Anti-HIV Effects of Chloroquine

Inhibition of Viral Particle Glycosylation and Synergism With Protease Inhibitors

Andrea Savarino, MD, PhD,† Mothanje B. Lucia, MD,* Elena Rastrelli, MD,* Sergio Rutella, MD,‡ Caterina Golotta, MD,* Emanuella Morra, PhD,§ Enrica Tamburrini, MD,* Carlo Federico Perno,‖ Johan R. Boelaert, MD,¶ Kirk Sperber, MD,‡ and Roberto Cauda, MD, PhD**

Objective: We tested the effects of chloroquine (CQ) on glycosylation of HIV particles and in combination with protease inhibitors (PIs) on HIV replication and on P-glycoprotein (P-gp)/multidrug resistance protein-1 (MRP1).

Design: $CD4^+$ cell lines were infected with laboratory strains and peripheral blood mononuclear cells were infected with primary isolates for evaluation of the anti-HIV effects. Peripheral blood lymphocytes were evaluated for of P-gp and MRP1 functions.

Methods: HIV replication was assessed by enzyme-linked immunosorbent assay. HIV glycosylation was measured by metabolic labeling of viral particles with [^{3}H] glucosamine. Synergism was tested using isobolograms. P-gp and MRP1 functions were assayed using rhodamine 123 (Rh123) and carboxyfluorescein (CF) efflux assays, respectively.

Results: CQ alone inhibited HIV replication and glycosylation in a dose-dependent manner. In combination with indinavir (IDV), ritonavir, or saquinavir (SQV), CQ had a synergistic effect at concentrations found in plasma of subjects receiving malaria prophylaxis. CQ decreased the 50% effective concentration of IDV in primary isolates from Africa and restored the response to IDV or SQV in 3 PI-resistant isolates. CQ increased the block of Rh123 and CF efflux activity exerted by PIs.

Conclusion: The inhibitory effects of CQ on HIV glycosylation are associated with synergistic effects in combination with PIs. The CQ/PI combination exerts combined inhibitory effects on P-gp and MRP1 function.

Key Words: antimalarial, protease inhibitors, synergism, P-glycoprotein, multidrug resistance protein-1, developing countries

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From the *Department of Infectious Diseases, Università Cattolica del Sacro Cuore, Rome, Italy; ‡ Department of Hematology, Università Cattolica del Sacro Cuore, Rome, Italy; †Department of Clinical Immunology, Mount Sinai School of Medicine, New York, NY; §Department of Genetics, Biology, and Biochemistry, University of Turin, Turin, Italy; ‖Laboratory of Virology, Department of Experimental Medicine, University of Rome "Tor Vergata," Rome, Italy; and ¶Unit of Infectious Diseases, AZ Sint-Jan, Brugge, Belgium.

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Reprints: Andrea Savarino, Laboratory of Viral Immunology, Department of Infectious Diseases, Università Cattolica del Sacro Cuore, Largo Agostino Gemelli 8, I-00168 Rome, Italy (e-mail: asavarino@medscape.com).

Most areas with high levels of HIV seroprevalence also have endemic malaria. Chloroquine (CQ) remains the antimalarial drug most widely used in the world, despite the increasing diffusion of drug-resistant *Plasmodium falciparum* strains. More information is therefore needed on the effects of CQ on HIV replication and on the interactions of CQ with antiretroviral drugs.

CQ and its hydroxy-analogue hydroxychloroquine (HCQ) are endowed with anti-HIV effects, inhibiting the production of infectious viral particles at doses nontoxic for human cultured cells.[1–4] We have recently shown that CQ at nontoxic clinically reachable concentrations has in vitro activity against primary isolates belonging to different HIV-1 and HIV-2 clades.[2] CQ reduces the infectivity of newly produced virions and has no activity on other postulated targets such as HIV-1 entry, reverse transcriptase (RT), or integrase.[3] The antiviral effects of CQ are associated with the reduced production of the heavily glycosylated epitope 2G12, which is located on the gp120 envelope glycoprotein surface and is fundamental for virus infectivity.[2] Although no direct proof for inhibition of HIV glycosylation by CQ has been reported, it is clear that CQ/HCQ inhibits viral replication by a mechanism different from those of currently used antiretroviral drugs.[3] These considerations have led to the testing of CQ/HCQ in combination with antiretrovirals in clinical trials.[5]

CQ/HCQ has an in vitro and in vivo anti-HIV effect similar to zidovudine (AZT) alone and exerts an additive effect with the combination of either didanosine or AZT and hy-

droxyurea.[6–9] The combined effect of CQ/HCQ and protease inhibitors (PIs) on HIV-1 replication is unknown at present. Both drug families inhibit HIV replication at a postintegration stage, resulting in production of immature virions.[1,10] Moreover, both CQ/HCQ and PIs are substrates and, at varying levels, inhibit important cell surface drug transporters, that is, the P-glycoprotein (P-gp) and the multidrug resistance protein-1 (MRP1), which belong to the adenosine triphosphate (ATP)–binding cassette family and transport the intracellular pools of antiretrovirals to the extracellular compartment.[11–17]

The aims of the present study are (1) to clarify further the mechanisms of HIV inhibition by CQ and (2) to determine the in vitro effects of CQ in combination with PIs on HIV replication and on P-gp and MRP1 function.

MATERIALS AND METHODS

Drugs

Chloroquine and AZT were purchased from Sigma (St. Louis, MO). Among the HIV PIs, we chose the first-generation PIs indinavir (IDV), ritonavir (RTV), and saquinavir (SQV), because their cellular effects are the best studied and they will probably be the first PIs available on a large scale in resource-poor areas. The PIs were kindly provided by the manufacturers (IDV: Merck Sharp and Dohme Ltd, Hoddesdon, Hertfordshire, UK; SQV: Roche Registration Ltd, Welwyn Garden City, Hertfordshire, UK; and RTV: Abbott Laboratories Ltd, Queenborough, Kent, UK). They were dissolved in dimethyl sulfoxide (DMSO; maximum content = 0.5% vol/vol).

