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 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 mu-

tant, resistance-conferring form of PfCRT (PfCRT[CQR]) transports CQ, whereas the wild-type form of the protein, found in CQ-sensitive parasites (PfCRT[CQS]), does not (20). These data support the hypothesis that PfCRT[CQR] confers CQ resistance by exporting CQ out of the digestive vacuole, away from its primary site of action. PfCRT[CQR]-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[CQR] (20). This finding raises the possibility that peptidomimetic drugs such as the APIs also interact with PfCRT[CQR] and that the CQ-API synergy observed in CQ-resistant parasites results from APIs blocking PfCRT[CQR]-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.

doi:10.1128/AAC.00166-12

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). $C4^{Dd2}$ and $C6^{7G8}$ were generated by replacing the wild-type *pfcrt* GCO3 allele with the CQ resistance-conferring Dd2 and 7G8 alleles, respectively. $C2^{GCO3}$, 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]hypoxanthine (0.5 μCi/well; Amersham International, England). After 48 h, cells were harvested onto filter mats (Harvester 96; Tomtec Incorporated), and the mats were counted in a model 1450 MicroBeta Plus liquid scintillation counter. All such experiments were performed on at least three separate occasions.

Isobolograms were constructed using data from all experiments. Briefly, the concentration of each drug that alone or in combination resulted in 50% inhibition of parasite growth (i.e., the IC_{50}) was plotted as the fractional inhibitory concentration (FIC) (3). Using the SAAM II program (SAAM Institute, Seattle, WA), a standard hyperbolic function (4), i.e., $Y_i = 1 - \{X_i/[X_i + e^{-I} \times (1 - X_i)]\}$ (where Y_i is the IC_{50} for drug A combined with drug B, X_i is the IC_{50} 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 are more effective against CQ-resistant lines than against CQ-sensitive parasites. Isobolograms describing the interactions of CQ with SQV against CQ-sensitive ($C2^{GCO3}$) and CQ-resistant ($C4^{Dd2}$ and

$C6^{7G8}$) 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 PfCRT^{CQR} allele, an additive interaction was observed (Fig. 1) ($I = -0.1$; $P > 0.1$). 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 $C2^{GCO3}$, $C4^{Dd2}$, and $C6^{7G8}$ lines in the presence or absence of verapamil, SQV, RTV, and LPV. Under control conditions, the CQ accumulation ratios for the CQ-resistant $C4^{Dd2}$ and $C6^{7G8}$ lines were 14.1 ± 2.0 and 13.7 ± 2.7 times lower (mean $\pm$ standard error of the mean [SEM]; $n = 4$), respectively, than that measured for the CQ-sensitive $C2^{GCO3}$ line (data not shown). Verapamil (1 μM) had no effect on CQ accumulation in the $C2^{GCO3}$ parasites but caused a 2.5- $\pm$ 0.2-fold increase in the CQ accumulation ratio in the $C4^{Dd2}$ line and a less dramatic, 1.8- $\pm$ 0.1-fold increase in the $C6^{7G8}$ 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 $C4^{Dd2}$ and $C6^{7G8}$ 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 $C2^{GCO3}$ 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 $C2^{GCO3}$ 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 $C2^{GCO3}$ line and to enhance its activity in $C4^{Dd2}$ and $C6^{7G8}$ parasites (Fig. 1).

