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

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\section*{Abstract}
Chloroquine was a cheap, extremely effective drug against \textit{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\textsubscript{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\textsubscript{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\textsubscript{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 \textit{pfmdr1} copy number and chloroquine IC\textsubscript{50} were determined. These data were compared with LynxTag-CQ\textsubscript{GREEN} uptake/fluorescence by multiple linear regression to identify genetic correlates of uptake. Uptake of the compound correlated with the logIC\textsubscript{50} of chloroquine and, more weakly, a mutation in Pgh1, F1226Y.

\section*{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 \textit{Plasmodium falciparum}. Chloroquine (CQ), once a spectacularly successful antimalarial drug, was first discovered by the German chemist Johann Andreas Gabriel Graebe in 1848 during his work on quinoline derivatives. It was rediscovered by Richard Atkinson in 1934 and then synthesized by the German chemists Erich Laschet and Ludwig Hagemann in 1935. Chloroquine was a cheap, extremely effective drug against \textit{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\textsubscript{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\textsubscript{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\textsubscript{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 \textit{pfmdr1} copy number and chloroquine IC\textsubscript{50} were determined. These data were compared with LynxTag-CQ\textsubscript{GREEN} uptake/fluorescence by multiple linear regression to identify genetic correlates of uptake. Uptake of the compound correlated with the logIC\textsubscript{50} of chloroquine and, more weakly, a mutation in Pgh1, F1226Y.
*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 crystallizes 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, avoiding it from escaping the DV; however, mutant PICRT 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 PICRT inhibitor or synthesizing “reversed” CQ analogues that cannot be effluxed by PICRT [18–24]. The ultimate goal is to reintroduce CQ as a viable treatment for malaria. Both development of PICRT 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-CQGREEN, in several laboratory strains and clinical isolates.

**Methods**

**Parasite culture and synchronization**

*P. falciparum* laboratory strains 3D7 (MRA-102), K1 (MRA-159), 7G8 (MRA-134), HB3 (MRA-155), CS2 (MRA-96), T9-94 (MRA-153), and Dd2 (MRA-156) were obtained from MRA, 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 northwestern 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 1% (Invitrogen), 0.005% (w/v) hypoxanthine, 0.03% (w/v) L-glutamate, 0.25% (w/v) gentamycin, and incubated at 37°C. Parasite culture and 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, propranolol hydrochloride, verapamil hydrochloride and CQ diposphate (all from Sigma-Aldrich) were dissolved in PBS to a working concentration of 1 mM. LynxTag-CQGREEN (BioLynx Technologies, Singapore; hereafter abbreviated to ‘CQGREEN’) was dissolved in DMSO to the same concentration. All compounds were stored at −20°C and protected from light. For microscopy uptake assays, methiothepin mesylate salt, metergoline, loperamide hydrochloride, octoclothepin maleate salt, mibefradil dihydrochloride hydrate, L703,606 oxalate salt hydrate, and chlorprophazine hydrochloride (all from Sigma-Aldrich) were dissolved in DMSO to 10 mM and stored at 4°C. Verapamil hydrochloride, adenosine triphosphate (ATP), and CQ diposphate (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 (3H-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/μmol.

**Reinvasion half-maximal inhibitory concentration (IC50)**

Synchronized ring-stage cultures at 1–2% parasitemia, 1.25% haematocrit were incubated with either CQ or CQGREEN 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). IC50s 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 CQGREEN 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 CQGREEN were excited at 405 nm and 488 nm with emissions captured at 430–470 nm and 505–525 nm respectively.

**Parasite CQGREEN uptake assay**

Synchronized trophozoite-stage cultures at 3–5% parasitemia were resuspended in 200 μl of MCM with 2 μM of CQGREEN 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 405 nm and 488 nm respectively. K1 chemoreversal assays were performed by pretreatment with 10 μM of the reversal agents for 30 min prior to the addition of CQGREEN.

