A Redox-Active Fluorescent pH Indicator for Detecting *P. falciparum* Strains with Reduced Responsiveness to Quinoline Antimalarial Drugs

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Supporting Information

ABSTRACT: Mutational changes in the *Plasmodium falciparum* chloroquine resistance transporter (*Pf*CRT) have been associated with differential responses to a wide spectrum of biologically active compounds including current and former quinoline and quinoline-like antimalarial drugs. *Pf*CRT confers altered drug responsiveness by acting as a transport system, expelling drugs from the parasite's digestive vacuole where these drugs exert, at least part, of their antiparasmodial activity. To preserve the efficacy of these invaluable drugs, novel functional tools are required for

epidemiological surveys of parasite strains carrying mutant *PfCRT* variants and for drug development programs aimed at inhibiting or circumventing the action of *PfCRT*. Here we report the synthesis and characterization of a pH-sensitive fluorescent chloroquine analogue consisting of 7-chloro-*N*-{2-[(propan-2-yl)amino]ethyl}quinolin-4-amine functionalized with the fluorochrome 7-nitrobenzofurazan, henceforth termed Fluo-CQ. In the parasite, Fluo-CQ accumulates in the digestive vacuole, giving rise to a strong fluorescence signal but only in parasites carrying the wild type *PfCRT*. In parasites carrying the mutant *PfCRT*, Fluo-CQ does not accumulate. The differential handling of the fluorescent probe, combined with live cell imaging, provides a diagnostic tool for quick detection of those *P. falciparum* strains that carry a *PfCRT* variant associated with altered responsiveness to quinoline and quinoline-like antimalarial drugs. In contrast to the accumulation studies, chloroquine (CQ)-resistant parasites were observed cross-resistant to Fluo-CQ when the chemical probe was tested in various CQ-sensitive and –resistant parasite strains. NBD derivatives were found to act as redox cyclers of two essential targets, using a coupled assay using methemoglobin and the NADPH-dependent glutathione reductase (GRs) from *Plasmodium falciparum*. This redox activity is proposed to contribute to the dual action of Fluo-CQ on redox equilibrium and methemoglobin reduction *via PfCRT*-mediated drug efflux in the cytosol and then continuous redox-dependent shuttling between food vacuole and cytosol. Taking into account these physicochemical characteristics, a model was proposed to explain Fluo-CQ antimalarial effects involving the contribution of *PfCRT*-mediated transport, methemoglobin reduction, hemozoin binding, and NBD reduction activity catalysed by *PfGR* in CQ-resistant *versus* CQ-sensitive parasites. Therefore, introduction of NBD fluorophore in drugs is not inert and should be taken into account in drug transport and imaging studies.

KEYWORDS: *chloroquine resistance, fluorescent pH indicator, malaria, PfCRT transporter, Plasmodium falciparum, redox*

Malaria continues to impose a heavy socio-economic and health burden on many nations. Particularly Sub-Saharan Africa and parts of Southeast Asia where the vast majority of the estimated 198 million malaria-related disease episodes and 584,000 deaths occur ache under the yoke of this infectious disease.¹ Relief is not in sight. The world malaria situation will remain tense not least because human malaria parasites have emerged and spread that are resistant to many currently used antimalarial drugs.² Of particular concern is the reduced responsiveness of *Plasmodium falciparum* to artemisinin, quinoline, and quinoline-like derivatives,^{3,4,5,6,7,8,9,10} which as artemisinin combination therapy carry the brunt of malaria chemotherapy.¹¹ In order to combat resistance and preserve the efficacy of these indispensable drugs, better tools are required for diagnosis of resistant strains and for identifying novel chemotherapeutic intervention strategies that prevent or circumvent established drug resistance mechanisms. Currently the surveillance of drug resistant *P. falciparum* strains relies on clinical observations, time-consuming and labor-intensive genotype analysis, and *in vitro* drug response testing. None of these approaches are particularly suitable for bedside diagnostics, although rapid and easy-to-perform tests to monitor drug resistant parasites would benefit patient care, particularly in areas of high prevalence of drug and multi-drug resistance.

Our understanding of the molecular mechanisms underpinning drug resistance in *P. falciparum* has substantially advanced in recent years. For instance, reduced susceptibility to quinoline antimalarial drugs, such as chloroquine (CQ), quinine, and amodiaquine, and quinoline-like drugs, such as lumefantrine and halofantrine, is associated, amongst other factors, with polymorphisms in a gene called the *P. falciparum* chloroquine resistance transporter gene (*pfcr*).^{12,13} This gene encodes a protein of 424 amino acids that is localized at the membrane of the parasite's digestive vacuole. The *P. falciparum* chloroquine resistance transporter protein (*PfCRT*) shares structural features with the drug/metabolite carrier family and was shown to transport the quinoline antimalarial drugs CQ,

quinine, and quinidine.^{14,15} However, *PfCRT* only alters drug transport when it contains the key mutational changes associated with quinoline resistance, such as the K76T substitution, whereas the wild type form does not act on these drugs.¹⁶ *PfCRT*-mediated drug transport is directed out of the digestive vacuole. Due to their acidotropic nature, quinoline antimalarial drugs accumulate in the acid digestive vacuole where they interfere with endogenous heme detoxification mechanisms, although they also might exploit other molecular targets. The digestive vacuole serves as the main proteolytic organelle for digestion of hemoglobin, which the parasite takes up from its host cell during intraerythrocytic development to meet its nutrient requirements. The large amounts of cytotoxic heme released as a consequence of hemoglobin degradation are biomineralized to inert hemozoin. Quinoline antimalarial drugs interfere with heme biomineralization, resulting in a build-up of toxic heme and heme:drug adducts. Thus, a key feature of quinoline resistant parasites is to reduce the intra-digestive vacuolar drug concentration via *PfCRT*-mediated efflux below levels required to inhibit heme biomineralization.^{17,18,19}

The information regarding the role of *PfCRT* in reduced quinoline responsiveness has inspired the search for, and development of, fluorescent CQ derivatives that differentiate between wild type strains and those mutant strains carrying *PfCRT* variants associated with reduced quinoline susceptibility.^{20,21,22,23} However, most of the currently available probes are compromise in one way or another either because they require expensive building blocks, are difficult to synthesize, have poor spectral properties, are not a validated substrate of *PfCRT*, or do not allow interactions with *PfCRT* to be studied directly without confounding effects such as the binding to heme.

