**Plasmodium falciparum** chloroquine resistance transporter is a \( \text{H}^+ \)-coupled polycpecific nutrient and drug exporter

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Extrusion of chloroquine (CQ) from digestive vacuoles through the *Plasmodium falciparum* CQ resistance transporter (PfCRT) is essential to establish CQ resistance of the malaria parasite. However, the physiological relevance of PfCRT and how CQ-resistant PfCRT gains the ability to transport CQ remain unknown. We prepared proteoliposomes containing purified CQ-sensitive and CQ-resistant PfCRTs and measured their transport activities. All PfCRTs tested actively took up tetraethylammonium, verapamil, CQ, basic amino acids, polypeptides, and polyamines at the expense of an electrochemical proton gradient. CQ-resistant PfCRT exhibited decreased affinity for CQ, resulting in increased CQ uptake. Furthermore, CQ competitively inhibited amino acid transport. Thus, PfCRT is a \( \text{H}^+ \)-coupled polycpecific nutrient and drug exporter.

**Results and Discussion**

To test the working hypothesis that PfCRT is a \( \text{H}^+ \)-coupled polycpecific organic cation transporter, we expressed and purified recombinant CQ-sensitive PfCRT\textsuperscript{His\textsuperscript{10}} or PfCRT\textsuperscript{Hist10} by fusing a soluble *Escherichia coli* protein (YbeL) to the N and C termini in *E. coli*, yielding His-YbeL-PfCRT-YbeL-His, which was used throughout this study (28, 29). We obtained a protein fraction containing three major protein bands on SDS–polyacrylamide gel electrophoresis (SDS/PAGE) (Fig. 1A). Western blotting analysis indicated that the major polypeptide with apparent molecular mass of 72 kDa was a PfCRT protein, and the lower-molecular-weight protein band was a proteolytic product (Fig. 1A and SI Appendix, Table S1).

**Significance**

Malaria caused by *Plasmodium falciparum* is a severe infectious disease with high mortality and morbidity rates worldwide. Chloroquine (CQ) is a widely used antimalarial agent, but the emergence and spread of CQ-resistant parasites is a growing global health problem. Although its physiological relevance remains unknown, *P. falciparum* CQ resistance transporter (PfCRT) confers CQ resistance through CQ egress from digestive vacuoles of *P. falciparum*. To address this issue, recombinant CQ-sensitive or CQ-resistant PfCRT proteins were purified and their transport activities were assessed. Both CQ-sensitive and CQ-resistant PfCRTs transported CQ, various antimalarial agents, and basic amino acids, indicating that PfCRT is a polycpecific drug and nutrient exporter.

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Accordingly, we established $\Delta pH$ and $\Delta \psi$ by the pH jump method and $\Delta \psi$ by the K+ diffusion potential with the K+ -selective ionophore, valinomycin, across the liposomal membranes (30). Liposomes containing PfCRT actively took up tetraethyl ammonium (TEA), a typical substrate for polyspecific organic cation transporters (31, 32), when both the outside-acidic $\Delta pH$ and outside-positive $\Delta \psi$ were applied (Fig. 1B). Bioenergetic analysis indicated that both $\Delta pH$ and $\Delta \psi$ were necessary for full uptake, and either $\Delta \psi$ or $\Delta pH$ alone resulted in low levels of TEA uptake (Fig. 1C). Opposite $\Delta pH$, i.e., inside-acidic $\Delta pH$ or inside-positive $\Delta \psi$, exhibited the background level of uptake and did not drive active transport.

