Inhibition of HIV-1 Replication by Hydroxychloroquine: Mechanism of Action and Comparison with Zidovudine

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ABSTRACT

We have previously described the inhibition of human immunodeficiency virus serotype 1 (HIV-1) using the antimalarial hydroxychloroquine (HCQ), a weak base that inhibits the posttranslational modification of glycoprotein 120 (gp120) in T cells and monocytes. The mechanism of inhibition of gp120 production was presumed to be the ability of HCQ to increase endosomal pH and therefore alter enzymes required for gp120 production. To further clarify this action, we have determined the effect of HCQ and its enantiomers on endosomal pH. Pretreatment of cells with HCQ and the levo- and dextro-enantiomers at concentrations demonstrated to suppress anti–HIV-1 activity increased endosomal pH to levels similar to increases seen with chloroquine and ammonium chloride, two other weak bases, and decreased gp120 production. The dextro- and levo-enantiomers suppressed HIV-1 replication to a similar extent and were no more toxic than racemic HCQ. We next compared the anti–HIV-1 effect of HCQ with zidovudine (ZDV) in both newly and chronically HIV-1–infected T-cell and monocytic cell lines (63 and 63_{HIV}). HCQ suppressed HIV-1 replication in a dose-dependent manner in both recently and chronically infected T-cell and monocytic cell lines. In contrast, ZDV pretreatment had potent anti–HIV-1 activity in the newly infected T and monocytic cells but not in chronically infected cells. An additive effect of HCQ with ZDV was observed in the newly infected T and monocytic cells but not in the chronically infected cells. Although the anti–HIV-1 effect of HCQ was less than that of ZDV, HCQ may still be potentially useful either as an alternative HIV-1 treatment or in combination with other anti–HIV-1 agents, especially in patients who have rheumatic manifestations of HIV-1 infection.

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INTRODUCTION

Numerous agents inhibit human immunodeficiency virus serotype 1 (HIV-1) replication in T cells and monocytes. These compounds are limited in their usefulness, however, because of significant toxicity and potential viral resistance associated with their long-term use.[1] Agents that are less toxic and are safe for long-term use would be of obvious importance in treating patients with HIV-1 infection both as a primary modality or in combination with other therapies. We have previously reported[2] that the lysosomotropic agent hydroxychloroquine (HCQ) can suppress HIV-1 replication in T-cell and monocytic cell lines as well as in primary T cells and monocytes by inhibiting post-transcriptional production of the virus. We have further demonstrated in a 2-month, placebo-controlled pilot study that HCQ reduced viral load by 50% in 38 asymptomatic HIV-1–infected patients with a T-cell count between 200 and 500/mm^3 and in two HIV-1–infected patients with active inflammatory arthritis.[3,4] Increased endosomal pH resulting from accumulation of weak bases, such as chloroquine (CQ) and ammonium chloride (NH_4Cl), inhibits the posttranslational modification of proteins.[5–7] The effect of HCQ on endosomal pH, however, has not been characterized. In this study we related the ability of HCQ to increase endosomal pH with its capacity to inhibit the production of glycoprotein 120 (gp120).

Dose-dependent eye toxicity has been associated with long-term HCQ use leading to accumulation of the drug in melanin-containing cells in the retina.[8] In our recently completed clinical trial,[3] we associated the antiviral effect of HCQ with higher tissue levels of the drug, indicating that higher doses might be necessary to achieve a better antiviral effect. Higher tissue levels of HCQ also correlate with more rapid and better responses in patients with rheumatoid arthritis.[9] HCQ is a racemic mixture of a dextro-enantiomer and a levo-enantiomer.[10] Recent data show that more of the dextro-enantiomer accumulates in the retina, causing the retinal toxicity associated with long-term drug use.[11] If the levo-enantiomer has antiviral activity similar to that of the dextro-enantiomer, this would be an advantage for therapeutic use. Therefore, we compared the ability of the levo- and dextro-enantiomers to increase endosomal pH and suppress HIV-1 replication.

