Structural and evolutionary analyses of the *Plasmodium falciparum* chloroquine resistance transporter

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Mutations in the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) confer resistance to several antimalarial drugs such as chloroquine (CQ) or piperaquine (PPQ), a partner molecule in current artemisinin-based combination therapies. As a member of the Drug/Metabolite transporter (DMT) superfamily, the vacuolar transporter PfCRT may translocate substrate molecule(s) across the membrane of the digestive vacuole (DV), a lysosome-like organelle. However, the physiological substrate(s), the transport mechanism and the functional regions of PfCRT remain to be fully characterized. Here, we hypothesized that identification of evolutionary conserved sites in a tertiary structural context could help locate putative functional regions of PfCRT. Hence, site-specific substitution rates were estimated over *Plasmodium* evolution at each amino acid sites, and the PfCRT tertiary structure was predicted in both inward-facing (open-to-vacuole) and occluded states through homology modeling using DMT template structures sharing <15% sequence identity with PfCRT. We found that the vacuolar-half and membrane-spanning domain (and especially the transmembrane helix 9) of PfCRT were more conserved, supporting that its physiological substrate is expelled out of the parasite DV. In the PfCRT occluded state, some evolutionary conserved sites, including positions related to drug resistance mutations, participate in a putative binding pocket located at the core of the PfCRT membrane-spanning domain. Through structural comparison with experimentally-characterized DMT transporters, we identified several conserved PfCRT amino acid sites located in this pocket as robust candidates for mediating substrate transport. Finally, in silico mutagenesis revealed that drug resistance mutations caused drastic changes in the electrostatic potential of the transporter vacuolar entry and pocket, facilitating the escape of protonated CQ and PPQ from the parasite DV.

Resistance to antimalarial drugs continues to threaten malaria control. Formerly used as first-line treatment for uncomplicated *Plasmodium falciparum* malaria in most endemic areas, chloroquine (CQ) has seen its efficacy reduced in the late 60s onwards after a decade of use. CQ resistance (CQR) was initially reported in Southeast Asia and South America, and then in Western Pacific and Africa¹. The spread of CQR has led to successive antimalarial drug replacements and since 2000 to the progressive adoption of artemisinin-based combination therapies (ACTs). CQ remains highly effective to treat malaria caused by the other human-infecting *Plasmodium* parasite species².

During the parasite intraerythrocytic development, hemoglobin from the host cytosol is internalized by the parasite and delivered into its digestive vacuole (DV), an acidic lysosome-like organelle. Degradation of hemoglobin in the DV generates toxic Fe²⁺-heme molecules that spontaneously oxidize to form membrane-disrupting Fe³⁺-heme³. To avoid this, the Fe³⁺-heme molecules are biocrystallized in the DV into non-toxic, chemically inert crystals known as hemozoin.

Quinoline-containing antimalarial drugs such as CQ, but also piperaquine (PPQ), amodiaquine and pyronaridine, are thought to primarily interfere with the conversion of toxic heme to non-toxic hemozoin crystal⁴. This process has been especially characterized for CQ, which is found in the DV at high concentrations⁵–⁷, leading to the accumulation of toxic Fe³⁺-heme and parasite death⁸.

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The main genetic determinant of CQR is the *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*)\(^{19}\). *Pf*cr\(t\) encodes a 424 amino acid protein (*PfCRT*), consisting of 10 transmembrane helices (TMs)\(^{10}\), which is essential to the parasite intraerythrocytic development\(^{14}\). At least six independent origins of CQR mutations were reported\(^{11}\), with various mutant *pfcrt* haplotypes harboring different sets of 4 to 10 non-synonymous mutations such as the Asian Dd2 haplotype that later spread to Africa\(^{2}\). Mutant *PfCRT* molecules have acquired the ability to expel CQ out of the DV\(^{13}\). All CQR haplotypes carry the key K76T mutation that removes a positive charge in TM 1, suggesting a charge-dependent transport mechanism as CQ is di-protonated in the acidic DV\(^{10,13}\). The K76T mutation is always accompanied by additional mutations which may increase the CQR level and/or attenuate the fitness cost of resistance\(^{11,15}\). *PfCRT* remains a protein of utmost clinical importance since both ancient and novel *PfCRT* mutations are associated with altered *in vitro* antimalarial activity of some ACT drugs (PPQ, amodiaquine, lumefantrine, and artemisinin derivatives) and with ACT treatment failures\(^{13,16,21}\). For most of these drugs, the *PfCRT*-dependent pharmaco-modulation might operate through different mechanisms than for CQ\(^{19,22,23}\).

After two decades of research, the physiological role of *PICRT* in the parasite biology is still questioned. Its secondary structure, location in the DV membrane and belonging to the Drug/Metabolite Transporter (DMT) superfamily support its participation in metabolite transport\(^{10,22}\). *PICRT* was reported as a proton-coupled transporter recognizing some cationic substrates\(^{26}\), a Fe\(^{2+}/Fe\(^{3+}\) transporter\(^{26}\), and playing a role in glutathione homeostasis\(^{27,28}\). Earlier studies have also reported *PICRT* as a Cl\(^{-}\) channel mediator, a proton pump regulatory and an activator of Na\(^{+}/H\(^{+}\) exchangers\(^{29–31}\).

Like DMT proteins\(^{32}\), *PICRT* may presumably operate by a mechanism known as the alternate access model\(^{33,34}\). In such a model, the binding of substrate, maintained inside the transporter through a so-called occluded state, triggers a structural transition between two other conformations, the inward- and outward-facing states, thereby inducing the translocation of the substrate across the membrane. In the case of *PICRT*, the binding site(s) of physiological substrate(s) and the different structural states of the transporter remain to be fully elucidated. Consistent with this, a *PICRT* mutant structure was recently determined in an inward-facing state by cryo-electron microscopy (named here *PICRT*\(^{cryo-EM}\), open-to-DV) and also predicted by modeling in an outward-facing state (*PICRT*\(^{OF}\), open-to-cytoplasm; which were published during the revision process of this work)\(^{35}\).

Here, we hypothesized that combining structural and evolutionary information could help identify putative functional regions of *PICRT* and also their link with drug resistance. We reasoned that codon or amino acid sites that are important for *PICRT* function are especially conserved. First, we investigated the variability of the conservation level across the CRT-coding DNA sequence and phylogeny over *Plasmodium* evolution. To get a finer map of evolutionary constraints in a structural context, we also built models of the tertiary structure of *PICRT* by homology modeling, based on high-resolution 3D structures of DMT proteins containing 10 TMs\(^{32,36}\). Two different *PICRT* conformations were predicted: an inward-facing state (*PICRT*\(^{IF}\) model; i.e. open-to-DV) and an occluded state (*PICRT*\(^{OC}\) model; i.e. both vacuolar and cytoplasmic gates closed) in which an open cavity and a binding pocket were identified, respectively. Combined evolutionary and structural analyses identified several slowly evolving amino acid sites located in the open cavity (for *PICRT*\(^{IF}\) and binding pocket (for *PICRT*\(^{OC}\)). Through a comparative structural approach with other experimentally-characterized DMTs, we further identified several amino acid sites of the binding pocket that may be involved in the physiological transport activity of *PICRT*. Finally, we explored the location and structural/physicochemical impacts of CQR and PPQ resistance (PPQ) mutations through a series of *in silico* mutagenesis from the *PICRT*\(^{IF}\) and *PICRT*\(^{OC}\) models.