Description of the Viruses Employed and Infection Assays

We used both laboratory-adapted strains and primary isolates. The laboratory-adapted HIV-1_{IIIB} and HIV-$2_{CBL/20}$ strains and the primary isolates HIV-1_{UG3} (clade A, R5) and HIV-$1_{VI\ 829}$ (clade C, R5) from antiretroviral-naive African subjects were obtained as previously described.[2,18] HIV-$1_{PAVIA11}$ and HIV-$1_{PAVIA12}$ (clade B, coreceptor usage undetermined) were isolated from Italian individuals with highly active antiretroviral therapy (HAART) failure and possess a genotypic profile of multidrug resistance.[19] Protease resistance mutations were L10I, M46I, L63P, G73T, V77IV, I84V, and L90M in HIV-$1_{PAVIA11}$ and L10I, M46L, I54V, L63V, A71T, V82A, and I93L in HIV-$1_{PAVIA12}$, whereas RT resistance mutations were M41L, E44D, D67N, T69D, K103N, V108I, V118I, M184V, L210W, T215Y, F227FL, and K238KT in HIV-$1_{PAVIA11}$ and M41L, E44D, D67N, M184V, L210W, and T215Y in HIV-$1_{PAVIA12}$. HIV-$1_{SQV\text{-}resistant}$ (clade B, X4) has in vitro SQV-induced PI resistance[20] as confirmed by protease resistance mutations L10IV, M36I, G48V, L63V, L90M, and I93L. HIV-$1_{14\ A018\text{-}G910\text{-}6}$ and HIV-$1_{8\ A012\text{-}G691\text{-}6}$ were isolated in the late 1980s from human subjects after AZT monotherapy.[21] The profile of RT resistance mutations of these viruses is typical for high-level AZT resistance: HIV-$1_{14\ A018\text{-}G910\text{-}6}$ displayed M41L, D67N, K70R, T215Y, and K219Q, and HIV-$1_{8\ A012\text{-}G691\text{-}6}$ displayed D67N, K70R, V118I, T215F, and K219Q. Viral stocks were obtained and titrated as described elsewhere.[2,18]

HIV-genotype analysis was performed on the same viral stock suspensions as used for the acute infection experiments by means of a commercially available kit (Abbott). In brief, viral RNA was extracted by the QIAmp Viral RNA kit (Qiagen, Valencia, CA), retrotranscribed by murine leukemia virus RT, and amplified with amplitaq-Gold polymerase enzyme (Applied Biosystems, Foster City, CA) by using 2 different sequence-specific primers for 40 cycles.[22] *Pol*-amplified products (containing the entire protease and the first 324 amino acids of the RT open reading frame) were full-length sequenced in sense and antisense orientations by using 7 different overlapping sequence-specific primers by an automated sequencer (ABI 3100; Applied Biosystems).[22] The quality end point was ensured by coverage of the entire protease and RT sequences by at least 3 sequence segments.

In acute infection assays, the different cell types were incubated at 37°C for 2 hours with the viral stock suspensions at a multiplicity of infection (MOI) of approximately 0.1, unless otherwise specified. After 3 washes, cells were incubated in fresh culture medium for 7 days at 37°C, and cell-free supernatants at different intervals after infection were harvested for enzyme-linked immunosorbent assay (ELISA) measurement of HIV-1 p24 (NEN Life Science Products, Boston, MA) or HIV-2 p27 (Coulter, Hialeah, FL).[2] Where necessary, cells were loaded with CQ before virus adsorption onto cells (method a) as previously described[6] or incubated, after virus adsorption, in the presence of concentrations of CQ reachable in plasma of individuals receiving CQ prophylaxis/treatment (method b).[2]

The $CD4^+$ $CXCR4^+$ MT-4 T-cell line was used to assess the effects of CQ and PIs on the X4 laboratory-adapted strains, whereas peripheral blood mononuclear cells (PBMCs) obtained by informed consent from healthy donors and stimulated for 3 days with 2 μg/mL of phytohemagglutinin (PHA; Difco Laboratories, Detroit, MI) were adopted in assays using primary isolates.

To measure synergism, cell pellets were resuspended in media containing different combinations of CQ and IDV after viral adsorption onto cells. A fractional inhibitory concentration (FIC) was then calculated as the ratio: 90% effective concentration (EC_{90}) of drug A in combination with drug B / EC_{90} of drug A alone. The effect was considered to be synergistic when the sum of FICs was ≤ 0.5.

Assays for Evaluation of Toxicity

Cell proliferation, viability, and apoptosis were analyzed by [^{3}H] thymidine incorporation, by the methyltetrazolium (MTT) method, and by propidium iodide/annexin

V fluorescein isothiocyanate (FITC) staining, respectively, as determined by techniques previously validated in our laboratories.[23–25]

Measurement of HIV Glycosylation

Lymphocytic H9 cells were infected with HIV-1_{IIIB} or HIV-$2_{CBL/20}$ and then plated (6×10^5 cells per well) in macrowell cultures (35-mm diameter; Falcon, Oxnard, CA). We used H9 cells rather than MT-4 cells, the latter being constitutively infected with HTLV-1. After 3 days, cells were treated with different concentrations of CQ (0.1–1000 μM) for 1 hour to achieve steady-state conditions and subsequently labeled with [^{3}H] glucosamine. For labeling, the medium was discarded and the cells were washed 3 times and incubated with RPMI-1640 containing 5% fetal calf serum (FCS), penicillin/streptomycin, and [^{3}H] glucosamine (1 μCi/mL; Amersham, Arlington Heights, IL). The labeling medium was removed after 16 hours. Virus was isolated from the supernatant of the H9 treated and untreated cells by ultracentrifugation in sucrose density gradients using a K-6 rotor with a 20% to 60% (wt/vol) sucrose gradient. The supernatant containing the virus was precipitated with 10% trichloroacetic acid (TCA; Sigma) and passed over a glass fiber filter (particle retention of 1.2 μm; Whatman, Maidstone, UK). The filter was washed 3 times with 5% vol/vol TCA, dried with 100% methanol, and counted in a scintillation counter.[26]

Internal Labeling With [^{35}S] Cysteine and [^{35}S] Methionine and Immunoprecipitation

The same HIV-1_{IIIB}–infected H9 cells used for [^{3}H] glucosamine labeling were washed 3 times after loading with CQ, as described previously. The cells were then resuspended at a density of 10^7 cells/mL in cysteine- and methionine-free RPMI-1640 (Sigma) plus 10% dialyzed FCS, 1% glutamine, and antibiotics. The cells were then metabolically labeled with [^{35}S] cysteine and [^{35}S] methionine (NEN) at 50 μCi at a rate of 5×10^6 cells for 16 hours. After incubation, the viable cells were counted again. The cell suspensions were then centrifuged, and the supernatants were subjected to immunoprecipitation with human anti-gp120 antibodies as previously described.[25] The immunoprecipitated material was then separated by electrophoresis on a 10% polyacrylamide gel and visualized by autoradiography.