Here we report a novel fluorescent reporter, called Fluo-CQ, based on a short CQ analogue (CQ1), functionalized at the side chain with 7-nitrobenzofurazan (NBD). The probe is easy to synthesize; it has improved physicochemical properties; and it serves as a fluorescence pH indicator. The probe appears to be a substrate of the *P. falciparum* Dd2 strain *PfCRT* (*PfCRT*^{Dd2}) variant and may

therefore allows the transport activity of this protein to be monitored in living parasites in real time using a fluorescence microscopic set-up. As the nitroaromatic NBD moiety is known to act as redox-cycler and substrate of many reductases a comprehensive study was performed to interrogate the interaction of the redox-active Fluo-CQ with four targets: *Pf*GR-catalysed NBD reduction, methemoglobin reduction, hematin binding, and *Pf*CRT-mediated transport. The physico(bio)chemical features of the new fluorophore Fluo-CQ were shown to contribute both to the antimalarial and fluorescent reporter activities of the chemical probe discriminating CQ-resistant *versus* CQ-sensitive parasites. The probe offers novel opportunities for diagnostic purposes, studies probing the mechanism of quinoline resistance, and efforts aimed at developing inhibitors of *Pf*CRT.

RESULTS

Design and synthesis of the fluorescent NBD-functionalized chloroquine analogue (Fluo-CQ).

We have previously described the synthesis of a family of chloroquine derivatives with altered amino alkyl side chains.²⁴ From these compounds we selected 7-chloro-*N*-{2-[(propan-2-yl)amino]ethyl}quinolin-4-amine (compound **2**) as a scaffold for functionalization with a fluorescent group. The reasons for choosing this compound were as follows: i) the compound is easy to synthesize in high yield;²⁴ ii) it is an amphiphilic diprotic weak base with p*K*_a values of 7.7 ± 0.2 and 9.6 ± 0.2 (Table S1, Supporting information) allowing it to cross membranes by passive diffusion as the free base and to accumulate in acidic compartments, such as the parasite's digestive vacuole; and iii) the compound is a substrate of *Pf*CRT.²⁴

Scheme 1. Structural formula of chloroquine (A) and synthetic route to prepare Fluo-CQ (6).

Scheme 1 shows the synthesis strategy to produce compound **6** functionalized with the fluorescent group 7-nitrobenzofurazan (NBD), henceforth called Fluo-CQ. As an initial building block we used *N*-(7-chloroquinolin-4-yl)ethyl-1,2-diamine (**1**), which reacted in a one-step reductive amination with propan-2-one to compound **2** in the presence of titanium isopropylate (IV) and sodium borohydride as reductive reagent (yield 92%). The subsequent step involved a peptide coupling reaction between compound **2** and *N*-(*tert*-butoxycarbonyl) glycine in the presence of *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as coupling reagents (yield 90%). The amide group of the resulting compound **3** was subsequently reduced to the amine using borane dimethyl sulfide complex (BH₃-Me₂S). BH₃-Me₂S was preferred over other reducing agents because of the better yield of the resulting product (**4**) (60% *versus* 0%, 16%, 25% when the reduction reaction was performed using sodium bis(2-methoxyethoxy) aluminium hydride (Red-Al), lithium aluminium

hydride (LAH), or borane tetrahydrofuran complex ($\text{BH}_3\text{-THF}$) with 1 M in tetrahydrofuran, respectively). Finally, the desired compound Fluo-CQ (**6**) was easily obtained in 60% yield (over 2 steps) by removal of the Boc protective group in the presence of HCl (6*N*), followed by a peptide coupling reaction between the intermediary product **5** and NBD-6-aminohexanoic acid (**7**) in the presence of hydroxybenzotriazole (HOBt) and excess of EDC as coupling reagents. For this purpose, the NBD-6-aminohexanoic acid precursor **7** was prepared in 77% yield by a nucleophilic substitution reaction between 6-aminohexanoic acid and NBD-chloride in the presence of excess of sodium bicarbonate.²⁵

Physicochemical properties of Fluo-CQ (6**).** We next investigated the physicochemical properties of Fluo-CQ and assessed the influence of pH on the emission properties (Figure S1-S11, Supporting information). Coupled absorption spectrophotometric (or spectrofluorimetric) and potentiometric titrations allowed us to calculate the pK_a values of the bifunctional molecule (Table S1, Supporting information). It was found that Fluo-CQ is a weak base with pK_a values of 6.4 ± 0.6 and 7.8 ± 0.4 close to those measured for its synthetic precursor compound **4** ($\text{pK}_{a1} = 6.9 \pm 0.3$ and $\text{pK}_{a2} = 8.5 \pm 0.2$). Although Fluo-CQ ($\text{pK}_a^{\text{av}} = 7.1$) was less basic than CQ (pK_a values of 8.4 ± 0.2 and 10.6 ± 0.2 ; $\text{pK}_a^{\text{av}} = 9.5$) and compound **2** (pK_a values of 7.7 ± 0.2 and 9.6 ± 0.2 ; $\text{pK}_a^{\text{av}} = 8.65$), it possesses, however, an enhanced lysosomotropic activity as indicated by the calculated LH_2^{2+} (pH=5)/ LH_2^{2+} (pH=7.4) ratio (L = CQ, **2**, or Fluo-CQ). The ratios were measured to be 14.4, 1.49, and 1.1 for Fluo-CQ, compound **2**, and CQ, respectively. This finding suggests an enhanced theoretical accumulation of the diprotic form of Fluo-CQ in the parasite's digestive vacuole as compared to CQ and compound **2**.

Figure 1. Spectral properties of Fluo-CQ. Panel A: Reconstituted relative fluorescence spectra of Fluo-CQ free base (L, red), mono-protonated (LH⁺, blue), and di-protonated (LH₂²⁺, black). F , relative fluorescence signal. Panel B: Distribution diagram showing the different forms of Fluo-CQ as a function of pH (color code as above). Overlaid is a graph showing the relative fluorescence signal (F) of quinoline and NBD at excitation and emission wavelengths of $\lambda_{exc.} = 265$ nm, $\lambda_{em.} = 378$ nm and $\lambda = 550$ nm, respectively, as a function of pH. $\lambda_{em.} = 378$ nm relates to the quinoline emission while $\lambda = 550$ nm is characteristics of the NBD emission. Solvent: H₂O/DMSO (1/1 v/v); $I = 0.1$ M NaClO₄, $T = 25.0^\circ\text{C}$; $l = 1$ cm; [Fluo-CQ]_{tot} = 1.16×10^{-5} M; excitation and emission slit widths = 5.5 nm; filter at 290 nm.