Results

**Crt sequence dataset and phylogenetic tree analysis.** To evaluate the selective regime acting on the coding-sequence and protein product of the *crt* gene, we first retrieved orthologous CRT sequences from public databases. Non-*Plasmodium* species have not been included in the study because we cannot confirm that *PICRT* homologous protein(s) in those organisms are truly orthologous to *PICRT*. As we had enough evolutionary information with CTRIs from *Plasmodium*, we choose this smaller dataset to avoid introducing any putative bias in our evolutionary analyses\(^{37}\). Among the retrieved 24 *Plasmodium* CRT sequence datasets, only that of the bird-infecting *P. relictum* appeared incomplete with an absence of twenty amino acid positions in the C-terminal region. The other sequences showed high conservation in length, ranging from 423 to 425 amino acids (Supplementary Table S1), resulting in the presence of few indels (8 amino acid positions) in the CRT multiple sequence alignment that were removed from subsequent analyses (Supplementary Fig. S1). The phylogenetic tree produced from the curated *crt* sequence alignment was highly congruent with the acknowledged phylogeny of *Plasmodium* species\(^{38}\), with four main monophyletic groups (bird parasites, primate parasites *Plasmodium*, rodent parasites and primate parasites *Laverania*) supported by high bootstrap values (>90%; Supplementary Fig. S2).

**Variable levels of conservation across the crt gene sequence and phylogeny.** The selective pressures acting on the CRT-coding DNA sequence were searched for by estimating the non-synonymous to synonymous substitution rate ratio $\omega = (d_{\text{S}}/d_{\text{N}})$ across lineages (branch model) and codon sites (random-site models) using the *codeml* program ($\omega > 1$ indicates positive selection whereas $\omega < 1$ reflects purifying selection)\(^{39}\). The entire *crt* gene was found to evolve under a variable selective regime across *Plasmodium* phylogeny as the free-ratio model (that allows $\omega$ to vary along branches in the *crt* phylogenetic tree) had better fit to the data than the one-ratio null model M0 (which supposes only one $\omega$ value for all branches; $p = 1.85 \times 10^{-32}$; Table 1). Although purifying selection ($\omega < 1$) was pervasive across the whole phylogeny, some specific branches showed evidence of positive selection ($\omega > 1$ in *P. vinckeii vinckeii* or in the common ancestor of *P. fragile*, *P. knowlesi* and *P. coatneyi*), suggesting few adaptive changes during *Plasmodium* evolution (Supplementary Fig. S3). The substitution rate ratio $\omega$ was also found to be heterogeneously distributed across *crt* codon sites as assessed by the M0:M3 comparison ($p = 1.45 \times 10^{-133}$; Table 1), but no specific codon site was detected to evolve under positive selection
Plasmodium served. Altogether, the data indicate that much of the CRT protein has evolved under purifying selection over comparison (Supplementary Table S5), homology modeling remains still feasible44 and the PfCRT tertiary structure is of high quality despite exhibiting low paired sequence identity (~15%), shared a similar fold and arrangement of 12 TMs 48, and respectively diminished to 4.5 Å and 5.4 Å after exclusion of the loops (Supplementary Table S5).

### Identification of DMT template structures to predict PfCRT tertiary structure by homology modeling

In order to get a finer map of evolutionary constraints in a structural context, we aimed to produce a tertiary structure of PfCRT by homology modeling. By reviewing the literature, we found that, since 2016, several high-resolution 3D structures of DMTs containing 10 TMs have been determined by X-ray diffraction in different conformational states: the nucleotide sugar GDP-mannose transporter Vrg4 resolved in an inward-facing state (PDB ID: 5oge)36, the triose-phosphate/phosphate translocator TPT resolved in an occluded state (in complex with 3-phosphoglycerate; PDB ID: 5y79)32, and the aromatic amino acids and exogenous toxic exporter YddG (PDB ID: 5z20)34. Phyre242 and HHpred43 interactive servers identified these same three DMT structures as the current best structures to model PfCRT, with an e-value criterion (which represents the probability that the match between the sequence and the template arises from a true relationship42) of 98.7% for the least acceptable of these three DMT structures (Y ddG; Supplementary Tables S3 and S4). Despite a low sequence identity between PfCRT and these transporters, reaching a maximum of 14% for the PfCRT-TPT pair, Phyre242 and HHpred43 identified these same three DMT structures as the best candidates DMT templates which structures were experimentally and therefore independently obtained. Of note, under the best-fitted model M3, the sequence encoding the cytoplasmic N- and C-terminal extremities of CRT were found to be much less conserved than most of the remaining protein-coding DNA sequence (also noticeable by visual inspection of the CRT multiple sequence alignment provided in Supplementary Figure S1). Those extremities contained several sites that were found phosphorylated in PICRT40, including T416 which notwithstanding appeared highly conserved. Altogether, the data indicate that much of the CRT protein has evolved under purifying selection over Plasmodium evolution.

| Models                  | 2Δℓ | df | p value | Conclusion                   |
|------------------------|-----|----|---------|------------------------------|
| M0:free-ratio          | 260.29 | 44 | 1.85 × 10^-32 | ω heterogeneous among lineages of the phylogenetic tree |
| M0:M3                  | 623.24 | 4 | 1.45 × 10^-133 | ω heterogeneous across codon sites |
| M1a:M2a                | 0.00 | 2 | 1.00     | No codon sites detected to evolve under positive selection |
| M7:M8                  | 0.00 | 2 | 1.00     | No codon sites detected to evolve under positive selection |

Table 1. Results of PAML analyses. ω, non-synonymous (d₂s) to synonymous (d₁s) substitution rate ratio (d₂s/d₁s); 2Δℓ, log-likelihood ratio of the two tested models; df, degrees of freedom; TMs, transmembrane helices; non-TMs, loops connecting two TMs. 2Δℓ was compared to a chi-squared table to determine the significance of the likelihood ratio tests. For the two series of partitioning tests shown here, codon sites were partitioned with PICRTIF model and we discarded the poorly conserved N- and C-terminal regions, both located in the cytoplasm (this alteration was conservative). Similar results were obtained when codon sites were partitioned with the PICRTIF model (data not shown).

since the positive selection models M2a and M8 provided no better fits to the data than the neutral models M1a and M7, respectively (p = 1.00 in both cases; Table 1 and Supplementary Table S2). Under the best-fitted model M3, the sequence encoding the cytoplasmic N- and C-terminal extremities of CRT were found to be much less conserved than most of the remaining protein-coding DNA sequence (also noticeable by visual inspection of the CRT multiple sequence alignment provided in Supplementary Figure S1). Those extremities contained several sites that were found phosphorylated in PICRT40, including T416 which notwithstanding appeared highly conserved. Altogether, the data indicate that much of the CRT protein has evolved under purifying selection over Plasmodium evolution.