Neither Na+ nor K+ gradients were effective. The $\Delta pH$- and $\Delta \psi$-driven TEA uptake showed an ordinary Michaelis–Menten type saturation curve with $K_m = 1.58$ mM and $V_{max} = 371$ nmol/min/mg protein, respectively (Fig. 1D). To date, eight individual mutations have been found in CQ-resistant PfCRTs (2, 8, 33–38). Among them, we expressed and purified two CQ-resistant PfCRTs (PfCRT$^{7G8}$ and PfCRT$^{Dd2}$) and PfCRT with a K76T mutation in the 3D7 background, PfCRT K76T, which is conserved in known resistant PfCRTs, and examined their TEA uptake capabilities (Fig. 1E) (2, 8, 33–38). The results indicated that all CQ-resistant PfCRTs also took up TEA, similar to CQ-sensitive PfCRT, although the activity tended to decrease in accordance with the degree of CQ resistance (Fig. 1E and SI Appendix, Fig. S1). These results demonstrated that PfCRT is a $\Delta pH$- and $\Delta \psi$-driven TEA transporter.

Subsequently, we examined the detailed transport properties of PICRT from cis-inhibition on TEA uptake by CQ-sensitive PfCRT. (A) Coomassie Brilliant Blue staining and Western blotting analysis of PfCRT$^{3D7}$. Purified protein (10 μg/lane) was applied. (B) Time course of TEA uptake after reconstitution of purified PfCRT$^{3D7}$. After addition of valinomycin (2 μM) or DMSO (control), the uptake assay was started by adding radioactive TEA to a final concentration of 100 μM. Samples were obtained at the indicated times, and the TEA taken up by the liposomes was measured. The TEA uptake by the liposomes in the absence of valinomycin is also shown to indicate the background level. (C) Proteoliposomes were prepared in buffer containing 0.15 M Na+ or K+ at the indicated pH. Then, the proteoliposomes were incubated in buffer containing 0.15 M Na+ or K+ at the indicated pH, and TEA uptake after 1 min of incubation was measured. Asterisks indicate statistical significance. *$P < 0.1$, **$P < 0.01$. (D) Concentration dependence of TEA uptake. K+ -trapped proteoliposomes were incubated in the presence (complete) or absence (no driving force) of valinomycin. Then TEA was added at the indicated concentration, and the TEA uptake after 1 min was measured. (E) TEA uptake of CQ-resistant PfCRTs. The uptake values at 1 min in the presence (complete) or absence (no driving force) of valinomycin are shown. Coomassie Brilliant Blue staining of CQ-resistant PfCRTs is also shown in the inset.

Fig. 1. TEA uptake by CQ-sensitive and CQ-resistant PfCRTs. (A) Coomassie Brilliant Blue staining and Western blotting analysis of PfCRT$^{3D7}$. Purified protein (10 μg/lane) was applied. (B) Time course of TEA uptake after reconstitution of purified PfCRT$^{3D7}$. After addition of valinomycin (2 μM) or DMSO (control), the uptake assay was started by adding radioactive TEA to a final concentration of 100 μM. Samples were obtained at the indicated times, and the TEA taken up by the liposomes was measured. The TEA uptake by the liposomes in the absence of valinomycin is also shown to indicate the background level. (C) Proteoliposomes were prepared in buffer containing 0.15 M Na+ or K+ at the indicated pH. Then, the proteoliposomes were incubated in buffer containing 0.15 M Na+ or K+ at the indicated pH, and TEA uptake after 1 min of incubation was measured. Asterisks indicate statistical significance. *$P < 0.1$, **$P < 0.01$. (D) Concentration dependence of TEA uptake. K+ -trapped proteoliposomes were incubated in the presence (complete) or absence (no driving force) of valinomycin. Then TEA was added at the indicated concentration, and the TEA uptake after 1 min was measured. (E) TEA uptake of CQ-resistant PfCRTs. The uptake values at 1 min in the presence (complete) or absence (no driving force) of valinomycin are shown. Coomassie Brilliant Blue staining of CQ-resistant PfCRTs is also shown in the inset.
and CQ-resistant PfCRTs. The TEA uptake by CQ-sensitive PfCRT

$^{3D7}$ was unaffected by neutral amino acids, acidic amino acids, and many organic anions (SI Appendix, Fig. S2). Consistent with these observations, PfCRT did not transport glatamic acids, GABA, leucine, t-serine, or p-aminohippuric acid (SI Appendix, Fig. S3). However, the TEA uptake by CQ-sensitive PfCRT