A pH-dependent spectrofluorimetric analysis of Fluo-CQ and its related analogues confirmed the pK_a values and revealed a strong pH effect on the fluorescence emission spectrum. To exclude any possibility of the self-folding of the two chromophores that are borne by Fluo-CQ (*i.e.* 4-aminoquinoline and NBD), its absorption and emission characteristics were compared to those of the model compounds (CQ, CQ1SPACboc, NBD). These studies established that the two chromophores that belong to Fluo-CQ are not self-interacting in solution meaning that the two chromophores retained their intrinsic properties within the Fluo-CQ dyad. At an acidic pH (<6), Fluo-CQ emits a strong fluorescence signal at the NBD-centred wavelengths ($\lambda_{max} = 543$ nm, $\Phi^{abs} =$

2.3% in PBS pH 7.5; $\lambda_{\text{max}} = 518 \text{ nm}$, $\Phi^{\text{abs}} = 14\%$ and $\tau = 3.56 \text{ ns}$ in CH_2Cl_2). This peak is attenuated 4 to 5-fold at neutral and alkaline pH values. At an alkaline pH, a new fluorescence peak arising from the 4-aminoquinoline subunit emerged at lower wavelengths ($\lambda_{\text{max}} = 378 \text{ nm}$, [Figure 1A](#)). The distribution of the different forms of Fluo-CQ as a function of the pH is shown in [Figure 1B](#). The complex pH-dependent emission spectrum might be explained as follows: Fluo-CQ contains two fluorescently active chromophores, the 4-amino-quinoline and the NBD moiety, which emit fluorescence light at approximately 373 nm and 535 nm, respectively ([Figure 1B](#)).^{26,27} As shown in [Figure 1B](#), the emission from the NBD unit is the highest at an acidic pH and then declines as the pH becomes more alkaline. Conversely, fluorescence emission by the quinoline unit increases with increasing pH. At an acidic pH, fluorescence emission by the quinoline chromophore may be disfavoured upon protonation of the 4-amino group due to an intramolecular charge transfer within the quinoline unit. This suppressive effect is removed at an alkaline pH. On the other hand, the fluorescence signal emitted by the NBD moiety is inhibited at alkaline pH values possibly by photoinduced electron transfer between the deprotonated tertiary amine present in the spacer region and the fluorescence emitter. These data show that Fluo-CQ is a fluorescent pH indicator, with its maximal fluorescence emission peak shifting from 543 nm to 374 nm as the pH changes from acid to basic and *vice versa*.

Hematin-binding properties of Fluo-CQ (6) and related 4-aminoquinolines. Up to now, 4-aminoquinolines are considered as antimalarial agents, targeting specific hemoglobin digestion pathways by interacting with hematin and thereby inhibiting the biocrystallisation process leading to hemozoin. For example, chloroquine accumulates in the acidic digestive vacuole of the parasite,^{28,29} and was proposed to interact with the μ -oxo dimer of oxidized heme (Fe^{III} PPIX), thereby preventing hemozoin formation.³⁰ CQ strongly binds to Fe^{III} PPIX leading to a 1:1 complex with association constants $\log K_A \sim 5\text{-}6$ ($K_D \sim 2\text{-}15 \text{ }\mu\text{M}$) at pH 7.5.³¹ The π - π interaction between CQ and the

conjugated π -system of hematin is believed to govern and stabilize the formation of adducts. In order to assess the capacity of the studied compounds, including Fluo-CQ, to interact with hematin, an ESI-MS approach coupled to collision induced dissociation (CID)³² was applied to reach the 1:1 hematin:drug stoichiometry of the complexes and to determine the dissociation voltage at which 50% of the complexes were dissociated (DV₅₀) (Table 1).

Table 1. Hematin binding and calculated DV₅₀ evaluated from stability responses of the [hematin.drug]⁺ complexes shown in the ESI-CID spectra (drug = CQ, CQ1 **2**, CQ1-SPAC-Boc **4**, and CQ1-SPAC-NBD **6**).

Compound	DV ₅₀ (V) ^[a] (<i>m/z</i>) of the [drug:hematin] ⁺ species	K _D (μM) ^[b]	pK _a values ^[c]
CQ	261 (935.5)	0.85	8.4 ± 0.2 10.6 ± 0.2
2 (CQ1)	236 (879.5)	0.41	7.71 ± 0.2 9.57 ± 0.2
4 (CQ1-SPAC-Boc)	267 (1022.65)	nd	6.94 ± 0.3 8.46 ± 0.2
6 (Fluo-CQ) (= CQ1-SPAC-NBD)	309 (1198.7)	nd	6.39 ± 0.6 7.82 ± 0.5 ^[d]

[a] Stability responses and calculated DV₅₀ of the [hematin.drug]⁺ complexes were obtained from an ESI-CID study with chloroquine (CQ) used as gold standard. ESI-MS⁺-CID test conditions: 10 μM hematin + 10 μM drug in CH₃CN/H₂O (1/1 v/v) + 0.1% formic acid; fragmentor voltage ranges from 20 V to 400 V with 20 V increments; see ref. (32). [b] 0.2 M sodium Hepes buffer pH 7.5, see ref. (31). [c] Absorption titrations; *I* = 0.1 M NaClO₄, *T* = 25.0°C; Error = 1σ with σ = standard deviation. [d] water/DMSO (1:1 v/v); *I* = 0.1 M NaClO₄.

The DV₅₀ sequence of the drugs:hematin species (7:hematin << 2:hematin (CQ1) < CQ:hematin < 4:hematin (CQ1-SPAC-Boc) < 6:hematin (**Fluo-CQ**, CQ1-SPAC-NBD)) shown in [Table 1](#) (and [Table S2, Supporting Information](#)) demonstrates the ability of 4-aminoquinolines to interact with hematin directly, with the strongest interaction found for Fluo-CQ.

Antimalarial Activities of Fluo-CQ (6) and related 4-aminoquinolines. The antimalarial activities of Fluo-CQ (6) and related 4-aminoquinolines 2, 4, and chloroquine as drug reference, were evaluated in a SYBR_{green}-based cell proliferation screening assay using red blood cells parasitized with the *P. falciparum* laboratory strains, the CQ-sensitive HB3 or the CQ-resistant Dd2.³³ In comparison to CQ, there is a trend in the half maximal inhibition (IC₅₀) values attesting for the higher antiparasmodial activities exerted by CQ1 (2) and Fluo-CQ (6) against the CQ-resistant strain Dd2 *versus* the CQ-sensitive strain HB3 ([Table S3, Supporting information](#)). These results with their hematin binding properties indicate that the primary target of 4-aminoquinolines CQ1 (2) and Fluo-CQ (6), is likely heme biocrystallisation in the CQ-sensitive strain. The synthetic intermediate CQ1-SPAC-Boc (4) displayed the lowest antimalarial activity, whatever the wild-type and CQ-resistant *P. falciparum* strains HB3 and Dd2 used, with comparable IC₅₀ of 95 nM and 77 nM, respectively. This latter observation suggested that the NBD moiety in Fluo-CQ might contribute *per se* to the antimalarial activity of the hybrid molecule (6).