Determination of P-Glycoprotein and Multidrug Resistance Protein-1 Expression

For P-gp immunodetection, peripheral blood lymphocytes (PBLs) were incubated with the mouse monoclonal antibody (mAb) MRK-16 (Kamiya Biomedicals Company, Seattle, WA) as previously reported.[5] For MRP1 detection, we used the specific mAb MRPm6 (Kamiya Biomedicals) according to the manufacturer's instructions. Fluorescence was acquired using a FACScan flow cytometer (Becton Dickinson). The mean of the fluorescence of the MRK-16– or MRP1-labeled cells was divided by the mean of the fluorescence of cells stained with a control-irrelevant antibody.

Flow Cytometric Detection of P-Glycoprotein and Multidrug Resistance Protein-1 Efflux Activity

For study of P-gp function, we used the dihydrorhodamine 123 (Rh123) efflux assay as previously reported.[27] MRP1 transport activity was investigated using the carboxyfluorescein (CF) accumulation/efflux capacity.[28–30] Carboxy-2′,7′-dichlorofluorescein diacetate (CFDA; Sigma) is a nonfluorescent compound that freely diffuses into cells, where it is cleaved by esterases to give CF, a fluorescent dye handled by MRP1. Briefly, for accumulation experiments, PBLs were incubated with 10 μM of Rh123 or 1.5 μM of CFDA in the presence or absence of either IDV or RTV (1–10 μM) and/or CQ (1–12.5 μM) in RPMI-1640 for 60 minutes at 37°C, 5% CO_2. The cells were then washed in ice-cold phosphate-buffered saline (PBS), and Rh123 or CF accumulation was determined using a FACScan flow cytometer (Becton Dickinson). For efflux experiments, cells were resuspended in Rh123- or CF-free medium with or without drugs and incubated for 60 minutes at 37°C, 5% CO_2, and then washed in ice-cold PBS. For dual and triple fluorescence, Rh123- or CF-stained cells were counterstained with phycoerythrin (PE)- and peridinin chlorophyll protein (PerCP)–conjugated mAbs. The mean retained intracellular CF fluorescence intensity was then estimated using flow cytometry as for the accumulation experiments. In general, FACS data were expressed as mean fluorescence intensity (MFI), where the fluorescence intensity distribution was approximately Gaussian on a linear scale, or as the percentages of $Rh123^{bright/dim}$ and $CF^{bright/dim}$ cells, where the fluorescence intensity distribution was bimodal.

RESULTS

Chloroquine Inhibits HIV-1 and HIV-2 Replication by Impairing Virus Glycosylation

The effects of CQ/HCQ on HIV replication have been studied by 2 different approaches: (a) by loading cells for 1 hour with CQ/HCQ concentrations mimicking those observed in lymphocytes of individuals under chronic treatment with CQ/HCQ[1,6] and (b) by keeping cells under constant incubation with CQ concentrations clinically reachable in plasma.[2]

In a first step, we confirmed that CQ dose dependently inhibited HIV-1 and HIV-2 replication either in H9 cells treated with method a (HIV-1: $P < 0.01$, HIV-2: $P < 0.05$, t test for regression) or in MT-4 cells treated with method b (HIV-1: $P < 0.05$, HIV-2: $P < 0.05$, t test for regression; Fig. 1A). Moreover, both treatments at any of the CQ concentrations tested caused no increases in cell death or apoptosis or inhibition of cellular proliferation as determined by MTT and propidium iodide staining as well as by [^{3}H] thymidine incorporation (not

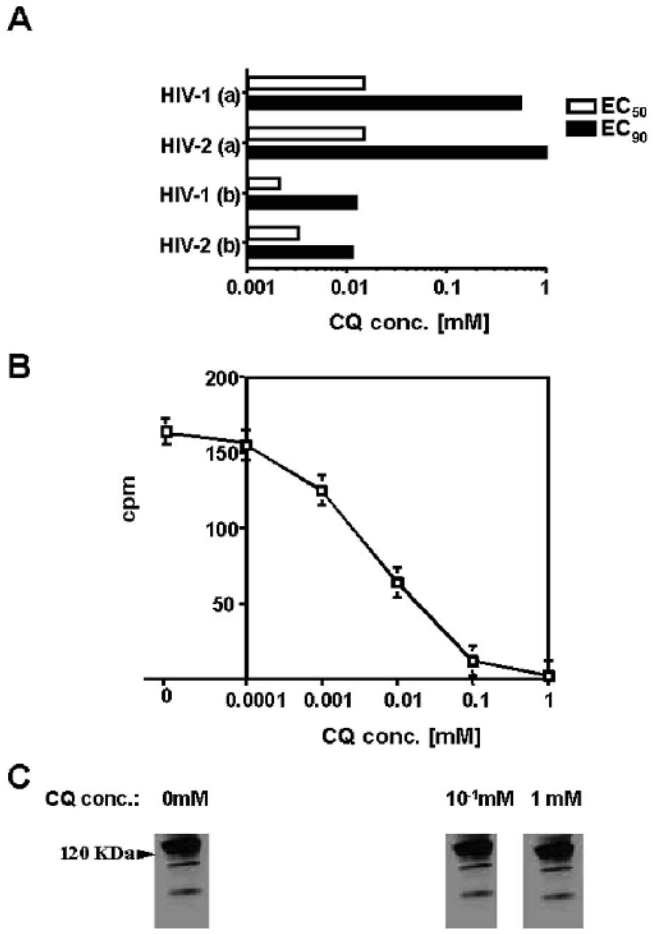

FIGURE 1. Anti-HIV effects of chloroquine (CQ). A, Concentrations of CQ inhibiting the 50% of viral replication (EC_{50}) and EC_{90} in acutely infected H9 cells preloaded with different CQ concentrations (method a) and in MT-4 cells constantly incubated with different CQ concentrations after viral adsorption onto cells (method b). B, Effects of CQ on HIV-1 glycosylation. C, Effects of CQ on the peptidic portion of the HIV-1 gp120 envelope glycoprotein. In (A), viral replication was measured 4 days after infection with HIV-1_{IIIB} or HIV-$2_{CBL/20}$ at a multiplicity of infection of 0.1. Results are presented as the EC_{50} and EC_{90} of CQ. Values represent medians from 3 independent experiments (ranges were <20% of the values reported). The method followed (method a or method b) is indicated in parentheses. In (B), 10^6 HIV-1_{IIIB}–infected cells were loaded with CQ according to method a and incubated for 16 hours with [^{3}H] glucosamine to label the carbohydrates. Virus was isolated from the supernatants and measured for ^{3}H activity in a scintillation counter. Results (mean ± SD from 3 experiments) are expressed as counts per minute and are normalized by the reverse transcriptase activity of supernatants from which the virus was isolated. In (C), 10^7 HIV-1_{IIIB}–infected cells were loaded with CQ according to method a and incubated for 16 hours with [^{35}S] cysteine/methionine to label the peptidic portion of gp120. The supernatants were then subjected to immunoprecipitation with anti-gp120 antibodies. The immunoprecipitated material was then separated by electrophoresis on a 10% polyacrylamide gel and visualized by autoradiography. One representative experiment of 3 experiments.