We also evaluated the ability of HCQ to inhibit replication of HIV-1 in acutely and chronically infected monocytic cell (63 and 63_{HIV})[12] and T-cell lines (SP and SPH)[13] compared with that of zidovudine (ZDV) by measuring reverse transcriptase activity and the amount of infectious virus from the culture supernatants of the acutely and chronically infected cells. As HCQ and ZDV act at different sites in the HIV-1 replication cycle,[1,2] we also compared the effectiveness of combination therapy with the single agents.

MATERIALS AND METHODS

Cell Lines and Primary Monocytes

The SP, SPH, 63, and 63_{HIV} cell lines have been previously described.[12–14] SP is a T-cell line derived from the pleural fluid of an HIV-1–infected individual.[13] Cell line 63 is a human macrophage hybridoma that retains characteristics of human macrophages.[14] Both 63 and 63_{HIV} were grown in Iscove's modified Dulbecco's medium (Gibco, Grand Island, New

York), supplemented with 10% fetal calf serum (FCS) (Gibco), 1% penicillin and streptomycin (Gibco), and 2 mmol/L glutamine (Gibco), henceforth called complete Iscove's. The SP and SPH cell lines were grown in minimal essential medium (MEM) (Gibco), supplemented with 10% FCS (Gibco), 1% penicillin and streptomycin (Gibco), and 2 mmol/L glutamine (Gibco), henceforth called complete MEM. To isolate monocytes, mononuclear cells were separated from buffy coats obtained from healthy blood donors by Ficoll-Hypaque (Pharmacia Biosystems, Piscataway, New Jersey) density centrifugation. The cells were washed three times with sterile phosphate-buffered saline (PBS) and resuspended in RPMI 1640 (Gibco) supplemented with 10% FCS (Gibco), 1% penicillin and streptomycin (Gibco), and 1% L-glutamine (Gibco), henceforth called complete RPMI. The peripheral blood mononuclear cells were allowed to adhere for 45 minutes at 37 °C in culture flasks in complete RPMI. The nonadherent cells were removed and the adherent cells were extensively washed with sterile PBS, harvested using a rubber-tubed policeman and stained with a monocyte-specific anti-CD14 monoclonal antibody (mAb) to assess the purity of the preparation. There were greater than 90% CD14-expressing cells.[14] The other cells did not stain with either anti-CD3 or anti-CD20 mAbs.

Measurement of Lysosomal pH

The pH in the endosomes of freshly isolated peripheral blood monocytes was determined by use of the pH sensitivity of endocytosed fluorescein isothiocyanate-labeled dextran (FD) (molecular weight, 70,000, Sigma Diagnostics, St. Louis, Illinois).[15–20] Cellular uptake of FD was accomplished by incubating monocytes, at a concentration of 10^6/mL in complete RPMI, in the presence of 1 mg/mL FD and lipopolysaccharide (10 μg/mL) (Sigma Diagnostics, St. Louis, Missouri) for 12 to 16 hours. Control cells were treated similarly, except FD was omitted from the incubation medium. The cells were then washed five times in PBS, resuspended in PBS at a concentration of 10^6 cells/mL, and allowed to recover for approximately 30 minutes before further manipulation. An aliquot of a stock solution of CQ, HCQ, NH_4Cl, or the levo- or dextro-enantiomers, was added for 1 hour to 200 μL of the monocyte suspension together with anti-fluorescein antibody (3 μg/mL; Molecular Probes, Eugene, Oregon) to quench the fluorescence of residual, uninternalized FD. After 2 to 5 minutes of equilibration, both the fluorescence excitation (emission at 450 nm) and emission (excitation at 485 nm) spectra were measured at room temperature by means of a laboratory-modified SLM spectrofluorometer (SLM Instruments, Inc., Urbana, Illinois) using a 3-mm–pathlength cuvette to minimize turbidity and scattering artifacts.