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As an initial validity control, we evaluated the variability in sequence and structure across the three candidate DMT templates which structures were experimentally and therefore independently obtained. Of note, in the phylogeny of the DMT superfamily, they are located in different branches45. Accordingly, the paired sequence identities for these three template transporters were low, reaching for example only 13% between TPT and Vrg4 (Supplementary Table S5). However, a visual inspection of paired structural superposition revealed a very similar fold (Supplementary Fig. S4). To quantify this, we computed for paired DMT structures the Ca-based RMSD including TMs-connecting loops ranging from 5.9 Å (LacY-GlpT) to 8.9 Å (GlpT-EmrD; Supplementary Fig. S5). Therefore, we assumed that PICRT may share a similar fold to the aforementioned 10 TMs-containing DMT proteins, at least regarding the membrane spanning region.
Predicting different conformational states of PfCRT tertiary structure by homology modeling.

Having identified robust DMT template structures, we modeled wild-type (WT) PfCRT tertiary structure in two different conformational states: an inward-facing state (PfCRTIF) and an occluded state (PfCRTOC), using respectively Vrg4 and TPT as templates (Supplementary Table S6). We discarded the YddG template because it had a lower sequence identity with PfCRT than Vrg4 (both in inward-facing state; Supplementary Table S5). We also discarded the PfCRT positions 269–313 corresponding to the long vacuolar loop connecting TM 7 and TM 8 and the cytoplasmic N- and C-terminal extremities (positions 1–55 and 399–424, respectively) because of insufficient amino acid coverage in DMT templates.

In homology modeling, alignment of the target-template sequences (here PfCRT-Vrg4 and PfCRT-TPT) is a critical step. Since hydrophobicity, which reflects the transmembrane topology, is typically conserved in TMs during evolution, the hydrophathy profile can contain similar global features even in very distantly related proteins. Consequently, we first produced a superfamily-averaged hydropathy profile alignment with AlignMe using the CRT multiple sequence alignment and an alignment of different members of the DMT superfamily. This profile-profile (CRTs-DMTs) alignment pinpointed some conserved amino acid positions that should be aligned in each final target-template alignment (Supplementary Figs. S6 and S7). We then produced a target-template alignment using the AlignMe PST algorithm which is designed for distantly related proteins (i.e. with a sequence identity <15%), and manually optimized it in order to align the conserved amino acid positions identified from the profile-profile alignment (see the final alignments for PICRT-Vrg4 and PfCRT-TPT in Supplementary Figs. S6 and S7 respectively).

After the refinement and minimization steps, we checked the quality of the two built PfCRT models using local stereochemistry (MolProbity). For comparison, we used as controls several high-resolution 3D structures: the two DMT template and the PfCRT cryo-EM-structured structure. The two PfCRT models exhibited similar quality indices than those experimentally-determined control structures as suggested by Ramachandran plots (Table 2). General (ERRAT, Prosna) and transmembrane-specialized (QMEANBrane) 3D quality metrics also provided high confidence in structure formation, energies and non-bonded interactions between atoms of the PfCRTOC and PfCRTIF models, when compared to the templates and other highly refined structures including PICRTcryo-EM from the Protein Data Bank (Table 2 and Supplementary Fig. S8). Finally, directional atomic contacts were evaluated to check the structures for correctness using the fine packing quality control implemented in the What IF server. For both PfCRT models, the average Z-score for all contacts was close to 0, confirming the normality of the local environment of amino acids, similarly to Vrg4 and TPT template structures and PICRTcryo-EM (Table 2).

We then found that the paired PICRTIF-Vrg4 and PICRTOC-TPT structures had a Cα based RMSD of 5.2 Å and 3.0 Å, respectively (3.0 Å and 2.5 Å when TMs-connecting loops were excluded; Supplementary Table S5). These RMSD values were similar to those obtained with paired DMT template structures (Supplementary Table S5). In all, different quality metrics and structural comparisons indicated that the PICRTIF and PICRTOC models can be reliably used for further structural analyses.

To evaluate the accuracy of the PICRTIF and PICRTOC models, we compared their secondary and tertiary structural features with those of PICRTcryo-EM. Our two models revealed symmetry-related structural repeats consisting of 10 TM bundle (with TM 1 to TM 5 and TM 6 to TM 10 distributed in anticlockwise and clockwise manners, respectively), approximately 30 Å in length, similarly to PICRTcryo-EM (Fig. 1A,B). In PICRTcryo-EM, two additional helices (JM 1 and JM 2) were identified, respectively located in the N-terminal region and the long vacuolar loop connecting TM 7 and TM 8 (two regions discarded in our modeling study; Fig. 2A). In the PICRTIF model, the structure was basket-shaped, similarly to Vrg4 and PICRTcryo-EM, with a deep cavity of ~3,200 Å³ opened at the vacuolar side and composed of amino acids from most TM helices except TM 5 and TM 10 (Fig. 1A). In the PICRTOC model, a pocket of ~1,050 Å³ was observed in the core of the structure (Fig. 1B; the 41 pocket positions are listed in Supplementary Table S7), which corresponds to the substrate-binding pocket in TPT. A structural comparison of PICRTIF and PICRTOC revealed a prominent difference in the orientation of TM 3 and TM 4 of ~30° at the vacuolar face (Fig. 1C), suggesting that these helices undergo rocker-switch movements to open and close the vacuolar gate. By estimating the electrostatic surface potential on the models (which have WT PfCRT haplotype), we noted that the vacuolar gate is slightly electropositive whilst the cavity and putative binding pocket are globally neutral (Fig. 1D). TMs boundaries between PICRTcryo-EM and models were similar, except TM 3, TM 7 and TM 10 that were longer in PICRTcryo-EM (Fig. 2A). In addition, the folds of the PICRTIF model and PICRTcryo-EM were highly similar as evidenced by a RMSD of 2.8 Å (2.4 Å without TMs-connecting loops; Fig. 2B), which provides a direct validation of our PICRTIF model. This result is consistent with the Co-based structural conservation profile produced between PfCRT models and PICRTcryo-EM using the Dali server, revealing a weaker structural conservation only on some TMs-connecting loops (Supplementary Fig. S9). Also, Co-based contact maps generated with CMWeb indicated overall the same intra-protein contacts between our PfCRT models and PICRTcryo-EM, with similar pairwise amino acid contact distributions (Supplementary Fig. S10). Finally, we used the orientation of side-chain amino acids related to CQR and PPQR mutations as another indicator of validity. After substituting mutant positions in PICRTcryo-EM (S72, T76, S220, D326 and L356) to WT (C72, K76, A220, N326 and I356) with UCSF Chimera, we noticed that most of drug resistance-associated amino acid sites were similarly oriented in the PfCRT models compared to PICRTcryo-EM (Fig. 2C), except a major difference for the amino acid I218 in PICRTIF. Altogether, the two models we produced were accurate and reliable for subsequent analyses.

