathways database). Full list of enriched pathways is available as **Supplementary Data 3**. **(D)** Overview of transcriptional changes in hemoglobin digestion genes and resistance-related genes upon antimalarial treatment. **(E)** Isobologram analysis for the combinations of CQ, MK, and PHEN in 3D7 and Dd2 strains. Isoboles representing FIC index of each drug in combination are plotted across a range of drug pair concentrations. Reference isobole indicating additivity is presented as a black dashed line. FIC indexes stated and error bars representing the standard deviation of the FICs are from 3 biological replicates.

Figure 5

Characterization of FLN overexpression line (A-C) and 3D7-FLN DD conditional knockdown line (D-F). (A) Timeline depicting the growth of FLN overexpression (FLN OE, top) and empty vector (control, bottom) strains after transfection. (B) Measurement of parasitaemia following parasites recovery from initial drug pressure in FLN overexpression (red) or empty vector (blue) strains. Each point represents the mean value of measurements from two transfection attempts in 3D7 strain. Error bars which represent standard

deviations are too small to be visible. **(C)** Merozoite count from the schizonts of 3D7 FLN overexpression (blue) and empty vector (red) strains. Lines represent the mean of merozoite count per schizont (10 schizonts for each strain) and error bars represent standard deviation. Numbers represent median merozoite count in each strain. *P*-values were obtained from non-parametric Mann-Whitney test. **(D)** Western blot analysis of FLN levels in 3D7 FLN-DD in the presence or absence of TMP stabilizing ligand. Cycle number refers to the number of life cycle the parasites have been cultured after the initial removal of TMP. FLN were probed with anti-FLN antibody, whereas anti-PfBIP was used as loading control. **(E)** Densitometry analysis of FLN levels obtained from **(D)**. Number in each bar in densitometry analysis represents average percentage decrease of FLN in parasites cultured without TMP stabilizing ligand compared to parasites cultured with TMP. Error bars represent standard deviation. Data are from 3 biological replicates. **(F)** Paired analysis of *in vitro* drug susceptibility of 3D7 FLN-DD against CQ, MK-4815, and dihydroartemisinin in the presence or absence of TMP in the ring (top) or trophozoite (bottom) stage of the parasites. Numbers below the dots represents mean IC₅₀. *P*-values were obtained from non-parametric Wilcoxon matched-pairs signed rank test, whereas *n* represent the number of biological replicates conducted for each experiment. * denotes statistically significant difference (i.e., *p*<0.05) between the pairs tested.

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