**Preparation of microsomes carrying PfCRT**

PICRT originating from *P. falciparum* strains Dd2 or 3D7 were expressed in *Pichia pastoris* KM71 and microsomes harvested as described previously [33]. Microsomal levels of PICRT 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\textsubscript{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 the concentration of CQ\textsubscript{GREEN} used, for 15 min at 37°C, before adding CQ\textsubscript{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\textsubscript{2}, and 5 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 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\textsubscript{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\textsubscript{max} and K\textsubscript{m} of specific uptake.

**CQ\textsubscript{GREEN} uptake in PfCRT microsomes**

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

**3\textsuperscript{H}-CQ uptake in PfCRT microsomes**

3\textsuperscript{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\textsuperscript{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\textsuperscript{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 RNaseasy 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 5X 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’- ATGGG-TAAAGAGCGAGAGAA and 5’- GCCGATACCCGGAGGACG- CAGT, or 5’- GTCAAGGGAGGATTTGGC and 5’- ATTCCTTCATTTGTCCAC. Pfcr-f-specific primers were 5’- GACGAAGCTGTTATAGAAG and 5’- CTTCGGAAATCTTT- GATTTTCT. PCR products were purified with the QIAquick PCR purification kit as per manufacturer’s instructions. Purified PCR products were sequenced by a commercial vendor (AI Labtech, Singapore). Copy number of pfmdr1 was assessed by real-time PCR as previously reported [34]. Briefly, mixture reactions were prepared with TaqMan universal PCR master mix (Applied Biosystems), 5.5 mM MgCl\textsubscript{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’- TGTCATGTATAAAGCAGTACGAA and 5’- TCGGTGTGTTCCATGTGACGTG, respectively, and TaqMan probe was 5’- VIC-TAGCAGATGCCGTTAATATCTCCATGCTT- TAMRA. A reference gene, β-tubulin, was also included; primers were 5’- TGATGTTGCCGAAGTGATCC and 5’- TCCTTTGTGGGACATTCCTCCTC, while the probe was 5’- VIC-TAGCAGATGCCGTTAATATCTCCATGCTT- TAMRA. The threshold cycle (Ct) was analysed by the comparative Ct method, based on DNA amplification efficiencies of the pfmdr1 and β-tubulin genes. Pfmdr1 copy number was calculated according to the following formula: ΔCt = C\textsubscript{R} - C\textsubscript{G}, where C\textsubscript{R} is the reference β-tubulin Ct, and C\textsubscript{G} 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. Chemoresistant assays were assessed with Student’s t test, 2-tailed. Multiple linear regression was performed with the stepwise method, using the log of IC\textsubscript{50} 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 Medicine, Singapore). All blood samples were collected after informed consent was obtained from the patients. The blood sample protocol was approved by the Research Ethics Committee of the National University of Singapore (NUS). The relevant ethical regulations were followed, and informed consent was obtained from all patients. The clinical isolates used were obtained under ethical guidelines in the approved protocol: OXTREC Reference Number 29-09 (Center for Clinical Vaccinology and Tropical Medicine, Singapore). All blood samples were collected after informed consent was obtained from the patients. The blood sample protocol was approved by the Research Ethics Committee of the National University of Singapore (NUS).

**Figure 1. CQ\textsubscript{GREEN} localization in P. falciparum 3D7.** Parasites were stained with CQ\textsubscript{GREEN} and Hoechst and visualized via confocal microscopy under a 100× objective. CQ\textsubscript{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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Results and Discussion

Validation of CQGREEN 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 CQGREEN in the parasite. Cultures of *P. falciparum* 3D7 were co-stained with Hoechst dye and CQ GREEN, revealing a preferential accumulation of CQGREEN in the parasite DV (Fig. 1). Interestingly, CQGREEN fluorescence was also observed in the parasite cytosol but not in the erythrocyte cytosol. As CQGREEN is a CQ analog, the antimalarial potency of CQGREEN should be similar to that of CQ. To assess this, reinvasion IC50s of CQ and CQGREEN on the laboratory strains 3D7, 7G8 and K1 were determined. CQGREEN 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).