To simultaneously determine both pH and HCQ and CQ concentrations, we devised two data analysis procedures that use the environmental sensitivity of the overall shape of the excitation and emission spectra. The FD excitation spectrum from the sample and all reference spectra are first normalized to their respective maxima. The sample spectrum is then compared with each individual calibration spectrum by calculating the sum of the squared values of the differences between their normalized intensity at each wavelength. The lowest of these sums identifies the calibration spectrum that

best matches the sample, meaning, therefore, the pH and HCQ and CQ concentrations that best approximate the intracellular conditions sensed by the FD.

The second procedure is based on an analytical description of the pH and HCQ and CQ concentration dependence of the spectral properties of FD, allowing a more precise determination of both variables. We chose the spectral center of mass (SCM), in units of nanometers, to condense the fluorescence spectral shape into a single numeric value: the SCM is calculated by $SCM = [\Sigma F(\lambda)\lambda]/\Sigma F(\lambda)$ where $F(\lambda)$ is the fluorescence intensity at wavelength λ in nanometers. The SCM values for the set of calibration excitation spectra are calculated, and their dependence with respect to pH and HCQ and CQ concentrations is assessed by nonlinear least-squares fitting of the data to an empiric equation consisting of a combination of Henderson-Hasselbach and hyperbolic binding functions. The emission spectra are analyzed in a similar manner by using different equations and parameter sets. Both of these equations and their parameter sets are incorporated into a computer program that takes as input the SCM values for the excitation and emission spectra of the sample and finds the values of pH and HCQ and CQ concentrations that simultaneously satisfy both nonlinear equations.

Reverse Transcriptase Assay

HIV-1 reverse transcriptase activity was measured in the culture supernatants in the recently infected SP and 63 cells and in SPH and 63_{HIV} after treatment with various concentrations of the study drugs at various times. Reverse transcriptase activity was detected by a modification of Hoffman's method using tritium (^3H)-labeled thymidine triphosphate incorporated onto a template primer of double-strand, polyadenylated RNA and oligodeoxythymidine (Poly(rA)•p(dT_{12-18}) (Pharmacia Biosystems), and is displayed as the mean counts per minute per milliliter of culture supernatant.[21] This represents the mean value from replicate cultures. For the reverse transcriptase assay, SP and 63 and SPH and 63_{HIV} were used as both the negative and positive controls, respectively.

Immunoprecipitation of gp120

Metabolic labeling of 63 and 63_{HIV} treated and untreated with HCQ and CQ was performed as follows: both clones were starved in methionine- and cysteine-free medium (RPMI 1640 Selectamine kit, Gibco), and pulsed for 4 hours with methionine and cysteine labeled with radioactive sulfur (^{35}S) at 50 μCi/5 $\times$ 10^6 cells.[22] The labeled cells were lysed and subjected to immunoprecipitation with polyclonal anti-gp120 antibodies, as previously described.[23] Nonimmune human serum was used as the negative control.

Drug Treatment and HIV-1 Infection of the T-Cell and Monocytic Cell Lines

SP, SPH, 63, and 63_{HIV} cells were incubated for 60 minutes with either sterile PBS, or serial 10-fold dilutions of racemic 1 mmol/L HCQ (Sanofi-Winthrop Pharmaceuticals, New York, New York), 1 mmol/L of the levo- and dextro-enantiomers (Sanofi-Winthrop Pharmaceuticals), or 1 mmol/L ZDV (Sigma Diagnostics), alone or in combination, for 1 hour before infection to achieve steady-state concentrations, and then were washed three times with sterile PBS. After treat-

ment, 0.5 × 10^6 SP and 63 cells were infected by cocultivation with an infectious supernatant of HIV-1_{IIIB}, standardized to 80,000 cpm/mL, for 60 minutes at 37 °C.[2] After washing to remove residual virus, SP and 63 cells were cultured at a final concentration of 0.5 × 10^6/mL in complete MEM and complete Iscove's, respectively, at 37 °C. Either ZDV or HCQ was maintained in culture at a concentration of 1 µmol/mL. The chronically infected lines, 63_{HIV} and SPH, also were treated for 1 hour with the study drugs as described above, and then washed with sterile PBS and maintained in culture at a concentration of 0.5 × 10^6 cells/mL in complete Iscove's and complete MEM, respectively. HCQ and ZDV were also maintained in culture at a concentration of 1 µmol/mL after the treatment.

