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Characterization of the Commercially-Available Fluorescent Chloroquine-BODIPY Conjugate, LynxTag-CQ_{GREEN}, as a Marker for Chloroquine Resistance and Uptake in a 96-Well Plate Assay

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Abstract

Chloroquine was a cheap, extremely effective drug against *Plasmodium falciparum* until resistance arose. One approach to reversing resistance is the inhibition of chloroquine efflux from its site of action, the parasite digestive vacuole. Chloroquine accumulation studies have traditionally relied on radiolabelled chloroquine, which poses several challenges. There is a need for development of a safe and biologically relevant substitute. We report here a commercially-available green fluorescent chloroquine-BODIPY conjugate, LynxTag-CQ_{GREEN}, as a proxy for chloroquine accumulation. This compound localized to the digestive vacuole of the parasite as observed under confocal microscopy, and inhibited growth of chloroquine-sensitive strain 3D7 more extensively than in the resistant strains 7G8 and K1. Microplate reader measurements indicated suppression of LynxTag-CQ_{GREEN} efflux after pretreatment of parasites with known reversal agents. Microsomes carrying either sensitive- or resistant-type PfCRT were assayed for uptake; resistant-type PfCRT exhibited increased accumulation of LynxTag-CQ_{GREEN}, which was suppressed by pretreatment with known chemosensitizers. Eight laboratory strains and twelve clinical isolates were sequenced for PfCRT and Pgh1 haplotypes previously reported to contribute to drug resistance, and *pfmdr1* copy number and chloroquine IC_{50}s were determined. These data were compared with LynxTag-CQ_{GREEN} uptake/fluorescence by multiple linear regression to identify genetic correlates of uptake. Uptake of the compound correlated with the logIC_{50} of chloroquine and, more weakly, a mutation in Pgh1, F1226Y.

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Competing Interests: KSWT and MJL are founding directors of BioLynx Technologies (Singapore), a private company that specializes in fluorophore-conjugated drug surrogates including LynxTag-CQGREEN. Other authors declare no competing interests. KSWT and MJL own minority shares in BioLynx Technologies (Singapore). LR and BR are PLOS ONE Editorial Board Members. This does not alter the authors' adherence to PLOS ONE Editorial policies and criteria.

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Introduction

Despite years of intense global effort to eradicate it, malaria is still one of the deadliest infectious diseases, killing more than 600 000 people in 2010 alone [1,2]. The severest form of malaria is caused by the protozoan parasite *Plasmodium falciparum*. Chloroquine (CQ), once a spectacularly successful antimalarial drug, was first discovered by the German chemist Johann Andersag but was mistakenly thought to be too toxic for therapeutic purposes, an incident which became known as "the resochin error" (resochin being the name given to the compound by Andersag) [3,4]. CQ was so effective that it inspired optimism for the eradication of malaria. However, resistance soon arose, first appearing along the Thai-Cambodian border in the 1950s. By the 1970s, CQ resistance had spread throughout the world [5,6]. This resistance is generally attributed to mutations in the *pfcrt* (*P.*

falciparum chloroquine resistance transporter) gene, which codes for a transporter situated on the membrane of the parasite digestive vacuole (DV).

During parasite development in the intraerythrocytic cycle, haemoglobin is digested in the DV and the toxic heme moiety is released, which the parasite crystalizes into non-toxic hemozoin [7]. CQ is generally thought to kill the parasite by inhibiting the formation of hemozoin and thus preventing the detoxification of free heme [8–10]. In wild-type parasites CQ diffuses through the DV membrane and is diprotonated in the acidic environment of the DV, acquiring a net positive charge which prevents it from escaping the DV; however, mutant PfCRT found in CQ-resistant parasites effluxes this charged CQ out of the DV, removing it from its site of action [11]. Although the current first line artemisinin-combination therapies are effective in clearing parasitaemia, resistance against artemisinins has emerged [12–17]. There is therefore an urgent need to develop novel antimalarial strategies. Several research groups, including our own, have tried different approaches to tackle the problem of CQ resistance by either reversing CQ resistance with a PfCRT inhibitor or synthesizing "reversed" CQ analogues that cannot be effluxed by PfCRT [18–24]. The ultimate goal is to reintroduce CQ as a viable treatment for malaria. Both development of PfCRT inhibitors and synthesis of "reversed" CQ analogues require a sensitive assay for CQ uptake which is typically performed by the use of radiolabelled CQ [22,25–28]. Such methods are difficult to adopt in a high-throughput screen and may raise concerns of safety. To overcome this technical difficulty, fluorescent derivatives of chloroquine have recently been developed and used for this purpose; fluorophores used include 6-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino)hexanoic acid (NBD) [29], coumarin [21,30], and 4, 4-difluoro-4-bora-3a, 4a-diaza-*s*-indacene (BODIPY) [31].

BODIPY derivatives typically exhibit strong fluorescence and are relatively inert in biological conditions [32]. Furthermore, their maximum emission wavelengths are in the green-red region [32], allowing them to be used with many DNA dyes that fluoresce blue, such as the DAPI and Hoechst stains. These properties make BODIPY a promising candidate as a marker for CQ uptake in *P. falciparum*. We therefore present here the characterization of a commercially-available BODIPY-CQ conjugate, LynxTag-CQ_{GREEN}, in several laboratory strains and clinical isolates.

Methods

Parasite culture and synchronization

P. falciparum laboratory strains 3D7 (MRA-102), K1 (MRA-159), 7G8 (MRA-154), HB3 (MRA-155), CS2 (MRA-96), T9-94 (MRA-153), and Dd2 (MRA-156) were obtained from MR4, ATCC Manassas Virginia. Strain T9/96 was obtained from The European Malaria Reagent Repository. A further twelve clinical isolates were collected from the Mae Sot district, Tak Province, in northwest Thailand at the Shoklo Malaria Research Unit; these isolates are prefixed 'SMRU'. Parasites were continuously cultured in complete malaria culture media (MCM) consisting of RPMI 1640 (Life Technologies) supplemented with 0.5% (w/v) Albumax I (Invitrogen), 0.005% (w/v) hypoxanthine, 0.03% (w/v) L-glutamate, 0.25% (w/v) gentamycin, with human erythrocytes at 2.5% haematocrit. Cultures were gassed with a mixture of 3% CO_2, 4% O_2 and 93% N_2 and incubated at 37°C. Synchronization of parasite cultures was performed by resuspending erythrocytes in 5% (w/v) D-sorbitol and incubating at 37°C for 10 min, after which the erythrocytes were washed twice, resuspended in MCM and returned to culture conditions. Thin Giemsa smears were made before each experiment to determine parasitemia and parasite stage.

Compound preparation

For work involving parasites, chlorpheniramine maleate salt, chlorpromazine hydrochloride, desipramine hydrochloride, promethazine hydrochloride, verapamil hydrochloride and CQ diphosphate (all from Sigma-Aldrich) were dissolved in PBS to a working concentration of 1 mM. LynxTag-CQ_{GREEN} (BioLynx Technologies, Singapore; hereafter abbreviated to 'CQ_{GREEN}') was dissolved in DMSO to the same concentration. All compounds were stored at −20°C and protected from light. For microsome uptake assays, methiothepin mesylate salt, metergoline, loperamide hydrochloride, octoclothepin maleate salt, mibefradil dihydrochloride hydrate, L703,606 oxalate salt hydrate, and chlorprothixene hydrochloride (all from Sigma-Aldrich) were dissolved in DMSO to 10 mM and stored at 4°C. Verapamil hydrochloride, adenosine triphosphate (ATP), and CQ diphosphate (all from Sigma-Aldrich) were dissolved in water to 7.5 mM, 50 mM and 0.1 M respectively and stored at −20°C. Tritiated CQ (^{3}H-CQ; from Moravek Biochemicals and Radiochemicals) was diluted in water to 5.32 µM and stored at −20°C; specific activity was 4.7 Ci/mmol.

