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 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) on 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.
INTRODUCTION

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/malaria co-infected 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 co-infection 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 \textit{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 \textit{P. falciparum} (14). However, analysis of the activity of CQ-API combinations against CQ-sensitive \textit{P. falciparum} parasites revealed that: (i) the degree of synergy varies amongst the APIs, with RTV showing the greatest degree of synergism; (ii) the synergistic effect of RTV on CQ activity against CQ-resistant \textit{P. falciparum} parasites is greater than that measured in CQ-sensitive parasites; and (iii) although LPV, SQV, nelfinavir and atazanavir behave synergistically with CQ against CQ-resistant \textit{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 be acting as CQ-resistance reversers in \textit{P. falciparum}. The primary determinant of CQ resistance in \textit{P. falciparum} is mutations in 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 \textit{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\(^{\text{CQR}}\) confers CQ resistance by exporting CQ out of the digestive vacuole, away from its primary site of action. PfCRT\(^{\text{CQR}}\)-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\(^{\text{CQR}}\) (20). This finding raises the possibility that peptidomimetic drugs such as the APIs also interact with PfCRT\(^{\text{CQR}}\), and that the CQ-API synergy observed in CQ-resistant parasites results from APIs blocking PfCRT\(^{\text{CQR}}\)-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 effect 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. (2002) and were generously provided by Dr David Fidock (Columbia University, New York). These transgenic lines were generated from GC03, a CQ-sensitive clone, derived from HB3 (36). C4Dd2 and C67G8 were generated by replacing the wild-type pfcrt GC03 allele with the CQ-resistance-conferring Dd2 or 7G8 alleles, respectively. C2GC03, the CQ-sensitive transgenic control, contains the wild-type GC03 pfcrt allele. All P. falciparum parasites were maintained in either static (34) or shaking culture (1) 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/combination assays

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

Antimalarial drug combinations were assessed by isobologram analysis as described previously (30). All assays were performed in 96-well micro-titre 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 hours cells were harvested onto filter mats (Harvester 96, Tomtec Incorporated, Orange) and the mats were counted in a 1450 MicroBeta Plus Liquid Scintillation Counter. All such experiments were performed on at least two separate occasions.

Isobolograms were constructed using data from all experiments. Briefly, the concentrations of each drug that alone, or in combination, resulted in 50% inhibition of parasite growth (i.e. the IC₅₀) were plotted as a ‘fractional inhibitory concentration’ (3). Using the SAAM II program (SAAM Institute, Seattle, WA), a standard hyperbolic function (4) \( Y_i = 1 - \left[ \frac{X_i}{(X_i + e^{-(i)}) \times (1 - X_i)} \right] \) (where \( Y_i \) is the IC₅₀
for drug A when combined with drug B, $X_i$ is the IC$_{50}$ for drug B when 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 $I = 0$ indicates that the effects of the two compounds are simply additive. The significance of the difference of $I$ from zero ($P > 0.01$ indicates an additive interaction) was assessed using Student’s $t$-test.

**Measurements of chloroquine accumulation in P. falciparum-infected erythrocytes**

The accumulation of [$^3$H]CQ (20 nM; 20 Ci/mm; American Radiolabeled Chemicals) in mature trophozoite-infected erythrocytes (~36 h post-invasion) was measured using a protocol described in full elsewhere (19).

**Measurements of chloroquine transport in X. laevis oocytes expressing PfCRT**

Expression of mutant and wildtype forms of PfCRT (from the 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 [$^3$H]CQ (0.3 μM; 15 Ci/mm; Moravek) was measured 4-6 days post-injection as described (20). Uptake measurements were made over 1-2 hr at 25°C, in medium that contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 10 mM MES, 10 mM Tris-base (pH 6.0) and 15 μM unlabelled CQ. Statistical comparisons were made with the Student’s $t$-test for unpaired samples or with ANOVA in conjunction with Tukey’s multiple comparisons test.
RESULTS

Combinations of chloroquine with saquinavir are more effective against CQ-resistant lines than CQ-sensitive parasites