shown). We conclude that CQ efficiently and dose dependently inhibits HIV-1 and HIV-2 replication in the absence of toxic effects independent of the CQ administration method. These data reconcile the different methods used in the past to evaluate the anti-HIV effects of CQ, even if the intracellular concentrations of CQ reached by both methods are likely to differ because of differences in the cell lines of choice. These results allowed us to use either method in the present study.

Previous studies indicated that CQ impairs the formation of the heavily glycosylated epitope 2G12 on gp120 and excluded other mechanisms of HIV inhibition by CQ/HCQ.[2,6] Here, we investigated whether CQ might interfere with the glycosylation of viral particles. HIV-1– and HIV-2–infected cells were loaded with CQ for 1 hour (method a) and metabolically labeled with [^{3}H] glucosamine. The ^{3}H signal in precipitated HIV-1_{IIIB} particles was decreased in a dose-dependent manner by CQ ($P < 0.05$, t test for regression; see Fig. 1B). Similar results were obtained with HIV-$2_{CBL/20}$ (not shown). In contrast, CQ (up to 1 mM) did not inhibit incorporation of [^{35}S] cysteine/methionine into gp120 (see Fig. 1C), thus confirming results previously obtained.[2] We conclude that CQ inhibits glycosylation of viral particles.

Chloroquine Increases HIV Inhibition in the Presence of Indinavir

Because CQ inhibits a posttranslational step in the HIV life cycle, we tested its effects in combination with a PI. We first investigated whether the addition of CQ to IDV might result in increased inhibition of HIV-1 replication compared with IDV alone. To test this hypothesis, HIV-1_{IIIB}–infected MT-4 cells were incubated with 10 nM of IDV in the presence or absence of increasing concentrations of CQ (1–6.25 μM according to method b). The IDV concentration chosen is close to the 50% effective concentration (EC_{50}) in HIV-1_{IIIB}–infected MT-4 cells as determined under our experimental conditions (not shown). Addition of CQ (0.78–6.25 μM) to IDV caused no significant impairment of cell viability (not shown) but significantly decreased the levels of HIV-1 p24 at day 5 after infection ($P < 0.05$, repeated-measures ANOVA; Fig. 2). This decrement was not dose dependent ($P = 0.7$, t test for regression) as shown by the loss of the CQ effects in the cell cultures treated with a clinically unreachable concentration of the antimalarial (12.5 μM). Such absence of dose dependence with the IDV/CQ combination differs from the dose-dependent anti-HIV effect ($P < 0.05$, t test for regression) observed with CQ alone (see Fig. 2).

Chloroquine Exerts a Synergistic Anti-HIV Effect in Combination With Protease Inhibitors

To explore this phenomenon better, we investigated whether the effects of different CQ/IDV combinations were additive, synergistic, or subsynergistic. HIV-1_{IIIB}–infected cells were treated with various concentrations of CQ (accord-

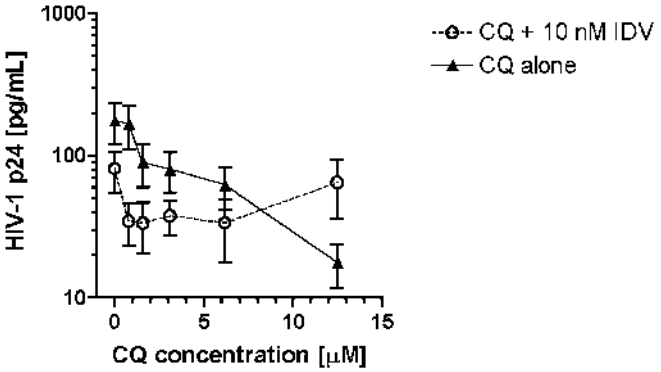

FIGURE 2. Combined effects of chloroquine (CQ) and indinavir (IDV) on HIV-1 replication. MT-4 cells were inoculated with HIV-1_{IIIB} and subsequently incubated with 10 nM of IDV with or without increasing CQ concentrations. In this case, cells were infected with a low multiplicity of infection (0.01) to better unmask any favoring effects of the IDV/CQ combination on HIV-1 replication. Viral replication is shown as the level of HIV-1 p24 in the culture medium at day 5 after infection (mean ± SEM in 3 experiments). The effects of CQ in the absence of IDV are also shown.

ing to method b) or IDV alone or in combination. We then determined which concentrations of each drug in the different combinations produced 90% inhibition of HIV-1 replication. For each drug, we calculated the FIC. Analysis using the isobologram method showed that the effect of CQ on the anti–HIV-1 activity of IDV was synergistic at the low FICs of CQ (corresponding to prophylactic antimalarial plasma concentrations, ie, ≃0.1–1 μM),[31] subsynergistic or additive at intermediate FICs (≃3.12–6.25 μM), and slightly antagonistic at the highest FICs (≥10 μM, clinically nonrelevant concentration) (Fig. 3A). Similar effects were obtained with HIV-$2_{CBL/20}$ (not shown) and using CQ in combination with RTV or SQV (Fig. 3B). We conclude that concentrations of CQ as found during malaria prophylaxis exert a synergistic anti-HIV effect in combination with PIs.

Chloroquine Increases the Susceptibility of Primary HIV Isolates to Protease Inhibitors

We then tested the effects of CQ on the susceptibility to PIs of PI-sensitive and resistant primary HIV-1 isolates and of a PI-resistant laboratory strain, (HIV-$1_{SQV\text{-}resistant}$; Table 1). For this purpose, MT-4 cells or PHA-stimulated PBMCs were infected with the viruses, washed, and incubated with increasing concentrations of IDV or SQV in the presence or absence of 1 μM of CQ.