Toxicity of HCQ, Dextro- and Levo-Enantiomers, and ZDV for the 63, 63_{HIV}, SP, and SPH Cell Lines

Serial 10-fold dilutions of 1 mmol/L HCQ (Sanofi-Winthrop Pharmaceuticals), 1 mmol/L dextro- and levo-enantiomers (Sanofi-Winthrop Pharmaceuticals), or 1 mmol/L ZDV (Sigma Diagnostics) were incubated with 0.5 × 10^6 of either SP, 63, 63_{HIV}, or the SPH cell lines for 1 hour at the beginning of the experiments, either alone or in combination. ZDV and HCQ were maintained in culture at a concentration of 1 µmol/mL, and the cells were kept in culture for 14 days. The cells were harvested daily for 14 days to further assess the toxicity of HCQ. Eighteen hours before daily harvesting, 1 µCi of [^{3}H]thymidine (ICN, Irvine, California) was added. The cells were harvested onto glass filters and the incorporated radiolabel was measured by scintillation counting.[2]

Endosomal pH

Weak bases such as CQ and NH_4Cl are known to accumulate in acidic cellular compartments (lysosomes and endosomes) and to increase their pH.[18] As HCQ is also a weak base,[12] we determined the effect of HCQ on endosomal pH by measuring changes in the fluorescence of FD endocytosed by primary monocytes. We used primary monocytes stimulated with lipopolysaccharide because of more efficient uptake of FD compared with both the T-cell and monocytic cell lines. T cells are not phagocytic and the monocytic cell lines, although retaining many monocytic characteristics, were nearly as phagocytic as primary monocytes. The cells were then treated with varying dilutions of HCQ, CQ, and NH_4Cl as a positive control. The fluorescence spectra were analyzed as described in the materials and methods section. The change in endosomal pH caused by the added compounds, as well as the estimated concentrations of the compounds within the endosomes, are given in the table. A dose-dependent increase in endosomal pH was observed for HCQ that was comparable to data for CQ and NH_4Cl (table). This effect lasted for 1 hour when HCQ was removed from the media.

gp120 Production

In 63_{HIV} cells treated with sterile PBS, a band with the molecular weight of 120 kDa corresponding to gp120 can be readily demonstrated in lysates and supernatants from [^{35}S]cysteine- and [^{35}S]methionine-labeled cells (Figure 1). The effect of HCQ is specific for gp120, since the production of other HIV-1 proteins is unaltered in the HCQ-treated cells. However, treatment of 63_{HIV} for 1 hour with 1

Table. The pH and chloroquine derivative concentrations within the endosomal compartments of peripheral blood monocytes* after a 5-minute exposure to the respective compounds.

Addition	pH	ΔpH	Endosomal mmol/L
None	5.6 ± 0.28		
30 mmol/L NH_4Cl	6.9 ± 0.16	1.3 ± 0.12	
0.1 mmol/L CQ	6.3 ± 0.09	0.7 ± 0.18	14 ± 0.28
1 mmol/L CQ	6.9 ± 0.47	1.3 ± 0.20	29 ± 6.6
0.1 mmol/L HCQ	6.1 ± 0.14	0.5 ± 0.13	11 ± 2.75
1 mmol/L HCQ	7.2 ± 0.48	1.6 ± 0.22	27 ± 3.45

ΔpH = the change in pH as compared with the value in the absence of any compound (the values represent mean values and the standard error using 3 different cell preparations); NH_4Cl = ammonium chloride; CQ = chloroquine; HCQ = hydroxychloroquine.
*With endocytosed fluorescein isothiocyanate-labeled dextran.