$^{3D7}$ was strongly inhibited by inhibitors of PfCRT, such as verapamil and quinidine, as well as CQ, and nutrients, such as arginine, lysine, and peptides (Fig. 2). Histidine also showed a strong inhibitory effect, whereas tryptophan had a weak effect. TEA uptake by PfCRT

$^{3D7}$ was also inhibited by dequalinium, Hoechst 33342, and rhodamine 123, which are typical substrates for multidrug transporters (SI Appendix, Fig. S4). In addition to TEA, we found that PfCRT

$^{3D7}$ transports cimetidine, histamine, and spermidine (SI Appendix, Fig. S5). These results suggested that PfCRT

$^{3D7}$ is a polyspecific transporter that can recognize a wide range of substrates, including organic cations, basic amino acids, polyamines, and peptides derived from the degradation of hemoglobin. Unexpectedly, aside from some exceptions, such as histidine, these compounds were not effective in inhibition of TEA uptake by PfCRT

$^{K76T}$ or CQ-resistant PfCRT

$^{7G8}$ and PfCRT

$^{Dd2}$, and their ineffectiveness tended to increase in accordance with the degree of resistance (Fig. 2 B–D). These observations suggest that CQ-resistant PfCRTs may have lost the ability to recognize these compounds as substrates and can no longer transport them or that CQ-resistant PfCRTs may have altered affinities to these compounds, resulting in changes in their kinetic parameters for these substrates.

Next, we investigated the mechanisms underlying the different responses between CQ-sensitive and CQ-resistant PfCRTs in cis-inhibition. We focused on CQ uptake by PfCRTs because oocytes of Xenopus laevis expressing resistant PfCRT

$^{Dd2}$ were reported to take up CQ, whereas oocytes expressing CQ-sensitive PfCRT

$^{7G8}$ and PfCRT

$^{Dd2}$ did not (16). In reconstituted liposomes, CQ-sensitive PfCRTs were reported to take up CQ, whereas oocytes expressing CQ-resistant PfCRT

$^{K76T}$ or PfCRT

$^{Dd2}$, and their ineffectiveness tended to increase in accordance with the degree of resistance (Fig. 2 B–D). We measured the uptake of radiolabeled amino acids, peptides at 1 mM, CQ at 1 or 10 µM, or antimalarials at 0.25 mM are shown as relative activities. TEA uptake (% of control)

$^{50}$ and $^{75}$ as in the case of CQ uptake, quinidine and verapamil strongly inhibited TEA uptake by CQ-sensitive PfCRT

$^{3D7}$ (Fig. 24). Furthermore, these compounds had less effect on TEA uptake by CQ-resistant PfCRTs. We postulated that PfCRT would transport these chemicals with changes in the kinetic properties, as in the case of CQ. As anticipated, we found that PfCRT transported verapamil. The transport ability was increased in the resistant variants, although that of PfCRT

$^{Dd2}$ was slightly lower than that of PfCRT

$^{7G8}$ (Fig. 3D). Taken together, these results indicated that PfCRT functions as a polyspecific drug transporter similar to Polycomb protein and MATE transporter (31, 32).

Quinidine and verapamil are known to restore CQ sensitivity in CQ-resistant strains of P. falciparum (39–45). Consistent with these observations, verapamil and quinidine inhibited CQ uptake by PfCRT

$^{3D7}$ and PfCRT

$^{Dd2}$, respectively, although the extent of inhibition of CQ uptake by PfCRT

$^{Dd2}$ was weak (Fig. 3E). These observations indicated that these agents are competitive inhibitors of PfCRT and inhibit CQ transport regardless of sensitivity to CQ. Although CQ uptake by CQ-resistant PfCRTs was inhibited by verapamil, CQ transport activity in the presence of verapamil was still higher than that of CQ-sensitive PfCRT

$^{3D7}$ due to increased CQ transport activity in the resistant strains. This may explain why the CQ susceptibility of CQ-resistant strains in the presence of verapamil is still lower than that of CQ-sensitive strains (39).