Reinvasion half-maximal inhibitory concentration (IC_{50})

Synchronized ring-stage cultures at 1–2% parasitemia, 1.25% haematocrit were incubated with either CQ or CQ_{GREEN} at a range of concentrations for 48 h in 96-well flat-bottomed plates at culture conditions. Following this, cells were stained with 1 µg/ml of Hoechst 33342 (Invitrogen) for 30 min at 37°C, washed twice and resuspended in PBS. Parasitemia was then assessed with the CyAn ADP flow cytometer (Beckman Coulter). IC_{50}s were determined by plotting the measurements in Graphpad Prism 5 using a variable slope logistic curve.

Confocal imaging

200 µl cultures of 3D7 at 3% parasitemia, 1.25% haematocrit were incubated with CQ_{GREEN} for 2 h at 2 µM in a 96-well plate format. Erythrocytes were then washed twice and stained with Hoechst 33342 as previously. Wet mounts of stained parasites were visualized under ×100 magnification with the Fluoview FV1000 confocal microscope (Olympus). Hoechst and CQ_{GREEN} were excited at 405 nm and 488 nm with emissions captured at 430–470 nm and 505–525 nm respectively.

Parasite CQ_{GREEN} uptake assay

Synchronized trophozoite-stage cultures at 3–5% parasitemia were resuspended in 200 µl of MCM with 2 µM of CQ_{GREEN} to 2.5% haematocrit in a 96-well plate format. The parasites were then incubated for 2 h at culture conditions, after which they were washed twice and resuspended in PBS. Cells were allowed to settle in a Nunc F96 MicroWell black non-treated polystyrene plate (Thermo Scientific) for 1 h. Fluorescence was then measured with the Infinite M200 microplate reader (Tecan) with excitation and emission wavelengths of 488 nm and 520 nm respectively. K1 chemoreversal assays were performed by pretreatment with 10 µM of the reversal agents for 30 min prior to the addition of CQ_{GREEN}.

Preparation of microsomes carrying PfCRT

PfCRT originating from *P. falciparum* strains Dd2 or 3D7 were expressed in *Pichia pastoris* KM71 and microsomes harvested as described previously [33]. Microsomal levels of PfCRT were

determined by western blot with standard curves generated from blots of purified PfCRT.

Uptake kinetics in microsomes

In order to assess the Michaelis-Menten kinetics of CQ_{GREEN} uptake by the microsomes, total or non-specific uptake was measured. Non-specific uptake was determined by pretreating microsomes with unlabelled CQ at 1000 times of the concentration of CQ_{GREEN} used, for 15 min at 37°C, before adding CQ_{GREEN}; total uptake was determined without the pretreatment. Reactions were carried out in accumulation buffer (0.25 M sucrose, 10 mM Tris–HCl, 5 mM $MgCl_2$, and 3 mM ATP, pH 7.5). Following this, microsomes were washed twice in accumulation buffer, then lysed in lysis buffer (0.75 M HCl, 1% Triton X-100, 77.5% isopropanol) on ice for at least one hour. Unlabelled CQ was excluded from the washing step as the addition of CQ was observed to cause displacement of CQ_{GREEN} from the microsomes, possibly due to the higher affinity of CQ for PfCRT. Measurement of the fluorescence intensity was performed using the FLUOstar Galaxy microplate reader (BMG Labtech) with excitation and emission wavelengths of 480 nm and 520 nm respectively. For each experiment, measurements were made in triplicate and the mean calculated. Specific uptake was then calculated by subtracting non-specific uptake from total uptake. Non-linear regression analysis with the Michaelis-Menten model (GraphPad Prism 5) was then applied to obtain the V_{max} and K_m of specific uptake.

CQ_{GREEN} uptake in PfCRT microsomes

Unless stated otherwise, microsomes were incubated with 15 μM CQ_{GREEN} at 37°C for 15 min. For uptake inhibition assays, microsomes were pre-incubated with chemoreversal compounds [21] for 15 min at 37°C prior to the addition of CQ_{GREEN}. All data presented are specific uptake based on the calculations stated above.

^{3}H-CQ uptake in PfCRT microsomes

^{3}H-CQ uptake was measured as described previously [33], with some modifications. Non-specific uptake was determined by pre-incubation with 200-fold unlabelled CQ. Incubation was performed with ^{3}H-CQ at 308 nM for 5 min, after which 200-fold unlabelled CQ was added to stop the reaction. Microsomes were then precipitated by the addition of polyethylene glycol (PEG) 8000 and washed twice with accumulation buffer containing 200-fold unlabelled CQ to remove excess ^{3}H-CQ. Microsomes were then resuspended in scintillation buffer and agitated overnight. Radioactivity was measured using the LS 5600 Scintillation Counter (Beckman). All data presented are specific uptake.

Genotyping of strains and isolates

To assess *pfmdr1* polymorphisms, parasite DNA from *in vitro* cultures was extracted with the QIAamp DNA Mini kit (Qiagen) as per the manufacturer's instructions. For *pfcrt* polymorphisms, total RNA was extracted with the RNeasy Mini Kit (Qiagen) and reverse transcription performed with SuperScript III (Invitrogen) as per manufacturers' instructions. Polymerase chain reaction (PCR) mixtures were made with 200 μM of each dNTP, 0.5 μM forward primer, 0.5 μM reverse primer, 0.02 U/μl Phusion DNA polymerase (Thermo Scientific), 6 μl of 5× Phusion HF buffer, and 1 μl of genomic DNA or cDNA to a total reaction volume of 30 μl. Thermocycler parameters were as follows: 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 1 min. Primers used for *pfmdr1* sequencing were 5′- ATGGGTAAAGAGCAGAAAGA and 5′- TCCACAATAACTTGCAACAGT, or 5′- GTCAAGCGGAGTTTTTGC and 5′- TATTCTCTGTTTTTGTCCAC. *Pfcrt*-specific primers were 5′- GACGAGCGTTATAGAGAAT and 5′- CTTCGGAATCTTCATTTTCT. PCR products were purified with the QIAquick PCR purification kit as per manufacturer's instructions. Purified PCR products were sequenced by a commercial vendor (AIT Biotech, Singapore). Copy number of *pfmdr1* was assessed by real-time PCR as previously reported [34]. Briefly, reaction mixtures were prepared with TaqMan universal PCR master mix (Applied Biosystems), 5.5 mM $MgCl_2$, 300 nM dNTPs, 300 nM each of forward and reverse primers, and 100 nM of the probe. Thermocycler parameters were 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Forward and reverse primers used were 5′- TGCATCTATAAAACGATCAGACAAA and 5′- TCGTGTGTTCCATGTGACTGT respectively, and TaqMan probe was 5′- 6FAM-TTTAATAACCCTGATCGAAATGGAACCTTTG-TAMRA. A reference gene, β-tubulin, was also included; primers were 5′- TGATGTGCGCAAGTGATCC and 5′- TCCTTTGTGGACATTCTTCCTC, while the probe was 5′- VIC-TAGCACATGCCGTTAAATATCTTCCATGTCT-TAMRA. The threshold cycle (C_t) was analysed by the comparative C_t method, based on DNA amplification efficiencies of the *pfmdr1* and β-tubulin genes. *Pfmdr1* copy number was calculated according to the following formula: $\Delta C_t = C_tR - C_tG$, where C_tR is the reference β-tubulin C_t, and C_tG is that of *pfmdr1*. Each TaqMan run included three reference DNA samples from clones 3D7, K1, and Dd2 having *pfmdr1* copy numbers of 1, 1, and 3 respectively.