Inhibition of chloroquine transport via PfCRT^{CQR} 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 PfCRT^{CQR}, as shown previously for verapamil (20). The expression of PfCRT in *X. laevis* oocytes enables direct measurements of CQ transport via PfCRT^{CQR}, 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-
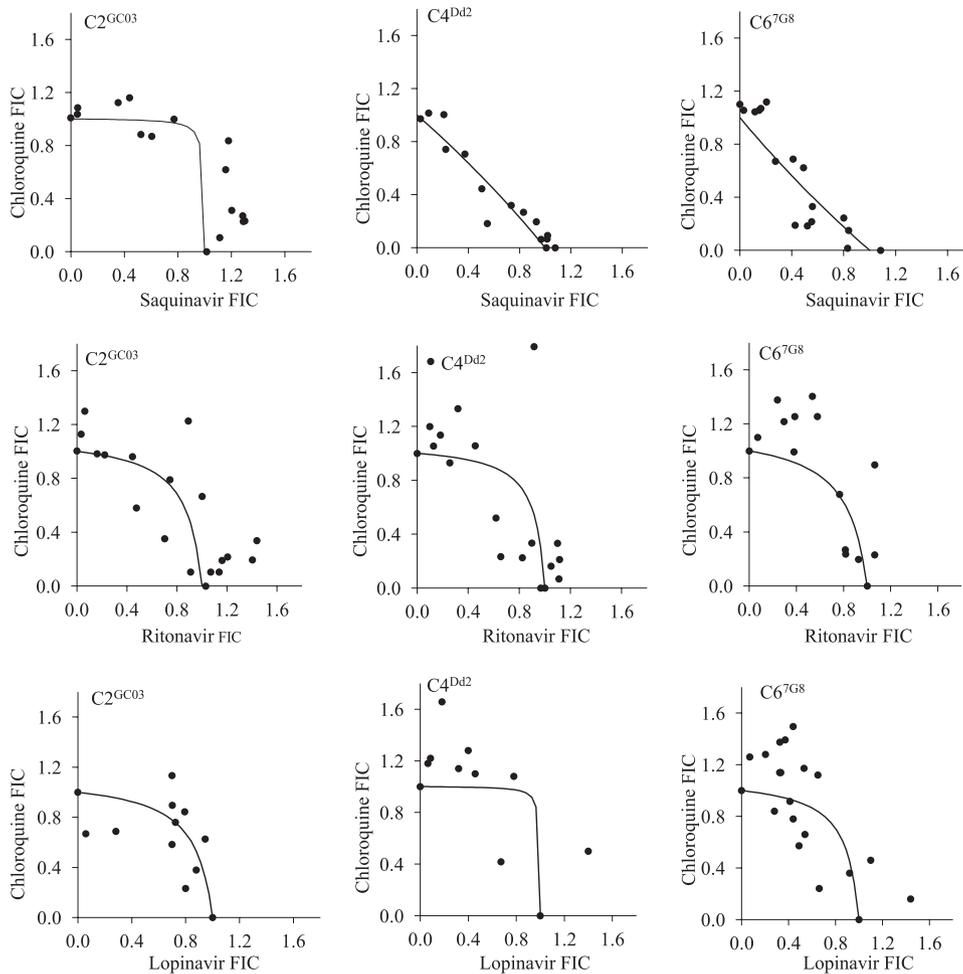
FIG 1 Isobolograms describing the interactions of SQV with CQ, RTV with CQ, and LPV with CQ against erythrocytes infected with C2[GCO3], C4[Dd2], or C6[7G8] parasites. Titration experiments were performed at least three times in triplicate. Data derived from all experiments are shown. Interaction values were derived from all data, and 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. Interaction values for chloroquine and saquinavir combinations were calculated to be -4.7 ($P < 0.0005$), -0.1 ($P > 0.1$), and 0.1 ($P > 0.1$) for C2[GCO3], C4[Dd2], and C6[7G8] parasites, respectively. Interaction values for chloroquine and ritonavir combinations were calculated to be -2.2 ($P < 0.01$), -2.5 ($P > 0.01$), and -1.9 ($P > 0.01$) for C2[GCO3], C4[Dd2], and C6[7G8] parasites, respectively. Interaction values for chloroquine and lopinavir combinations were calculated to be -2.1 ($P < 0.001$), <-5.0 ($P > 0.001$), and -2.3 ($P > 0.05$) for C2[GCO3], C4[Dd2], and C6[7G8] parasites, respectively.