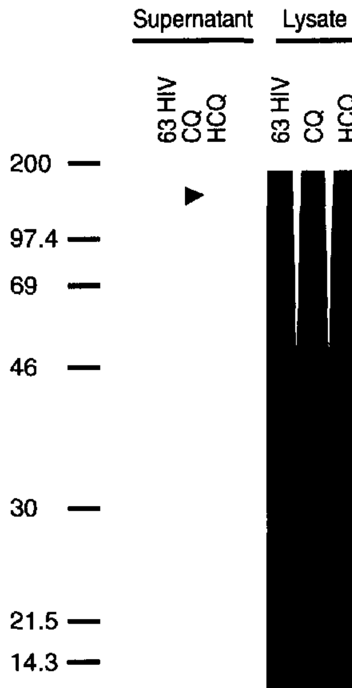

Figure 1. Macrophages chronically infected with human immunodeficiency virus (63_{HIV}) were cultured with radiolabeled cysteine and methionine and treated with 1 mmol/L hydroxychloroquine (HCQ) or 1 mmol/L chloroquine (CQ). After immunoprecipitation with a human polyclonal antiglycoprotein (gp120) antibody, gp120 (arrow) can be demonstrated in both the lysate and the supernatant of the labeled cells.

mmol/L of racemic HCQ or CQ resulted in the disappearance of gp120 from both the cell lysates and from the supernatants of the treated 63_{HIV} cells.

HIV-1 Replication in Recently and Chronically Infected T and Monocytic Cells

Pretreatment with racemic HCQ and both enantiomers resulted in a dose-dependent suppression of reverse transcriptase activity in the recently infected SP and 63 cells (Figure 2). The effective concentration of HCQ to reduce viral replication by 50% (ED_{50}) was 0.01 mmol/L in 63 and 0.1 mmol/L in the SP cells. To exclude the possibility that the levo- and dextro-enantiomers caused a shift in the kinetics of expression of HIV-1 proteins in 63 and SP cells, viral replication was measured at multiple times. In agreement with results obtained with the racemic mixture, viral replication was inhibited at all times tested (as far out as 14 days) in both the SP and 63 cells infected with HIV-1_{IIIB} compared with that in the untreated cells.

Both chronically HIV-1–infected cell lines, 63_{HIV} and SPH, were treated with serial dilutions of racemic HCQ and the levo- and dextro-enantiomers (1 mmol/L to 0.001 mmol/L for 1 hour). As HCQ renders HIV-1 passaged on treated cells noninfectious, we isolated virus from the HCQ-treated 63_{HIV} and SPH cells and tested its ability to infect uninfected SP and 63 cells. HCQ reduced the amount of infectious virus from the treated cells in a dose-dependent manner (Figure 3).

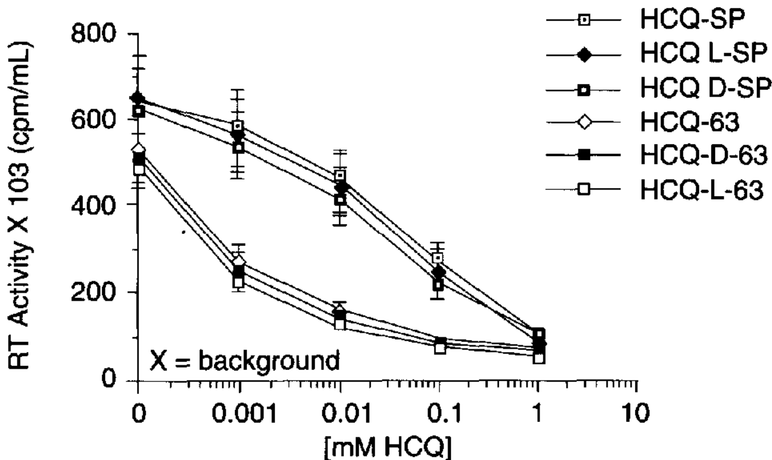

Figure 2. The levo-enantiomer (HCQ L) and dextro-enantiomer (HCQ D) of hydroxychloroquine (HCQ) can suppress human immunodeficiency virus serotype 1 (HIV-1) replication to a similar extent at different times. The SP (T-cell) and 63 (monocytic) cell lines were treated with either 1 mmol/L of levo- or dextro-enantiomer and infected with HIV-1_{IIIB}. RT = reverse transcriptase. Data pooled from 5 separate experiments; error bars represent the standard error of the mean. Background = 2.4×10^3 cpm/mL.

