

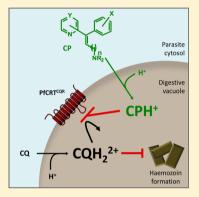
Chlorpheniramine Analogues Reverse Chloroguine Resistance in Plasmodium falciparum by Inhibiting PfCRT

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Supporting Information

ABSTRACT: The emergence and spread of malaria parasites that are resistant to chloroquine (CQ) has been a disaster for world health. The antihistamine chlorpheniramine (CP) partially resensitizes CQ-resistant (CQR) parasites to CQ but possesses little intrinsic antiplasmodial activity. Mutations in the parasite's CQ resistance transporter (PfCRT) confer resistance to CQ by enabling the protein to transport the drug away from its site of action, and it is thought that resistance-reversers such as CP exert their effect by blocking this CQ transport activity. Here, a series of new structural analogues and homologues of CP have been synthesized. We show that these compounds (along with other in vitro CQ resistance-reversers) inhibit the transport of CQ via a resistanceconferring form of PfCRT expressed in Xenopus laevis oocytes. Furthermore, the level of PfCRT-inhibition was found to correlate well with both the restoration of CQ accumulation and the level of CQ resensitization in CQR parasites.



KEYWORDS: Malaria parasite, chloroquine resistance, chemosensitization, chlorpheniramine, PfCRT, Xenopus oocytes

The malaria parasite Plasmodium falciparum has proven largely refractory to the vaccine approaches trialled to date and reliance on chemotherapy is under serious threat with the recent emergence of parasites that are resistant to the current mainstay of malaria treatment (the artemisinin-based combination therapies). As a result, malaria remains a global health problem; there are around 225 million clinical cases and over 1.2 million deaths each year,² and the disease also imposes considerable socio-economic burdens upon afflicted countries. Chloroquine (CQ, Figure 1A) was a cheap, safe, and efficacious treatment for the disease until the eventual emergence and spread of resistant parasites. Resistance to CQ is caused primarily by mutations in the P. falciparum CO resistance transporter, PfCRT.3 Within the P. falciparum-infected erythrocyte, PfCRT is found in the membrane of the parasite's digestive vacuole⁴ (DV; pH 5-5.5); the organelle in which CQ accumulates and exerts its antimalarial effect. Mutations in PfCRT that confer CQ resistance result in a marked reduction in the accumulation of CQ within the DV. Using the Xenopus *laevis* oocyte expression system, a CQ resistance-conferring form of PfCRT (PfCRT^{CQR}) was shown to transport CQ, whereas the wild-type form found in CQ-sensitive (CQS) parasites (PfCRT^{CQS}) lacked this ability.⁵

Mutations in PfCRT have been associated with decreases or increases in the parasite's susceptibility to other antimalarials,⁶ and the transporter has been shown to be one of the key determinants of the parasite's response to a diverse set of novel antiplasmodial compounds. Whether these effects are due to PfCRT-mediated drug efflux and/or the inhibition of the transporter's normal function (which is essential for the survival

of the parasite, but currently unknown) remains unclear.8 A better understanding of the interactions between PfCRT^{CQR} and its substrates and inhibitors could inform the development of new antimalarial chemotherapies and strategies.

CQ-resistant (CQR) parasites can be partially resensitized to CQ in vitro by a range of weak bases including the calcium channel blocker verapamil (VP), the antihistamines promethazine and chlorpheniramine (PZ and CP, respectively), and the liver-stage antimalarial primaquine (PQ) (Figure 1A). The phenomenon of CQ resistance-reversal is characterized by both an increase in CQ accumulation and a decrease in the CQ IC₅₀ in CQR parasites (the CQ IC50 is the concentration of CQ at which the inhibition of parasite growth is half-maximal).1 Reversers of CQ resistance generally have no effect on either CQ accumulation or the CQ IC₅₀ in CQS parasites¹⁰ and possess little or no intrinsic antimalarial activity against the intraerythrocytic stage of the parasite. It is thought that resistance-reversers accumulate in the acidic environment of the DV via weak-base trapping and that they may exert their CQ resistance-reversing activity by binding to PfCRT^{CQR} and thereby inhibiting the efflux of CQ from the DV.9 Consistent with this mechanism of action, VP and a series of dibemethin derivatives have recently been shown to interact directly with PfCRT^{CQR} to inhibit CQ transport in the oocyte system. 5,11