Statistical analyses

All statistical analyses were performed with SPSS 21. Chemoreversal assays were assessed with Student's t test, 2-tailed. Multiple linear regression was performed with the stepwise method, using the log of IC_{50}s and with dummy coded values for the respective amino acid residues.

Ethics statement

The blood collection protocol for *in vitro* malaria culture was approved by the Institutional Review Board (NUS-IRB Reference Code: 11–383, Approval Number: NUS-1475) of the National University of Singapore (NUS). All participants provided written informed consent. The clinical isolates used were obtained under ethical guidelines in the approved protocol: OXTREC Reference Number 29–09 (Center for Clinical Vaccinology and Tropical

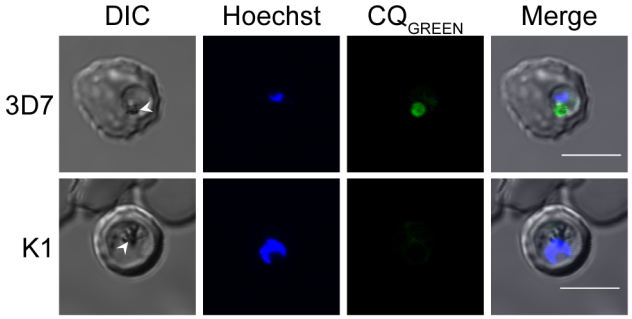

Figure 1. CQ_{GREEN} localization in *P. falciparum* 3D7. Parasites were stained with CQ_{GREEN} and Hoechst and visualized via confocal microscopy under a 100× objective. CQ_{GREEN} accumulates in the DV but also slightly stains parasite cytosol; erythrocyte cytosol is not stained. Arrowheads denote the DV. Scale bars represent 5 μm.
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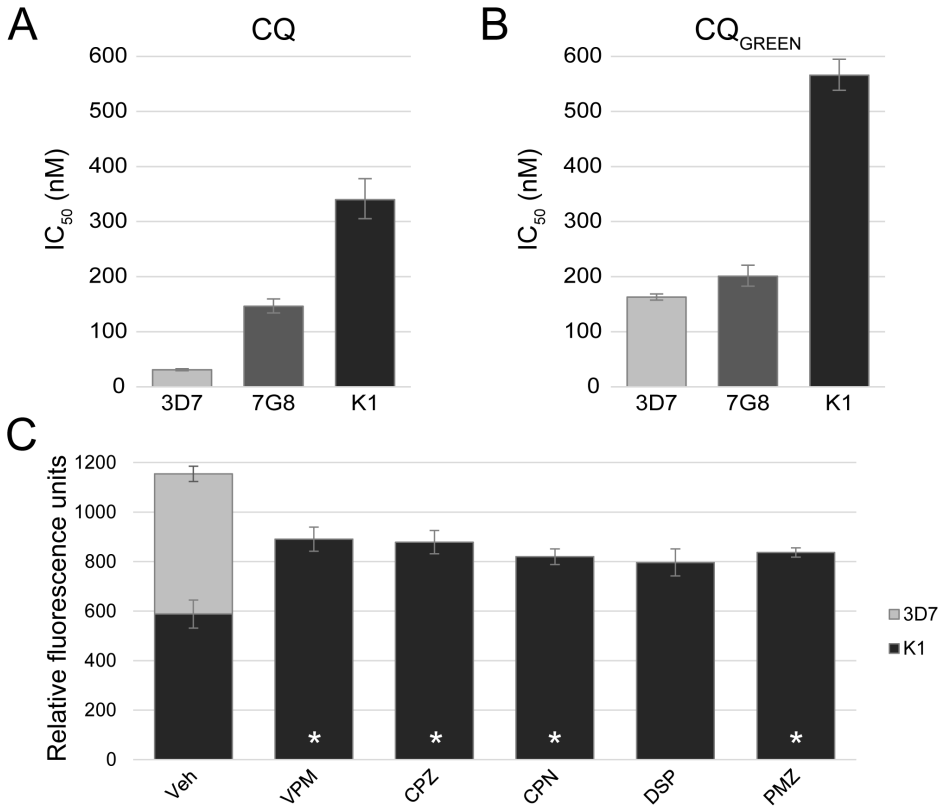

Figure 2. Validation of CQ_{GREEN} activity and uptake in laboratory strains. (A, B) CQ_{GREEN} IC_{50}s recapitulates CQ IC_{50}s in three *P. falciparum* laboratory strains, 3D7, 7G8 and K1. 3D7 is a CQ-susceptible strain, 7G8 is moderately resistant, while K1 is highly CQ-resistant. Data shown are geometric means from at least 3 experiments. As the error from IC_{50}s are not symmetrical, error bars indicate 95% confidence intervals instead of the standard error of the mean (S.E.M.). (C) CQ_{GREEN} uptake as measured by fluorescence is increased in CQ-resistant K1 when pretreated with chemoreversal agents. 3D7 exhibits highest uptake of CQ_{GREEN}. Data are means from at least 3 experiments; error bars are S.E.M. Veh: vehicle control; VPM: verapamil; CPZ: chlorpromazine; CPN: chlorpheniramine; DSP: desipramine; PMZ: promethazine. *: $p<0.05$.
doi:10.1371/journal.pone.0110800.g002

Medicine, University of Oxford, Oxford, United Kingdom). Use of field isolates in NUS was in accordance with NUS IRB (Reference Code: 12–369E).