Isobolograms describing the interactions of CQ with SQV against the CQ-sensitive (C2GCO3) and CQ-resistant (C4Dd2 and C67G8) lines demonstrate that the antiplasmodial activity of these drugs when used in combination is dependent on the PfCRT allele expressed. When combinations of SQV and CQ were assessed against parasites expressing the Dd2 PfCRTCQR allele, an additive interaction was observed (Figure 1; I=-0.1; P>0.1). However, this same combination behaved antagonistically in control parasites expressing the wild-type GC03 allele (Figure 1; I=-4.7; P<0.0005). By contrast, combinations of CQ with RTV or LPV behaved antagonistically against all transgenic parasites (Figure 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 re-sensitize 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 verapamil and other resistance-reversers, CQ accumulation was measured in the C2GCO3, C4Dd2, and C67G8 lines in the presence or absence of verapamil, SQV, RTV, and LPV. Under control conditions, the CQ accumulation ratios for the CQ-resistant C4Dd2 and C67G8 lines were 14.1 ± 2.0 and 13.7 ± 2.7 times lower, respectively, than that measured in the CQ-sensitive C2GCO3 line (mean ± SEM, n = 4; data not shown). Verapamil (1 µM) had no effect on CQ accumulation in the C2GCO3 parasites, but caused a 2.5 ± 0.2 fold increase in the CQ accumulation ratio in the C4Dd2 line, and a less dramatic 1.8 ± 0.1 fold increase in the C67G8 line (P < 0.05, paired t tests; Figure. 2A-C), a finding that is consistent with previous reports (20). SQV (0.5 or 1 µM) also increased CQ accumulation in the CQ-resistant lines, with a similar fold-increase (1.5-1.7 fold) in both the C4Dd2 and C67G8 parasites (P >
By 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 a similar fold across all three stains. Indeed, all of the APIs tested – including SQV – significantly decreased the CQ accumulation ratio in C2GCO3 parasites (P < 0.01, paired t tests), the one exception being the 1 µM RTV treatment, which was not statistically different from the C2GCO3 control. Thus, SQV decreased the CQ accumulation ratio in the CQ-sensitive line by 1.3-1.4 fold, yet caused a 1.5-1.7 fold increase in the resistant lines, consistent with the observed ability of SQV to antagonize CQ activity in the C2GCO3 line and to enhance its activity in C4Dd2 and C67G8 parasites (Figure 1).

Inhibition of chloroquine transport via PfCRT\textsuperscript{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\textsuperscript{CQR}, as has previously been shown for verapamil (20). The expression of PfCRT in X. laevis oocytes enables direct measurements of CQ transport via PfCRT\textsuperscript{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 5-5.5; (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 effects such as the binding of drugs to haem 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\textsuperscript{CQR}, RTV reduced PfCRT\textsuperscript{CQR}-mediated transport by ~66%, whereas LPV had no effect (Figure 3A). An analysis of the concentration-dependent inhibition of CQ transport by SQV yielded an IC\textsubscript{50} value of 13 ± 1 µM (mean ± s.e.m.; n = 4; Figure. 3B). This value is significantly lower than that measured for verapamil (30 ± 3 µM (20); P < 0.01, unpaired t-test), which makes SQV the most potent inhibitor of PfCRT\textsuperscript{CQR} identified to date.
DISCUSSION

Understanding the pharmacokinetic and pharmacodynamics 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 two CQ-resistant parasite lines C4Dd2 and C67G8 (Figure 1). These three transgenic cell lines differ only in the version of pfCRT they harbor – wild-type, 7G8, or Dd2. 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. By 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 work reporting synergy between CQ and SQV, or CQ and RTV, was generated using Dd2 parasites. Likewise, the additive effect of the CQ-SQV combination in CQ-sensitive parasites has only been shown with 3D7. Although GCO3 is a progeny of a HB3 X Dd2 cross, it nevertheless contains key differences from its CQ-resistant Dd2 parent. For example, GCO3 possesses a single pfmdr1 (P. falciparum multidrug resistance transporter 1) allele whereas Dd2 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 variant 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 the API in the compartment in which it can compete with CQ for efflux via PfCRT.
and that this route is diminished or abolished in GCO3 parasites. It should also be noted that the version of PfMDR1 expressed by 3D7 differs from that carried by GCO3 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 \textit{P. falciparum} strains from different backgrounds and/or geographical regions (38). Further analysis of the differential sensitivity patterns to LPV revealed that the magnitude of its IC$_{50}$ value was significantly associated with mutations in PfCRT and PfMDR1, and that \textit{P. falciparum} lines which differed only in the version of PfMDR1 they carried showed significantly different susceptibilities to LPV. These findings support the idea that the response of GCO3 lines to APIs are likely to differ from that of Dd2, and 3D7, and that this could be due, at least in part, to the different forms and copy numbers of \textit{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 activities upon these strains. This is the first study that has attempted to control for these variables by testing CQ-API combinations against the C$_{2}$GCO3, C$_{4}$Dd2, and C$_{6}$7G8 lines, which differ only in the version of PfCRT 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 \textit{pfmdr1}.