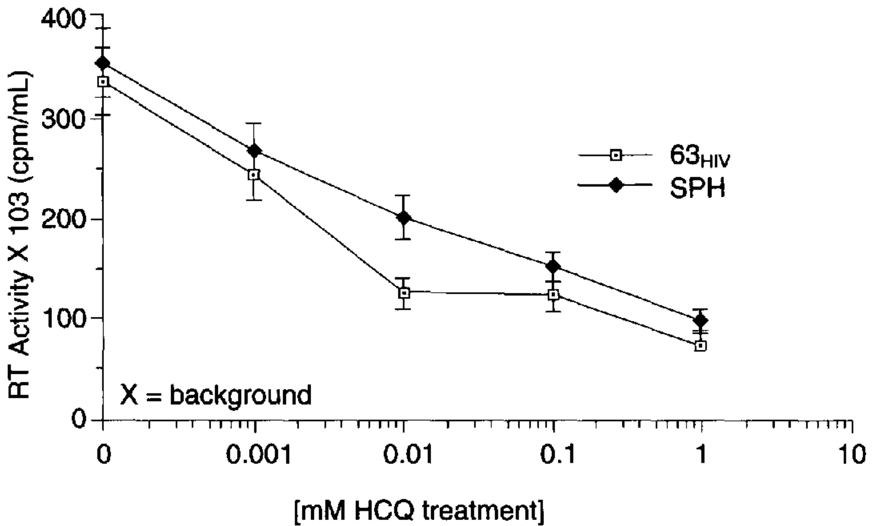

Figure 3. Hydroxychloroquine (HCQ) suppresses human immunodeficiency virus serotype 1 (HIV-1) replication in chronically infected T cells (SPH) and monocytic cells (63_{HIV}) treated with varying concentrations of HCQ for 1 hour before infection with HIV-1_{IIIB}. After removal of excess virus, the cells were cultured for 7 days. Virus harvested from the treated cells was assessed for its ability to infect CEM cells. RT = reverse transcriptase. Data pooled from 5 separate experiments; error bars represent the standard error of the mean. Background = 2.4×10^3 cpm/mL.

Comparison of HCQ Anti–HIV-1 with ZDV in Recently Infected T-Cell and Monocytic Cell Lines

Both HCQ and ZDV had anti–HIV-1 activity in the pretreated, recently infected SP and 63 cells (Figure 4), although HCQ was less effective. The ED_{50} for ZDV was 0.001 mmol/L, while the ED_{50} for HCQ was 0.01 mmol/L. In contrast to HCQ, ZDV did not have anti–HIV-1 activity in the chronically infected 63_{HIV} and SPH cells (data not shown). Both SP and 63 cells were treated with varying concentrations of racemic HCQ, in combination with ZDV, for 1 hour to achieve steady-state intracellular concentrations. Although no additive effect was observed in the chronically infected SPH and 63_{HIV} cell lines (data not shown), there was an additive effect of HCQ with ZDV in the recently infected SP and 63 cells. Viral replication was inhibited to background reverse transcriptase levels when both drugs were used together as pretreatment in the recently infected SP and 63 cells (Figure 5).