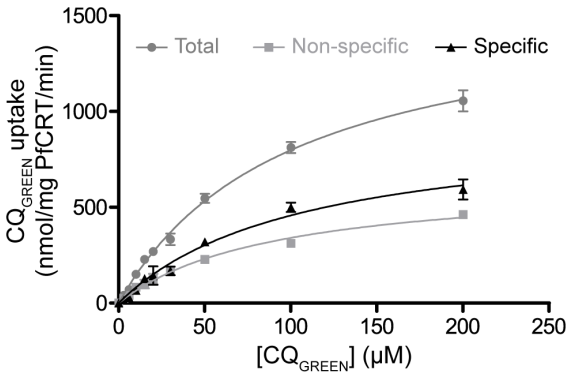

Figure 3. CQ_{GREEN} uptake by Dd2 PfCRT microsomes. Total uptake of CQ_{GREEN} was measured at various CQ_{GREEN} concentrations in microsomes carrying Dd2 PfCRT. Non-specific uptake of CQ_{GREEN} was measured with pre-treatment of excess unlabelled CQ. Specific uptake was estimated as the difference between total and non-specific uptake. V_{max} and K_m of the specific uptake was 938.5 nmol/mg PfCRT/min and 105.1 μM respectively. Data are means ± S.E.M.; n≥3.
doi:10.1371/journal.pone.0110800.g003

Results and Discussion

Validation of CQ_{GREEN} localization and antimalarial activity

CQ is generally believed to accumulate in the DV as a result of ion-trapping [35,36]. Confocal microscopy was therefore performed to ascertain the localization of CQ_{GREEN} in the parasite. Cultures of *P. falciparum* 3D7 were co-stained with Hoechst dye and CQ_{GREEN}, revealing a preferential accumulation of CQ_{GREEN} in the parasite DV (Fig. 1). Interestingly, CQ_{GREEN} fluorescence was also observed in the parasite cytosol but not in the erythrocyte cytosol. As CQ_{GREEN} is a CQ analog, the antimalarial potency of CQ_{GREEN} should be similar to that of CQ. To assess this, reinvasion IC_{50}s of CQ and CQ_{GREEN} on the laboratory strains 3D7, 7G8 and K1 were determined. CQ_{GREEN} showed the same general trend of antimalarial activity as CQ, in that it is most potent against 3D7, followed by 7G8, then K1 (Fig. 2A, 2B).

CQ_{GREEN} fluorescence as a proxy for CQ uptake in parasites

Next, we determined if CQ_{GREEN} uptake by the highly CQ-resistant strain K1 can be increased by pre-treatment with chemosensitizers. Verapamil, chlorpromazine, chlorpheniramine, desipramine, and promethazine have previously been reported to reverse CQ resistance and increase CQ uptake in CQ-resistant

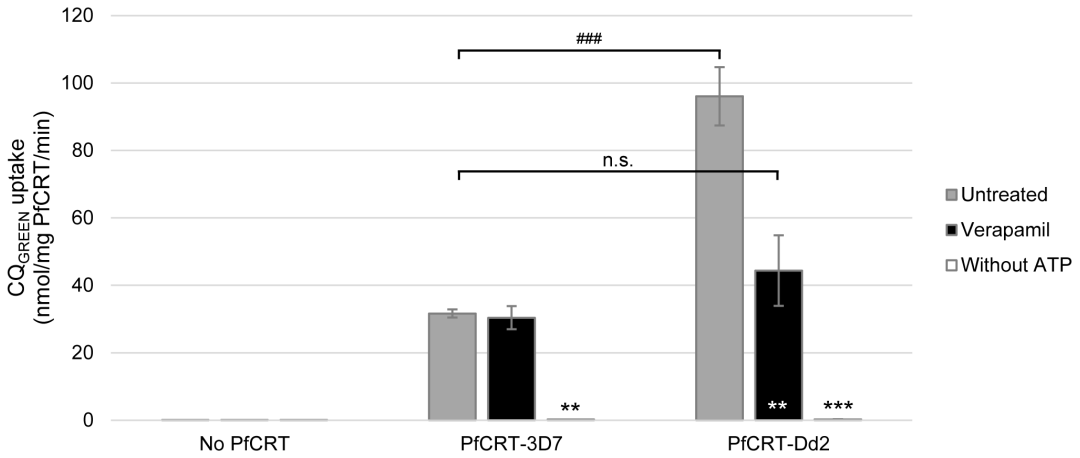

Figure 4. ATP-dependent, verapamil-sensitive uptake of CQ_{GREEN} in microsomes. Yeast microsomes expressing CQ-sensitive or -resistant PfCRT ("PfCRT-3D7" and "PfCRT-Dd2" respectively), or microsomes from plasmid vector control ("No PfCRT"), were incubated with CQ_{GREEN} under different conditions. Preincubation with 150 μM verapamil abrogated CQ_{GREEN} uptake from PfCRT-Dd2 but did not affect uptake in PfCRT-3D7 microsomes. Removal of ATP from buffer abolished CQ_{GREEN} uptake entirely. **, ***: $p<0.005$ and $p<0.001$ respectively, against untreated control. ###: $p<0.001$. N.s.: not significant. Data presented are means ± S.E.M.; $n\geq3$.
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strains [37–41]. CQ-sensitive 3D7 was included as a reference for complete reversal. All reversal agents except desipramine induced a significant increase in CQ_{GREEN} fluorescence (Fig. 2C). Desipramine is in fact a less potent reversal agent compared to verapamil when tested in the resistant strain Dd2, and two CQ-resistant field isolates [42]; this may explain why desipramine's effect on CQ_{GREEN} uptake did not achieve statistical significance. Taken together with the CQ_{GREEN} IC_{50} data, we believe that the reversibility of CQ_{GREEN} uptake by known chemoreversal agents suggests that CQ_{GREEN} shares similar structural properties with CQ.

Uptake of CQ_{GREEN} in microsomes bearing PfCRT

To test if CQ_{GREEN} can be transported by PfCRT, we have expressed PfCRT in *Pichia pastoris* and used the microsomes derived to study CQ_{GREEN} uptake. Figure 3 shows that CQ_{GREEN} uptake in Dd2 PfCRT-expressing microsomes is specific. At the highest concentration of CQ_{GREEN} used (200 μM), uptake was close to saturated. Michaelis-Menten approximation of CQ_{GREEN} uptake kinetics in Dd2 microsomes (Figure 3) yields a V_{max} and K_m of 938.5 nmol/mg PfCRT/min and 105.1 μM respectively, which are approximately 2000 and 500 times higher compared to when ^{3}H-CQ was used [33]. Conjugation of CQ with the BODIPY fluorophore may have altered the affinity of PfCRT for the molecule. However, both the high (micromolar) K_m and non-saturation of CQ_{GREEN} transport are consistent with a previous report in a *Xenopus* oocyte system using ^{3}H-CQ [43]. We have also compared CQ_{GREEN} uptake in microsomes with PfCRT originating from either CQ-sensitive 3D7 or CQ-resistant Dd2. CQ_{GREEN} uptake in Dd2 PfCRT-expressing microsome was 96.07 nmol/mg PfCRT/min, which was about three times that of 3D7 PfCRT (31.64 nmol/mg PfCRT/min) (Figure 4). PfCRT-

