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Saquinavir Inhibits the Malaria Parasite’s Chloroquine Resistance Transporter

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The antiretroviral protease inhibitors (APIs) ritonavir, saquinavir, and lopinavir, used to treat HIV infection, inhibit the growth of Plasmodium falciparum at clinically relevant concentrations. Moreover, it has been reported that these APIs potentiate the activity of chloroquine (CQ) against this parasite in vitro. The mechanism underlying this effect is not understood, but the degree of chemosensitization varies between the different APIs and, with the exception of ritonavir, appears to be dependent on the parasite exhibiting a CQ-resistant phenotype. Here we report a study of the role of the P. falciparum chloroquine resistance transporter (PfCRT) in the interaction between CQ and APIs, using transgenic parasites expressing different PfCRT alleles and using the Xenopus laevis oocyte expression system for the heterologous expression of PfCRT. Our data demonstrate that saquinavir behaves as a CQ resistance reverser and that this explains, at least in part, its ability to enhance the effects of CQ in CQ-resistant P. falciparum parasites.

Certain antiretroviral protease inhibitors (APIs) inhibit the growth of malaria parasites at clinically relevant concentrations (2, 21, 23, 25, 32). This observation may have clinically significant implications for the treatment of HIV- and malaria parasite-coinfected patients as well as for the development of a potent class of antimalarial agents possessing a novel mode of action. While detailed clinical trials are required to determine whether the antiplasmodial activity of the APIs is beneficial in a coinfection setting, reports that these drugs act synergistically with selected antimalarial drugs both in vitro and in vivo (13, 14, 31) are promising, and further studies are clearly warranted.

Initial studies demonstrated that chloroquine (CQ) acts synergistically with the APIs ritonavir (RTV) and saquinavir (SQV) against CQ-resistant Plasmodium falciparum parasites (31). These findings were confirmed and extended by the observation that CQ also acts synergistically with lopinavir (LPV), nelfinavir, and atazanavir against CQ-resistant P. falciparum (14). However, analysis of the activity of CQ-API combinations against CQ-sensitive P. falciparum parasites revealed that (i) the degree of synergy varies among the APIs, with RTV showing the greatest degree of synergism; (ii) the synergistic effect of RTV on CQ activity against CQ-resistant P. falciparum parasites is greater than that measured against CQ-sensitive parasites; and (iii) although LPV, SQV, nelfinavir, and atazanavir behave synergistically with CQ against CQ-resistant P. falciparum parasites, these combinations are additive when assessed with CQ-sensitive parasites (14).

The mechanism underlying the different effects of CQ-API combinations against CQ-resistant parasites compared to CQ-sensitive parasites is not well understood, but one plausible explanation is that the APIs may act as CQ resistance reversers in P. falciparum. The primary determinant of CQ resistance in P. falciparum is mutation of the CQ resistance transporter (PfCRT) (5, 8, 29, 36). PfCRT is an integral membrane protein located on the parasite’s digestive vacuole (8), the organelle in which CQ exerts its antimalarial effects. Using the Xenopus laevis oocyte expression system, it has been shown that a mutant, resistance-conferring form of PfCRT (PfCRT<sup>CQR</sup>) transports CQ, whereas the wild-type form of the protein, found in CQ-sensitive parasites (PfCRT<sup>CQS</sup>), does not (20). These data support the hypothesis that PfCRT<sup>CQR</sup> confers CQ resistance by exporting CQ out of the digestive vacuole, away from its primary site of action. PfCRT<sup>CQR</sup>-mediated transport of CQ is inhibited by the resistance reverser verapamil (VP) (20), thus providing a mechanistic explanation for the ability of this compound to enhance CQ activity in CQ-resistant strains. A number of other compounds, including peptides (ranging from 4 to 10 residues in length), have also been found to inhibit CQ transport via PfCRT<sup>CQR</sup> (20). This finding raises the possibility that peptidomimetic drugs such as the APIs also interact with PfCRT<sup>CQR</sup> and that the CQ-API synergy observed in CQ-resistant parasites results from APIs blocking PfCRT<sup>CQR</sup>-mediated CQ efflux from the digestive vacuole.