The most important characteristic of PfCRT that can be inferred from the analysis of TEA uptake is that it is inhibited by basic amino acids, such as arginine or lysine, and by peptides, suggesting that PfCRT transports basic amino acids and peptides (Fig. 2). We measured the uptake of radiolabeled amino acids by PfCRTs. As expected, PfCRTs transported arginine in a pH- and Δψ-dependent manner with $K_m$ and $V_{max}$ of 0.57 mM and 44.7 nmol/min/mg protein for PfCRT

$^{Dd2}$, and 0.71 mM and 75.9 nmol/min/mg protein for PfCRT

$^{7G8}$, respectively (Fig. 4 A and B). In the case of lysine, these values were 0.43 mM and 105 nmol/min/mg protein for PfCRT

$^{Dd2}$ and 0.74 mM and 154 nmol/min/mg protein for PfCRT

$^{7G8}$ (Fig. 4B and SI Appendix, Fig. S7). The $K_m$ and $V_{max}$ values of histidine transport were 0.085 mM and 26.0 nmol/min/mg protein for PfCRT

$^{3D7}$ and 0.097 mM and 37.8 nmol/min/mg protein for PfCRT

$^{Dd2}$, respectively (Fig. 4C and SI Appendix, Fig. S9). Moreover, CQ strongly inhibited the transport of these amino acids, whereas this inhibition in CQ-resistant PfCRT

$^{Dd2}$ was relatively weak (Fig. 4D and E).

Based on the information regarding the transport properties presented here, we concluded that PfCRT possesses two significant activities as a polyspecific drug exporter and a polyspecific drug importer.
nutrient exporter. PfCRT exports CQ and organic amine drugs, such as verapamil, from the DV. K76T is a common mutation in CQ-resistant strains and is the most important mutation for CQ susceptibility (8). In agreement with detailed drug susceptibility testing reported by Johnson et al. (46), our results indicate that the K76T mutation confers CQ insensitivity but does not provide sufficient CQ resistance without other mutations found in Dd2 and 7G8. The CQ-resistant variant showed decreased affinity to CQ and increased transport, which caused a decrease in CQ level inside the vacuole, thus resulting in resistance.

The observation that PfCRT transports amino acids and peptides, which are generated in the DV, suggests a physiological role of PfCRT in nutrient supply (SI Appendix, Fig. S10). It is possible that inhibition of amino acid transport by CQ is correlated with its antimalarial effect, and this should be examined in future studies. The observation that PfCRT recognizes a variety of cationic substrates, similar to polyspecific drug transporters, suggests that inhibitors of multidrug transporters may be good candidates for antimalarial drug discovery.

The functions of PfCRT clarified in this study provide insight into overcoming drug resistance by malaria parasites: noncompetitive inhibitors of PfCRT, such as quinine dimers, may inhibit both export of CQ and supply of nutrients and thus result in the development of more potent drugs that may cause malfunction of the DV (47). The assay system used in this study with purified PfCRT will be useful in screening for such drugs.

Materials and Methods

Construction of PfCRT Expression Vectors. A cDNA encoding CQ-sensitive PfCRT3D7 was synthesized with optimized codons using the OptimumGene algorithm for expression in E. coli (GenScript). The codon adaptation index and GC contents of synthesized cDNA were 0.88% and 45%, respectively, and did not contain a Shine-Dalgarano sequence. The synthesized cDNA was amplified by PCR with the forward primer 5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′ and the reverse primer 5′-CTTGGTGATCCTCGAGTTGGATATCATGCTGTCCA-3′. The amplified DNA fragment was cloned into an expression vector using an infusion cloning kit (TaKaRa). The resultant plasmid was named β-pET-28a(+)-β-pET-28a(+)2, which was designed for overexpression of eukaryotic membrane proteins in E. coli, was used in this study (28). The resultant plasmid was named β-pET-jpFCTR3D7β-β.