CQGREEN fluorescence as a proxy for CQ uptake in parasites

Next, we determined if CQGREEN 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 parasites. 
strains [37–41]. CQ-sensitive 3D7 was included as a reference for complete reversal. All reversal agents except desipramine induced a significant increase in CQGREEN 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 CQGREEN uptake did not achieve statistical significance. Taken together with the CQGREEN IC50 data, we believe that the reversibility of CQGREEN uptake by known chemoreversal agents suggests that CQGREEN shares similar structural properties with CQ.

Uptake of CQGREEN in microsomes bearing PfCRT

To test if CQGREEN can be transported by PfCRT, we have expressed PfCRT in *Pichia pastoris* and used the microsomes derived to study CQGREEN uptake. Figure 3 shows that CQGREEN uptake in Dd2 PfCRT-expressing microsomes is specific. At the highest concentration of CQGREEN used (200 μM), uptake was close to saturated. Michaelis-Menten approximation of CQGREEN uptake kinetics in Dd2 microsomes (Figure 3) yields a Vmax and Km of 938.5 nmol/mg PfCRT/min and 105.1 μM respectively, which are approximately 2000 and 500 times higher compared to when 3H-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) Km and non-saturation of CQGREEN transport are consistent with a previous report in a *Xenopus* oocyte system using 3H-CQ [43]. We have also compared CQGREEN uptake in microsomes with PfCRT originating from either CQ-sensitive 3D7 or CQ-resistant Dd2. CQGREEN 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 4.** ATP-dependent, verapamil-sensitive uptake of CQGREEN 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 CQGREEN under different conditions. Preincubation with 150 μM verapamil abrogated CQGREEN uptake from PfCRT-Dd2 but did not affect uptake in PfCRT-3D7 microsomes. Removal of ATP from buffer abolished CQGREEN 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≥3.

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**Figure 5.** CQGREEN 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 CQGREEN. At the highest concentration of 10 μM, mibefradil drastically suppressed CQGREEN 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≥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 CQGREEN 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 CQGREEN 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 CQGREEN still allows CQGREEN to be differentially recognized by resistant versus sensitive PfCRT and these transport activities are still sensitive to verapamil. To demonstrate the usefulness of CQGREEN in screening for PfCRT inhibitors, we found that CQGREEN uptake by Dd2 PfCRT microsomes can be inhibited by mibebradil, a novel potent chemosensitizer [21], in a dose-dependent manner (Figure 5). Inhibition of CQGREEN uptake by a panel of known chemoreversal agents was compared to that using 3H-CQ (Figure 6). Mean uptake of CQGREEN was positively correlated with that of 3H-CQ ($R^2 = 0.766$). However, 3H-CQ uptake was roughly 100 times greater than that of CQGREEN.

Polymorphisms and copy number variation in pfcr and pfmdr1

Eight laboratory strains and twelve clinical isolates were sequenced for polymorphisms in PfCRT and Pfgh1, the proteins encoded by the genes pfcr 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 Pfgh1. 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 Pfgh1 mutations and pfmdr1 copy numbers were more varied (Table 1).