**Vacuolar and TM sites have evolved under strong purifying selection.** We used our new PICRT models to test whether some PICRT regions, apart from the N- and C-terminal extremities, were differentially conserved. We used the fixed-site models and partitioning function implemented in the codeml program. We tested two different partitioning of *crt* codon sites: 1) cytoplasmic-half versus vacuolar-half sides of the transporter and 2) non-TMs (i.e. loops) versus TM. In the two sets of partitioning, model B provided a better fit to
Table 2. Quality metrics for PfCRT models and templates. *The experimentally-determined tertiary structures of two DMTs (Vrg4 and TPT) were used as templates for homology modeling of PfCRT structure. PfCRT_WoES-EM corresponds to a PfCRT mutant structure recently determined in an inward-facing state by cryo-electron microscopy. PfCRT_IF and PfCRT_OC correspond to PfCRT wild type models in inward-facing and occluded states, respectively. **ProSA II generates an overall quality score compared with the ones from all experimentally determined protein chains available in the Protein Data Bank. MolProbity indicates the accuracy of a macromolecular structure model and diagnoses local problems by all-atom contact analysis, complemented by updated versions of covalent-geometry and torsion-angle criteria. ERRAT analyzes the statistics of non-bonded interactions between different atom types, then plots error values as a function of the position of a 9-residue sliding window, calculated by a comparison with statistics of non-bonded atom interactions from refined structures. *QMEANBrane is a structure quality assessment tool dedicated to membrane proteins. It determines a local quality score per residue (the mean per-residue quality score is shown), by combining statistical potentials trained on membrane protein structures with a per-residue weighting scheme. The fine packing quality control, implemented in the What IF server, checks the normality of the local environment of amino acids. Values are expressed as Z-scores; a value $<-2$ or $>2$ indicates a wrong model or an incorrect structure; a Z-score close to 0 represents a high quality structure.

| Protein | ProSA II | MolProbity | Poor rotamers, n (%) | Favored rotamers, n (%) | Ramachandran favored, n (%) | Ramachandran outliers, n (%) | Bad bonds, n (%) | Bad angles, n (%) | ERRAT* | QMEAN Brane* | Fine packing quality control |
|---------|----------|-------------|---------------------|-----------------------|-----------------------------|-----------------------------|----------------|----------------|--------|--------------|-----------------------------|
| Vrg4    | -1.76    | 21/268 (7.84)| 218/268 (81.34) | 2/298 (0.67) | 278/298 (93.29) | 0/2415 (0.00) | 4/3278 (0.12) | 92.32 | 0.80          | 0.47                  |
| TPT     | -3.17    | 5/251 (1.99) | 239/251 (95.22) | 0/303 (0.00) | 300/303 (99.01) | 0/2423 (0.00) | 0/3313 (0.00) | 96.61 | 0.84          | 0.85                  |
| PICRT_IF | -2.75    | 3/312 (0.96) | 289/312 (92.63) | 0/346 (0.00) | 334/346 (96.53) | 0/2876 (0.00) | 0/3898 (0.00) | 89.10 | 0.81          | -0.60                 |
| PICRT_OC | -2.72    | 4/279 (1.43) | 269/279 (96.42) | 0/301 (0.00) | 287/301 (95.35) | 0/2550 (0.00) | 0/2446 (0.00) | 98.70 | 0.79          | 0.24                  |
| PICRT_OC | -2.11    | 0/272 (0.00) | 270/272 (99.26) | 0/294 (0.00) | 287/294 (97.62) | 4/2487 (0.16) | 3/3366 (0.09) | 99.01 | 0.81          | 0.43                  |

The cavity of PfCRT contains highly conserved amino acid sites. We next searched for the most conserved amino acid positions in the PfCRT_OC and PfCRT_IF models as indicators of putative functional sites. We used the FuncPatch server which jointly analyses phylogenetic tree, protein tertiary structure and conservation information. FuncPatch infers site-specific substitution rates at the protein level by taking into account their spatial location in the tertiary structure, and is especially useful in the case of highly conserved proteins37,60. Site-specific substitution rates were very similar using either the PfCRT_IF or PfCRT_OC models (Spearman’s rank correlation: $r = 0.99$, $p < 0.001$; Fig. 3A and Supplementary Data S1), so only the data obtained with PfCRT_IF will be presented. We detected a significant spatial correlation of site-specific substitution rates in the PfCRT_IF model as evidenced by a log Bayes factor of 47.6, suggesting the presence of a functional patch. We then mapped onto the tertiary structure the 10% most conserved amino acid sites of the 297 PfCRT positions included in the FuncPatch analysis. Remarkably, almost the whole TM 9 that lines the PfCRT_IF cavity was highlighted (Fig. 3B and Supplementary Table S9). Other highly conserved positions were located on TM 4, TM 5, in the middle of TM 8 and on TM 10 (Fig. 3B). It is noteworthy that TM 9 has been reported to play a major role in substrate binding in other DMTs consisting of 10 TMs24, and especially in Vrg4 and TPT proteins (Supplementary Fig. S11)32,36,41. Evolutionary rates at the protein level were also estimated using the Consurf web server34, and this analysis revealed again the high conservation of the PfCRT_IF cavity and especially TM 9 (Supplementary Fig. S12).

PfCRT amino acids Y68, D329, Y345 and S349 might play a role in physiological substrate trafficking. Some of the most highly conserved PfCRT amino acid sites might be under strong functional – rather than structural – constraints. Being a member of the DMT superfamily, PfCRT may bind a substrate through amino acids located in its central cavity, and more particularly in the binding pocket. Consequently, we focused on amino acid positions strictly conserved across Plasmodium species and whose side-chains line the binding pocket. After these filtering steps, we listed 24 amino acid positions (Supplementary Table S10) that were further analyzed in three paired structural alignments, PfCRT_IF, Vrg4, PfCRT_IF-YddG and PfCRT_OC-TPT. We searched for structurally aligned positions that have been shown experimentally to modulate substrate trafficking in DMT template proteins (Supplementary Fig. S13)32,36,41. Two tyrosines of PfCRT_IF (Y68 in TM 1 and Y345 in TM 9) paired with two tyrosines of Vrg4 (Y28 in TM 1 and Y281 in TM 9; Fig. 4A) reported to be essential for the translocation of...
GDP–mannose and GMP molecules. One aspartic acid of PfCRTOC (D329 in TM 8) paired with a tyrosine of the TPT protein (Y339 in TM 8, Fig. 4B) which is crucial for phosphate recognition and transport. Finally, PfCRTIF S349 (TM 9) paired with both S244 from the bacterial Y ddG protein and G285 from Vrg4 (Fig. 4C), which play a role in the transport of threonine/methionine and both GDP–mannose and GMP molecules, respectively. Of those four candidate PfCRT positions, three belonged to the conserved patch evidenced by the FuncPatch server (D329, Y345 and S349; Supplementary Table S9). In PfCRTcryo-EM (inward-facing state), the side-chains of these four positions were also oriented towards the cavity (Supplementary Fig. S14). Overall, this comparative evolutionary and structural analysis identified amino acid sites putatively involved in the physiological transport activity of PfCRT (Fig. 4D).

