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# Saquinavir-Mediated Inhibition of Human Immunodeficiency Virus (HIV) Infection in SCID Mice Implanted with Human Fetal Thymus and Liver Tissue: an In Vivo Model for Evaluating the Effect of Drug Therapy on HIV Infection in Lymphoid Tissues

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**Treatment with protease inhibitors alone or in combination with inhibitors of reverse transcriptase potently suppresses levels of human immunodeficiency virus (HIV) RNA in plasma and thereby may significantly delay the progression of HIV-mediated disease. To investigate the effect of treatment with the protease inhibitor saquinavir on HIV replication in the lymphoid tissues, we used a SCID-hu mouse model that we developed, in which human thymic and liver tissues (hu-thy/liv) were implanted under both kidney capsules in SCID mice (thy/liv-SCID-hu mice). These mice are populated in the periphery with large numbers of human T cells and develop disseminated HIV infection after intraimplant injection. thy/liv-SCID-hu mice with established HIV infection that were treated for 1 month with saquinavir had a significantly lower viral load present in the implanted hu-thy/liv and mouse spleen than did the untreated HIV-infected thy/liv-SCID-hu mice. To examine the capacity of acute treatment with saquinavir to prevent HIV infection, some thy/liv-SCID-hu mice were inoculated with HIV and then immediately started on saquinavir. Although treated mice had markedly lower viral loads in the thy/liv implants and spleens, HIV infection was not completely prevented. Thus, the effect of antiviral therapy on HIV infection in the major site of HIV replication, the lymphoid tissues, can be readily evaluated in our thy/liv-SCID-hu mice. These mice should prove to be a useful model for determining the in vivo effectiveness of different therapeutic interventions on acute and chronic HIV infection.**

A new class of agents that inhibits human immunodeficiency virus (HIV) protease activity has been developed that potently suppresses the in vitro infectivity of HIV and HIV-infected cells (9). One of them, saquinavir, is a hydroxyethylamine transition-state analog of an HIV protease cleavage site which, by inhibiting proteolysis of the Gag and Gag-Pol proteins, renders newly formed HIV particles noninfectious (33). Saquinavir has a broad anti-HIV effect, as evidenced by its ability to inhibit in vitro HIV infection of cultured cells with a broad range of clinical isolates (5). Clinical trials have demonstrated that treatment of HIV-infected individuals with saquinavir alone resulted in increased peripheral $CD4^+$ T-cell counts and decreased HIV load (14). The rise in the $CD4^+$ T-cell count and the reduction of the plasma HIV levels induced by treatment with saquinavir could be enhanced significantly by the addition of two other anti-HIV agents, zidovudine and zalcitabine, to the therapeutic regimen (1). Recent reports have indicated that combinations of other protease inhibitors with reverse transcriptase inhibitors can reduce plasma HIV RNA to undetectable levels and may even abort HIV infection when given acutely (3, 29).

After infection with HIV, systemic dissemination of HIV and HIV-infected cells into lymphoid tissues such as the lymph nodes, spleen, and thymus occurs (10, 22). Based on analysis of dynamic changes in plasma HIV RNA levels, the subsequent course of infection has been demonstrated to be characterized by a continuous level of highly productive HIV replication (2, 12, 27, 28, 34). Because lymphoid tissues are the major site of HIV replication (7, 25, 26), they are a crucial location wherein anti-HIV drugs should be effective in suppressing HIV replication. However, investigation of the activity of anti-HIV drugs in these lymphoid compartments has been hampered by the difficulties of routinely accessing lymphoid tissues in HIV-infected humans. Development of an experimental mouse model that displays a high degree of HIV infection in lymphoid tissues that is responsive to anti-HIV therapy should greatly facilitate these studies. C.B-17 *scid/scid* mice (SCID mice) implanted with human fetal thymus and liver tissue (hu-thy/liv [thy/liv-SCID-hu mice]) are an attractive small animal model for studying in vivo HIV-1 infection (23, 24). By altering the technique for implanting the hu-thy/liv under the kidney capsule of the SCID mice, we increased the numbers of human T cells in the murine peripheral lymphoid compartment so that disseminated HIV infection occurred after intraimplant or intraperitoneal inoculation (16). In addition to being populated in the periphery with human T cells, these modified thy/liv-SCID-hu mice are also populated with human monocytes and can be infected with a broad range of T-cell-tropic and monocyte-tropic HIV isolates (17, 18). Because high levels of HIV infection occur in the implanted human thymus and the mouse spleen of these modified thy/liv-SCID-hu mice (16), we postulated that these modified thy/liv-SCID-hu mice would be a useful model for assessing the in vivo effectiveness of drug treatment to suppress established HIV infection as well as to prevent acute HIV infection in the lymphoid compartment. Therefore, we investigated the effectiveness of oral adminis-

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tration of saquinavir to either suppress established HIV infection in the lymphoid tissues of these modified thy/liv-SCID-hu mice or to prevent HIV infection in these mice if started immediately after HIV inoculation.