Toxicity of HCQ and the Levo- and Dextro-Enantiomers

No inhibition of proliferation in any cell line was seen at concentrations of HCQ between 1 to 0.1 mmol/L, where an-

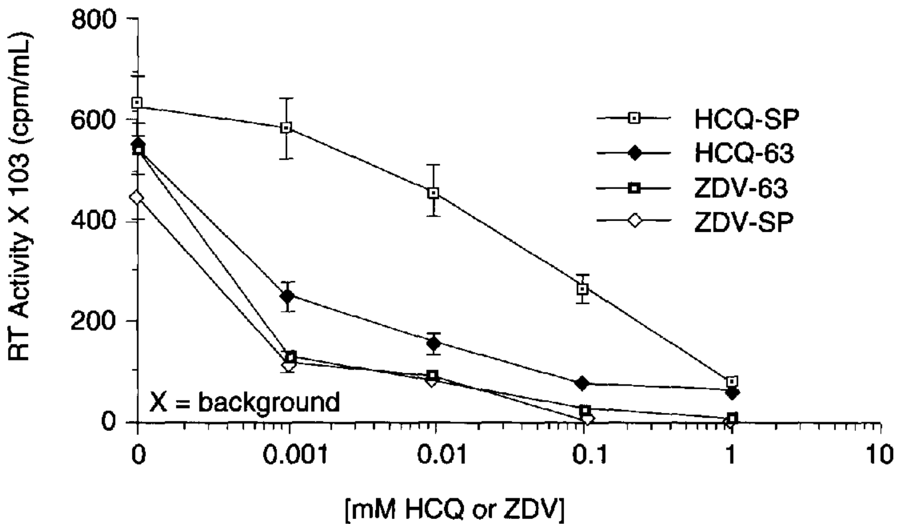

Figure 4. Hydroxychloroquine (HCQ) has antiretroviral activities similar to those of zidovudine (ZDV). T-cell (SP) and macrophage (63) cell lines were treated with serial dilutions of HCQ and ZDV followed by infection with human immunodeficiency virus serotype 1_{IIIB}. Reverse transcriptase (RT) activity was determined 7 days after infection. Background = 2.4 × 10^3 cpm/mL. Data pooled from 5 separate experiments; error bars represent the standard error of the mean.

tiviral activity was observed (data not shown). Cell death of 100% was observed when concentrations of 100 mmol/L were used to treat the cells for both the enantiomers and the racemic mixture.

DISCUSSION AND CONCLUSION

This report clarifies the anti–HIV-1 effect of HCQ by demonstrating that the drug increases endosomal pH, which inhibits the posttranscriptional production of gp120. Although other weak bases, such as CQ and NH_4Cl, have been recognized to increase endosomal pH,[15–20] there have been no studies to date investigating the effect of HCQ. We demonstrate that HCQ increases pH in a dose-dependent fashion similar to that of CQ (table). Unlike previously reported studies, this study correlates the increase in pH induced by these drugs with the intracellular concentrations of CQ and HCQ (table). We used primary lipopolysaccharide-stimulated monocytes to determine the effect on endosomal pH, because of difficulties in achieving sufficient internalization of FD in primary T-cell, SP, and 63 cell lines for accurate measurement. The effect of the elevated endosomal pH on HIV-1 replication was to decrease the synthesis of gp120 (Figure 1), leading to defective virion production. The production of other HIV-1 proteins was not affected, however, by the HCQ treatment. We have previously demonstrated that HIV-1 passaged in HCQ-treated cells poorly infects other cells,[2] and that in vivo therapy with HCQ re-