Here we sought to gain insights into the synergistic interplay between CQ and APIs in CQ-resistant parasites by examining the activity of these combinations in previously described transgenic parasites (29) that share the same genetic background but express different PfCRT alleles. We also investigated the effects of SQV, RTV, and LPV on CQ accumulation in these parasites and employed the X. laevis expression system to test the ability of these APIs to inhibit CQ transport via PfCRT (20). The results provide new insights into the mechanism underpinning the interplay between CQ and the APIs in their effects on CQ-resistant parasites.
MATERIALS AND METHODS

Parasites and culture. The three transgenic parasites used in this study were generated by Sidhu et al. (29) and were generously provided by David Fidock (Columbia University, New York, NY). These transgenic lines were generated from GCO3, a CQ-sensitive clone derived from HB3 (36). C4ΔDd2 and C67G8 were generated by replacing the wild-type pfCRT GCO3 allele with the CQ resistance-conferring Dd2 and 7G8 alleles, respectively. C2ΔGCO3, the CQ-sensitive transgenic control, contains the wild-type GCO3 pfCRT allele. All P. falciparum parasites were maintained in either static (34) or shaking (1) cultures, as described previously. While transgenic parasites were maintained in the presence of 5 μM blasticidin and 5 nM WR99210, these selection agents were not present during any of the experimental procedures.

In vitro antimalarial activity and combination assays. Stock solutions of RTV, SQV, and LPV (obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were prepared in 100% dimethyl sulfoxide (DMSO). CQ (diphosphate salt; Sigma) was prepared in autoclaved distilled water. Dilutions of all drugs were prepared from stock solutions by use of culture medium when required.

Antimalarial drug combinations were assessed by isobologram analysis as described previously (30). All assays were performed in 96-well microtiter plates. Each well contained 100 μl of cell culture and 100 μl of each drug dilution or control. Plates were then labeled with [3H]chloroquine (3) using the fractional inhibitory concentration (FIC) (3). Using the SAAM II program (SAAM Institute, Seattle, WA), a standard hyperbolic function (4), i.e., \( Y_i = 1 - \left[ X_i / \left( X_i - \text{IC}_{50} \times (1 - X_i) \right) \right] \) (where \( Y_i \) is the IC50 for drug A combined with drug B, \( X_i \) is the IC50 for drug B combined with drug A, and \( I \) is the interaction value), was fitted to the data. Positive values of \( I \) indicate synergism, negative values indicate antagonism, and an \( I \) value of 0 indicates that the effects of the two compounds are simply additive. The significance of the difference of \( I \) from zero (\( P \) values of >0.01 indicate an additive interaction) was assessed using Student’s \( t \) test.

Measurements of chloroquine accumulation in P. falciparum-infected erythrocytes. The accumulation of [3H]CQ (20 nM [20 Ci/mmol]; American Radiolabeled Chemicals) in mature trophozoite-infected erythrocytes (36 h postinvasion) was measured using a protocol described in full elsewhere (19).

Measurements of chloroquine transport in X. laevis oocytes expressing PfCRT. Expression of mutant and wild-type forms of PfCRT (from strains Dd2 and D10, respectively) at the plasma membrane of X. laevis oocytes was achieved as described previously (20). Briefly, oocytes were injected with cRNA encoding PfCRT (30 ng per oocyte), and the uptake of [3H]CQ (0.3 μM [15 Ci/mmol]; Moravek) was measured at 4 to 6 days postinjection as described previously (20). Uptake measurements were made over 1 to 2 h at 25°C in medium that contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 10 mM morpholineethanesulfonic acid (MES), 10 mM Tris base (pH 6.0), and 15 μM unlabeled CQ. Statistical comparisons were made with Student’s \( t \) test for unpaired samples or with analysis of variance (ANOVA) in conjunction with Tukey’s multiple comparison test.

RESULTS

Combinations of chloroquine with saquinavir or ritonavir are more effective against CQ-resistant lines than against CQ-sensitive parasites. Isobolograms describing the interactions of CQ with SQV against CQ-sensitive (C2ΔGCO3) and CQ-resistant (C4ΔDd2 and C67ΔG8) lines demonstrate that the antiplasmodial activity of these drugs used in combination is dependent on the PfCRT allele expressed. When combinations of SQV and CQ were assessed against parasites expressing the Dd2 PfCRTΔCQR allele, an additive interaction was observed (Fig. 1) (\( I = -0.01; P > 0.1 \)). However, the same combination behaved antagonistically in control parasites expressing the wild-type GCO3 allele (Fig. 1) (\( I = -4.7; P < 0.0005 \)). In contrast, combinations of CQ with RTV or LPV behaved antagonistically against all transgenic parasites (Fig. 1).