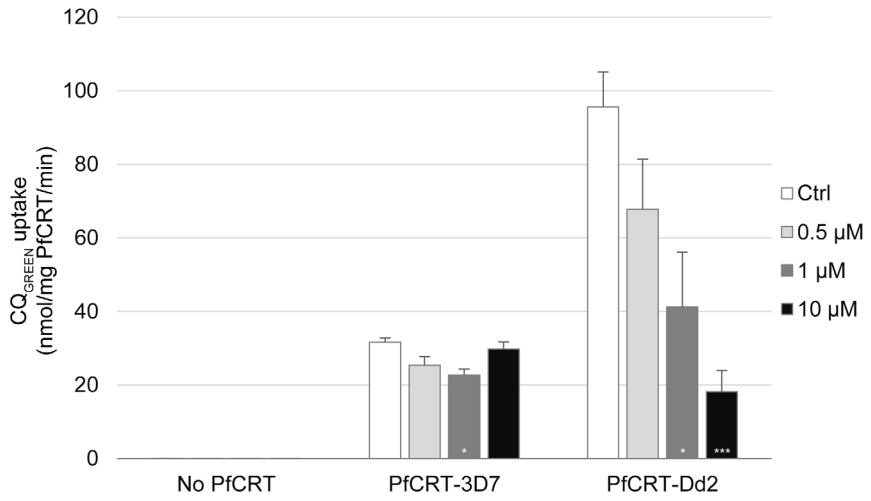

Figure 5. CQ_{GREEN} uptake by resistant-type PfCRT is inhibited by mibefradil in a dose-dependent manner. Microsomes were preincubated with varying concentrations of the PfCRT inhibitor mibefradil prior to addition of CQ_{GREEN}. At the highest concentration of 10 μM, mibefradil drastically suppressed CQ_{GREEN} uptake in PfCRT-Dd2 microsomes but had no significant effect on uptake in PfCRT-3D7 microsomes. *, ***: $p<0.05$ and $p<0.001$ respectively, against no mibefradil control (Ctrl). Data presented are means ± S.E.M.; $n\geq3$.
doi:10.1371/journal.pone.0110800.g005

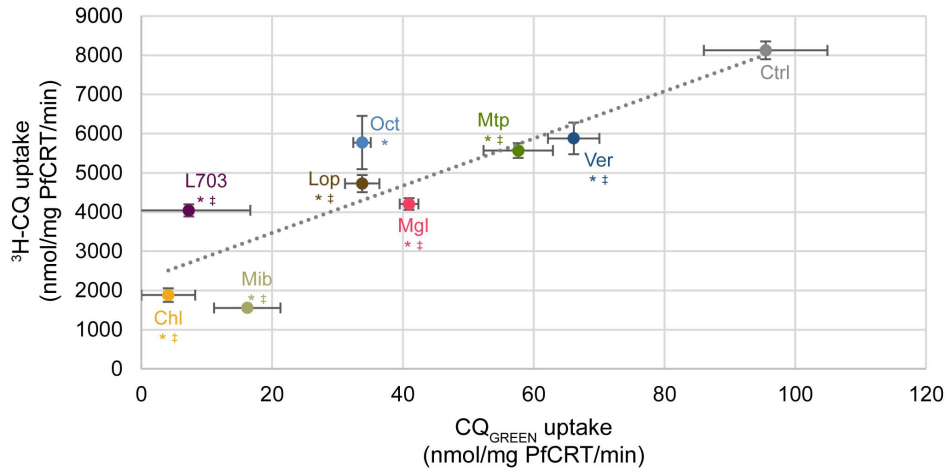

Figure 6. Accumulation of CQ_{GREEN} and ^{3}H-CQ in PfCRT-Dd2 microsomes. Microsomes were incubated with 10 μM of chemosensitizers before addition of CQ_{GREEN} or ^{3}H-CQ. Ctrl: negative control; Ver: verapamil; Mtp: methiothepin; Mgl: metergoline; Lop: loperamide; Oct: octoclothepin; Mib: mibefradil; L703: L703,606; Chl: chlorprothixene. *: $p<0.05$, comparing CQ_{GREEN} uptake against control. ‡: $p<0.05$, comparing ^{3}H-CQ uptake against control. Data presented are means ± S.E.M.; n≥3.
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mediated transport of CQ is thought to be ATP-dependent [25,44]. Here we demonstrated that removal of ATP completely abolished CQ_{GREEN} uptake in both Dd2 and 3D7 PfCRT microsomes (Figure 4). Verapamil, a known CQ resistance chemosensitizer which has no effect on CQ-sensitive strains, can reverse CQ_{GREEN} uptake in Dd2 PfCRT to almost that of 3D7 level but has almost no effect on 3D7 PfCRT (Figure 4). These results suggest that CQ_{GREEN} is similar to CQ in that (1) it is differentially recognized by CQ-resistant versus CQ-sensitive PfCRT, (2) its uptake by PfCRT is ATP-dependent, and (3) its uptake by CQ-resistant PfCRT is verapamil-reversible. Others have shown that extensive CQ side-chain modifications can render the CQ analogues not transportable by PfCRT and abolish their verapamil sensitivity [45,46]. Our findings show that the additional BODIPY moiety in CQ_{GREEN} still allows CQ_{GREEN} to be differentially recognized by resistant versus sensitive PfCRT and these transport activities are still sensitive to verapamil. To demonstrate the usefulness of CQ_{GREEN} in screening for PfCRT inhibitors, we found that CQ_{GREEN} uptake by Dd2 PfCRT microsomes can be inhibited by mibefradil, a novel potent chemosensitizer [21], in a dose-dependent manner (Figure 5). Inhibition of CQ_{GREEN} uptake by a panel of known chemoreversal agents was compared to that using ^{3}H-CQ (Figure 6). Mean uptake of CQ_{GREEN} was positively correlated with that of ^{3}H-CQ (β of 60.24, $p = 0.002$), showing moderately good agreement between CQ_{GREEN} and ^{3}H-CQ uptake ($R^2 = 0.766$). However, ^{3}H-CQ uptake was roughly 100 times greater than that of CQ_{GREEN}.

Polymorphisms and copy number variation in *pfcrt* and *pfmdr1*

Eight laboratory strains and twelve clinical isolates were sequenced for polymorphisms in PfCRT and Pgh1, the proteins encoded by the genes *pfcrt* and *pfmdr1* respectively. Residues examined were 72, 74, 75, 76, 220, 271, 326, 356, and 371 for PfCRT, and 86, 184, 1034, 1042, 1226, and 1246 for Pgh1. These residues were chosen for analysis as they were previously implicated in modulating multidrug resistance as well as resistance against CQ [47,48]. Copy number of *pfmdr1* was also determined for each strain and isolate. All clinical isolates showed Dd2-type PfCRT mutations, whereas Pgh1 mutations and *pfmdr1* copy numbers were more varied (Table 1).

Genetic correlates of CQ_{GREEN} uptake

All strains and isolates were assayed for CQ_{GREEN} uptake in the trophozoite stage, and their CQ IC_{50}s determined with the standard reinvasion assay. For the entire data set, CQ_{GREEN} uptake was inversely correlated to the log of CQ IC_{50}, with an R^2 of 0.53 (Fig. 7A). However, multiple linear regression with sequencing and copy number data revealed that CQ_{GREEN} uptake was significantly correlated with not only CQ logIC_{50} but also a F1226Y substitution in Pgh1 (β of -587.32 and 178.70, $p<0.001$ and $p = 0.024$ respectively; adjusted R^2 of 0.615). None of the other mutations was significantly correlated with CQ_{GREEN} uptake. Separating the data set to two subpopulations improved the R^2, to 0.72 in the Pgh1 1226F group and 0.676 in the Pgh1 1226Y group (Fig. 7B). It is tempting to conclude that the Pgh1 F1226Y substitution plays a significant causal role in modulating CQ_{GREEN} uptake, given that it is also correlated with resistance to artemisinin, mefloquine and lumefantrine [48] and Pgh1's putative sequestration of cytosol-active drugs in the DV [49]. However, it must be kept in mind that the F1226Y mutation was only detected in the clinical isolates, and given the localized collection of these isolates within a small geographical region, F1226Y is likely to be strongly correlated with other undiscovered mutations. In fact, the PfCRT and Pgh1 haplotypes of the F1226Y mutants examined were identical, apart from SMRU0501 which had an additional Pgh1 Y184F substitution (Table 1). It is notable that for the F1226Y mutants, CQ_{GREEN} uptake could range as high as that of the CQ-susceptible strains (Fig. 7B) while still maintaining CQ resistance. One possible gene modulating CQ_{GREEN} uptake could be *pfmrp*, which has been proposed to efflux drugs across the parasite plasma membrane into the parasitophorous vacuolar lumen [49], which would still contribute to CQ_{GREEN} fluorescence but sequester the drug from its site of action. Knock-out mutants of this gene exhibit increased susceptibility to CQ, quinine, artemisinin, piperaquine, and primaquine [50]. Alternative mechanisms besides efflux of CQ could perhaps also contribute to CQ resistance, such as decreased susceptibility to CQ-induced apoptosis-like cell death [51].