**Figure 1.** 3D-fold and structural features of the wild type (WT) PfCRTIF and PfCRTOC models. Predicted tertiary structure of PfCRTIF (A) and PfCRTOC (B) models. Each TM is highlighted in a specific color. In PfCRTIF, the cavity is open from the vacuolar face. In PfCRTOC, a substrate-binding pocket constitutes the core of the transporter. (C) Superposition of PfCRTIF and PfCRTOC models. TMs are shown as cylinders. A prominent shift of ~30° (red arrows) was observed for both TM 3 and TM 4 between PfCRTIF (blue) and PfCRTOC (green). (D) Electrostatic surface potential of PfCRTIF. Values are in units of kT/e at 298 K, on a scale of -8 kT/e (red) to +8 kT/e (blue). White color indicates a neutral potential. The structure is shown from the side (left structure) and the vacuolar face (right structure). The location of the key position K76, involved in CQR when mutated (K76T mutation), is indicated.

Most PfCRT mutations conferring drug resistance are located in the cavity or binding pocket. Mutations in PfCRT confer drug resistance, chiefly to CQ and PPQ, but also alter sensitivities to other ACT drugs. To better understand the underlying resistance mechanisms, we mutated the PfCRTOC and PfCRTIF models in...
Figure 2. Structural comparison of PfCRTcryo-EM with both PfCRTIF and PfCRTOC models. (A) Delineation of PfCRT secondary structures according to cryo-EM (PfCRTcryo-EM) or modeling (PfCRTIF and PfCRTOC). TMs are colored in black, whilst TMs-connecting loops are indicated with dashes. Non-TM helices are shown in grey. Regions that are not covered by the PfCRT structures are colored in salmon. Strictly conserved CRT amino acid positions across Plasmodium species are written in red. Positions associated with CQR or PPQR mutations are surrounded in purple. (B) Structural superposition of PfCRTcryo-EM with the PfCRTIF model. For ease of representation, the long vacuolar loop connecting TM 7 and TM 8 was removed in PfCRTcryo-EM. The structures are shown as cartoon from the digestive vacuole (left structure) and the side (right structure). PfCRTcryo-EM and PfCRTIF are respectively colored in red and blue. The labels of TMs are also indicated. Both superposition and RMSD calculation were based on all Ca atoms (including TMs-connecting loops) using the MatchMaker function implemented in UCSF Chimera. When TMs-connecting loops were excluded, the RMSD was 2.4 Å. (C) Schematic representation of the side-chain orientation of the positions associated with CQR or PPQR mutations. The side-chain orientations were noted by visual inspection of a structural superposition of PfCRTcryo-EM, PfCRTIF and PfCRTOC. Mutant positions in PfCRTcryo-EM (S72, T76, S220, D326 and L356) were substituted to WT (C72, K76, A220, N326 and I356) using the swapaa function of UCSF Chimera. TM 5 and TM 10 are not involved in the architecture of the transporter cavity.

Figure 3. Location of the most conserved amino acid sites in the PfCRT tertiary structure. (A) Positive correlation of site-specific substitution rates estimated by the FuncPatch server between PfCRTIF and PfCRTIF models. Each point corresponds to one amino acid site. (B) Location of the 10% most conserved amino acid sites (inferred with FuncPatch) in the PfCRTIF model, which are colored in dark pink. The structure is shown as cartoon from the side (left structure) and as surface from the digestive vacuole (right structure).

In silico. We introduced jointly several mutations to produce Dd2, GB4 and 7G8 mutant structures which confer CQR and correspond to Asian, African and South American CQR haplotypes, respectively (Table 3). From an evolutionary view, five of the nine CQR-related positions investigated here were strictly conserved during...
Plasmodium evolution including the position 76 related to the key CQR K76T mutation (Supplementary Fig. S1). We noted that some positions related to CQR mutations (M74I, N75E and K76T) were markedly more conserved when site-specific substitution rates were computed by taking into account their spatial location in the tertiary structure (FuncPatch versus PAML data in Fig. 5A; Supplementary Data S1), indicating that these three amino acid sites were spatially close to conserved ones. Remarkably, the CQR-related positions we analyzed were directly located in the cavity of the PfCRT IF model, except position 74 (mutation M74I) which lies in TM 1 but on the outer surface of the transporter and position 371 (mutation R371I) in the TM 9-TM 10 vacuolar loop next to the entry of the cavity (Figs. 2C and 5A). Based on the PfCRT OOC model, six positions related to CQR mutations were predicted to be involved in the architecture of the putative binding pocket (C72S, N75E, K76T, A220S, N326S/D and I356T/L), including five of the eight CQR mutations carried by the canonical Dd2 haplotype (Table 3). As the key CQR K76T mutation removes a positive charge in the cavity, we investigated in silico the changes in the electrostatic surface potential of the PfCRT IF and PfCRT OOC Dd2, GB4 and 7G8 models. While the surface potential of PfCRT IF WT cavity and PfCRT OOC WT binding pocket were neutral (Fig. 5B), they turned highly electronegative in all CQR mutant structures (Fig. 5C and Supplementary Fig. S15A).

Next, the recently identified, additive PfCRT mutations conferring PPQR on a CQR genetic background (namely T93S, H97Y, C101F, F145I, I218F, M343L and G353V, found in PfCRT Dd2; and C350R found in PfCRT 7G8) were individually introduced in the PfCRT IF and PfCRT OOC Dd2 or 7G8 CQR mutant models. While the surface potential of PfCRT IF WT cavity and PfCRT OOC WT binding pocket were neutral (Fig. 5B), they turned highly electronegative in all CQR mutant structures (Fig. 5C and Supplementary Fig. S15A).
rendered neutral a large part of the cavity except in the proximity of K76T (note that this was found only in the PfCRT<sup>7</sup> model; Supplementary Fig. S15B). Six of the eight PPQR-related positions were strictly conserved during Plasmodium evolution (T93, C101, I218, M343, C350, G353; Supplementary Fig. S1). Altogether, we concluded that PfCRT positions associated with mutations conferring resistance to quinoline-containing antimalarial drugs were globally conserved over Plasmodium evolution, mostly located in the cavity/binding pocket where physiological substrate trafficking is supposed to take place, and made the surface potential of the cavity and binding pocket more electronegative, as reported using the high-resolution PfCRT<sup>cryo-EM</sup> structure.<sup>39</sup>

**Discussion**

Based on our evolutionary results, we found that PfCRT is on average more conserved on its vacuolar-half side and its membrane-spanning domain. Therefore, the vacuolar side of PfCRT may play an important role in substrate recognition and/or transport, which supports the hypothesis of a physiological substrate being expelled by PfCRT out of the parasite DV. This is in line with the observation that recombinant PfCRT, either reconstituted in proteoliposomes<sup>25</sup> or expressed at the surface of *Xenopus laevis* oocytes<sup>13,26</sup>, directionally transports CQ, peptides, basic amino acids, and ferric/ferrous iron. In the parasite, these solutes are known to accumulate in the DV from which they could be transported out either solely by mutant PfCRT (CQ and Ppq in their protonated forms) or by WT and/or mutant PfCRT (peptides, basic amino acids, and ferric/ferrous iron).