Saquinavir partially restores chloroquine accumulation in CQ-resistant P. falciparum, whereas ritonavir and lopinavir do not. CQ resistance-reversing agents such as verapamil can be used in vitro to resensitize resistant parasites to CQ (albeit partially). This verapamil-induced chemosensitization is associated with an increased accumulation of CQ in resistant parasites, but no such change in CQ accumulation occurs in CQ-sensitive parasites treated with verapamil (20). To investigate the possibility that SQV potentiates the effects of CQ via the same mechanism as that for verapamil and other resistance reversers, CQ accumulation was measured in the C2ΔGCO3, C4ΔDd2, and C67ΔG8 lines in the presence or absence of verapamil, SQV, RTV, and LPV. Under control conditions, the CQ accumulation ratios for the CQ-resistant C4ΔDd2 and C67ΔG8 lines were 14.1 ± 2.0 and 13.7 ± 2.7 times lower (mean ± standard error of the mean [SEM]; \( n = 4 \)), respectively, than that measured for the CQ-sensitive C2ΔGCO3 line (data not shown). Verapamil (1 μM) had no effect on CQ accumulation in the C2ΔGCO3 parasites but caused a 2.5-± 0.2-fold increase in the CQ accumulation ratio in the C4ΔDd2 line and a less dramatic, 1.8-± 0.1-fold increase in the C67ΔG8 line (\( P < 0.05 \); paired \( t \) tests) (Fig. 2a to c), consistent with previous reports (20). SQV (0.5 or 1 μM) also increased CQ accumulation in the CQ-resistant lines, with similar fold increases (1.5- to 1.7-fold) in both the C4ΔDd2 and C67ΔG8 parasites (\( P > 0.05 \); paired \( t \) tests). In contrast, RTV (1 or 2.25 μM) and LPV (0.5, 1, or 4.3 μM) had either little or no effect on the CQ accumulation ratio or caused it to decrease by similar levels across all three strains. Indeed, all of the APIs tested, including SQV, significantly decreased the CQ accumulation ratio in C2ΔGCO3 parasites (\( P < 0.01 \); paired \( t \) tests), with the one exception being the 1 μM RTV treatment, which gave a ratio that was not statistically different from the C2ΔGCO3 control ratio. Thus, SQV decreased the CQ accumulation ratio in the CQ-sensitive line by 1.3- to 1.4-fold yet caused a 1.5- to 1.7-fold increase in the resistant lines, consistent with the observed ability of SQV to antagonize CQ activity in the C2ΔGCO3 line and to enhance its activity in C4ΔDd2 and C67ΔG8 parasites (Fig. 1).

Inhibition of chloroquine transport via PfCRTΔCQR by saquinavir and ritonavir. The finding that SQV increased CQ accumulation in CQ-resistant parasites led us to investigate whether this effect is mediated by blockade of CQ transport via PfCRTΔCQR, as shown previously for verapamil (20). The expression of PfCRT in X. laevis oocytes enables direct measurements of CQ transport via PfCRTΔCQR, and thus an assessment of inhibition of CQ transport by potential resistance reversers. The direction of CQ transport in the PfCRT expression system is from the mildly acidic extracellular medium (pH 6.0) into the oocyte cytosol (pH 7.2), which corresponds to the efflux of CQ from the acidic digestive vacuole (pH 5 to 5.5) (12) into the parasite cytosol (pH 7.3) (27). A key advantage of the oocyte system is that it allows PfCRT to be studied directly and in isolation, without confounding ef-
effects such as the binding of drugs to heme or interactions of the compound with other targets or transporters.

In an initial experiment in which the APIs were tested at an extracellular concentration of 500 μM, SQV abolished CQ transport via PfCRT CQR, RTV reduced PfCRT CQR-mediated transport by 66%, and LPV had no effect (Fig. 3a). An analysis of the concentration-dependent inhibition of CQ transport by SQV yielded an IC₅₀ of 13 μM (mean ± SEM; n = 4) (Fig. 3b). This value is significantly lower than that measured for verapamil (30 ± 3 μM [20]; P < 0.01 by unpaired t test), which makes SQV the most potent inhibitor of PfCRT CQR identified to date.