fects 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 IC$_{50}$ of 13 $\pm$ 1 μM (mean $\pm$ SEM; $n =$ 4) (Fig. 3b). This value is significantly lower than that measured for verapamil (30 $\pm$ 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 C2[GCO3] parasite line, whereas an additive interaction occurred against the CQ-resistant parasite lines C4[Dd2] and C6[7G8] (Fig. 1). These three transgenic cell lines differ only in the version of *pfcrt* that 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
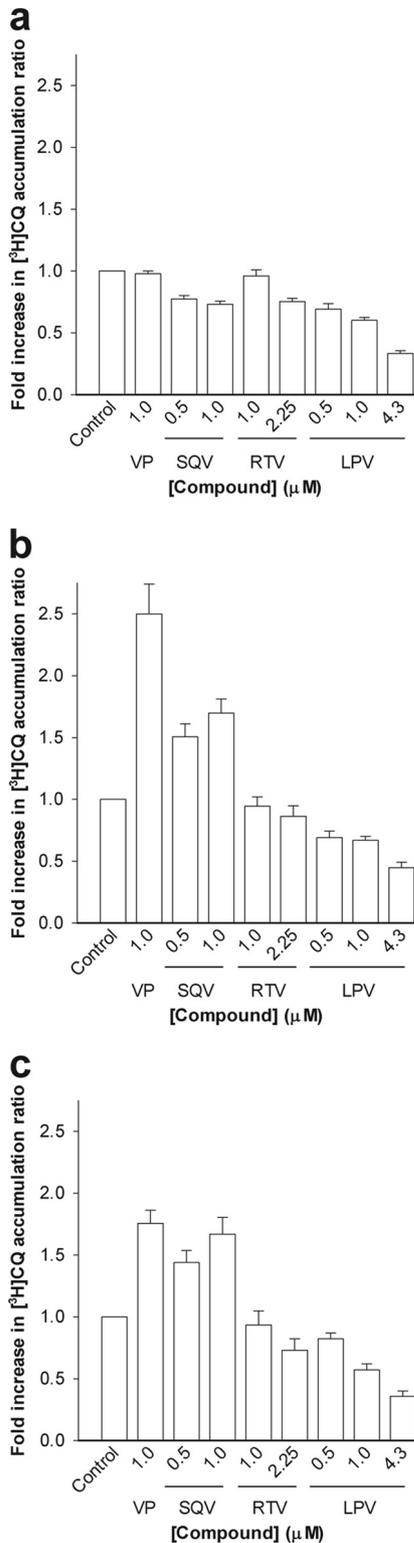
FIG 2 Effects of SQV, RTV, and LPV on [^{3}H]CQ accumulation by erythrocytes infected with mature trophozoite-stage parasites. Panels a, b, and c show data for the parasite lines C2^{GCO3}, C4^{Dd2}, and C6^{7G8}, respectively. The accumulation assays were performed over 1 h at 37°C and with an initial [^{3}H]CQ concentration of 20 nM. CQ accumulation is expressed in terms of the fold difference in the [^{3}H]CQ accumulation ratio (i.e., the concentration of radiolabeled CQ within the infected cells relative to the concentration in the extracellular medium). The data represent the means plus SEM for four independent experiments.

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* multidrug 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 antiplasmodial 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 due, at least in part, to the different forms and copy numbers of *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 C2^{GCO3}, C4^{Dd2}, and C6^{7G8} lines, which differ only in the version of PfCRT that they express. Given that PfMDR1 is also likely to play a role in the parasite's response to CQ-API combinations, it would be interesting to evaluate these combinations in lines of parasites that differ only in the version and/or copy number of *pfmdr1*.