A high-resolution 3D PfCRT mutant structure determined in an inward-facing state by single-particle cryo-electron microscopy (PfCRT<sup>cryo-EM</sup>) was recently published<sup>35</sup>. The authors also modeled an outward-facing conformation of this mutant PfCRT (PfCRT<sup>OF</sup>), considering the pseudo-symmetrical arrangement of the TMs. Here, we complete the putative cycle of the PfCRT transporter by producing a model of PfCRT in an occluded state (PfCRT<sup>OC</sup>), where any substrate is engulfed by the residues of the binding pocket. Altogether, the results are compatible with the hypothesis that PfCRT may operate by the alternate access model<sup>33,34</sup>. Such a transporter does not significantly alter CQR but enhances parasite fitness in *pfcrt* initially mutated in the canonical Dd2 haplotype that initially invaded Southeast Asia and Africa. Their removal TM 9 (I356) from the highly conserved patch are located in the PfCRT binding pocket. N326 and I356 were TM 4 undergo rocker-switch movements which may contribute to open (in PfCRT IF/PfCRT<sup>cryo-EM</sup>) and close state (PfCRT<sup>OC</sup>), where any substrate is engulfed by the residues of the binding pocket. Altogether, the results here provide some insights into quinoline-containing drug resistance, which are fully congruent with those recently reported<sup>39</sup>. The most striking result is that most

Table 3. PfCRT haplotypes investigated in this study. *Geographic origin of the haplotype. SE Asia, Southeast Asia; S America, South America.*<sup>1</sup> For Dd2 and 7G8 parasite lines, one additional mutation was found to reverse CQR but confer PPQR<sup>18,19</sup>. <sup>2</sup>CQS, chloroquine sensitivity; CQR, chloroquine resistance; PPQS, piperaquine sensitivity; PPQR, piperaquine resistance. In this study, the Q271E mutation was not introduced in the mutant structures and subsequent analyses since it is located in the long TM 7-TM 8 vacuolar loop that was discarded for tertiary structure prediction. The location in the structure (TM, transmembrane helix; L, loop) is indicated above each position.

| Origin region<sup>1</sup> | Parasite line<sup>2</sup> | Drug response<sup>3</sup> | TM 1 | TM 1 | TM 1 | TM 1 | TM 2 | TM 2 | TM 2 | TM 3 | TM 3 | TM 3 | TM 6 | L7-8 | TM 8 | TM 9 | TM 9 | TM 9 | TM 9 | L9- 10 |
|---------------------|---------------------|---------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| All regions         | 3D7                 | CQS, PPQS           | C     | M     | N     | K     | T     | H     | C     | F     | I     | A     | Q     | N     | M     | C     | G     | I     | R     |
| SE Asia             | Dd2                 | CQR, PPQS           | C     | I     | E     | T     | T     | H     | C     | F     | I     | S     | E     | S     | M     | C     | G     | I     | T     |
| SE Asia             | Dd2 + T93S          | CQS, PPQR           | C     | I     | E     | T     | S     | H     | C     | F     | I     | S     | E     | S     | M     | C     | G     | T     | 1     |
| SE Asia             | Dd2 + H97Y          | CQS, PPQR           | C     | I     | E     | T     | T     | Y     | C     | F     | I     | S     | E     | S     | M     | C     | G     | T     | 1     |
| SE Asia             | Dd2 + C101F         | CQS, PPQR           | C     | I     | E     | T     | T     | H     | F     | F     | I     | S     | E     | S     | M     | C     | G     | T     | 1     |
| SE Asia             | Dd2 + F145I         | CQS, PPQR           | C     | I     | E     | T     | T     | H     | C     | I     | I     | S     | E     | S     | M     | C     | G     | T     | 1     |
| SE Asia             | Dd2 + I218F         | CQS, PPQR           | C     | I     | E     | T     | T     | H     | C     | F     | F     | S     | E     | S     | M     | C     | G     | T     | 1     |
| SE Asia             | Dd2 + M343L         | CQS, PPQR           | C     | I     | E     | T     | T     | H     | C     | F     | I     | S     | E     | S     | L     | C     | G     | T     | 1     |
| SE Asia             | Dd2 + G353V         | CQS, PPQR           | C     | I     | E     | T     | T     | H     | C     | F     | I     | S     | E     | S     | M     | C     | V     | T     | 1     |
| S America           | 7G8                 | CQR, PPQS           | S     | M     | N     | T     | T     | H     | C     | F     | I     | S     | Q     | D     | M     | C     | G     | L     | R     |
| S America           | 7G8 + C350R         | CQS, PPQR           | S     | M     | N     | T     | T     | H     | C     | F     | I     | S     | Q     | D     | M     | R     | G     | L     | R     |
| Africa              | GB4                 | CQR, PPQS           | C     | I     | E     | T     | T     | H     | C     | F     | I     | S     | E     | N     | M     | C     | G     | I     | 1     |
positions mutated in CQR, including the key K76T, are located in the cavity of the PfCRT\textsuperscript{IF} model, and more particularly in its predicted substrate-binding pocket (PfCRT\textsuperscript{TOC} model). Several of the mutated positions (C72S, M74I, N75E, N326S/D, I356T/L) are in close proximity with K76T, suggesting that all these mutations work together. These amino acid changes jointly cause drastic alterations of the electrostatic surface potential in the vacuolar entry of the cavity and in the binding pocket that become highly electronegative in the CQR PfCRT mutant structures. This radical shift in electrostatic surface potential is very likely a major contributor to the dramatically increased uptake of di-protonated CQ in \textit{X. laevis} oocytes expressing CQR versus WT PfCRT\textsuperscript{13} and the decreased accumulation of CQ in the DV of CQ-resistant parasites\textsuperscript{6,64}.