Resistance-reversers generally feature a secondary or tertiary amine tethered to two aromatic hydrophobic residues by a

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Figure 1. Chemical structures and synthesis. (A) The structures of chloroquine (CQ), verapamil (VP), chlorpheniramine (CP), promethazine (PZ), and primaquine (PQ). (B) Retrosynthesis of CP analogues. (C) Synthesis of ketones 1–6. Reagents and conditions: (a) n-BuLi, THF, ether, -85 °C warming to -10 °C, 15 min, then -110 °C warming to r.t., 24 h (50–77%); (b) n-BuLi, ether, -78 °C, 1 h warming to r.t., 1.5 h (91%); (c) MnO₂ 11 days (78%); (d) ether, toluene, 80 °C, 1.75 h then 2 M H₂SO₄, 0.5 h (64–66%). (D) Synthesis of CP analogues 12–21c. Reagents and conditions: (e) R₂NH_(aq), MeOH, 80 °C, 18 h (96–99%); (f) LiN(SiMe₃)₂, THF, 0 °C, 15 min, then 1–6, r.t., 24 h (33–83%); (g) Pd/C, H₂ EtOH, 6.5–13 h (46–50%); (h) PtO₂, H₂, EtOH, 3.5–6.5 h (77–99%).

flexible aliphatic side chain.9 The failure of early in vivo animal trials with VP12 and the apparent lack of activity of the in vitro resistance-reversers cyproheptadine¹³ and desipramine¹⁴ in human trials have been attributed to a combination of (1) the low availability of the resistance-reversers due to binding to serum protein, (2) the inability to achieve the concentrations required for efficacy without risking toxic side-effects, and (3) low potency of the resistance-reversing effect. Some of these properties may be inherent to weakly basic hydrophobic compounds. However, the successful treatment of CQR parasite infections with the combination of CQ and CP in Nigeria 16-18 suggests that these obstacles may be overcome. Given the promising clinical use of CP as a CQ resistancereverser, we used the molecular framework of this compound as a basis for a series of derivatives, with the aim of developing structure-activity relationships, investigating their mode of action, and potentially increasing the resistance-reversing activity of CP. The structural changes included increases to the side-chain length, alterations to the heteroaromatic ring, and changes to the position of attachment of the chlorine atom on the phenyl ring.

The desired CP analogues were prepared using a Wittig olefination reaction to install the amine side-chain from pyridylor pyrazinyl- chlorophenyl ketones (Figure 1B). This enabled the synthesis of a range of analogues with the desired changes. The 2-pyridyl ketones 1–3 were prepared using the method described by Popova et al.¹⁹ from 2-bromopyridine and methyl 2-, 3-, or 4-chlorobenzoate (Figure 1C). The 3-pyridyl ketone 4 was prepared in a two-step procedure from the reaction of lithiated 3-bromopyridine with 3-chlorobenzaldehyde, followed

by manganese dioxide oxidation,²⁰ which was slow but highyielding and clean. Because of the problems with the stability of 4-bromopyridine, 5 was prepared using an alternate strategy from the Grignard reaction of 4-cyanopyridine with 3chlorophenylmagnesium bromide and acidic aqueous workup.²¹ Pyrazine ketone 6 was prepared in a similar fashion from 2cyanopyrazine.

The Wittig salts 7–8 were prepared using the method of Rao and Reddy, 22 via (2-methoxyethyl)triphenylphosphonium bromide (Figure 1B). Compounds 9–11 were prepared from the corresponding (ω -bromoalkyl)triphenyl phosphonium bromides using the procedure of Sano et al. 23