In order to confirm that CQ-resistant parasites are cross-resistant to Fluo-CQ the strain susceptibilities of two additional strains, 3D7 (CQ^S) and K1 (CQ^R), to both compounds CQ1 (2), and Fluo-CQ (6) were also measured in comparison to CQ ([Table 2](#)). First, the IC₅₀ values clearly indicated that both compounds showed decreased antiparasmodial activities against the CQ-resistant parasite strains Dd2 and K1, but to a lesser extent than CQ. These findings suggested that i) CQ-resistant parasites are cross-resistant to Fluo-CQ and that Fluo-CQ and CQ share at least one

primary molecular target (hemozoin formation); ii) the most parsimonious explanation of the reduced activity of Fluo-CQ against the CQ-resistant parasites is one that would evoke Fluo-CQ being effluxed by PfCRT-Dd2 from the vacuole into the cytosol, where it can no longer target hemozoin formation. Therefore, the antimalarial action of Fluo-CQ **6** deserved to be discussed with cautious considerations. The potent antimalarial activities of **2** and Fluo-CQ in the CQ-resistant strains led us to perform a detailed investigation of their interactions with potential targets, beside the PfCRT variants. The strongest antiplasmodial effects of CQ1 (**2**) and Fluo-CQ (**6**) against the CQ-resistant strain Dd2 compared to CQ suggested that the difference is at least partially attributed to the higher ability of **2** and **6** to cross the membranes of infected cells as a consequence of their modified physicochemical properties. These latter might led to a higher lipophilicity value, lower flexibility of the side chain, or lower ionization constant (pK_a) of the amine groups, resulting in improved antimalarial effects, as confirmed in the physicochemical study. Indeed, as reported above, introduction of the lipophilic ethylenediamine chain, and the NBD hexanoic acid moiety modified the physicochemical properties of the 4-aminoquinolines, as attested by the calculated clogP values for the final compounds **2** and **6** being equal to 3.76 and 6.89 *versus* 5.06 for CQ, respectively (calculated from ChemBioDraw Ultra 14.0).

Table 2. Averaged IC₅₀ values (nM) for CQ, CQ1 and Fluo-CQ determined from growth inhibition assays with *Plasmodium falciparum* strain HB3 (CQ^S), 3D7 (CQ^S) and Dd2 K1 (CQ^R) and K1 (CQ^R).^[a]

Strain \ Compound	IC ₅₀ ± SEM (nM)		
	CQ	2 (CQ1)	6 (Fluo-CQ)
HB3 (CQ ^S)	7 ± 2	5 ± 2	25 ± 8
3D7 (CQ ^S)	6 ± 2	7 ± 1	21 ± 8
Dd2 (CQ ^R)	150 ± 30	46 ± 7	70 ± 18
K1 (CQ ^R)	160 ± 25	40 ± 9	81 ± 23

^[a] Test conditions: synchronous cultures, SYBR_{green} assay, 72 h incubation. ^[b] Values are means of at least two independent determinations in triplicate. The IC₅₀ value of the antimalarial drug chloroquine (CQ) is indicated as a reference.

Fluo-CQ inhibits PfCRT-Dd2 mediated CQ uptake in *X. laevis* oocytes. We further investigated the interactions between PfCRT and Fluo-CQ by performing direct measurements of CQ transport in the PfCRT^{HB3} or PfCRT^{Dd2} expressing *Xenopus laevis* oocyte system.¹⁵ We observed that Fluo-CQ interacts with PfCRT and inhibits PfCRT in a concentration dependent manner (Figure 2). Preliminary attempts to detect the accumulation of Fluo-CQ in the PfCRT-expressing oocytes were positive but not confirmed because Fluo-CQ intercalates into the oocyte membrane resulting in equally high fluorescence signals in water-injected oocytes and in oocytes expressing PfCRT^{HB3} or PfCRT^{Dd2}. In spite of numerous trials we were not able to demonstrate a

highly reproducible transport of Fluo-CQ via *Pf*CRT hampering the determination of a K_m value for Fluo-CQ nor inhibition parameters of Fluo-CQ uptake by verapamil. However, it was possible to use the native fluorescence of Fluo-CQ to visualize the accumulation of Fluo-CQ in *Pf*CRT^{HB3} or *Pf*CRT^{Dd2} expressing *P. falciparum*-infected erythrocytes.

Figure 2. Fluo-CQ inhibits the transport of radiolabeled CQ in *Pf*CRT^{Dd2} expressing *Xenopus laevis* oocytes. *Pf*CRT^{Dd2}-expressing oocytes were incubated for 60 min in buffer containing 40 nM [³H]CQ, 10 μM cold CQ, and Fluo-CQ at the concentrations indicated. Water-injected oocytes were analysed in parallel. *Pf*CRT-mediated CQ uptake was calculated by subtracting the amount of CQ taken up by water-injected oocytes from that of *Pf*CRT^{Dd2} expressing oocytes. The means ± SEM are shown for at least three independent replications, with each single measurement being made from 10 oocytes.

Fluo-CQ as a reporter for *Pf*CRT-mediated CQ flux. We next examined Fluo-CQ accumulation in *P. falciparum*-infected erythrocytes. Fluo-CQ accumulated in the digestive vacuole of the CQ-sensitive strain HB3, as indicated by a strong food vacuolar fluorescence signal (Figure 3A).

Accumulation of Fluo-CQ in the digestive vacuole of strain HB3 did not occur in glucose starved cells. In the absence of metabolic energy, a diffuse fluorescence signal was observed throughout the parasite's cytoplasm (Figure 3A). This finding is explained by the requirement of an energy input to acidify the digestive vacuole *via* the combined action of a H^{+} -ATPase and a H^{+} -pyrophosphatase,³⁴ which in turn is a prerequisite for accumulation and trapping of Fluo-CQ in this organelle. Also note that the green Fluo-CQ's fluorescence signal is the strongest at an acidic pH (Figure 1). Accumulation of Fluo-CQ in the digestive vacuole was independent of verapamil (30 μ M), a chemosensitizer of CQ resistance and a partial mixed type inhibitor of *PfCRT*.^{15,35} Differential fluorescence images were observed for erythrocytes infected with the CQ-resistant strain Dd2 (Figure 3B). The food vacuolar Fluo-CQ fluorescence signal that was very prominent in vital HB3 parasites was only observed in Dd2 following treatment with verapamil (30 μ M) causing inhibition of *PfCRT*. In untreated cells and in glucose starved cells, the Fluo-CQ fluorescence was weak and distributed throughout the parasite's cytoplasm. Quantification of the fluorescence signals confirmed the differential partitioning behaviour of Fluo-CQ in the CQ-sensitive and CQ-resistant *P. falciparum* strain (Figure 3C). These findings are consistent with *PfCRT* from the CQ-resistant strain being able to transport Fluo-CQ out of the digestive vacuole, whereas *PfCRT* from the CQ-sensitive strain does not act on this fluorochrome - and hence, the differential accumulation and fluorescence phenotypes. Therefore, Fluo-CQ might accurately reflect CQ transport via *PfCRT* without confounding effects such as the binding to heme because the time frame of the antimalarial potency experiments, i.e. drug incubation with synchronized ring stage parasite cultures for 72 h, excludes to be confounded with the interactions with *PfCRT* studied directly by fluorescence of Fluo-CQ in very short time, i.e. following 1 h incubation with the parasitized erythrocytes.