Similarly to CQR mutations, most of the novel PPQR mutations that occurred in Dd2 or 7G8 CQR genetic backgrounds\textsuperscript{19,21,62,63} are located in the cavity and binding pocket in our PfCRT\textsuperscript{IF} and PfCRT\textsuperscript{TOC} models. However, the addition of PPQR mutations on CQR mutant structures (Dd2 and 7G8 haplotypes) did not alter the electronegative surface potential. This was moderately surprising because some PPQR mutations (such as M343L or G353V) attenuate but do not fully reverse CQR\textsuperscript{19}. Remarkably, substantially increased PPQ transport rates by PPQ-resistant PfCRT variants were found in proteoliposomes, which were then confirmed in \textit{pfcrt}-edited parasites\textsuperscript{35}. Hence, we hypothesize that PPQ-conferring mutations provide additional changes in the cavity/binding pocket which could affect interactions with PPQ.

In conclusion, we showed that our bioinformatics analysis of PfCRT led to results similar to those obtained with the recently obtained experimental structure\textsuperscript{35} regarding the overall fold, architecture of the open cavity and
alteration of its electrostatic surface potential in drug-resistant PfCRT mutant structures. Such results strengthen the usefulness of tertiary structure prediction of transmembrane proteins through homology modeling, even when a protein to model shares less than 15% sequence identity than a similarly folded template structure. Furthermore, we complement the experimental work of Kim et al. (2019) by providing an occluded state model of PfCRT and identifying a putative binding pocket where a substrate is likely maintained and could trigger a structural transition between the inward- and outward-facing states. This pocket, of smaller volume than the open cavity, also provides with a shortened list of highly conserved putative functional sites to be experimentally tested.

Materials and Methods

Collection of PfCRT orthologous sequences. The PfCRT amino acid sequence (PlasmoDB database identifier: PF3D7_0709000) was queried against the specialized Plasmodium database (PlasmoDB, release 38) and the non-redundant protein database of NCBI using blastp and tblastn searches (e-value cutoff = 10^{-6}, BLASTNUM62 scoring matrix)^{39}. Twenty four CRT protein and corresponding cDNA sequences from distinct Plasmodium species were retrieved (Supplementary Table S1). For P. ovale spp. and P. inui sequences, exon skipping was manually performed as so as to reconstruct the full cDNA and amino acid sequences.

Multiple protein and codon sequence alignments. The CRT multiple sequence alignment was first generated with TM-aligner^66 using the transmembrane-specific substitution matrix PHAT^67 and with gap opening and extension penalties set to 10 and 1, respectively. The output alignment was visually inspected and manually edited using BioEdit v7.2.5^68. The positions of the CRT multiple alignment containing gaps in ≥30% of all sequences were removed. Then, a c rt nucleotide sequence alignment was generated with PAL2NAL^69 using the cleaned CRT protein sequence alignment as template.

Phylogenetic analysis. A phylogenetic tree was built from the c rt nucleotide sequence alignment by using the maximum likelihood method implemented in PhyML v3.0^70, after determining the best-fitting nucleotide substitution model using the Smart Model Selection (SMS) package^71. A general time-reversible model with optimized equilibrium frequencies, gamma distributed among-site rate variation and estimated proportion of invariant sites (GTR optimized equilibrium frequencies, gamma distributed among-site rate variation and estimated proportion of invariant sites; GTR + G + I) was used, as selected by the Akaike Information Criterion. The nearest neighbor interchange approach implemented in PhyML was chosen for tree improving, and branch supports were estimated using the approximate likelihood ratio aLRT SH-like method^72. The c rt phylogenetic tree was rooted using the most distant bird-infecting Plasmodium species as outgroup and displayed using the iTOl server^73.

Analysis of selective pressures acting on c rt. To investigate the evolutionary regime that has shaped the CRT-coding DNA sequence during Plasmodium evolution, the nucleotide sequence alignment and the maximum likelihood phylogenetic tree were submitted to the codeml tool of PAML v4.9h^74,75. The level of coding sequence conservation was measured by calculating the non-synonymous (d_{NS}) to synonymous (d_S) substitution rate ratio \( \frac{d_{NS}}{d_S} \). Theoretically, positive selection at codon sites is indicated by \( \frac{d_{NS}}{d_S} > 1 \), while codon sites associated with \( \frac{d_{NS}}{d_S} < 1 \) suggest neutral evolution and purifying selection, respectively. We investigated the heterogeneity of \( \frac{d_{NS}}{d_S} \) among lineages and along the c rt sequence; then we searched for codons evolving under positive selection using a set of random-site and branch models (free-ratio, M0, M1a, M2a, M3, M7 and M8)^76,77. Model M0 supposes the same \( \frac{d_{NS}}{d_S} \) ratio for all branches of the phylogeny, while the "free-ratio" branch model assumes independent \( \frac{d_{NS}}{d_S} \) ratio for every branch. Model M1a allows two classes of sites under neutral evolution (\( \frac{d_{NS}}{d_S} = 1 \)) and purifying selection (\( \frac{d_{NS}}{d_S} < 1 \)), whereas model M2a adds a third class of sites to model M1a as positive selection (\( \frac{d_{NS}}{d_S} > 1 \)). In the discrete model M3, several classes (\( k = [3–5] \)) in this study) of independent \( \omega \) were estimated, each of them being associated with a specific proportion. Model M7 assumes a \( \beta \)-distribution of ten \( \omega \) ratios limited to the interval [0, 1] with two shape parameters \( p \) and \( q \). In model M8, an additional site class is estimated, with \( \omega \) possibly > 1 as M2a does. The heterogeneity of \( \omega \) among codon sites was tested by comparing model M0 to model M3, while paired models M1a:M2a and M7:M8 allow to detect positively selected sites^73. Candidate sites for positive selection were identified in M2a and M8 models using the Bayes empirical Bayes inference (EBE)^77, which calculates the posterior probability that every codon site belongs to a site class affected by positive selection; and using the naive empirical Bayes (NEB) inference in model M3 (in which no EBE approach is implemented yet), considered as less powered than the EBE inference. For models considering k classes of \( \frac{d_{NS}}{d_S} \) ratio, we used the mean \( \omega \) value at each codon site, calculated as the sum of the \( \omega \) values of each k class weighted by their estimated probabilities (Supplementary Data S1)^73.

Statistical analyses based on structural information of the PfCRT^{78} and PfCRT^{79} models were performed using the partitioning function implemented in codeml in order to test i) whether the vacuolar-half side is more or less conserved than the cytoplasmic-half side; and ii) whether TMs are more or less conserved than non-TMs (or loops). Partitionings are based on fixed-site models A to E. Model A – that corresponds to model M0 – assumes identical parameters for transition/transversion (\( \kappa \)) rate ratio, branch lengths and equilibrium nucleotide frequencies, and unique \( \omega \) ratio for partitions. Model B allows homogeneity among partitions in codon frequencies, \( \kappa \) and \( \omega \) ratios but different rates of evolution, whereas model C further accounts for potential variation in codon frequency. Model D uses different \( \kappa \) and \( \omega \) ratios based on the same codon frequencies for partitions, and model E ultimately allows different \( \kappa \), \( \omega \) and codon frequencies between partitions. The A:B comparison tests for different nucleotide substitution rates among partitions, while both B:D and C:E comparisons evaluate the variation in \( \kappa \), \( \omega \), and codon frequencies among partitions^80.