Wittig olefination utilizing LiHMDS proceeded smoothly to produce olefins 12-21a,b, although yields decreased with increasing chain length (Figure 1D). Hydrogenation was performed in excellent conversion, utilizing palladium on carbon or platinum dioxide catalysts, as illustrated by the disappearance of the distinctive olefinic triplet between 6.23 and 6.87 ppm and the appearance of a benzylic triplet between 4.07 and 4.72 ppm. The use of Pd/C promoted cleavage of the chloro group, whereas platinum dioxide could be used to catalyze hydrogenation of alkenes, suppressing cleavage of the chloro groups. Compounds 12a-21a, 12b-21b, 12c-21c, and 22 were prepared in this manner, and the compounds tested are presented in Table 1. Compounds 23a and 23b were prepared from the Horner-Wadsworth-Emmons (HWE) olefination between diethylcyanomethyl phosphonate and 2benzoyl-pyridine.²⁴ Diastereomers were separated by either flash column chromatography or HPLC, and all CP analogues

Table 1. Analogues Prepared and Tested in This Study

	config	subst	Y	X	n	Z
12a	E	2	CH	4-Cl	1	NEt_2
12b	Z	2	CH	4-Cl	1	NEt_2
12c	rac	2	CH	4-Cl	1	NEt_2
13a	E	2	CH	3-Cl	1	NMe_2
13b	Z	2	CH	3-C1	1	NMe_2
13c	rac	2	CH	3-Cl	1	NMe_2
14a	E	2	CH	4-Cl	2	NMe_2
14b	Z	2	CH	4-Cl	2	NMe_2
14c	rac	2	CH	4-Cl	2	NMe_2
15a	E	2	CH	3-Cl	2	NMe_2
15b	Z	2	CH	3-Cl	2	NMe_2
15c	rac	2	CH	3-Cl	2	NMe_2
16a	E	2	CH	2-Cl	2	NMe_2
16c	rac	2	CH	2-Cl	2	NMe_2
17a	E	2	CH	4-Cl	3	NMe_2
17b	Z	2	CH	4-Cl	3	NMe_2
17c	rac	2	CH	4-Cl	3	NMe_2
18c	rac	2	CH	4-Cl	4	NMe_2
19c	rac	3	CH	3-Cl	2	NMe_2
20c	rac	4	C	3-Cl	2	NMe_2
21c	rac	2	N	3-Cl	2	NMe_2
22	rac	2	CH	H	2	NMe_2
23a	E	2	CH	H	0	CN
23b	Z	2	CH	H	0	CN
CP	rac	2	CH	4-Cl	1	NMe_2
CP	(+)	2	СН	4-Cl	1	NMe ₂

were purified by HPLC prior to testing (see Supporting Information for details).

The ability of the CP analogues to inhibit the PfCRT mediated transport of CQ was assessed using the *Xenopus* oocyte system. When present at an extracellular concentration of 100 μ M, CP, PQ, PZ, and VP all inhibited the transport of

[3H]CQ via PfCRT^{CQR} (Figure 2A). Each of the 24 CP analogues also inhibited PfCRT^{CQR}-mediated [³H]CQ transport when tested at 100 μ M, albeit to different degrees (Figure 2B; P < 0.01) and with less potency than the benchmark resistance-reverser VP (P < 0.05). None of the compounds affected the diffusion of CQ into oocytes expressing PfCRT^{CQS} (P > 0.05). The structural changes investigated here mostly resulted in reduced PfCRT^{CQR} inhibition relative to CP, with the exception of 13c (the 3-chlorophenyl derivative of CP) and 19c (containing 3-chlorophenyl and 3-pyridyl groups). Both of these compounds appeared to be slightly more potent than CP, but the differences were not statistically significant (P > 0.05). The general trends derived from this data were (1) the reduced compounds (present as racemates) were more active than their olefinic counterparts (e.g., 15a-c), (2) lengthening the sidechain was not beneficial (e.g., 14c, 17c, and 18c), and (3) having a 3-chlorophenyl group was preferable to 2-chloro, 4chloro, or unsubstituted phenyl groups (e.g., 15c, 14c, 16c, and

An analysis of the concentration-dependent inhibition of PfCRT^{CQR} by a selection of representative derivatives (12a, 13c, 15c, and 17c; Figure S1 d) and resistance reversers (CP, PZ, and PQ; Figure S1 a–c) yielded the IC₅₀ values reported in Table 2. The IC₅₀ value of 12a was significantly greater than those of the other compounds tested (P < 0.05). Compound 17c was less potent than PZ or PQ, but more active than 12a. The activities of 13c and 15c were comparable to that of CP but were significantly lower than that of VP (30 \pm 3 μ M; 5 P < 0.01).