Table 1. CQ IC_{50}s, PfCRT and Pgh1 polymorphisms, and *pfmdr1* copy number.

		PfCRT residue no.									Pgh1 residue no.						*pfmdr1* copy number
Laboratory strains	**CQ IC_{50} (nM)**	**72**	**74**	**75**	**76**	**220**	**271**	**326**	**356**	**371**	**86**	**184**	**1034**	**1042**	**1226**	**1246**	
T9/96	24	C	M	N	K	A	Q	N	I	R	N	Y	S	N	F	D	1
3D7	31	C	M	N	K	A	Q	N	I	R	N	Y	S	N	F	D	1
HB3	42	C	M	N	K	A	Q	N	I	R	N	**F**	S	**D**	F	D	1
CS2	115	C	**I**	**E**	**T**	**S**	**E**	**S**	I	**I**	**Y**	Y	S	N	F	D	3
T9-94	146	C	**I**	**E**	**T**	**S**	**E**	**S**	I	**I**	**Y**	Y	S	N	F	D	3
7G8	146	**S**	M	N	**T**	**S**	Q	**D**	**L**	R	N	**F**	**C**	**D**	F	**Y**	1
Dd2	276	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	**Y**	Y	S	N	F	D	3
K1	340	C	**I**	**E**	**T**	**S**	**E**	**S**	I	**I**	**Y**	Y	S	N	F	D	1
Clinical isolates	**CQ IC_{50} (nM)**	**72**	**74**	**75**	**76**	**220**	**271**	**326**	**356**	**371**	**86**	**184**	**1034**	**1042**	**1226**	**1246**	***pfmdr1* copy number**
SMRU0233	142	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	**Y**	D	2
SMRU0116	147	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	**Y**	D	2
SMRU0101	156	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	F	D	2
SMRU1116	172	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	F	D	1
SMRU0270	178	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	F	D	2
SMRU0402	196	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	**Y**	D	2
SMRU1093	231	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	**Y**	D	2
SMRU0201	249	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	F	D	1
SMRU0501	287	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	**F**	S	N	**Y**	D	1
SMRU0272	332	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	**Y**	D	3
SMRU0002	377	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	**Y**	D	1
SMRU0279	473	C	**I**	**E**	**T**	**S**	**E**	**S**	**T**	**I**	N	Y	S	N	**Y**	D	1

Bolded residues indicate deviation from 3D7 haplotype. CQ IC_{50}s are geometric means of at least 3 measurements.

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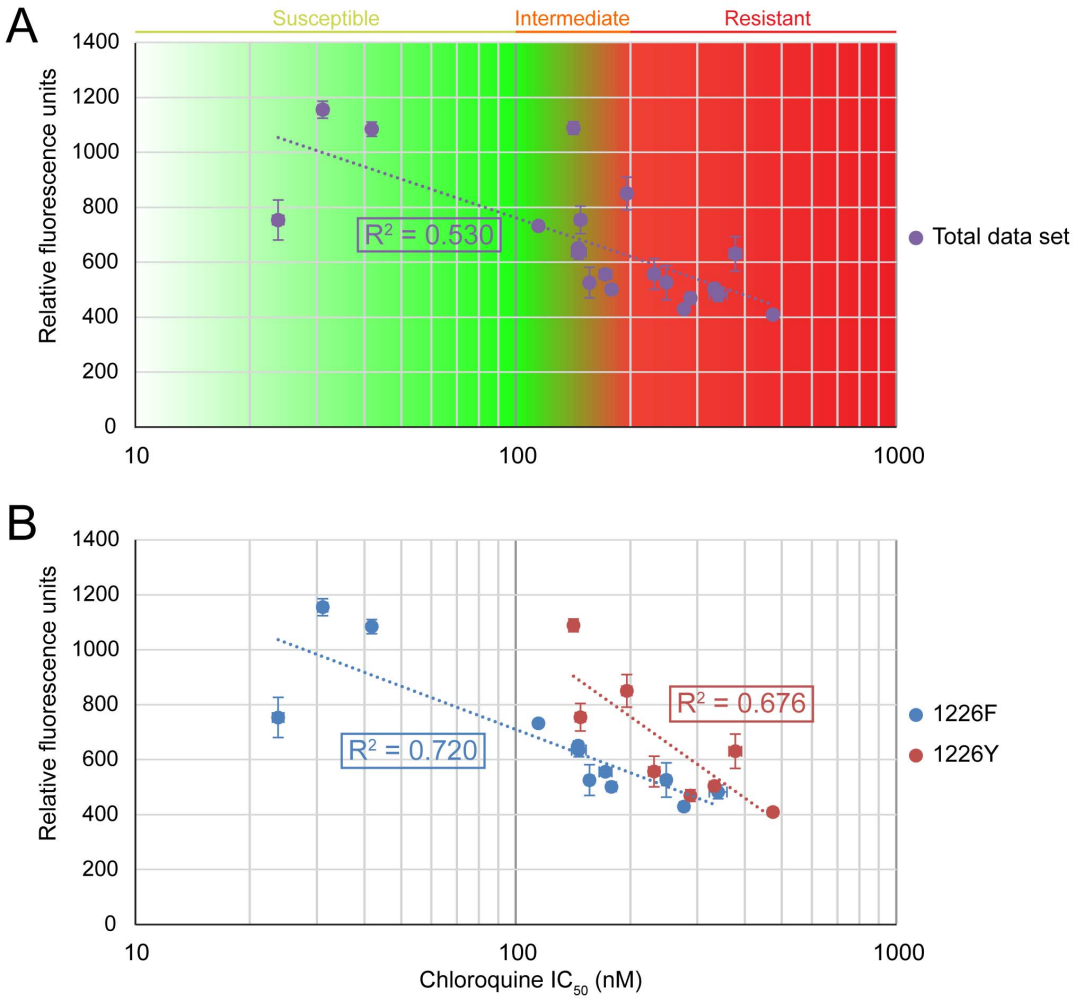

Figure 7. CQ_{GREEN} uptake correlates with CQ resistance. (A) CQ_{GREEN} fluorescence is inversely correlated with CQ $logIC_{50}$. Total data set shows moderate R^2 of 0.53. (B) When split into subpopulations on the basis of Pgh1 residue 1226, R^2 is improved. Data shown are means of at least 3 experiments. Error bars represent S.E.M.
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Conclusions

CQ_{GREEN} is a commercially available fluorescent CQ analog that interacts with the parasite in a similar fashion to CQ. CQ_{GREEN} presents some advantages over traditional radiolabelled CQ in uptake studies: it is safer to handle as it is not radioactive, and its fluorescence properties allows it to be monitored by common fluorescence equipment. Using a defined microsomal platform, we showed that CQ_{GREEN} interacts with PfCRT in a manner similar to CQ. Its use as a predictor of CQ susceptibility is enhanced if residue 1226 of Pgh1 is known. Unlike a typical reinvasion assay which may require 48 h or more, the use of CQ_{GREEN} allows for measurement of CQ susceptibility within several hours. We believe that CQ_{GREEN} could be a valuable tool in future drug discovery projects or used in the identification of factors involved in drug resistance.