Expression plasmids encoding CQ-resistant PfCRTs were generated by site-directed mutagenesis using a plasmid encoding PfCRT3D7. The expression plasmids harboring 7G8 (C725K/K76T/A220S/D763E/I786F), Dd2 (M74I/N75E/V270A/R371I/Q380H), and 7G8 (C725K/K76T/A220S/D763E/I786F), were constructed using the following primer pairs: K76T, forward—5′-AATACCTATTTGCGAACGGCCGCTGTAAAGAATT-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′; M74I and N75E, forward—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′; A220S, forward—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′; D763E, forward—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′; I786F, forward—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′; K76T/A220S, forward—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′; D763E/I786F, forward—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′; N75E/V270A/R371I, forward—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′; V270A/R371I/Q380H, forward—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′; M74I/N75E/V270A/R371I, forward—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′; M74I/N75E/V270A/R371I/Q380H, forward—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′, reverse—5′-CCGGGATCCGAAATCTAGAATTCGGCAAGTAAAA-3′.
A Tomy UD 200 tip sonifier at OUTPUT level 4 (Tomy). To remove large influence (PMSF). The cell suspension was then disrupted by sonication with harvested by centrifugation and suspended in buffer containing 20 mM 4 °C for 10 min, and the supernatant was then recentrifuged at 150,000 · HCl (pH 7.5), 100 mM NaCl, 10 mM KCl, and 2 mM PMSF, and then applied to a column containing 1 mL of nickel-NTA Superflow resin (Qiagen) equilibrated with a buffer consisting of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM KCl. After protein binding, the column was washed with washing buffer consisting of 20 mM Tris-HCl (pH 8.0), 20 mM imidazole, 100 mM NaCl, 10 mM KCl, and 1% octyl glucoside (Dojin). The PfCRT protein was eluted with buffer consisting of 20 mM Tris-HCl (pH 8.0), 300 mM imidazole, 100 mM NaCl, 10 mM KCl, and 1% octyl glucoside and then stored at –80 °C, at which temperature it was stable without loss of activity for at least several months.

**Expression and Purification of PfCRT.** The protocol used was essentially the same as that described previously by Levanian et al. (28). The expression vector was transformed into E. coli C43(DE3) cells. Transformed cells were grown in Terrific Broth medium containing 20 μg/mL kanamycin sulfate until OD600 = 0.6–0.8, and then isopropyl β-D-thiogalactopyranoside was added at a final concentration of 1 mM. After incubation for 16 h at 18 °C, cells were harvested by centrifugation and suspended in buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM KCl, and 2 mM phenylmethanesulfonyl fluoride (PMSF). The cell suspension was then disrupted by sonication with a Tomy UD 200 tip sonifier at OUTPUT level 4 (Tomy). To remove large influence (PMSF). The cell suspension was then disrupted by sonication with harvested by centrifugation and suspended in buffer containing 20 mM 4 °C for 10 min, and the supernatant was then recentrifuged at 150,000 × g for 1 h at 4 °C. The pellet was suspended and adjusted to 10 mg/mL with the same buffer, followed by addition of 1.5% (wt/vol) Fos–choline 14 (Affymetrix) and centrifuged at 150,000 × g at 4 °C for 1 h. The supernatant containing PfCRT was obtained, diluted twofold with buffer consisting of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM KCl, and 2 mM PMSF, and then applied to a column containing 1 mL of nickel-NTA Superflow resin (Qiagen) equilibrated with a buffer consisting of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM KCl. After protein binding, the column was washed with washing buffer consisting of 20 mM Tris-HCl (pH 8.0), 20 mM imidazole, 100 mM NaCl, 10 mM KCl, and 1% octyl glucoside (Dojin). The PfCRT protein was eluted with buffer consisting of 20 mM Tris-HCl (pH 8.0), 300 mM imidazole, 100 mM NaCl, 10 mM KCl, and 1% octyl glucoside and then stored at –80 °C, at which temperature it was stable without loss of activity for at least several months.