Figure 3. Fluo-CQ live cell imaging in the CQ-sensitive *P. falciparum* strain HB3 (Panel A) and the CQ-resistant strain Dd2 (Panel B). *P. falciparum*-infected erythrocytes at the trophozoite stage were incubated with 0.5 μ M Fluo-CQ for 20 min at 37°C before images were taken using a confocal fluorescence microscope. Where indicated, cells were glucose starved (-glucose), treated with 30

μM verapamil (+VP), or incubated without Fluo-CQ (-Fluo-CQ). DNA was stained using Hoechst 33342 (blue channel). (Panel C) Ratio of the digestive vacuolar and total parasite fluorescence signals ($F_{\text{vacuole}} / F_{\text{parasite}}$). The means $\pm$ SEM of over 20 independent determinations are shown. ** $P < 0.01$ compared to Fluo-CQ treated vital cells, as determined using the Holm-Sidak ANOVA test. Bar, 1 μm .

***P. falciparum* GR-catalyzed 7-nitrobenzofurazan reductase Activity.** The ability of *P. falciparum* GR to reduce the nitroaromatic group of the 7-nitrobenzofurazan (NBD) derivatives was then investigated in the methemoglobin (metHb) reduction assay coupled to the *Pf*GR/NADPH system *in vitro* under quasi-physiological conditions (equations 1-4). This assay was recently established as a relevant *in vitro* model in our laboratory and allowed to assess redox characteristics of the *P. falciparum* GR-catalyzed nitroreduction kinetics.^{31,36,37} The UV-visible absorption spectrum of metHb(Fe^{III}) between 300 nm and 700 nm is characterized by an intense Soret band of the Fe^{III} heme whose maximum absorbance is centred at 405 nm and was used as a valuable spectroscopic probe. Upon metHb(Fe^{III}) reduction by the enzymatically-generated reduced species of the redox-active compounds, the occurrence of the oxyHb(Fe^{II}) species is associated to a bathochromic shift of the maximum of absorption from 405 nm to ~ 410 nm (now corresponding to a low spin hexacoordinated ferrous ion), as well as the formation of two new less intense absorptions lying at ~ 536 nm and ~ 576 nm, respectively (Figure 4, Figure S12-S13, Supporting information). The nitroaromatic reductase activity of *P. falciparum* GR was observed as long as NADPH is present in the cuvette and compared to the intrinsic NADPH oxidation activity of the enzyme in the absence of the NBD derivative (equation 2). As an example, Figure 4 depicts the spectral absorption variation recorded for Fluo-CQ (Panels A-B), and **7** (Panel C) under these experimental conditions. The Table in Figure 4 reports on the determined MetHb(Fe^{III}) reduction kinetic parameters determined in the presence of the compounds, 4-aminoquinolines (**2** and **4**), and

the NBD derivatives (Fluo-CQ, **6** and the NBD-hexanoic acid, **7**). The kinetic analysis clearly evidenced the presence of two rate-limiting steps. The first one did not induce any classical shift of the Soret band usually observed for metHb reduction, and the kinetic rates are seemingly independent of the nature of the compounds within the experimental errors. The second one is clearly associated with the metHb reduction as evidenced by the bathochromic shift of the Soret band. Marked effects can be seen on the values of these second pseudo-first order rate constants depending on the nature of the system. With the most efficient redox-cycling activity to convert metHb to oxyHb, we confirmed the ability of the NBD-hexanoic acid (**7**) to behave as an efficient substrate of *Pf*GR in the direct enzymic assay using the NADPH-dependent *P. falciparum* GR. The nitroreductase activity of *P. falciparum* GR was compared to the intrinsic NADPH oxidation activity of the enzyme in the absence of the NBD derivative (**7**) (equation 2). The flavoenzyme displayed a 2.9-fold higher NADPH oxidation activity in the presence of 150 μ M the NBD-hexanoic acid (**7**) (equations 1 or 2); the observed NADPH oxidation rates were 0.013 U/mg protein in the absence of the NBD derivative and 0.347 U/mg protein in the presence of 150 μ M NBD-hexanoic acid (**7**).

The K_m and k_{cat} values, 508.7 μ M and $14 \pm 0.004 \text{ s}^{-1}$ corresponding to $V_{\text{max}} = 14.629 \pm 6.52 \text{ U/mg}$ protein were derived from two measurements at seven different substrate concentrations in duplicate (Figure 4D). When following NADPH consumption, the NBD-hexanoic acid (**7**) was reduced by *P. falciparum* GR with a catalytic efficiency k_{cat}/K_m of $27.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, a half-value only when compared to that determined for methylene blue ($k_{\text{cat}}/K_m = 50.0 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$).³⁸ These

data are also in good agreement with previous observations on various nitroaromatics reported by Cenás *et al.*,^{39,40,41} Blumenstiel *et al.*,⁴² and Biot *et al.*⁴³

Compound	k_{obs1} ($\times 10^{-3} \text{ s}^{-1}$) ($\pm\sigma$)	k_{obs2} ($\times 10^{-5} \text{ s}^{-1}$) ($\pm\sigma$)
2 (CQ1)	1.39 ± 0.04	5.2 ± 0.5
4 (CQ1-SPAC-Boc)	1.44 ± 0.05	11.6 ± 0.4
6 (Fluo-CQ, = CQ1-SPAC-NBD)	0.98 ± 0.02	3.3 ± 0.11
7 (NBD-hexanoic acid)	2.5 ± 0.4	48 ± 0.4

Figure 4. Kinetics of metHb(Fe^{III}) reduction in an established coupled assay with *Pf*GR/NADPH in the presence of the NBD derivatives, Fluo-CQ (**6**) (Panels A and B) and NBD-hexanoic acid (**7**) (Panel C), and rate constants of metHb(Fe^{III}) reduction kinetics observed for the NBD derivatives (Table). Initial velocities of the nitroreductase activity of the NADPH-dependent *Pf*GR in the presence of NBD-hexanoic acid (**7**) as substrate (Panel D).