## MATERIALS AND METHODS

**Implantation of hu-thy/liv into SCID mice.** hu-thy/liv obtained from 17- to 21-gestational-week fetuses within 8 h after the elective termination of pregnancy was implanted into SCID mice (6 to 8 weeks old) as described previously (16, 17). Briefly, SCID mice were anesthetized with pentobarbital (40 to 80 mg/kg of body weight), and the left and right kidneys were sequentially exteriorized and subcapsularly implanted with at least 10 pieces (1 $mm^3$) of syngeneic thymus and liver tissue. Over 95% of the mice were successfully implanted, and by 3 months after implantation, a greater than 20-fold increase of the implanted tissue from its original size was observed. The consent forms and procedures used in this study were reviewed and approved by the Albert Einstein College of Medicine Committee on Clinical Investigation.

**Flow cytometric analysis.** Flow cytometric analysis of mononuclear cells harvested from the hu-thy/liv implants and spleens of the thy/liv-SCID-hu mice was performed as described previously (16). After staining with peridinin chlorophyll protein-conjugated monoclonal antibody to human CD45, fluorescein isothiocyanate-conjugated monoclonal antibody to human CD4, and phycoerythrin-conjugated monoclonal antibody to human CD8 (Becton Dickinson, Mountain View, Calif.), expression of human CD4 and CD8 by human lymphocytes present in the thy/liv-SCID-hu mice was determined by three-color flow cytometric analysis with a FACScan cell analyzer with LYSIS-II software (Becton Dickinson). The expression of human CD45 was used to confirm the human origin of the cells. With lymphocyte gates set for control human lymphocytes, nonviable cells and unlysed erythrocytes were gated out based on their forward and side scatter profiles. Overlaps of phycoerythrin versus fluorescein isothiocyanate versus peridinin chlorophyll protein emissions were compensated for, and cutoff values for the quadrants were set with the appropriate positive and negative controls.

**Measurement of plasma saquinavir levels.** Quantitative analysis of saquinavir in plasma samples was carried out by means of a specific high-performance liquid chromatography-UV method. "Unknown," spiked standard, and quality control samples were extracted from plasma with a Varian AASP PrepStation with $C_8$ cassettes. The cassettes were primed with methanol and conditioned with aqueous ammonium acetate (0.01 M [pH 3]), and the sample was loaded and washed with more buffer. The cassettes were transferred to the AASP PrepStation, and the cartridges were purged with a buffer consisting of 1:1 methanol-distilled water. The samples were then washed onto the high-performance liquid chromatography system with an eluent made up of methanol-aqueous ammonium acetate (~0.01 M)-acetic acid (90:9.75:0.25) at a flow rate of 2 ml/min. Separation of saquinavir from endogenous compounds was achieved with a phenyl cartridge (8 by 100 mm) in a Waters radial compression module, and the drug was detected by UV $A_{238}$. The retention time of saquinavir was dependent on the individual cartridge, but variation of the ammonium acetate concentration between 0.006 and 0.019 M ensured that the optimum value of approximately 10 min was obtained. Standard curves were constructed from the ratios of the peak height of the drug to those of an internal standard by weighted linear regression analysis. Levels of quality control samples and unknowns in plasma were then calculated with respect to the standard curve.

**Infection of thy/liv-SCID-hu mice with HIV.** The SCID-hu mice were infected by direct injection of 300 50% tissue culture infective doses ($TCID_{50}$) of the HIV-$1_{59}$ isolate in a volume of 30 μl into the left hu-thy/liv implant. HIV-$1_{59}$ is a primary, monocyte-tropic, nonsyncytium-inducing strain isolated from the peripheral blood of a 17-month-old HIV-1-infected child by coculture with phytohemagglutinin (PHA)-activated donor peripheral blood mononuclear cells (PBMCs) as described previously (17). After expansion by another round of coculture with PHA-activated PBMCs, the viral stock was divided into aliquots that were frozen in liquid nitrogen.