We found that CQ not only decreased the PI's EC_{50} values in PI-susceptible isolates but in 3 PI-resistant viruses as well ($P < 0.05$, Wilcoxon rank sum; see Table 1), partially restoring PI sensitivity in viruses such as HIV-$1_{PAVIA11}$ and HIV-$1_{PAVIA12}$ (HIV-$1_{PAVIA11}$: $P < 0.01$, HIV-$1_{PAVIA12}$: $P < 0.05$, *t* test for regression), which display no dose-dependent response to PIs in the absence of CQ (HIV-$1_{PAVIA11}$: $P = 0.660$, HIV-$1_{PAVIA12}$: $P = 0.583$, *t* test for regression; see Figs. 3C, D). The effects of the CQ/PI combination were synergistic, the sum of FICs being ≤0.5 in the 3 sets of experiments allowing this calculation (see Table 1). This result cannot be attributed to toxic effects of the CQ/IDV combination, because the PBMC viability was similar whether cell treatment consisted of IDV with or without 1 μM of CQ (not shown).

In contrast, CQ had no effect on the response to AZT in AZT-resistant isolates (see Table 1). In these experiments, the sum of FICs of the CQ/AZT combination lay within values of 1 to 1.2, indicating an additive but not a synergistic effect in keeping with previous studies.[6,9]

Chloroquine Enhances the Inhibitory Effect Exerted by Protease Inhibitors on the P-Glycoprotein– and Multidrug Resistance Protein-1–Mediated Efflux Activity

Because both CQ and PIs inhibit P-gp and MRP1,[14–17,24] we tested the combined effects of these drugs on the efflux of Rh123 (indicator of P-gp function) and of CF (indicator of MRP1 function in lymphocytes).[27–30]

To confirm the effects of CQ on P-gp– and MRP1-mediated efflux, intracellular retention of Rh123 and CF was determined in normal human PBLs in the presence or absence of various concentrations of CQ. CQ was found to enhance Rh123 and CF accumulation by inhibiting their efflux in a dose-dependent manner (Fig. 4). PIs alone at concentrations of 3 μM had little or no inhibitory effect on Rh123 efflux, depending on the baseline efflux activity of the donor and on the PI adopted (ritonavir > saquinavir ≃ indinavir). These effects are milder than those observed using higher PI concentrations (10 μM), which produce more sustained inhibition of Rh123 efflux, which is evident in PBLs from all donors and with all the PIs adopted.[27] The effects of PIs (3 μM) on CF efflux were mild but consistent in PBLs from all donors tested (n = 10). The combination of CQ with IDV, SQV, or RTV inhibited Rh123 and CF efflux in PBLs more strongly than each drug alone ($P < 0.05$, Kolmogorov-Smirnov statistics; see Fig. 4). These effects were highly reproducible in lymphocytes from all donors tested (n = 4). Three-color immunofluorescence demonstrated that the IDV/CQ combination significantly ($P < 0.05$, Kolmogorov-Smirnov statistics) inhibited Rh123 and CF efflux not only in the memory but in the naive $CD4^+$ subpopulation showing higher efflux activity (Fig. 5). We conclude that CQ increases the blockade of P-gp– and MRP1-mediated efflux activity exerted by PIs.

Finally, we examined the impact of CQ and IDV on P-gp and MRP1 expression. Our results confirm the previously reported lack of effect of PIs on P-gp and MRP1 expression.[27] CQ treatment (1 μM) increased P-gp and MRP1 expression by