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Author Contributions

Conceived and designed the experiments: KSWT BR FHN LR MJL LMCC. Performed the experiments: YQL CCYL KWKC KYC RS. Analyzed the data: YQL CCYL RS. Contributed reagents/materials/analysis tools: KSWT LR FHN. Wrote the paper: YQL KSWT BR FHN LR MJL LMCC CCYL KWKC KYC RS.

References

1. Murray CJ, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, et al. (2012) Global malaria mortality between 1980 and 2010: a systematic analysis. The Lancet 379: 413–431. doi:10.1016/S0140-6736(12)60034-8.

2. World Health Organization (2012) World Malaria Report 2012. Available: http://www.who.int/malaria/publications/world_malaria_report_2012/en/index.html. Accessed 2013 Mar 11.

3. Krafts K, Hempelmann E, Skórska-Stania A (2012) From methylene blue to chloroquine: a brief review of the development of an antimalarial therapy. Parasitol Res 111: 1–6. doi:10.1007/s00436-012-2886-x.
4. Coatney GR (1963) Pitfalls in a Discovery: The Chronicle of Chloroquine. Am J Trop Med Hyg 12: 121–128.
5. Butler A, Khan S, Ferguson E (2010) A brief history of malaria chemotherapy. J R Coll Physicians Edinb 40: 172–177. doi:10.4997/JRCPE.2010.216.
6. Wellems TE, Plowe CV (2001) Chloroquine-Resistant Malaria. J Infect Dis 184: 770–776. doi:10.1086/322858.
7. Sullivan DJ Jr, Gluzman IY, Goldberg DE (1996) Plasmodium hemozoin formation mediated by histidine-rich proteins. Science 271: 219–222.
8. Hempelmann E (2007) Hemozoin Biocrystallization in *Plasmodium falciparum* and the antimalarial activity of crystallization inhibitors. Parasitol Res 100: 671–676. doi:10.1007/s00436-006-0313-x.
9. Gildenhuys J, Roex T le, Egan TJ, de Villiers KA (2013) The Single Crystal X-ray Structure of β-Hematin DMSO Solvate Grown in the Presence of Chloroquine, a β-Hematin Growth-Rate Inhibitor. J Am Chem Soc 135: 1037–1047. doi:10.1021/ja308741e.
10. Rathore D, Jani D, Nagarkatti R, Kumar S (2006) Heme detoxification and antimalarial drugs – Known mechanisms and future prospects. Drug Discov Today Ther Strateg 3: 153–158. doi:10.1016/j.ddstr.2006.06.003.
11. Summers RL, Martin RE (2010) Functional characteristics of the malaria parasite's "chloroquine resistance transporter": implications for chemotherapy. Virulence 1: 304–308. doi:10.4161/viru.1.4.12012.
12. Pradines B, Bertaux L, Pomares C, Delaunay P, Marty P (2011) Reduced *in vitro* susceptibility to artemisinin derivatives associated with multi-resistance in a traveller returning from South-East Asia. Malar J 10: 268. doi:10.1186/1475-2875-10-268.
13. Miotto O, Almagro-Garcia J, Manske M, MacInnis B, Campino S, et al. (2013) Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. Nat Genet 45: 648–655. doi:10.1038/ng.2624.
14. Takala-Harrison S, Clark TG, Jacob CG, Cummings MP, Miotto O, et al. (2013) Genetic loci associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment in Southeast Asia. Proc Natl Acad Sci 110: 240–245. doi:10.1073/pnas.1211205110.
15. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois A-C, et al. (2014) A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature 505: 50–55. doi:10.1038/nature12876.
16. Mok S, Imwong M, Mackinnon MJ, Sim J, Ramadoss R, et al. (2011) Artemisinin resistance in *Plasmodium falciparum* is associated with an altered temporal pattern of transcription. BMC Genomics 12: 391. doi:10.1186/1471-2164-12-391.
17. Witkowski B, Amaratunga C, Khim N, Sreng S, Chim P, et al. (2013) Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: *in-vitro* and *ex-vivo* drug-response studies. Lancet Infect Dis. doi:10.1016/S1473-3099(13)70252-4.
18. Peyton DH (2012) Reversed chloroquine molecules as a strategy to overcome resistance in malaria. Curr Top Med Chem 12: 400–407.
19. Martin SK, Oduola AMJ, Milhous WK (1987) Reversal of Chloroquine Resistance in *Plasmodium falciparum* by Verapamil. Science 235: 899–901.
20. Burgess SJ, Selzer A, Kelly JX, Smilkstein MJ, Riscoe MK, et al. (2006) A Chloroquine-like Molecule Designed to Reverse Resistance in *Plasmodium falciparum*. J Med Chem 49: 5623–5625. doi:10.1021/jm060399n.
21. Ch'ng J-H, Mok S, Bozdech Z, Lear MJ, Boudhar A, et al. (2013) A Whole Cell Pathway Screen Reveals Seven Novel Chemosensitizers to Combat Chloroquine Resistant Malaria. Sci Rep 3. doi:10.1038/srep01734.
22. Martin RE, Butterworth AS, Gardiner DL, Kirk K, McCarthy JS, et al. (2012) Saquinavir Inhibits the Malaria Parasite's Chloroquine Resistance Transporter. Antimicrob Agents Chemother 56: 2283–2289. doi:10.1128/AAC.00166-12.
23. Adovelande J, Delèze J, Schrével J (1998) Synergy between two calcium channel blockers, verapamil and fantofarone (SR33557), in reversing chloroquine resistance in *Plasmodium falciparum*. Biochem Pharmacol 55: 433–440. doi:10.1016/S0006-2952(97)00482-6.
24. Kelly JX, Smilkstein MJ, Cooper RA, Lane KD, Johnson RA, et al. (2007) Design, Synthesis, and Evaluation of 10-N-Substituted Acridones as Novel Chemosensitizers in *Plasmodium falciparum*. Antimicrob Agents Chemother 51: 4133–4140. doi:10.1128/AAC.00669-07.
25. Sanchez CP, Stein W, Lanzer M (2003) Trans Stimulation Provides Evidence for a Drug Efflux Carrier as the Mechanism of Chloroquine Resistance in *Plasmodium falciparum*. Biochemistry (Mosc) 42: 9383–9394. doi:10.1021/bi034269h.
26. Sanchez CP, McLean JE, Stein W, Lanzer M (2004) Evidence for a Substrate Specific and Inhibitable Drug Efflux System in Chloroquine Resistant *Plasmodium falciparum* Strains. Biochemistry (Mosc) 43: 16365–16373. doi:10.1021/bi048241x.