**Treatment of the mice with saquinavir.** Saquinavir was mixed with powdered animal feed and then added to feeding jars specially designed to minimize spillage. The dosage was calculated based on the average oral intake by the mice (each weighing about 25 g) of 5 g of diet/day, resulting in a predicted saquinavir dosage of either 250 or 2,500 mg/kg/day. The thy/liv-SCID-hu mice were housed singly, so that drug consumption could be confirmed by measurement of the quantity of feed consumed.

**Titration of HIV-infected mononuclear cells in the hu-thy/liv implant and spleen by limiting dilution coculture.** The degree of HIV-1 infection in the hu-thy/liv implants and spleens was measured by quantitative coculture as described previously (16, 17). Fivefold dilutions of mononuclear cells isolated from the hu-thy/liv implants and spleens (ranging from $1 \times 10^6$ cells to $3.2 \times 10^2$ cells) were cultured in quadruplicate in 24-well culture plates at 37°C with PHA-activated PBMCs ($10^6$) in a total volume of 2.0 ml of RPMI 1640 with added fetal calf serum (10% [vol/vol]) and interleukin 2 (32 U/ml). One to 2 weeks later, the p24 antigen content of the culture supernatant was measured with the HIV-1 p24 core profile enzyme-linked immunosorbent assay (Dupont-NEN, Wilmington, Del.). The lowest dilution of added mononuclear cells that productively infected at least one of the quadruplicate cultures with HIV-1 was taken as the TCID, and the data are presented as TCID/$10^6$ mononuclear cells as described previously (11).

**HIV-1-specific DNA and RNA PCR.** The presence of HIV DNA *gag*-encoded sequences and spliced *tat*/*rev* mRNA sequences was assessed by PCR as described previously (15, 32). Briefly, mononuclear cells from the hu-thy/liv implant or spleens were lysed in guanidine isothiocyanate (4 M) buffer, and then cellular DNA and RNA were separated by cesium chloride (5.7 M) density gradient centrifugation and precipitated with ethanol. For detection of *gag* DNA, DNA (1 μg) was amplified for 35 cycles with a primer pair specific for the HIV *gag* gene segment (SK38-SK39) and electrophoresed through a 1.5% NuSieve–0.5% SeaKem agarose (FMC, Rockland, Maine) gel containing ethidium bromide, and the amplified product was visualized under UV light. HIV *tat*/*rev*-spliced RNA, which is associated with active viral replication (32), was detected by reverse transcription followed by PCR amplification (RT-PCR) as described previously (16, 32). Briefly, RNA (7 μg) in 7 ml of double-distilled $H_2O$ was mixed with 4 μl of 5× buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$), 2 μl of dithiothreitol (100 mM), 1 μl of random hexamers (BRL-Gibco, Gaithersburg, Md.), and 5 μl of mixed deoxynucleoside triphosphates (2 mM [each]). After being heated to 65°C for 10 min, the samples were cooled on ice for 5 min. One microliter (200 U) of Superscript reverse transcriptase (BRL-Gibco) was then added, and the mixture was incubated at 37°C for 60 min and then placed on ice. HIV-1 cDNA was amplified with a primer pair specific for *tat*/*rev*-spliced mRNA sequences (TR-5 and TR-3). The identity of the amplified product was confirmed by hybridization of a Southern blot of the amplified DNA with a biotin-labeled internal probe, SK19, specific for the SK38-SK39 product or a biotin-labeled internal probe, TR-4, specific for the TR-5–TR-3 product. The hybridized probe was detected with the Genius luminescent detection kit (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.). A given sample was regarded as positive if PCR amplification resulted in a DNA product of the predicted size that hybridized to the specific internal probe, and the assay is sensitive to 10 copies of HIV DNA. Positive and negative controls were included in all runs, and suggested guidelines for PCR quality control were followed to prevent contamination (19). For RT-PCR, the specificity for RNA was verified by demonstrating the absence of an amplified product following PCR amplification of samples that had not been reverse transcribed.