**Reconstitution.** Purified PfCRT was reconstituted into proteoliposomes using the freeze-thaw method described previously (28). Briefly, 20 μg of purified PfCRT was mixed with liposomes (0.5 mg lipid), frozen at –80 °C, and then left at this temperature for at least 10 min. The mixture was then thawed quickly and diluted 60-fold with reconstitution buffer consisting of 20 mM Tricine-KOH (pH 8.0), 5 mM magnesium chloride, and 100 mM KCl. Proteoliposomes were precipitated by centrifugation at 200,000 × g for 1 h at 4 °C and suspended in 0.4 mL of reconstitution buffer.

**Transporter Assay.** The transporter assay was carried out as described previously (28). Briefly, proteoliposomes (~1.5 μg of reconstituted protein/assay) were suspended in high pH buffer containing 20 mM tricine-KOH (pH 8.0), 5 mM magnesium chloride, and 100 mM NaCl or in low pH buffer containing 20 mM MES-KOH (pH 5.0), 5 mM magnesium chloride, 100 mM KCl, and 2 μM valinomycin and then incubated for a further 3 min at 27 °C. The assay was initiated by addition of 100 μM [1-14C] TEA (0.5 MBq/μmol), 1 μM [Ring-3H] chloroquine (0.5 MBq/μmol), 100 μM [4,5,3H] lysine (0.5 MBq/μmol), 100 μM [2,3,4-3H] arginine (0.5 MBq/μmol), 100 μM [3H] histidine (0.5 MBq/μmol), 10 μM [N-methyl-3H] verapamil (0.5 MBq/μmol), 100 μM [N-methyl-3H] citemidine (0.5 MBq/μmol), 100 μM [methyl-3H] methyl-4-phenypprondinium iodide, 100 μM [3H(N)] spermidine trihydrochloride (0.5 MBq/μmol), or 100 μM [Ring, methylens-3H(N)] histamine (0.5 MBq/μmol), and 130-μL aliquots were obtained at the indicated times and centrifuged through Sephadex G-50 (fine) spin columns at 760 × g for 2 min. Radioactivity in the eluate was measured with a liquid scintillation counter, TEA

**Fig. 4.** Amino acid uptake by CQ-sensitive and CQ-resistant PfCRTs. Uptake of unlabeled or radiolabeled amino acids in the presence or absence of driving force (Δψ and ΔpH) into PfCRT proteoliposomes. The listed amino acids were used as substrates at 100 μM for arginine (A) and lysine (B) or at 10 μM for histidine (C). Uptake was measured at 1 min. Dose-dependent inhibition of PfCRT-mediated uptake of amino acids by CQ. The assay was carried out in the presence or absence of CQ. Dose-dependent inhibition of PfCRT-mediated uptake of amino acids by CQ (D and E). The assay was carried out in the presence of indicated concentrations of CQ. CQ uptake by PfCRT(D7) (D) and PfCRT(D2) (E).
The mass spectrometer was externally calibrated using peptide calibration preparation method. Mass spectrometric analysis was performed with an acetonitrile by evaporation, samples were reduced by addition of 10 mM DTT and then alkylated by 55 mM iodoacetamide. In-gel digestion was performed with 5 mg/ml trypsin (Promega) at 37 °C for 24 h. Samples were concentrated by evaporation and were spotted onto an MTP AnchorChip 600/384 TF plate using the thin-layer affinity α-cyanohydroxyacridine AnchorChip preparation method. Mass spectrometric analysis was performed with an UltraflexXTM MALDI-TOF/TOF Mass Spectrometer System (Bruker Daltonics). The mass spectrometer was externally calibrated using peptide calibration standard II (Bruker Daltonics). Peptide masses were acquired with a range of 300–6000 m/z. The MS/MS spectra were assigned using BioTools software v.3.2.1 (Bruker Daltonics), and the results were searched for matches to the PfCRT protein sequence by the Mascot search engine (Matrix Science).

Miscellaneous Procedures. Site-specific rabbit polyclonal antibodies against PfCRT were prepared by repeated injection of GST fusion polypeptides encoding amino acid residues M1–L35 of the N terminus of PfCRT. Polyacrylamide gel electrophoresis in the presence of SDS and Western blotting was performed as described previously (28).