The comparisons of models were performed using Likelihood Ratio Tests (LRTs)^78. For every LRT, twice the log-likelihood difference between alternative and null models (2\( \Delta \ell \)) was compared to critical values from a
Modeling the PfCRT tertiary structure. When the analyses were performed, no experimental structure was determined for PfCRT. Consequently, we aimed at modeling it in different conformational states using a template-based protein structure modeling approach. The PfCRT sequence was submitted to interactive Phyre2 servers and HHpred servers to find the best available, appropriate templates. The nucleotide sugar GDP-mannose transporter Vrg4 (inward-facing state, PDB ID: 5oge) and the triose-phosphate/phosphate translocator TPT (occluded state, PDB ID: 5y79) were identified as sharing putative similar structural folds with PfCRT with confidence >99%. The very low sequence identity of these protein with PfCRT, reaching only 14% with TPT, makes homology modeling challenging but still feasible. In homology modeling, the sequence alignment of the target (here PfCRT) and the template (here other DMT proteins) is a critical step. Because hydrophobicity reflects the transmembrane topology and is typically conserved in TMs during evolution, we first produced a superfamily-averaged hydropathy profile alignment with AlignMe using the CRT multiple sequence alignment and an alignment of different members of the DMT superfamily. The profile (CRT)-profile (DMT) alignment pinpointed some amino acids which are supposed to be important to align in the final target-template sequence alignment (Supplementary Figs. S6 and S7). Finally, the target-template alignment was produced using the AlignMe PST algorithm, designed for distantly related proteins, using the AlignMe PST algorithm, designed for distantly related proteins (i.e. with a sequence identity <15%) and, then manually optimized it in order to align the putative important amino acid positions identified from the superfamily-averaged hydropathy profile alignment (Supplementary Figs. S6 and S7). The long TM 7-TEM 8 vacuolar loop and the cytoplasmic N- and C-terminal extremities were not considered for PfCRT modeling because of too few numbers of amino acid positions available from templates to properly cover them. Initially, we built 1,000 3D models for each PfCRT state satisfying the spatial restraints of the template structures using MODELLER. The best models among them were selected based on scores calculated from discrete optimized protein energy (DOPE) and GA341 functions. Then, the best PfCRTOC and PfCRTCIF models were submitted to GalaxyRefine for atomic-level, high-resolution refinement, which helped to achieve significant improvement in physical quality of the local structure. Finally, the refined models were subjected to an energy minimization to get the most stable conformations using the YASARA server.

The refined, energy-minimized PfCRT models were then validated using i) MolProbity for local stereochemistry; ii) fine packing quality control implemented in the What IF server by estimating directional atomic contacts; and iii) ERRAT, ProSA II and QMEANBrane for tertiary fold and global 3D quality metrics. C-based intra-protein contacts and pairwise amino acid contact distributions were investigated with the CMWeb server (default parameters). Finally, the Dal server was used to study the C-based structural conservation between the PfCRT models and the high-resolution PfCRTCryoe-EM structure. The PDB files of the refined, energy-minimized PfCRTOC and PfCRTCIF models are provided in Supplementary Files S1 and S2, respectively.

Analyses of structural features. Protein electrostatic surface potential was calculated using Adaptive Poisson-Boltzmann Solver (APBS), after determining the per-atom charge and radius of the structure with PDB2PQR v.2.1.1. The Poisson-Boltzmann equation was solved at 298 K using a grid-based method, with solute and solvent dielectric constants fixed at 2 and 78.5, respectively. We used a scale of $-8 \ kT/e$ to $+8 \ kT/e$ to map the electrostatic surface potential in a radius of 1.4 Å.

The cavity/pocket volume of the PfCRT models was analytically calculated using the Connolly’s surface (or molecular surface model) with CASTp 3.0 (default parameters). Putative functional patches (i.e. highly conserved amino acid positions that are in close physical proximity in the tertiary structure) in the two PfCRT models were searched for using the FuncPatch server by estimating site-specific substitution rates, possibly spatially correlated. We submitted to the server the PfCRTOC and PfCRTCIF models along with the CRT multiple sequence alignment and the maximum-likelihood crt phylogenetic tree. The spatial correlation of the site-specific amino acid substitution rates in the PfCRT predicted structures was tested using a Bayesian model comparison, where a null model (model 0), in which no spatial correlation of site-specific substitution rates is present, is compared to the alternative model (model 1). As suggested by the FuncPatch’ authors, a spatial correlation was considered as significant if the estimated log Bayes factor (model 1 versus model 0) was larger than the conservative cutoff value of 8. Finally, evolutionary rates were also estimated at each amino acid site of the PfCRT sequence and then mapped onto the tertiary structure with Consurf (default parameters), a widely used server implementing advanced probabilistic evolutionary models. Contrary to FuncPatch, Consurf does not include the spatial correlation of site-specific substitution rates attributed to tertiary structure.

Identifying candidate functional sites of PfCRT. In order to identify candidate functional amino acid sites possibly involved in PfCRT physiological substrate(s) trafficking, a comparative evolutionary and structural analysis was performed to filter step-by-step PfCRT amino acid sites. Because functional amino acids are usually very conserved over evolutionary time, only the strictly conserved amino acid positions (i.e. the absence of non-synonymous substitutions for a given position) of PfCRT during Plasmodium evolution were kept. From this subset of positions, we then focused on those involved in the putative substrate-binding pocket in the PfCRTOC model since it is widely accepted that DMT proteins transport substrates through binding sites located in their pocket. Finally, the DMT template structures used to predict the conformational states of PfCRT tertiary structure (Vrg4 and TPT), in addition with another DMT protein (YddG) were aligned with the PfCRTOC and PfCRTCIF models. By focusing on the amino acid sites from these three structures that have been shown to be important or essential for substrate binding or transport, we listed PfCRT candidate positions that might contribute to substrate trafficking (Supplementary Fig. S13).
In silico production of PfCRT mutant structures. Some in silico mutagenesis from the refined, energy-minimized PfCRT models were performed. We generated three PfCRT mutant models per PfCRT conformational state based on the Dd2, GB4 and 7G8 haplotypes which are associated with CQR (Table 3)\(^{10,48}\). The CQR-resistant PfCRT\(^{26}\), Dd2 and 7G8 structures then subjected to individual additional mutations that attenuate CQR but confer PPQR (Table 3)\(^{19,21,62,63}\). Mutations were introduced using the swapfail function of UCSF Chimera by substituting the residue with the most probable rotameric conformation\(^{57}\).

Structure visualization. Molecular drawings were produced using UCSF Chimera\(^{57}\).

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Author contributions
R.C., A.S. and J.C. designed the research. A.S. and J.C. supervised the whole work. R.C. performed the building of datasets, statistical and evolutionary analyses, homology modeling and structural visualization. All the authors participated in data interpretation and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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