Consistent with its ability to act in concert with CQ in the C4^{Dd2} and C6^{7G8} lines and to antagonize its activity in the C2^{GCO3} 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 C6^{7G8} 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 C2^{GCO3} parasites within the 1-h
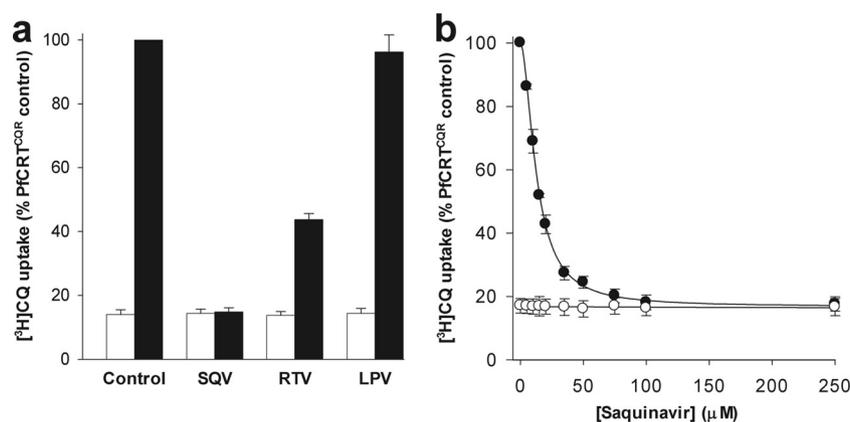
FIG 3 Effects of APIs on CQ uptake via PfCRT^{CQR}. (a) [^{3}H]CQ uptake into oocytes expressing Dd2 PfCRT^{CQR} (black bars) or D10 PfCRT^{CQS} (white bars) in the presence of 500 μM unlabeled SQV, RTV, or LPV. SQV and RTV ($P < 0.001$; ANOVA), but not LPV ($P > 0.05$; ANOVA), caused significant reductions in CQ uptake via PfCRT^{CQR}. Rates of CQ uptake (pmol h^{-1} per oocyte; $n = 4$) under control conditions were 0.87 ± 0.05 and 6.4 ± 0.5 (mean $\pm$ SEM) for PfCRT^{CQS}- and PfCRT^{CQR}-expressing oocytes, respectively. (b) Concentration-dependent inhibition by SQV of the uptake of [^{3}H]CQ into oocytes expressing Dd2 PfCRT^{CQR} (solid circles) or D10 PfCRT^{CQS} (open circles). The IC$_{50}$ derived from these data (13 ± 1 μM) was obtained by least-squares fit of the equation $Y = Y_{min} + \{(Y_{max} - Y_{min})/(1 + ([\text{inhibitor}]/\text{IC}_{50})^C)\}$, where Y is PfCRT^{CQR}-mediated CQ transport, Y_{min} and Y_{max} are the minimum and maximum values of Y, and C is a constant. PfCRT^{CQR}-mediated CQ transport was calculated by subtracting the uptake measured in oocytes expressing D10 PfCRT^{CQS} from that in oocytes expressing Dd2 PfCRT^{CQR}. In both panels, uptake is shown as the mean $\pm$ SEM for four independent experiments, within which measurements were made for 10 oocytes per treatment. Note that noninjected oocytes and oocytes expressing PfCRT^{CQS} take up [^{3}H]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 PfCRT^{CQR} to inhibit CQ transport. SQV displayed significant activity against Dd2 PfCRT^{CQR}, with an IC$_{50}$ that was about half that measured for verapamil (Fig. 3) (20). The fact that SQV acts as a CQ resistance reverser in C6^{7G8} parasites (Fig. 2) suggests that it also has the ability to inhibit the transport of CQ via 7G8 PfCRT^{CQR}. 