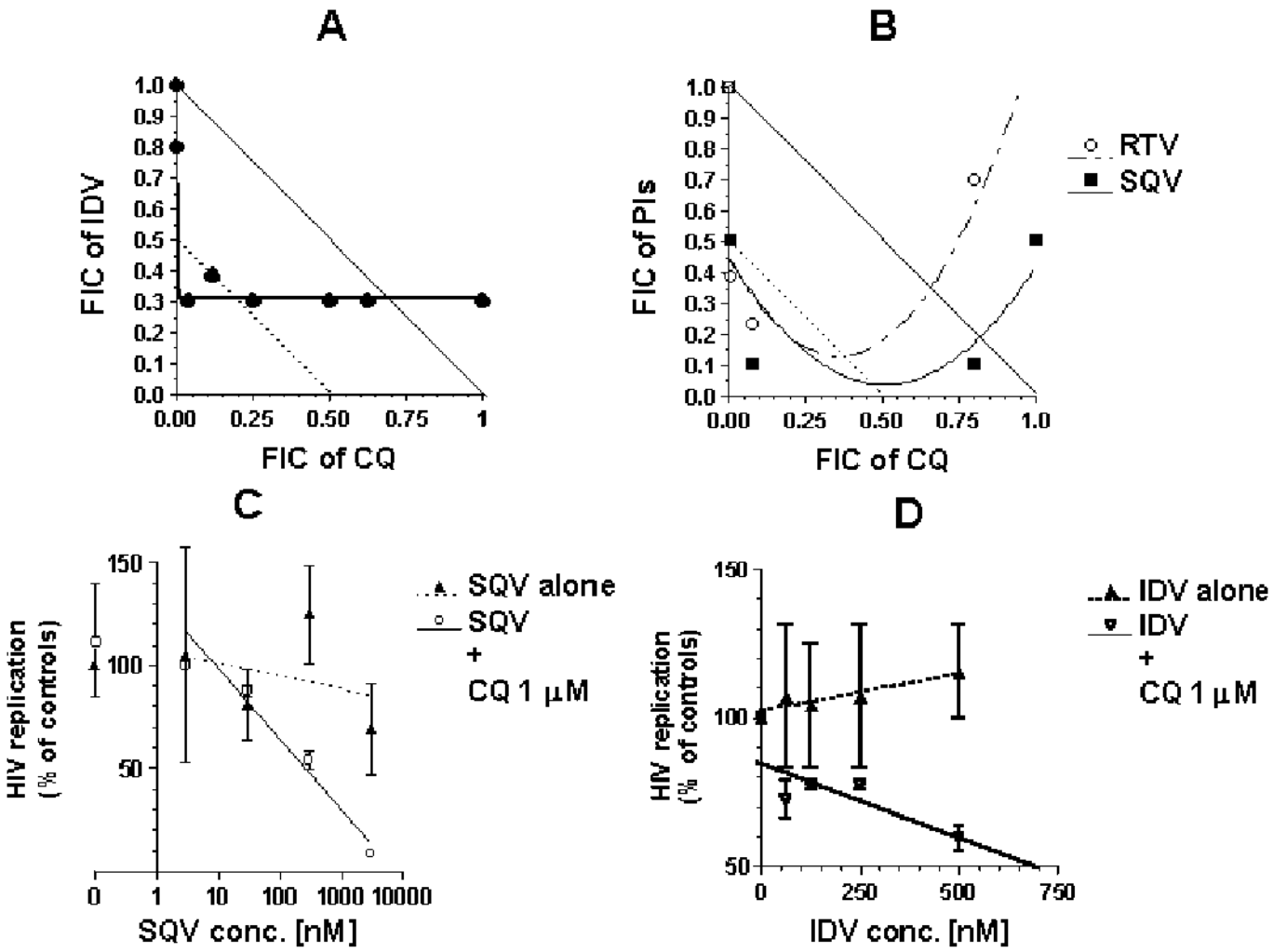

FIGURE 3. Combined effects of chloroquine (CQ) and HIV protease inhibitors (PIs) on viral replication. A, Isoboles showing the fractional inhibitory concentrations (FICs) of the combination of CQ plus indinavir (IDV) against HIV-1_{IIIB} replication. B, Isoboles showing the FICs of the combination of CQ with saquinavir (SQV) or with ritonavir (RTV) against HIV-1_{IIIB} replication. (C,D) Partial restoration by CQ (1 µM) of the response to PIs of the HIV-$1_{PAVIA11}$ (C) and HIV-$1_{PAVIA12}$ (D) multidrug resistant isolates in peripheral blood mononuclear cells (PBMCs). Briefly, MT-4 cells or primary PBMCs were inoculated with laboratory strains or primary isolates, respectively, at a multiplicity of infection of 0.1. The HIV-infected cells were then incubated with selected concentrations of PIs and/or CQ. Panels (A) and (B) show the FICs of the CQ/PI combination capable of inhibiting viral replication by 90% as calculated on the basis of 3 independent experiments (ranges were <20% of the values reported). The curves best fitting the data points were calculated according to a nonlinear regression model. The graphs show the expected line in case of simply additive effects (the straight line connecting the 1.0 FIC values on the *x* and *y* axes) as well as the threshold between subsynergism and true synergism (ie, the dotted line connecting the 0.5 FIC values). In panels (C) and (D), the data points (mean ± SEM) show the replication rates of the viral isolates in the presence of IDV or SQV in the presence or absence of CQ (1 µM). Results are expressed as a percentage of the viral replication levels obtained in the absence of drugs. The regression lines best fitting the data points demonstrate the CQ-induced restoration of a dose-dependent HIV inhibition by PIs. The results of 3 experiments using PBMCs from 3 different donors are shown (mean ± SEM in 3 experiments).

approximately 2-fold after 48 hours. When these cells were washed and tested for Rh123 and CF efflux in CQ-free media, however, the Rh123 and CF efflux was not significantly increased when compared with untreated control cells (not shown).

DISCUSSION

The results of this study show (1) that CQ inhibits viral particle glycosylation and (2) that CQ in combination with PIs carries out synergistic anti-HIV effects and combined inhibitory effects on P-gp and MRP1 function.

TABLE 1. Effects of the Antiretroviral Drugs IDV, SQV, and AZT on Different Viral Strains and Isolates in the Presence or Absence of CQ

Virus	Drug Resistance Profile*	MOI†	Antiretroviral Drug Used	EC_{50} of the Antiretroviral Drug Adopted (nM)‡		EC_{90} of the Antiretroviral Drug Adopted (nM)		Sum of FICs	Cell Type, Donor No.
				Alone	+CQ (1 μM)	Alone	+CQ (1 μM)		
VI 829	HIV-1 clade A isolate from an antiretroviral-naive African individual	0.1	IDV	37 (23–168)	6 (3–53)	355 (206–678)	126 (68–563)	0.48§	PBMC, 3
UG3	HIV-1 clade C isolate from an antiretroviral-naive African individual	0.1	IDV	291 (146–436)	80 (40–120)	550 (325–1050)	200 (150–250)	0.46§	PBMC, 2
Pavia11	APV H, IDV H, LPV I, NFV H, RTV H, SQV H, 3TC H, ABC H, AZT H, d4T H, ddC H, ddI H, TDF I, NRTIs H	0.1	SQV	>3000	251 (140–>3000)	ND	>3000	ND	PBMC, 3
Pavia12	APV I, ATV I, IDV H, LPV I, NFV H, RTV H, SQV I, 3TC H, ABC H, AZT H, d4T I, ddC I, ddI I, TDF I, susceptible to NNRTIs	0.1	IDV	>1000	695 (565–1892)	ND	>1000	ND	PBMC, 3
SQV-resistant	APV I, IDV I, LPV L, NFV H, RTV I, SQV H susceptible to NRTIs and NNRTIs	0.1	SQV	2294	813	3000	1122	0.5§	MT-4, —
14 A018 G910-6	3TC S, ABC I, AZT H, d4T H, ddC L, ddI L, TDF I, susceptible to NNRTIs and PIs	0.01	AZT	5500	4800	>10,000	>10,000	1‖	MT-4, —
8 A012 G69106	3TC S, ABC I, AZT H, D4T I, ddC L, ddI L, TDF L, susceptible to NNRTI and PIs	0.1	AZT	4600	5250	>10,000	>10,000	1.2‖	MT-4, —

CQ inhibits HIV-1 and HIV-2 replication by a dose-dependent mechanism as shown (a) by acute (1 hour) CQ loading of cells using high concentrations of the drug to reach steady-state conditions[6] or (b) by keeping HIV-infected cells in continuous contact with CQ.[2,3] The EC_{50}/EC_{90} values are apparently 10- to 100-fold lower in MT-4 cells treated with method b than in H9 cells loaded with method a; this discrepancy can be reconciled in light of the kinetics of intracellular accumulation of CQ.[32] Indeed, MT-4 cells treated by constant incubation (method b) accumulate CQ intracellularly 10- to 100-fold (E. Morra, G. P. Pescarmona, unpublished results).