DISCUSSION

Understanding the pharmacokinetic and pharmacodynamic interactions between APIs and antimalarial drugs is important if such combinations are to form the basis of treatment regimens. In this study, we found an antagonistic interaction between CQ and SQV against the CQ-sensitive C₂GCO₃ parasite line, whereas an additive interaction occurred against the CQ-resistant parasite lines C₄DM₂ and C₆₇G₈ (Fig. 1). These three transgenic cell lines differ only in the version of pfcrt they harbor—the wild type or the 7G8 or Dd2 variant. The data therefore indicate that the variants of PfCRT encoded by the Dd2 and 7G8 alleles influence the parasite’s response to CQ-SQV combinations. In contrast, the activities of the CQ-LPV and CQ-RTV combinations were not dependent on the pfcrt allele carried by the parasite, as an antagonistic interaction was observed with these combinations against all transgenic parasites.

These findings differ somewhat from those of previous studies, which reported synergistic and additive (rather than additive and antagonistic) interactions when SQV-CQ combinations were assessed against CQ-resistant and CQ-sensitive strains of P. falciparum, respectively (13, 14, 31). This apparent anomaly may be due to the genetic background shared by the
transgenic parasite lines. All of the previous data reporting synergy between CQ and SQV or CQ and RTV were generated using Dd2 parasites. Likewise, the additive effect of the CQ-SQV combination in CQ-sensitive parasites has been shown only with 3D7 parasites. Although GCO3 parasites are the progeny of an HB3 × Dd2 cross, they nevertheless contain key differences from their CQ-resistant Dd2 parent. For example, the GCO3 line possesses a single pfmdr1 (P. falciparum multi-drug resistance transporter 1) allele, whereas the Dd2 line contains four (7, 36). Moreover, the Dd2 PfMDR1 protein differs from the GCO3 version at positions 86 (Y instead of N) and 1042 (N instead of D) (28, 33, 36). Since PfMDR1 is thought to play a role in mediating drug resistance in the parasite (26, 28), it is conceivable that the single copy number and sequence variation harbored by the GCO3 lines affected the ability of SQV and RTV to potentiate CQ activity. For instance, perhaps overexpression of the Dd2 variant of PfMDR1 serves to provide an additional route for the influx of APIs into the digestive vacuole—leading to increased levels of APIs in the compartment where they can compete with CQ for efflux via PfCRT—and this route is diminished or abolished in GCO3 parasites. It should also be noted that the version of PfMDR1 expressed by the 3D7 line differs from that carried by the GCO3 lines, at position 1042 (N instead of D) (9). Thus, the activity of the CQ-API combinations had not previously been tested against the form of PfMDR1 carried by the GCO3 lines. In this regard, it is worth noting that Yuan and colleagues reported that LPV and, to a lesser extent, SQV displayed a 5-fold or more difference in antimalarial activity between P. falciparum strains from different backgrounds and/or geographical regions (38). Further analysis of the differential sensitivity patterns for LPV revealed that the magnitude of its IC_{50} was significantly associated with mutations in PfCRT and PfMDR1 and that P. falciparum lines which differed only in the version of PfMDR1 that they carried showed significantly different susceptibilities to LPV. These findings support the idea that the response of GCO3 lines to APIs is likely to differ from those of Dd2 and 3D7 and that this could be, at least in part, to the different forms and copy numbers pfmdr1 harbored by these parasites. Other differences between GCO3 and Dd2 (and between GCO3 and 3D7) may also cause CQ-API combinations to exert differential activity upon these strains. This is the first study that has attempted to control for these variables by testing CQ-API combinations against the C2GCO3, C4Dd2, and C67G8 lines, which differ only in the version and/or copy number pfmdr1.

Consistent with its ability to act in concert with CQ in the C4Dd2 and C67G8 lines and to antagonize its activity in the C2GCO3 line, SQV was found to increase the accumulation of CQ in the former and cause a reduction in CQ accumulation in the latter (Fig. 2). Indeed, SQV displayed resistance reversal activity in the C67G8 line that was comparable to that of verapamil. The presence of RTV or LPV caused modest to significant reductions in the accumulation of CQ in the three GCO3 lines, which also correlated well with their antagonistic interactions with CQ in these parasites. The finding that SQV, RTV, and LPV all caused a decrease in CQ accumulation in C2GCO3 parasites within the 1-h
independently experiments, within which measurements were made for 10 oocytes per treatment. Note that noninjected oocytes and oocytes expressing PfCRTCQS take up [3H]CQ to similar (low) levels via simple diffusion of the neutral species; this represents the “background” level of CQ accumulation in oocytes (refer to reference 20 for full data and a detailed discussion).