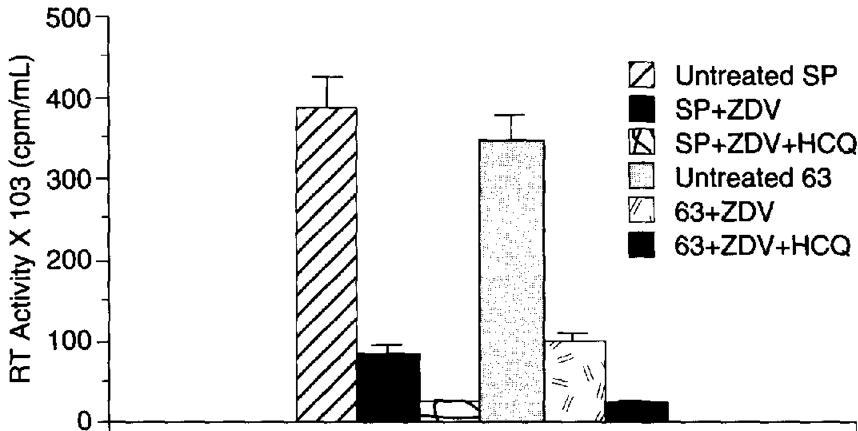

Figure 5. Hydrochloroquine (HCQ) suppresses human immunodeficiency virus serotype 1_{IIIB} (HIV-1_{IIIB}) replication in combination with zidovudine (ZDV). T-cell (SP) and macrophage (63) cell lines were pretreated with either 1 mmol/L HCQ or 0.001 mmol/L ZDV, either alone or in combination, and then infected with HIV-1_{IIIB}. Reverse transcriptase (RT) activity was determined 7 days after infection. Data pooled from 5 separate experiments; error bars represent the standard error of the mean.

duced plasma levels of HIV-1 ribonucleic acid by 50% in a 2-month study treating asymptomatic HIV-1–infected patients.[3] Other weak bases have been reported to inhibit the production of gp120. Tsai et al[24] have reported that chloroquine inhibits gp120 production in the CEM T-cell line, and Pal et al[25] have demonstrated that monensin, an iontophore that also increases endosomal pH, selectively inhibited gp120 production in another T-cell line.

Dose-dependent retinal toxicity has been recognized as the major side effect of long-term HCQ therapy, but recent studies[8] have demonstrated the safety of higher doses over time. Other investigators using the levo- and dextro-enantiomers of HCQ have revealed that more of the dextro-enantiomer accumulates in the retina,[11] presumably causing the retinal toxicity.[26] In our system, there was no difference in toxicity between the levo- and dextro-enantiomers even after a 5-hour exposure (data not shown). There was no difference in the ability of the levo- and dextro-enantiomers to inhibit HIV-1 replication in recently infected SP and 63 cell lines (Figure 2). The efficacy of the levo-enantiomer in inhibiting HIV-1 replication may permit treatment of HIV-1 patients with higher levels of drug. This may be important, as in our pilot clinical study the best response to HCQ occurred in the patient with the highest blood concentration of drug. This is also consistent with recent observations in patients with rheumatoid arthritis, whose increased blood concentrations of HCQ were associated with more rapid and better clinical responses.[9] A further benefit

of HCQ in treating HIV-1–infected patients is its ability to suppress viral replication in the chronically infected T-cell and monocytic cell lines (63_{HIV} and SPH) (Figure 3). This anti–HIV-1 effect contrasts with other nucleoside antagonists that are only effective in recently, but not in chronically, infected cells.[27]

We compared the anti–HIV-1 effects of HCQ with those of ZDV in recently infected T cells and monocytic cells (Figure 4). The inhibitory effect of HCQ on HIV-1 replication was less than that of ZDV in the recently infected SP and 63 cells (ED_{50} of 0.1 mmol/L vs 0.01 mmol/L). There was an additive effect of HCQ and ZDV that resulted in a reduction of reverse transcriptase activity to baseline levels in the recently infected 63 cells and SP cells (Figure 5). However, no additive effect was observed in the chronically infected 63_{HIV} and SPH cell lines. Our data suggest that combination therapy of HCQ with ZDV may be potentially useful to treat HIV-1–infected patients. In support of this concept, we have previously described the antiviral and anti-inflammatory effects of HCQ in two HIV-1–infected patients with inflammatory arthritis, both of whom were taking antiretroviral therapy.[4] Although HIV-1 ribonucleic acid polymerase chain reaction data were only available for one patient who was taking 400 mg of dideoxyinosine and 600 mg of HCQ daily, there was a 10-fold reduction in the number of viral copies with the combined therapy.

In conclusion, HCQ has antiviral and anti-inflammatory properties and potential additive anti–HIV-1 effects when combined with ZDV, which may make it the drug of choice for patients who are HIV-1 infected and have inflammatory arthritis.

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