We previously demonstrated that the sole mechanism explaining the anti-HIV activity of CQ is a decrease in the infectivity of the newly produced virus associated with defective production of the heavily glycosylated 2G12 epitope of gp120.[2] This structure is highly conserved on the gp120 surface and plays a pivotal role in virus infectivity.[33] The present study provides direct evidence that CQ is an inhibitor of HIV glycosylation as evidenced by metabolic labeling of virions produced in the presence of the drug. This observation is in line with reports that CQ affects the glycosylation of some viral and cellular proteins, likely by increasing the pH of the Golgi vesicles.[1,34] Of interest is the fact that these effects occur in the absence of toxic effects on cultured cells. HIV glycosylation may therefore represent a new target for antiretroviral therapy that could lend itself to combination with other well-known

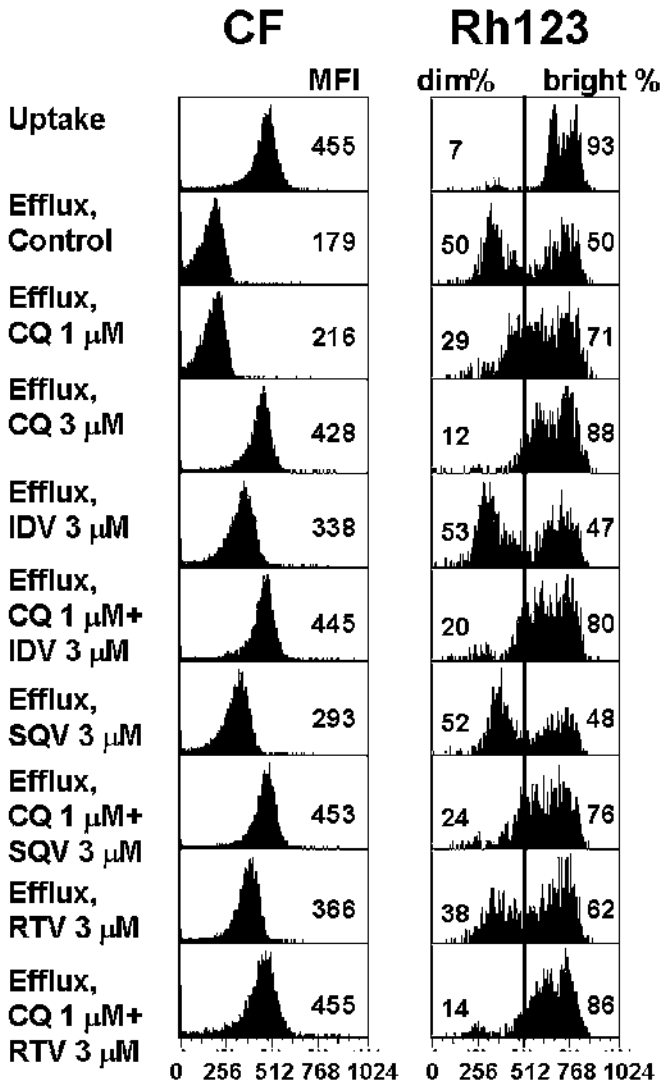

FIGURE 4. Effect of various concentrations of chloroquine (CQ; 1 μM and 3 μM) and of protease inhibitors (3 μM) on intracellular accumulation and efflux of rhodamine (Rh123; right panels) and carboxyfluorescein (CF; left panels) in normal peripheral blood lymphocytes (PBLs). Cells were stained with Rh123 (specific substrate for P-glycoprotein [P-gp] or CF (specific substrate for multidrug resistance protein-1 [MRP1]). To estimate Rh123 and CF uptake, P-gp and MRP-1 activity was blocked by transferring cells to 4°C immediately after staining with Rh123 and CF. P-gp and MRP1 function was inferred from a decrease in fluorescence after incubation in Rh123- and CF-free medium for 1 hour. CQ dose dependently inhibited Rh123 and CF efflux. Indinavir (IDV; 3 μM), saquinavir (SQV; 3 μM), or ritonavir (RTV; 3 μM) further increased the retention of the dyes. The fluorescence histograms illustrate 1 of 4 experiments with similar results using PBLs from 4 different donors. The number of events is indicated by the *y* axis. Note that because of the different fluorescence distribution, unimodal for CF and bimodal for Rh123, results are expressed as mean CF fluorescence intensities and percentages of $Rh123^{dim/bright}$ cells, respectively. The latter were established by means of a cursor (vertical line) positioned on histograms referring to control uptake samples.