Consistent with its ability to act in concert with CQ in the C$_{4}$Dd2 and C$_{6}$7G8 lines and to antagonize its activity in the C$_{2}$GCO3 line, SQV was found to increase the accumulation of CQ in the former and cause a reduction in CQ accumulation in the latter (Figure 2). Indeed, SQV displayed resistance-reversal activity in the C$_{6}$7G8 line that was comparable to that of verapamil. The presence of RTV or LPV caused modest to significant reductions in the accumulation of CQ in the three GC03 lines, which also correlated well with their antagonistic interactions with CQ in these parasites. The finding that SQV, RTV, and LPV all cause a decrease in CQ accumulation in C$_{2}$GCO3 parasites within the one-hour incubation of the assay is intriguing. Perhaps the APIs reduce the amount of haem (the target of CQ) in the digestive vacuole by
inhibiting haemoglobin digestion, which in turn would lead to a decrease in the accumulation of haem-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\textsuperscript{CQR} to inhibit CQ transport. SQV displayed significant activity against Dd2 PfCRT\textsuperscript{CQR}, with an IC\textsubscript{50} value that is about half that measured for verapamil (Figure 3) (20). The fact that SQV acts as a CQ resistance-reverser in C6\textsuperscript{7G8} parasites (Figure 2) suggests that it also has the ability to inhibit the transport of CQ via 7G8 PfCRT\textsuperscript{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\textsubscript{a} 1.1 and 7.1), where K\textsubscript{a} is the acid dissociation constant; (10, 16) (Figure 4), and is therefore expected to accumulate in the acidic environment of the vacuole via ‘weak-base trapping’. By contrast, the pKa values for LPV and RTV are below 3 (10, 18) (Figure 4), and hence these compounds are expected to be largely non-ionized 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 would be 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 in part explain its superior ability in blocking PfCRT\textsuperscript{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\textsuperscript{CQR} in the oocyte system (Figure 3) (20), but less effective than verapamil in increasing the accumulation of CQ in C4\textsuperscript{DAG} parasites (Figure 2). For instance, verapamil (pK\textsubscript{a} ~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\textsuperscript{C03} line makes it difficult to compare its resistance-reversing activities in the C4\textsuperscript{DAG} and C6\textsuperscript{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 its ability to increase accumulation via inhibition of PfCRT\textsuperscript{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 \textit{P. falciparum} parasites than in 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\textsuperscript{CQR}. Hence, SQV can be considered as a potential dual-function antimalarial that could be used in combination with CQ (or other quinoline antimalarials) against \textit{P. falciparum}. The clinical consequences of this CQ resistance-reverser activity are 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) – 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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Conflict of interests:

The authors of this article do not have a commercial or other association that may pose a conflict of interest.

REFERENCES:


FIGURE LEGENDS

Figure 1: Isobolograms describing the interaction of SQV with CQ, RTV with CQ, and LPV with CQ against erythrocytes infected with C2GC03, C4Dd2 or C67G8 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>0.01 indicates 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 C2GC03, C4Dd2 and C67G8 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 C2GC03, C4Dd2 and C67G8 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 C2GC03, C4Dd2 and C67G8 respectively.

Figure 2: Effects of SQV, RTV, and LPV on [3H]CQ accumulation by erythrocytes infected with mature trophozoite-stage parasites. Panels (A), (B), and (C) show data for the parasite lines C2GC03, C4Dd2, and C67G8, respectively. The accumulation assays were performed over 1 h at 37°C and with an initial [3H]CQ concentration of 20 nM. CQ accumulation is expressed in terms of the fold difference in the [3H]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 (+ s.e.m.) of four independent experiments.

Figure 3: Effects of APIs on CQ uptake via PfCRTCQR. (A) [3H]CQ uptake into oocytes expressing Dd2 PfCRTCQR (black bars) or D10 PfCRTCQS (white bars) in the presence of 500 μM unlabelled 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 PfCRTCQR. Rates of CQ uptake (pmol hour⁻¹ per oocyte; n = 4 ± s.e.m.) under control conditions were 0.87 ± 0.05 and 6.4 ± 0.5 for PfCRTCQS- and PfCRTCQR-expressing...
Concentration-dependent inhibition by SQV of the uptake of $[^3H]$CQ into oocytes expressing Dd2 PfCRT$^{CQR}$ (solid circles) or D10 PfCRT$^{CQS}$ (open circles). The IC$_{50}$ value derived from these data (13 ± 1 µM) was obtained by least-squares fit of the equation $Y = Y_{\min} + \left(\frac{Y_{\max} - Y_{\min}}{1 + ([\text{inhibitor}]/IC_{50})^{C}}\right)$, 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 ± s.e.m. from four independent experiments, within which measurements were made from 10 oocytes per treatment. Note that non-injected oocytes and oocytes expressing PfCRT$^{CQS}$ 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 (20) for full data and a detailed discussion).

Figure 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)). By contrast, both RTV (B1) and LPV (C1) would be predominantly in an uncharged form at pH 7.3 and pH 5.0. Verapamil would be for the most part positively charged at pH 7.3 (~96% species D2) and at pH 5.0 would be present almost exclusively as the positively charged species (D2).
SQV
A1

RTV
B1

Verapamil
D1

A2

LPV
C1

D2