Panels A and B: UV-visible absorption spectra recorded as a function of time in the coupled assay in the presence of Fluo-CQ (**6**); Panel A inset: Absorbance variation at 405 nm as a function of time. Panel C: UV-visible absorption spectra recorded as a function of time in the coupled assay in the presence of the NBD-hexanoic acid (**7**); Panel C inset: Absorbance variation at 405 nm as a function of time. Solvent: water (47 mM phosphate buffer pH 6.9 + 1 mM EDTA + 200 mM KCl); $T = 25.0^{\circ}\text{C}$; 120 μM NADPH + 1.72 nM *Pf*GR + 8 μM metHb + 20 μM GSSG + 40 μM Fluo-CQ (**6**) or NBD-hexanoic acid (**7**); (1) $t = 0$; (2) $t = 120$ min. Table: Kinetic parameters determined in the coupled MetHb(Fe^{III}) reduction assay in the presence of the compounds, 4-aminoquinolines (**2** and **4**), and the NBD derivatives (Fluo-CQ, **6** and the NBD-hexanoic acid, **7**). Panel D: Nitroreductase Activity of *P. falciparum* Glutathione Reductase. The nitroreductase activity of *P. falciparum* GR (Panel D) in the presence of the NBD-hexanoic acid, **7** (—●—) was tested by monitoring the oxidation of NADPH at 340 nm, at 25 °C, and pH 6.9, under steady-state conditions. The NBD-hexanoic acid was dissolved in DMSO and the NADPH-oxidation activity was measured at seven different concentrations in duplicate (0 – 150 μM) in the presence of 1% DMSO. The initial rate for aerobic NADPH oxidation activity of *Pf*GR was subtracted from the rates measured in the presence of the nitroaromatic derivative. For the determination of K_m and V_{max} values, the steady-state rates were fitted by using nonlinear regression analysis software (Kaleidagraph) to the

Michaelis-Menten equation,⁴⁴ and the turnover number k_{cat} and the catalytic efficiency $k_{\text{cat}}/K_{\text{m}}$ were therefore calculated.

In our work, the NBD-moiety from Fluo-CQ **6** showed a GR-catalyzed 7-nitrobenzofurazan reductase activity *per se*, i.e. a redox activity with a validated target in *P. falciparum*, which has been shown in several reports to be relevant to explain the antimalarial activity of redox-active agents.^{31,45,46,47,48} a 29-fold higher value compared to that of the carboxylic acid counterpart **M₅** ($k_{\text{cat}}/K_{\text{m}} = 945.0 \text{ M}^{-1} \text{ s}^{-1}$).⁴³ In previous works, the combination of various nitroaromatics^{41,49} and quinones,^{24,50} such as the carboxylic acid **M₅**, a menadione alkylated in C-2 by the hexanoic acid chain, and 4-aminoquinolines resulted in hybrid drugs, called Trojan horse drugs, displaying a higher antimalarial activity than both starting materials, probably through a dual action in the parasite. This effect seems to be specifically pronounced in infected red blood cells since cytotoxicity against hMRC-5 cells was observed neither for Fluo-CQ (data not shown), nor for the menadione-hexanoic acid **M₅**⁵⁰ and the NBD-hexanoic acid **7** (data not shown).

DISCUSSION

The malaria parasite *P. falciparum* degrades most of host cell hemoglobin to provide aminoacids essential for its intraerythrocytic growth. Endocytosis is a fundamental process of eukaryotic cells and fulfils numerous functions, most notably, that of macromolecular nutrient uptake. Malaria parasites invade red blood cells and during their intracellular development endocytose large amounts of erythrocyte cytosol for digestion in a specialized lysosomal compartment, the food vacuole, via a complex structure constituted by the cytostome (an invagination in the parasitophorous vacuolar membrane) and transport vesicles. The food vacuole contains a number of proteolytic enzymes identified in the process of hemoglobin digestion as potential drug targets, including the aspartic acid proteases (plasmepsins) that initiate the cleavage of hemoglobin,

cysteine proteases (falcipains), the metalloprotease falcilysin and a histioaspartic protease that catalyze further degradation into small peptides. These enzymes are active under the acidic conditions prevailing in the food vacuole, which was observed to have a pH between 5.0 and 5.5. In this context, it has been known for long that the antimalarial drug CQ, a dicationic weak base belonging to the 4-aminoquinoline series, penetrates and accumulates in the food vacuole of plasmodial parasites through its lysosomotropic properties, and exerts its action by interfering with heme metabolism. Hemoglobin digestion is accompanied by intensive pulses of reactive oxygen species (ROS), which are important cell signalling molecules. The redox homeostasis results from a subtle interplay between the thiol network controlled by various redox enzymes, heme biocrystallisation, and transport of redox-active components. CQ-resistance phenotype associated to reduced susceptibility of the strains to CQ has been corroborated with the presence of mutations in the food vacuole membrane protein *PfCRT*, and with lower levels of glutathione and hemozoin. A novel role for mutated *PfCRT* in CQ-resistant parasites has been recently discovered with strong data supporting a dual mechanism for *PfCRT* facilitating both influx of glutathione in the food vacuole and efflux of CQ from the food vacuole.⁵¹ As mutated *PfCRT* has also been associated with transport of redox-active agents (e.g. methylene blue)⁵² we investigated in this study the possible contribution of *PfCRT*-mediated transport of the redox-active nitroaromatic component of Fluo-CQ, which discriminates CQ-sensitive and -resistant parasites.

Well-known redox-cyclers (or subversive substrates of various members of the disulfide reductase family) are nitrofurans displaying notable antiparasitic activities. The molecular basis of nitroaromatics toxicity is the enzyme-catalyzed reduction to nitroradicals which then reduce oxygen to superoxide anion radicals thereby regenerating the redox-cycler (eq. 2).⁵³ This futile redox cycling and oxygen activation lead to increased levels of hydrogen peroxide (eq. 8) and GSSG. Electrochemical studies have demonstrated that the kinetics of the reaction with molecular oxygen

is very fast, thus privileging the reaction pathway (eq. 5).⁵⁴ The electrochemical potential remains one of the relevant descriptor that has to be taken into consideration in any attempts to design redox-active nitroaromatics, including the differently functionalized NBD derivatives.⁵⁵

Redox cycling is known to occur when a redox-active agent (e.g. phen(othi)azines, pyocyanin, naphthoquinones, nitroaromatics) undergoes alternating reduction and oxidation reactions, resulting in the transfer of electrons from the reductant (e.g. NAD(P)H) to the oxidant (e.g. oxygen).^{56,57}

Redox-cyclers, such as phenazines, can cross cell membranes, enabling the transfer of reducing equivalents from inside the cell to the extracellular environment.⁵⁸ In the case of *P. falciparum*-infected red blood cells the compartment where are present the powerful oxidants (metHb, oxygen) is the food vacuole. The antimalarial methylene blue/pyocyanin/plasmodione, in the oxidized form behave as oxidants accepting the electrons produced upon NADPH-dependent GR-catalyzed reduction in the cytosol along with concomitant NADPH oxidation to NADP⁺. The generated reduced form of the redox-active mediators (leucomethylene blue, reduced pyocyanin, reduced benzylmenadiones, reduced nitroaromatics) might also be membrane-permeable and diffuse away from the cytosol – possibly *via* iron complexation – to the food vacuole where, the reduced species is then re-oxidized in the course of the redox-cycling activity of methemoglobin(Fe^{III}) into oxyhemoglobin(Fe^{II}). The leakage of the reduced species out of the cytosol was exemplified by the antimalarial redox-active indolone *N*-oxide derivatives.⁵⁹ The oxidized mediator is then ready to repeat the redox-dependent shuttling between the parasitized erythrocytic cytosols and the food vacuole. This cycling continually drains off metabolic reducing power from *P. falciparum*-infected erythrocytes to give “electrical power at the electrodes” (Figure 5, panel B).⁴⁶ Thus, the antimalarial nitroaromatics (chinifur, NBD) derivatives are proposed to act catalytically as redox-active mediators that are shuttled – *via* mutated *Pf*CRT – in and out the food vacuole in parasitized red blood cells, thereby oxidizing major intracellular reductants (like NADPH in the cytosol) and subsequently reducing the oxidants (oxygen, hematin and metHb).