Data Analysis. All numerical values are shown as the means ± SEM; n = 3–6, unless otherwise specified. Results were analyzed by Student’s t test.

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Detected peptide (Residue No.) | m/z
---|---
Upper band (~100 k) EIVFK (232 – 236) | 635
 | NTVVENCGLGMMAK (295 – 307) | 1392
 | QLHLPYNEIWTNIK (271 – 284) | 1768
 | LSFETQEENSIIFNLVLISALIPVCFSNMTREIVFK (201 – 236) | 4202
Center band (~72 k) EIVFK (232 – 236) | 635
 | NTVVENCGLGMMAK (295 – 307) | 1392
 | QLHLPYNEIWTNIK (271 – 284) | 1768
Bottom band (~50 k) EIVFK (232 – 236) | 635
 | NTVVENCGLGMMAK (295 – 307) | 1392
 | QLHLPYNEIWTNIK (271 – 284) | 1768
 | YHLYNYLGAVIVVTIALVEMK (179 – 200) | 2522

Supplementary Table I. Mass spectrometric analysis of purified PfCRT protein.

Purified PfCRT protein was analyzed after separation by SDS-PAGE. Upper, center, and bottom bands shown in Fig. 1A were subjected to in-gel trypsin digestion and analyzed by UltrafleXtreme™ MALDI-TOF/TOF-Mass Spectrometer system (Bruker Daltonics). Amino acid sequences identified by MS/MS analyses are shown with residue numbers in parentheses.
Figure S1. Dose dependence of TEA uptake by PfCRT<sup>Dd2</sup>.

TEA uptake at 1 minutes was measured as described in Fig. 1D.

$K_m = 0.47 \text{ mM}$

$V_{max} = 85.3 \text{ nmol/mg/min}$
Figure S2. *cis*-Inhibition of TEA uptake by CQ-sensitive PfCRT<sup>3D7</sup>.

Δψ- and ΔpH-driven TEA uptake by CQ-sensitive PfCRT<sup>3D7</sup> at 1 minute in the presence of the listed amino acids and candidates for the PfCRT substrate at 1 mM.
Figure S3. Uptake of various radiolabeled compounds by CQ-sensitive PfCRT<sup>3D7</sup>.

Uptake of the listed compounds at 0.1 mM was measured 1 minute after starting the reaction in the presence or absence of driving force (Δψ and ΔpH).
Figure S4. *cis*-Inhibition of TEA uptake by CQ-sensitive PfCRT<sup>3D7</sup>.

TEA uptake in the presence of the listed compounds at 1 mM was measured as described in Fig. 2. TEA concentration was 100 µM.
Figure S5. PfCRT\textsuperscript{3107} uptakes cimetidine, histamine and spermidine.

Substrate uptake at 100 µM was measured as described in Fig. 1E.
Figure S6. Energetics of arginine uptake.

Arginine uptake by PfCRT^{3D7} was measured as described in Fig. 1C.
Figure S7. Dose dependence curves of arginine uptake.

Arginine uptake by PfCRT\textsuperscript{3D7} (A) and PfCRT\textsuperscript{Dd2} (B) was measured as Fig. 1D. Values after subtraction by liposome (-PfCRT) background are plotted.
Figure S8. Dose dependence curves of lysine uptake.

Concentration dependence of lysine uptake was measured as described in Fig. S7. The uptake values at 1 minute are shown.
Figure S9. Dose dependence curves of histidine uptake.

Concentration dependence of histidine uptake was measured as described in Fig. S7.

The uptake values at 1 minute are shown.
Figure S10. Physiological role of PfCRT.

PfCRT exports not only CQ but also supplies nutrients from digestive vacuole. PfCRT transports amino acids and peptides produced in digestive vacuole to cytoplasm. CQ inhibits conversion of toxic hematin to hemozoin.

CQ-resistant PfCRT possesses enhanced transport activity for CQ and other antimalarias and nutrients by changing affinities for these compounds. This reduces CQ concentration in the digestive vacuole and promotes conversion of toxic hematin.