CP derivatives 12a, 15c, 17c, and 23a were tested for the ability to behave as CQ resistance-reversers in CQR parasites. In the first set of experiments, CQ accumulation was measured in the C2^{GCO3}, C4^{Dd2}, and C6^{7G8} parasite lines in the presence or absence of VP, CP, and the CP analogues. These isogenic parasite lines express either the wild-type *pfcrt* allele (C2^{GCO3}) or the CQ-resistance-conferring *pfcrt* alleles from the CQR strains Dd2 or 7G8 (C4^{Dd2} and C6^{7G8}, respectively). Under control conditions, the CQ accumulation ratios for the CQR C4^{Dd2} and C6^{7G8} lines were 13.3 \pm 2.8 and 11.6 \pm 1.6 times lower, respectively, than that measured in the CQS C2^{GCO3} line (Figure 2C). VP (1 μ M) had no effect on CQ accumulation in

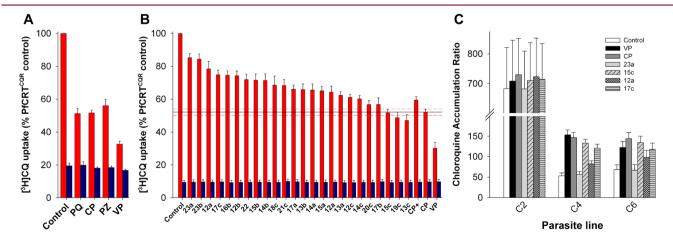


Figure 2. Inhibition of PfCRT^{CQR}-mediated CQ transport by the resistance-reversers PZ, CP, PQ, VP, and by analogues of CP. (A) [3 H]CQ uptake into oocytes expressing PfCRT^{CQR} (red bars) or PfCRT^{CQS} (blue bars) in the presence of 100 μ M PQ, CP, PZ, or VP. (B) [3 H]CQ uptake into oocytes expressing PfCRT^{CQR} (red bars) or PfCRT^{CQS} (blue bars) in the presence of CP analogues (100 μ M). The level of inhibition by CP is shown as a solid line with SEM (n = 5) represented by red dashed lines. (C) Effects of VP, CP, and the CP analogues 23a, 15c, 12a, and 17c on [3 H]CQ accumulation by erythrocytes infected with C2^{GCO3}, C4^{Dd2}, or C6^{7G8} parasites. [See Supporting Information for further details.]

Table 2. Inhibition of PfCRT^{CQR}-Mediated CQ Transport in Oocytes, Intrinsic Antiplasmodial Activity, and in Vitro Resistance-Reversal Activity of Four CP Analogues

		parasite growth relative to control $(\%)^b$			$CQ IC_{50} (nM) (RMI)^c$		
compd	${ m IC}_{50}$ values ($\mu{ m M}$) for the inhibition of PfCRT ${ m ^{CQR}}{}^a$	C2 ^{GC03}	C4 ^{Dd2}	C6 ^{7G8}	C2 ^{GC03}	C4 ^{Dd2}	C6 ^{7G8}
CQ					$14.5 \pm 1.7 \ (1.00)$	154 ± 8 (1.00)	110 ± 15 (1.00)
VP	33 ± 3	43 ± 6	46 ± 3	55 ± 5^{d}	$13.1 \pm 1.6 \ (0.91)$	$36.2 \pm 2.8 \ (0.23)$	$71 \pm 8 \ (0.65)$
CP	54 ± 5	97 ± 2	78 ± 5	76 ± 1^{d}	$15.6 \pm 1.7 (1.09)$	$36.6 \pm 0.7 \ (0.24)$	$39.8 \pm 3.1 \ (0.37)$
15c	66 ± 8	99 ± 2	67 ± 3^{d}	72 ± 4^{d}	$13.8 \pm 1.8 \ (0.95)$	$41 \pm 2 \ (0.27)$	$36.8 \pm 1.9 \ (0.35)$
12a	321 ± 24	100 ± 4	84 ± 5^{d}	90 ± 1	$14.8 \pm 2.4 (1.01)$	$75 \pm 2 \ (0.49)$	$75 \pm 5 \ (0.69)$
17c	180 ± 29	100 ± 4	74 ± 5^{d}	79 ± 4^{d}	$13.4 \pm 1.9 \ (0.92)$	$44 \pm 2 \ (0.28)$	$42 \pm 3 \ (0.39)$
13c	63 ± 7	99 ± 4	75 ± 2^{d}	74 ± 3^{d}	$14.3 \pm 1.6 \ (0.99)$	$42 \pm 4 \ (0.27)$	$38.3 \pm 4.1 \ (0.35)$
PZ	85 ± 7						
PQ	68 ± 8						