27. Martin RE, Marchetti RV, Cowan AI, Howitt SM, Broer S, et al. (2009) Chloroquine Transport via the Malaria Parasite's Chloroquine Resistance Transporter. Science 325: 1680–1682. doi:10.1126/science.1175667.
28. Zishiri VK, Hunter R, Smith PJ, Taylor D, Summers R, et al. (2011) A series of structurally simple chloroquine chemosensitizing dibemethin derivatives that inhibit chloroquine transport by PfCRT. Eur J Med Chem 46: 1729–1742. doi:10.1016/j.ejmech.2011.02.026.
29. Cabrera M, Natarajan J, Paguio MF, Wolf C, Urbach JS, et al. (2009) Chloroquine Transport in *Plasmodium falciparum*. 1. Influx and Efflux Kinetics for Live Trophozoite Parasites Using a Novel Fluorescent Chloroquine Probe. Biochemistry (Mosc) 48: 9471–9481. doi:10.1021/bi901034r.
30. Ch'ng J-H, Kotturi SR, Chong AG-L, Lear MJ, Tan KS-W (2010) A programmed cell death pathway in the malaria parasite *Plasmodium falciparum* has general features of mammalian apoptosis but is mediated by clan CA cysteine proteases. Cell Death Dis 1: e26. doi:10.1038/cddis.2010.2.
31. Alcantara LM, Kim J, Moraes CB, Franco CH, Franzoi KD, et al. (2013) Chemosensitization potential of P-glycoprotein inhibitors in malaria parasites. Exp Parasitol 134: 235–243. doi:10.1016/j.exppara.2013.03.022.
32. Tram K, Yan H, Jenkins HA, Vassiliev S, Bruce D (2009) The synthesis and crystal structure of unsubstituted 4, 4-difluoro-4-bora-3a, 4a-diaza-s-indacene (BODIPY). Dyes Pigments 82: 392–395. doi:10.1016/j.dyepig.2009.03.001.
33. Tan W, Gou DM, Tai E, Zhao YZ, Chow LMC (2006) Functional reconstitution of purified chloroquine resistance membrane transporter expressed in yeast. Arch Biochem Biophys 452: 119–128. doi:10.1016/j.abb.2006.06.017.
34. Price RN, Uhlemann A-C, Brockman A, McGready R, Ashley E, et al. (2004) Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. The Lancet 364: 438–447. doi:10.1016/S0140-6736(04)16767-6.
35. Homewood CA, Warhurst DC, Peters W, Baggaley VC (1972) Lysosomes, pH and the anti-malarial action of chloroquine. Nature 235: 50–52.
36. Yayon A, Cabantchik ZI, Ginsburg H (1984) Identification of the acidic compartment of *Plasmodium falciparum*-infected human erythrocytes as the target of the antimalarial drug chloroquine. EMBO J 3: 2695–2700.
37. Van Schalkwyk DA, Walden JC, Smith PJ (2001) Reversal of Chloroquine Resistance in *Plasmodium falciparum* Using Combinations of Chemosensitizers. Antimicrob Agents Chemother 45: 3171–3174. doi:10.1128/AAC.45.11.3171–3174.2001.
38. Kyle DE, Oduola AMJ, Martin SK, Milhous WK (1990) *Plasmodium falciparum*: modulation by calcium antagonists of resistance to chloroquine, desethylchloroquine, quinine, and quinidine *in vitro*. Trans R Soc Trop Med Hyg 84: 474–478. doi:10.1016/0035-9203(90)90004-X.
39. Martiney JA, Cerami A, Slater AFG (1995) Verapamil Reversal of Chloroquine Resistance in the Malaria Parasite *Plasmodium falciparum* Is Specific for Resistant Parasites and Independent of the Weak Base Effect. J Biol Chem 270: 22393–22398. doi:10.1074/jbc.270.38.22393.
40. Oduola AM, Sowunmi A, Milhous WK, Brewer TG, Kyle DE, et al. (1998) *In vitro* and *in vivo* reversal of chloroquine resistance in *Plasmodium falciparum* with promethazine. Am J Trop Med Hyg 58: 625–629.
41. Egan T, Kaschula C (2007) Strategies to reverse drug resistance in malaria. Curr Opin Infect Dis 20: 598–604. doi:10.1097/QCO.0b013e3282f1673a.
42. Bayoumi RAL, Babiker HA, Arnot DE (1994) Uptake and efflux of chloroquine by chloroquine-resistant *Plasmodium falciparum* clones recently isolated in Africa. Acta Trop 58: 141–149. doi:10.1016/0001-706X(94)90053-1.
43. Summers RL, Dave A, Dolstra TJ, Bellanca S, Marchetti RV, et al. (2014) Diverse mutational pathways converge on saturable chloroquine transport via the malaria parasite's chloroquine resistance transporter. Proc Natl Acad Sci 111: E1759–E1767. doi:10.1073/pnas.1322965111.
44. Krogstad DJ, Gluzman IY, Herwaldt BL, Schlesinger PH, Wellems TE (1992) Energy dependence of chloroquine accumulation and chloroquine efflux in Plasmodium falciparum. Biochem Pharmacol 43: 57–62. doi:10.1016/0006-2952(92)90661-2.
45. De D, Krogstad FM, Cogswell FB, Krogstad DJ (1996) Aminoquinolines That Circumvent Resistance in *Plasmodium falciparum in Vitro*. Am J Trop Med Hyg 55: 579–583.
46. Lakshmanan V, Bray PG, Verdier-Pinard D, Johnson DJ, Horrocks P, et al. (2005) A critical role for PfCRT K76T in Plasmodium falciparum verapamil-reversible chloroquine resistance. EMBO J 24: 2294–2305. doi:10.1038/sj.emboj.7600681.
47. Valderramos SG, Valderramos J-C, Musset L, Purcell LA, Mercereau-Puijalon O, et al. (2010) Identification of a Mutant PfCRT-Mediated Chloroquine Tolerance Phenotype in *Plasmodium falciparum*. PLoS Pathog 6: e1000887. doi:10.1371/journal.ppat.1000887.
48. Veiga MI, Ferreira PE, Jörnhagen L, Malmberg M, Kone A, et al. (2011) Novel Polymorphisms in *Plasmodium falciparum* ABC Transporter Genes Are Associated with Major ACT Antimalarial Drug Resistance. PLoS ONE 6: e20212. doi:10.1371/journal.pone.0020212.
49. Sanchez CP, Dave A, Stein WD, Lanzer M (2010) Transporters as mediators of drug resistance in *Plasmodium falciparum*. Int J Parasitol 40: 1109–1118. doi:10.1016/j.ijpara.2010.04.001.
50. Raj DK, Mu J, Jiang H, Kabat J, Singh S, et al. (2009) Disruption of a *Plasmodium falciparum* Multidrug Resistance-associated Protein (PfMRP) Alters Its Fitness and Transport of Antimalarial Drugs and Glutathione. J Biol Chem 284: 7687–7696. doi:10.1074/jbc.M806944200.
51. Ch'ng J-H, Liew K, Goh AS-P, Sidhartha E, Tan KS-W (2011) Drug-induced permeabilization of parasite's digestive vacuole is a key trigger of programmed cell death in *Plasmodium falciparum*. Cell Death Dis 2: e216. doi:10.1038/cddis.2011.97.