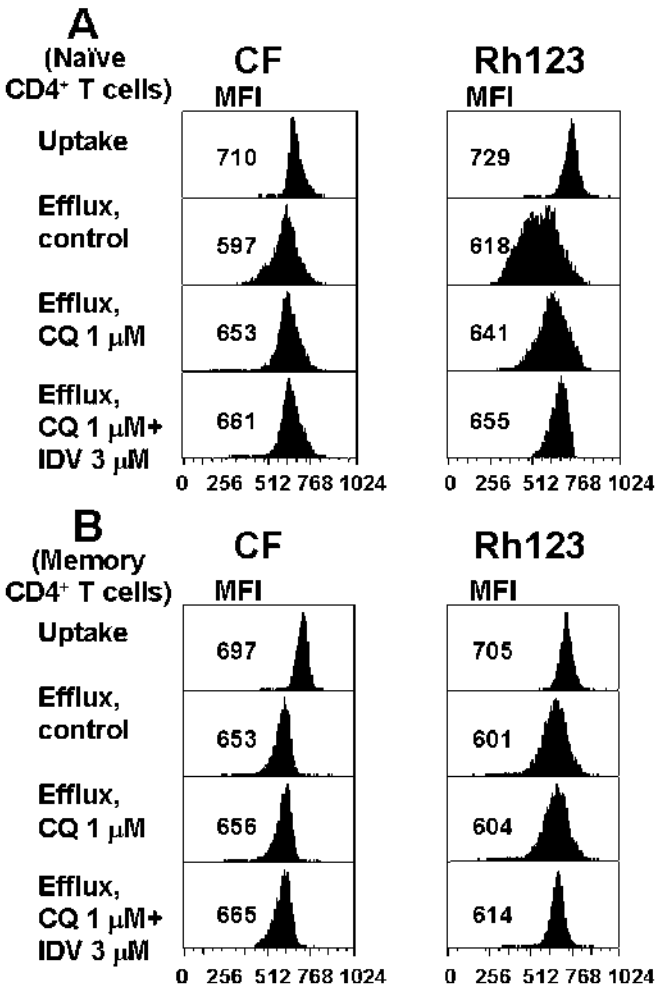

FIGURE 5. Effect of chloroquine (CQ) on rhodamine (Rh123) and carboxyfluorescein (CF) efflux from $CD45RA^+$ naive (A) and $CD45RO^+$ memory (B) $CD4^+$ cells in the presence or absence of indinavir (IDV). Peripheral blood mononuclear cells from a healthy control subject were stained with peridinin chlorophyll protein–conjugated monoclonal antibodies (mAbs) directed against CD4 and with phycoerythrin-conjugated mAbs to CD45RA and CD45RO. P-glycoprotein (P-gp) and multidrug resistance protein-1 (MRP1) function was inferred, respectively, from a decrease in fluorescence of Rh123- and CF-loaded cells after 1 hour of incubation in fluorochrome-free media. Inhibitory effects on Rh123 and CF efflux were observed in the presence of CQ (1 μM), and higher inhibition rates were observed in the presence of CQ plus IDV (3 μM). Rh123 and CF uptake was measured as explained in the legend of Figure 4. Events were gated on side scatter and $CD4^+$ fluorescence. The population of interest was further defined on the basis of the CD45 isoform. The histograms refer to the fluorescence intensity of the cell subpopulations of interest. The number of events is indicated by the *y* axis. Histograms represent 1 of 3 experiments with similar results using lymphocytes from 3 different donors.

therapeutic targets in the life cycle of HIV. Because viral envelope glycosylation is mediated by cellular enzymes, its inhibition may explain the broad spectrum of the in vitro antiviral activity of CQ inhibiting isolates from all major subtypes of HIV-1 and HIV-2.[2] The anti-HIV activity of CQ thus provides a paradigm exploitable for designing strategies against HIV or other enveloped viruses.

The effect of CQ on HIV glycosylation may account for its effects in combination with a major class of antiretroviral drugs (ie, the PIs). In this article, we show that the addition of increasing CQ concentrations to IDV results in increased inhibition of HIV-1 replication compared with IDV alone. This decrement is not dose dependent as opposed to that observed using CQ alone. The lack of dose dependency is explained by the isobologram analysis of the results obtained with various concentrations of CQ or PIs alone or in combination. According to this analysis, the combined effect of CQ and a PI is synergistic at the lowest CQ concentrations, additive at higher CQ concentrations, and antagonistic at supratherapeutic concentrations of the antimalarial drug. The loss of a synergistic effect paralleling the increase in CQ concentrations thus accounts for the lack of a dose-dependent HIV inhibition by CQ in the presence of IDV. Interestingly, the synergistic activity of CQ/PIs was observed using a CQ concentration of 0.1 to 1 μM, which is similar to the CQ concentrations found in the plasma of individuals receiving malaria prophylaxis.[31] Both CQ and PIs affect the production of infectious viral particles, although they act on different targets.[3,10] Moreover, PIs inhibit virion-associated HIV proteases, which play an important role in virus infectivity.[35] In this light, it is not surprising that the anti-HIV effects of a PI are amplified by an inhibitor of HIV glycosylation such as CQ, because the only HIV components subjected to glycosylation are the envelope glycoproteins (fundamental for virus infectivity). The synergism between CQ and PIs was associated with decreased thresholds of susceptibility to PIs in resistant isolates. That the CQ/PI synergism is the basis for this phenomenon is evident from the observation that CQ partially reverts resistance to IDV or SQV but not to AZT, in keeping with previous observations that the combined effects of CQ and AZT are additive but not synergistic.[9]

CQ increases P-gp and MRP1 blockade by PIs, which was recently postulated to have important consequences for the intracellular concentrations of antiretrovirals.[11–14,27] P-gp and MRP1 blockade occurred in HIV-target cells such as $CD4^+$ lymphocytes and was also evident in naive subpopulations, which have higher P-gp– and MRP1-mediated efflux activity than memory cells. Interestingly, the combined CQ/PI effects were more evident on MRP1, whose efflux activity was recently suggested by Speck et al[36] to favor HIV-1 replication. Our unpublished finding that specific MRPs inhibitors downmodulate HIV replication (M. B. Lucia et al, manuscript submitted) is consistent with the data of Speck et al.[36] The precise mechanisms behind these phenomena merit further investigation, however. If these effects are confirmed, the fact that the CQ/PI combination efficiently inhibits a protein favoring HIV replication may have interesting repercussions in vivo, because MRP1 blockade occurs using clinically achievable PI concentrations. Nevertheless, we doubt that the combined effects of CQ and PIs contribute to the anti-HIV synergism of these drugs in our in vitro model, where PIs were used at lower concentrations.

A major concern regarding the effects of CQ on P-gp and MRP1 is the possibility of an upregulation of these proteins. In the present study, the increased anti–P-gp– and anti-MRP1–antibody binding capacity of cells treated with CQ for 48 hours was not associated with increased Rh123 and CF efflux, suggesting a conformational change of both P-gp and MRP1 because of the presence of CQ rather than a true induction of their expression. This hypothesis is in line with observations of others reporting that (1) substrates induced conformational changes in surface pumps, increasing binding of specific antibodies,[37,38] and (2) CQ did not induce the surface expression of P-gp.[39] Our results concerning the unchanged efflux activities despite upmodulation of anti–P-gp– and anti-MRP1–antibody binding capacity were obtained using 0.5 to 1 μM of CQ. Such concentrations are found in blood/plasma at an antimalarial prophylactic dosage (100 mg of CQ base per day). We recently reported blood concentrations of 1 μM, whereas plasma concentrations may be slightly lower, approximately 0.2 to 0.5 μM.[40] Higher concentrations of CQ might have other consequences on efflux, however, as suggested by the slight antagonism between the effects of CQ and PIs on HIV-1 replication using supraprophylactic and even supratherapeutic plasma CQ concentrations $\geq$10 μM. We speculate that the high levels of inhibition of P-gp and especially MRP1 using CQ in combination with PIs at clinically relevant concentrations may counterbalance the effects of a possible upregulation of these proteins by CQ. We are presently devising experiments to examine the effect of CQ on the intracellular concentrations of PIs both in vitro and in vivo.

In conclusion, this study provides the first evidence of synergistic anti-HIV activity between CQ and PIs. These in vitro results warrant in vivo confirmation in HIV-positive individuals by comparing the long-term antiviral effect of PI-containing HAART regimens with and without CQ or HCQ. If such studies provide confirmatory results, they may lead to a strategy whereby coadministration of CQ/HCQ allows lowering of the PI dosage, lessening cost and possibly toxicity. Cost lessening would of course be particularly welcome in developing countries. Moreover, the potential ability of CQ to overcome resistance to PIs could be important in the treatment of drug-experienced HIV-positive subjects who have developed multiple resistances to antiretroviral drugs and thus have limited therapeutic options.

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