 $^{a}\text{IC}_{50}$ values (mean \pm SEM) were determined from the data presented in Figure S1, Supporting Information. The IC $_{50}$ value for VP is from Martin et al. 5 Proliferation of *pfcrt* transfectant parasite lines in the presence of the test compound (800 nM), relative to the control. $^{c}\text{IC}_{50}$ of CQ (mean \pm SEM) against the *pfcrt* transfectant lines in the presence of the test compound (800 nM), relative to the control. The response modification index (RMI) equals the IC $_{50}$ of CQ determined in the presence of the compound/the IC $_{50}$ of CQ measured in the absence of the compound. d Significantly different from C2 GC03 (P < 0.05). [See Supporting Information for further details.]

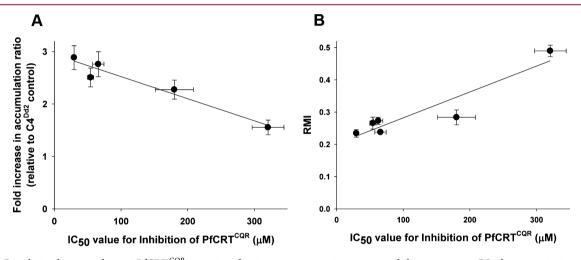


Figure 3. Correlation between the anti-PfCRT^{CQR} activities of resistance-reversers in oocytes and their respective CQ-chemosensitizing activities in C4^{Dd2} parasites. The IC₅₀ values for the inhibition of PfCRT^{CQR}-mediated CQ transport by CP, VP, **12a**, **13c**, **15c** and **17c** were plotted against (A) the fold increase in the CQ accumulation ratio ($R^2 = 0.94$, P = 0.0064) or (B) the CQ resistance modification index (RMI) ($R^2 = 0.869$, P = 0.0067).

the C2^{GCO3} parasites, but caused a 3.1 ± 0.5 fold increase in the CQ accumulation ratio in the C4^{Dd2} line and a 1.9 ± 0.2 fold increase in the C6^{7G8} line (P < 0.05; Figure 2C). These findings are consistent with previous reports.

Both CP and **15c** caused marked increases in the accumulation of CQ in the CQR lines; CQ levels were increased 2.7–2.9-fold in the C4^{Dd2} parasites and 2.0–2.2-fold in the C6^{7G8} parasites (P > 0.05). Compounds **17c** and **12a** had less dramatic effects on CQ accumulation, causing increases of 2.4- and 1.6-fold, respectively, in the C4^{Dd2} line, and fold-increases of 1.8 and 1.4 in the C6^{7G8} line.

Compound **23a** had no effect on CQ accumulation in either the CQS or CQR lines (P > 0.05), which is consistent with our finding that it is a poor inhibitor of CQ transport via PfCRT^{CQR} (Figure 2B). None of the compounds tested altered CQ accumulation in the C2^{GC03} parasite line (P > 0.05).