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 (pK$_a$ values of 1.1 and 7.1 [K_a is the acid dissociation constant] [10, 16]) (Fig. 4) and is therefore expected to accumulate in the acidic environment of the vacuole via weak-base trapping. In contrast, the pK$_a$ values for LPV and RTV are below 3 (10, 18) (Fig. 4), and hence these compounds are expected to be largely nonionized within the digestive vacuole and therefore not subject to weak-base 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 PfCRT^{CQR} (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 PfCRT^{CQR} in the oocyte system (Fig. 3) (20) but less effective than verapamil at increasing the accumulation of CQ in C4^{Dd2} parasites (Fig. 2). For instance, verapamil (pK$_a$ 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 C2^{GCO3} line makes it difficult to compare its resistance-reversing activities in the C4^{Dd2} and C6^{7G8} 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 PfCRT^{CQR} 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 PfCRT^{CQR}. 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
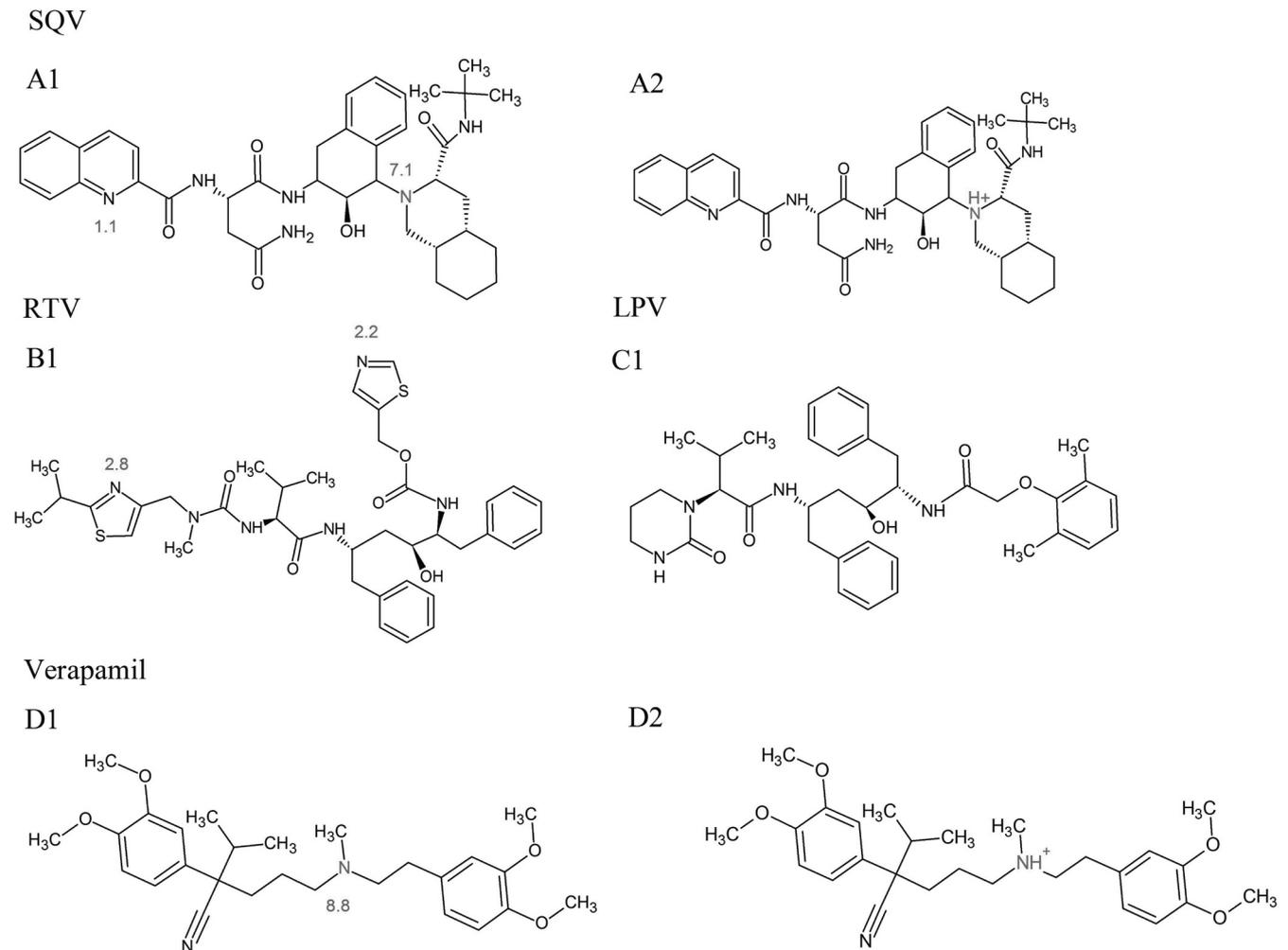
FIG 4 Chemical structures for SQV, RTV, LPV, and verapamil, with protonation sites relevant to digestive vacuole accumulation via weak-base trapping. At pH 7.3 (cytosolic pH of the parasite [27]), approximately half of the SQV would be positively charged (species A2), but this would increase to ~100% at pH 5.0 (the approximate pH of the parasite digestive vacuole [12]). In contrast, both RTV (B1) and LPV (C1) would be predominantly in an uncharged form at pH 7.3 and pH 5.0. Verapamil would for the most part be positively charged at pH 7.3 (~96% species D2), and at pH 5.0, it would be present almost exclusively as the positively charged species (D2).

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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