Coming back to the redox-active fluorescent pH indicator and likely substrate of *Pf*CRT, the observations might be rationalized as followed. In both the CQ-sensitive and -resistant parasite HB3 and Dd2 strains Fluo-CQ is proposed to be internalized in the food vacuole of parasitized red blood cells. The antimalarial activity of Fluo-CQ against the CQ-sensitive strain corroborates with the visualization of the pH-dependent fluorophore concentrating in the food vacuole where the 4-aminoquinoline moiety interacts with hemozoin (Figure 5, Panel A). The efflux is not supposed to occur since the *Pf*CRT gene is not mutated in CQ-sensitive parasites. In the CQ-resistant parasite Dd2 strain Fluo-CQ might rapidly be extruded by the mutated *Pf*CRT transporter to the cytosol where the NBD moiety may be reduced by the NADPH-dependent *Pf*GR. The reduced NBD species is proposed to leave the cytosol to penetrate the acidic compartment to transfer electrons to oxidants. Thereby, in agreement with the images (Figure 3) Fluo-CQ is more visualized out of the food vacuole in the *P. falciparum* Dd2 strain with reduced responsiveness to the quinoline antimalarial drug (Figure 5, Panel B). However, the mechanism of the antimalarial action of Fluo-CQ might result from its dual action, i.e. inhibition of hemozoin crystallisation upon 1e-transfer to (Fe^{III}) species and disturbance of the redox equilibrium in the infected red blood cells.

With respect to the literature, the redox-active pH indicator acridine orange (AO) has also been used as a fluorescent dye for the diagnosis of malaria⁶⁰ and as a probe for estimating the differential accumulation of, and susceptibility to, CQ between CQ-sensitive and CQ-resistant parasites.^{61,62,63} However, while the observed difference in the accumulation of AO has been linked to a lower pH of the food vacuole in CQ-resistant parasites *versus* their CQ-sensitive counterparts the interpretation has alternatively been suggested to be due to its export from the food vacuole *via* mutant *Pf*CRT.⁶⁴ Furthermore, recent studies broadened the known substrate range of mutant *Pf*CRT to include tricyclic compounds and established that the *Pf*CRT-mediated efflux of quinacrine and methylene blue from the food vacuole decreases the parasite's susceptibility to these

molecules, adding therefore further support to the hypothesis that *Pf*CRT behaves as a multidrug resistance carrier.⁵² The behaviour of redox-active pH-dependent fluorophores should be considered when used to measure changes in the pH of acidic compartments containing oxidants since *Pf*CRT seems to be a transporter responsible for efflux of many redox-active agents.

Figure 5. Models for how the antimalarial Fluo-CQ affects the growth of CQ-sensitive and -resistant *P. falciparum* parasites.

Conclusions. Fluo-CQ extends the repertoire of tools available to study altered responsiveness of *P. falciparum* to quinoline and quinoline-like drugs. Fluo-CQ is a probable substrate of *PfCRT* and thus a likely competitor of *PfCRT*'s drug transport activity. Fluo-CQ could be useful as a pH-dependent fluorescent probe for functional studies on *PfCRT* in the parasite. While it is very difficult to ascertain the essential requirement of the redox component of the chemical probe in the applications to discriminate CQ-sensitive from CQ-resistant parasites our work allowed us to measure the NBD derivatives as redox cyclers interacting with four essential targets: i) in metHb(Fe^{III}) reduction, ii) in hematin crystallisation, iii) in mutated *PfCRT*-mediated drug transport, and iv) in NADPH-dependent *PfGR* reduction. We provide here data that suggests that the fluorescent NBD moiety may contribute to altered redox equilibrium, *PfCRT*-mediated efflux of Fluo-CQ, and reduction of (Fe^{III})species (metHb, heme) via continuous redox-dependent shuttling of the fluorescent chemical probe between food vacuole and cytosol. Taking into account these characteristics, a model was proposed to explain the Fluo-CQ antimalarial effects involving the contribution of *PfCRT*-mediated transport, the hematin binding, the NBD reduction activity catalysed by GR in CQ-resistant *versus* CQ-sensitive parasites. Introduction of a NBD fluorophore in drugs is therefore not innocent and should be taken into account in drug transport and imaging studies. In this study, a robust fluorescent probe was designed and characterized, with several lines of evidence suggesting that it is a substrate of *PfCRT*-Dd2. Despite being redox active, this compound could be used to visually detect *P. falciparum* strains with reduced susceptibilities to quinoline antimalarial drugs. More work will be now developed to set up an *in cellulo* diagnostic test that might be advantageous in large screens, such as drug development projects and epidemiological surveys.

METHODS

Enzymes. Recombinant *Plasmodium falciparum* glutathione reductases (GR, E.C.1.6.4.2) was purified as previously reported.^{65,66} One unit of GR activity is defined as the consumption of 1 μmol NADPH per min ($\epsilon_{340\text{ nm}} = 6.22\text{ mM}^{-1}\text{ cm}^{-1}$) under conditions of substrate saturation. The enzyme (*P. falciparum* GR) stock solutions used for kinetic determinations were > 98% pure as judged from silver stained SDS-PAGE and had specific activities of 155 U/mg. All other reagents were of the highest available purity and were purchased from Biomol, Boehringer, and Sigma.