In the second set of experiments, the CP analogues 12a, 13c, 15c, and 17c were tested for the ability to modulate CQ susceptibility in the *pfcrt* transfectant lines using parasite proliferation assays. In the absence of CQ, 800 nM VP was found to have a negative effect on proliferation in all three lines (Table 2; P < 0.05). By contrast, at the same concentration, CP

and its derivatives had no effect on the proliferation of CQS $C2^{GC03}$ parasites, yet all displayed significant inhibitory activity against the CQR $C4^{Dd2}$ and $C6^{7G8}$ lines (Table 2; P < 0.05). This finding is consistent with previous observations of resistance-reversers displaying modest but significant levels of intrinsic antiplasmodial activity against CQR strains²⁶ (while exerting little or no effect in CQS strains) and provides further support for the idea that PfCRT^{CQR} could be viewed as a drug target.⁸

None of the test compounds affected the IC₅₀ of CQ against CQS C2^{GC03} parasites (Table 2; P > 0.2). However, VP, CP, and the four CP analogues each caused a significant decrease in the IC₅₀ of CQ against the CQR lines (Table 2; P < 0.01). In all cases, the resistance modification index (RMI) calculated for the C6^{7G8} line was greater than that measured for the C4^{Dd2} line, indicating a weaker resistance-reversing effect against the C6^{7G8} line; this was particularly noticeable for VP (Table 2).

The haplotype of $PfCRT^{CQR}$ expressed in the oocyte system is the same as that carried by the $C4^{Dd2}$ parasite line. It was therefore relevant to compare the IC_{50} values obtained for each compound in the oocyte system with the corresponding (1) fold-increases in CQ accumulation and (2) RMI values for CQ

susceptibility determined for the C4^{Dd2} parasites. As shown in Figure 3A, there was a strong positive correlation between the ability to inhibit PfCRT^{CQR}-mediated CQ transport in oocytes and the restoration of CQ accumulation in erythrocytes infected with C4^{Dd2} parasites ($R^2 = 0.94$, P = 0.0064). Likewise, the IC₅₀ values for PfCRT^{CQR} inhibition were strongly correlated with the CQ RMI values derived for C4^{Dd2} parasites (Figure 3B; $R^2 = 0.869$, P = 0.0067). These analyses indicate that the degree of anti-PfCRT^{CQR} activity exhibited by a CQ resistance-reverser is an important determinant of its in vitro chemosensitization activity. While the number of compounds compared here was small, these findings suggest that the extent to which a compound inhibits PfCRT-mediated CQ transport in the oocyte system is a relatively reliable indicator of its ability to inhibit PfCRT^{CQR} in situ.

In summary, a library of CP analogues was produced, and all of these molecules were shown to be inhibitors of PfCRT^{CQR} in the oocyte system. The most active of these compounds carried a 3-chlorophenyl group in place of the 4-chlorophenyl group of CP and had in vitro resistance-reversing activity similar to that of CP. Within this series of analogues, the extent of PfCRT^{CQR} inhibition observed in the oocyte system was found to correlate well with the compound's ability to modulate the CQsusceptibility of CQR parasites. This finding indicates that the degree of anti-PfCRT activity exhibited by a resistancereverser is an important determinant of its in vitro chemosensitization activity in CQR parasites. Moreover, the oocyte system was able to detect relatively small differences in the inhibitory activities of the 24 CP analogues, indicating that this assay is well suited to assessing potential blockers of PfCRT^{CQR}. The fact that previous work²⁷ has shown that CP, PZ, and PQ appear to be substrates of PfCRT^{CQR} in situ, together with the finding here that they inhibit the transporter in a concentrationdependent manner, indicates that these compounds exert their CQ resistance-reversing activity by competing with CQ for transport via PfCRT^{CQR}. CQ remains a first-line treatment and prophylactic in 21 countries and a CQ-azithromycin combination is in phase IIb/III development for preventative treatment in pregnant women. It is therefore possible that inhibitors of mutant PfCRT could play a role in CQ-based combination therapies, or in therapies based on related quinolines such as amodiaquine, to boost the activity of the quinoline partner drug against CQR parasites. Alternatively, inhibitors of mutant PfCRT could be combined with the antimalarial pharmacophore of CO^{28,29} or with other antimalarial moieties to create dual-function antimalarials that are active against drug-resistant parasites.30

ASSOCIATED CONTENT

S Supporting Information

Experimental details for the synthesis and purification of the compounds, the full spectral data, the in vitro assay conditions, and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CQ, chloroquine; CP, chlorpheniramine; CQR, chloroquine-resistant; CQS, chloroquine-sensitive; PfCRT, *Plasmodium falciparum* chloroquine resistance transporter; PQ, primaquine; PZ, promethazine; VP, verapamil

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