Incubation time of the assay is intriguing. Perhaps the APIs reduce the amount of heme (the target of CQ) in the digestive vacuole by inhibiting hemoglobin digestion, which in turn would lead to a decrease in the accumulation of heme-CQ complexes in the vacuole. Alternatively, the APIs may affect CQ accumulation by influencing the pH gradient and/or membrane potential across the vacuole membrane.

Experiments using the Xenopus oocyte system revealed that SQV and, to a lesser extent, RTV interact directly with Dd2 PfCRTCQR to inhibit CQ transport. SQV displayed significant activity against Dd2 PfCRTCQR, with an IC50 that was about half that measured for verapamil (Fig. 3) (20). The fact that SQV acts as a CQ resistance reverser in C67G8 parasites (Fig. 2) suggests that it also has the ability to inhibit the transport of CQ via 7G8 PfCRTCQR. It is likely that in order to reverse CQ resistance, SQV gains access to and accumulates within the digestive vacuole via simple diffusion and/or carrier-mediated transport. Like verapamil and the quinoline drugs, SQV can behave as a weak base (pKa values of 1.1 and 7.1 [10, 16] (Fig. 4) and is therefore expected to accumulate in the acidic environment of the vacuole via weak-base trapping. In contrast, the pKa values for LPV and RTV are below 3 (10, 18) (Fig. 4), and hence these compounds are expected to be largely nonionized in the acidic environment of the vacuole due to weak-acid trapping. As a result, the APIs may affect CQ accumulation by decreasing the amount of heme (the target of CQ) in the digestive vacuole and therefore not subject to weak-acid trapping within this compartment. LPV and RTV are also less lipophilic than SQV and are less likely to transverse membranes via simple diffusion in their uncharged states (10). Furthermore, SQV more closely mimics the peptidomimetic design of classic protease inhibitors (whereas RTV and LPV were designed to be more stable and less peptidomimetic in nature); this may explain in part its superior ability in blocking PfCRTCQR (37), since this protein has previously been shown to be inhibited by a number of peptides (20).

Differences in the physicochemical properties of SQV and verapamil may also explain why SQV is the more potent inhibitor of Dd2 PfCRTCQR in the oocyte system (Fig. 3) (20) but less effective than verapamil at increasing the accumulation of CQ in C4Dd2 parasites (Fig. 2). For instance, verapamil (pKa of ~9 [11]) is expected to reach a higher concentration than SQV within the vacuole via weak-base trapping. In any case, the fact that SQV caused a decrease in CQ accumulation in the C2CQS line makes it difficult to compare its resistance-reversing activities in the C4Dd2 and C6G8 parasites with those of verapamil; the observed level of CQ accumulation in CQ-resistant parasites treated with SQV is likely to be the net result of SQV’s ability to increase accumulation via inhibition of PfCRTCQR while simultaneously causing a decrease in accumulation via an as yet unknown mechanism.

Taken together, these findings provide new insights into why CQ-SQV combinations are more effective against CQ-resistant P. falciparum parasites than against CQ-sensitive strains. The data demonstrate that in addition to its intrinsic antiplasmodial activity, SQV can act as a CQ resistance reverser, and that it exerts this effect (at least in part) by inhibiting the transport of CQ via PfCRTCQR. Hence, SQV can be considered a potential dual-function antimalarial that could be used in combination with CQ (or other quinoline antimalarials) against P. falciparum. The clinical consequences of this CQ resistance-reversing activity have yet to be explored. CQ and SQV are both largely discarded drugs—the emergence of resistant parasites has rendered CQ largely ineffective (except when double-dose regimens are employed [35]), and SQV has been superseded by next-generation APIs. Nevertheless, new CQ-like drugs that are effective against CQ-resistant parasites are being developed (6, 15, 17, 24) and are undergoing clinical trials (22), and these could be paired with a next-generation SQV that possesses intrinsic antimalarial activity as well as the ability to inhibit mutant PfCRT. This would place an additional...
selection pressure on the parasite and its quinoline resistance mechanism and may thereby extend the longevity of both the quinoline antimalarial and its API partner drug.

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