***P. falciparum* GR-catalyzed 7-nitrobenzofurazan reductase activity.** The ability of *P. falciparum* GR to reduce the nitroaromatic derivative **7** was assayed by monitoring the oxidation of NADPH at 340 nm ($\epsilon_{340\text{ nm}} = 6.22\text{ mM}^{-1}\text{ cm}^{-1}$). The assay mixtures, in a total volume of 1 mL, contained 100 mM phosphate buffer pH 6.9, 200 mM KCl, 100 μM NADPH, and 24.2 nM *P. falciparum* GR (0.22 units/assay in the cuvette). The nitroaromatic derivative **7** was dissolved in DMSO and the NADPH-oxidation activity was measured at seven different inhibitor concentrations (0 - 150 μM) in the presence of 1% DMSO. For the determination of K_m and V_{max} values, the steady-state rates were graphically fitted by using nonlinear regression analysis software (Kaleidagraph) to the Michaelis-Menten equation,⁴⁴ and the turnover number k_{cat} and the catalytic efficiency k_{cat}/K_m were calculated. The initial rate for intrinsic NADPH oxidation activity of *P. falciparum* GR was not subtracted from the rates measured in the presence of the nitroaromatic derivative when it proved negligible in comparison to GR-catalyzed nitroreductase activity.

Antimalarial activities of Fluo-CQ (6**) and related 4-aminoquinolines.** The antimalarial activities were evaluated in the SYBR_{green} assays³³ using parasitized red blood cells in culture. To assess this, the 50% inhibitory concentration (IC_{50}) for each compound was

determined in the presence of two *P. falciparum* laboratory strains, the CQ-sensitive HB3 or the CQ-resistant Dd2.

Drug Transport Assays. *X. laevis* adult females were purchased from Xenopus Express and kept as described.⁶⁷ Anesthesia was administered by immersion in ice water containing 0.1% ethyl-3-aminobenzoate methanesulfonate salt. Ovarian fragments were surgically removed and incubated in Ca²⁺-free medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES; pH 7.6). Oocytes follicular envelopes were removed by enzymatic treatment with 1mg/ml (0.2-0.3 U/mg) collagenase A (Sigma-Aldrich) for 30-40 minutes. Oocytes (Dumont stage V-VI) were incubated in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES; pH 7.5) supplemented with 0.1 mg/ml gentamicin and 2.5 mM sodium pyruvate. Expression of PfCRT^{Dd2} at the plasma membrane of Xenopus oocytes was achieved as described previously (Martin et al. Science 325:1680-1682).¹⁴ Briefly, cRNA was prepared by *in vitro* transcription from the SP6 promoter (mMessage mMachine *in vitro* transcription kit, Ambion). 30 ng cRNA (in 40 nl water) were injected into each *X. laevis* oocyte, using a pneumatic microinjector (5246, Eppendorf-Netheler-Hinz). Oocytes injected with 40 nl water served as controls. After injection of the cRNA, the oocytes were incubated in ND96 medium for 3 days at 18°C before uptake studies were performed. For inhibition studies, oocytes expressing PfCRT^{Dd2} and water-injected oocytes were incubated for 60 min at 25 °C in ND96 buffer pH 6.0 containing 40 nM [³H]CQ, 10 µM cold CQ, and Fluo-CQ at concentrations ranging from 0 to 500 µM. The uptake assays were terminated by removing the reaction medium and washing the oocytes three times with 3 ml of ice-cold ND96 buffer. Each oocyte was transferred to a separate scintillation vial and lysed by the addition of 5% sodium dodecyl sulfate (200 µl). The radioactivity in the sample solution was measured using a β-scintillation counter. PfCRT^{Dd2}-mediated drug transport was calculated by subtracting the uptake measured in water-injected oocytes from that in oocytes expressing PfCRT^{Dd2}. At least three

independent biological replicates were performed using oocytes from different frogs, and each single measurement was made from 10 oocytes.

Electrospray Mass Spectrometric Measurements: Electrospray mass spectra of heme complexes were obtained with a Bruker Daltonics MicroTOF spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an orthogonal electrospray (ESI) interface. Calibration was performed using Tuning mix (Agilent Technologies). CID experiments were performed with a capillary exit (cone voltage) ranging from 120 to 400V with 20V increments.⁶⁸ Stock solution of hematin ($[\text{Fe}^{\text{III}}\text{PIX}(\text{OH}_2)]^{3+}$ or $[\text{Fe}^{\text{III}}\text{PIX}(\text{OH})]^{2+}$) was freshly prepared from hemin (ferriprotoporphyrin chloride, $[\text{Fe}^{\text{III}}\text{PIX}(\text{Cl})]^{2+}$) just before use in 50% ammonia. Stock solution of benzoxanthone 4c (5 mM) was prepared in CH_3CN , while CQ (CQ, 2.91 mM) and amodiaquine (AQ, 2.28 mM) were prepared in water. Hematin and the substrate (**2**, **4**, **6** and **7**) were mixed together in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (50:50 v:v) in order to obtain equimolar concentrations of 100 μM . Prior to analyses, the samples were further diluted at 50 μM in $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{HCO}_2\text{H}$ (50:50:1 v:v:v). The sample solutions were then introduced into the spectrometer source with a syringe pump (Harvard type 55 1111: Harvard Apparatus Inc., South Natick, MA, USA) with a flow rate of 5 $\mu\text{L}\cdot\text{min}^{-1}$.

Live cell imaging. Live *P. falciparum*-infected erythrocytes were imaged at the trophozoite stage (22–26 h post invasion), as previously described.⁶⁹ Briefly, *P. falciparum*-infected erythrocytes were washed twice with RPMI 1140 medium (without NaHCO_3 , pH 7.3 adjusted at 37°C) or RPMI medium (without NaHCO_3 and glucose, supplemented with 10 mM Hepes-Na, pH 7.3 adjusted at 37°C) and then loaded with 0.5 μM Fluo-CQ for 20 min at 37°C. Where indicated 30 μM verapamil was added together with Fluo-CQ. Nuclear staining was carried out using Hoechst33342 (5 mM). Confocal laser scanning fluorescence microscopy was performed using a Zeiss LSM510 (Carl Zeiss) equipped with UV and visible laser lines (351 nm, 364 nm, 458 nm, 488 nm). Images were

deconvoluted using Huygens Essential 3.4 (Scientific Volume Imaging) and analyzed using Image J.

Ethical Statement. Ethical approval of the work performed with the *X. laevis* frogs in Heidelberg University was obtained from the Regierungspräsidium Karlsruhe (Aktenzeichen 35-9185 81/G-31/11) in accordance with the German “Tierschutzgesetz.”

ASSOCIATED CONTENT

Supporting Information

Procedures and additional data : The synthetic and physico-chemical protocols as well as the absorption and emission titrations *versus* pH of the investigated compounds are available in the Supplementary Material. This material is available free of charge on the ACS Publications website at DOI:

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Author Contributions

[#] M. J. and C. P. S. contributed equally to this article. E. D.-C. and M. L designed the molecules and the project, respectively. M. J., C. P. S., M. E., M. L., and E. D.-C. analyzed the data and wrote the manuscript. S. M., K. U., and A. G. performed the synthesis of the derivatives. M. E. supervised the

physico-chemical investigations of the derivatives. K. E. tested the compounds in the parasite assays *in vitro*. All authors edited the manuscript.

Notes

The authors declare no competing financial interest.

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