Genetic correlates of CQGREEN uptake

All strains and isolates were assayed for CQGREEN uptake in the trophozoite stage, and their CQ IC50s determined with the standard reinvasion assay. For the entire data set, CQGREEN uptake was inversely correlated to the log of CQ IC50, with an $R^2$ of 0.53 (Fig. 7A). However, multiple linear regression with sequencing and copy number data revealed that CQGREEN uptake was significantly correlated with not only CQ logIC50 but also a F1226Y substitution in Pfgh1 ($\beta$ 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 CQGREEN uptake. Separating the data set to two subpopulations improved the $R^2$, to 0.72 in the Pfgh1 1226G group and 0.676 in the Pfgh1 1226Y group (Fig. 7B). It is tempting to conclude that the Pfgh1 F1226Y substitution plays a significant causal role in modulating CQGREEN uptake, given that it is also correlated with resistance to artemisinin, mefloquine and lumefantrine [48] and Pfgh1’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 more prevalent within the population. 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 Pfgh1 haplotypes of the F1226Y mutants examined were identical, apart from SMRU0501 which had an additional F1226Y substitution (Table 1). All strains and isolates within a small geographical region, F1226Y is likely to be strongly correlated with other undiscovered mutations. In fact, the PfCRT and Pfgh1 haplotypes of the F1226Y mutants examined were identical, apart from SMRU0501 which had an additional F1226Y substitution (Table 1). All strains and isolates were assayed for CQGREEN uptake in the trophozoite stage, and their CQ IC50s determined with the standard reinvasion assay. For the entire data set, CQGREEN uptake was inversely correlated to the log of CQ IC50, with an $R^2$ of 0.53 (Fig. 7A). However, multiple linear regression with sequencing and copy number data revealed that CQGREEN uptake was significantly correlated with not only CQ logIC50 but also a F1226Y substitution in Pfgh1 ($\beta$ 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 CQGREEN uptake. Separating the data set to two subpopulations improved the $R^2$, to 0.72 in the Pfgh1 1226G group and 0.676 in the Pfgh1 1226Y group (Fig. 7B). It is tempting to conclude that the Pfgh1 F1226Y substitution plays a significant causal role in modulating CQGREEN uptake, given that it is also correlated with resistance to artemisinin, mefloquine and lumefantrine [48] and Pfgh1’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 Pfgh1 haplotypes of the F1226Y mutants examined were identical, apart from SMRU0501 which had an additional F1226Y substitution (Table 1). All strains and isolates were assayed for CQGREEN uptake in the trophozoite stage, and their CQ IC50s determined with the standard reinvasion assay. For the entire data set, CQGREEN uptake was inversely correlated to the log of CQ IC50, with an $R^2$ of 0.53 (Fig. 7A). However, multiple linear regression with sequencing and copy number data revealed that CQGREEN uptake was significantly correlated with not only CQ logIC50 but also a F1226Y substitution in Pfgh1 ($\beta$ 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 CQGREEN uptake. Separating the data set to two subpopulations improved the $R^2$, to 0.72 in the Pfgh1 1226G group and 0.676 in the Pfgh1 1226Y group (Fig. 7B). It is tempting to conclude that the Pfgh1 F1226Y substitution plays a significant causal role in modulating CQGREEN uptake, given that it is also correlated with resistance to artemisinin, mefloquine and lumefantrine [48] and Pfgh1’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 Pfgh1 haplotypes of the F1226Y mutants examined were identical, apart from SMRU0501 which had an additional F1226Y substitution (Table 1). It is notable that for the F1226Y mutants, CQGREEN uptake could range as high as that of the CQ-susceptible strains (Fig. 7B) while still maintaining CQ resistance. One possible gene modulating CQGREEN 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 CQGREEN fluorescence but sequester the drug from its site of action. Knock-out mutants of this gene exhibit increased susceptibility to CQ, quinine, artesiminin, 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].

Figure 6. Accumulation of CQGREEN and 3H-CQ in PfCRT-Dd2 microsomes. Microsomes were incubated with 10 μM of chemosensitizers before addition of CQGREEN or 3H-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 CQGREEN uptake against control. **: p<0.05, comparing 3H-CQ uptake against control. Data presented are means ± S.E.M.; n≥3.

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## Table 1. CQ IC₅₀s, PfCRT and Pgh1 polymorphisms, and pfmdr1 copy number.

| Laboratory strains | CQ IC₅₀ (nM) | PFRT residue no. | Pgh1 residue no. | pfmdr1 copy number |
|--------------------|-------------|-----------------|-----------------|--------------------|
|                    | 72 74 75 76 220 271 326 356 371 | 86 184 1034 1042 1226 1246 | 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                  |
| 3D7                | 31          | C M N K A Q N I R | N Y S N 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                  |
| 3D7                | 31          | C M N K A Q N I R | N Y S N 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                  |

Bolded residues indicate deviation from 3D7 haplotype. CQ IC₅₀s are geometric means of at least 3 measurements.

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Conclusions

CQ\textsubscript{GREEN} is a commercially available fluorescent CQ analog that interacts with the parasite in a similar fashion to CQ. CQ\textsubscript{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 allow it to be monitored by common fluorescence equipment. Using a defined microsomal platform, we showed that CQ\textsubscript{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\textsubscript{GREEN} allows for measurement of CQ susceptibility within several hours. We believe that CQ\textsubscript{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.

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