**Statistical analysis of the data.** The statistical significance of the data was evaluated by Student's *t* test.

## RESULTS

**Pharmacokinetic analysis of orally administered saquinavir in mice.** We have previously examined the toxicokinetics of saquinavir in mice after administration by oral gavage during safety studies with the compound. The drug has a short half-life, and so it was necessary to dose the animals twice a day in order to reduce the period at which plasma drug levels were low. It was not considered feasible to administer the drug by gavage more than twice a day, and a suitable compromise which produced reasonably consistent exposure with a convenient dosing regimen involved a first dose at the start of the working day and a second dose 6 h later (Fig. 1A). The necessity to avoid "drug holidays" for antiviral treatment and to reduce the handling of HIV-infected mice meant that the administration of saquinavir by frequent oral gavage was inappropriate, and we therefore examined the multiple-dose pharmacokinetics of the compound when incorporated into the animal's diet. Levels of saquinavir in plasma obtained after treatment of mice either by dietary admixture (2,500 mg/kg/day) or by twice-daily gavage dosing (2,000 mg/kg/day) were compared to those obtained in humans after oral treatment with either the standard dose of 1,800 mg/day (600 mg three times per day [t.i.d.]) or the investigational dose of 7,200 mg/day (31) given in six divided doses (Fig. 1A). Evaluation of plasma drug levels demonstrated that mice dosed with saquinavir by dietary admixture displayed consistent 24-h exposure (e.g., $C_{max}$ and $C_{min}$ = 1,400 and 320 ng/ml, respectively, at 2,500 mg/kg/day,) while mice treated by twice-daily gavage dosing of 1,000 mg/kg exhibited wide fluctuations in plasma drug concentrations, which ranged from a peak level of 2,100 ng/ml to trough levels that were undetectable (<20 ng/ml). Plasma drug levels exhibited by mice treated with saquinavir by dietary admixture with 2,500 mg/kg/day were comparable to

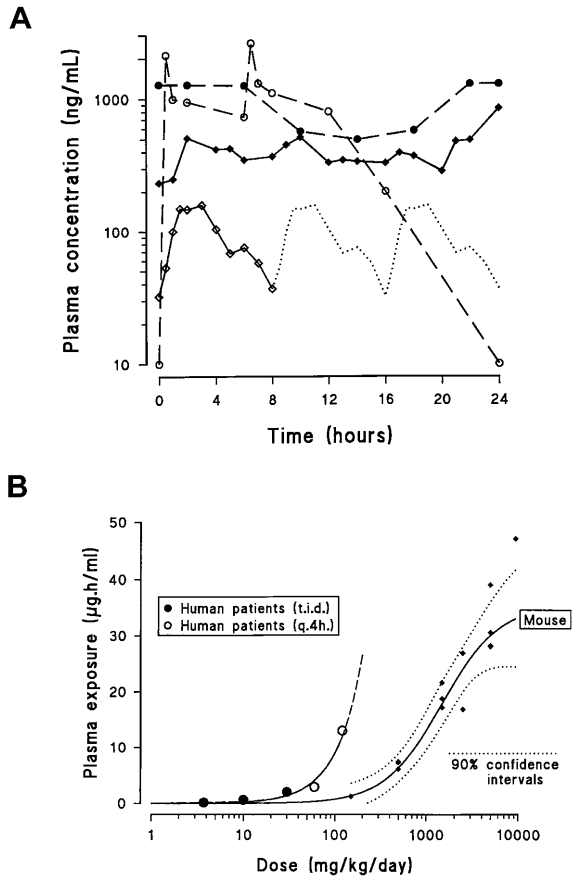


FIG. 1. Pharmacokinetic profiles of saquinavir in mice and humans. (A) Saquinavir was administered to the mice either by oral gavage of a suspension in 10% aqueous succinylated gelatin at alternating 6- and 18-h intervals or by dietary admixture for at least 2 weeks. Plasma drug levels were determined for two animals of each sex at 0.5, 1, 2, and 6 h after the morning gavage dose (○ [first of two 1,000-mg/kg/day doses) or at 4-h intervals from three mice of each sex during dietary administration (● [2,500 mg/kg/day). Each mouse was bled twice on any one day; thus, a group of 18 mice yielded 36 data points toward a single composite pharmacokinetic profile for dietary administration. The human patients (20 subjects per treatment) were dosed with saquinavir either at 1,800 mg/day divided into three doses (◇) or 7,200 mg/day divided into six doses (◆) for 28 days as described previously (31). Blood samples for analysis were drawn on 11 occasions during one dosing interval in the first study and on 19 occasions throughout the 24 h in the second. Levels of saquinavir in plasma were assayed as described in Materials and Methods. (B) The relationship between the saquinavir dose and exposure to mice and humans, reported as the daily area under the curve (AUC) for 24 h, was estimated with the trapezoidal rule, assuming a linear change in plasma drug concentration between adjacent time points (31). q.4h, once every 4 h. A total of 14 profiles were used for the calculation of the dose-exposure relationship in the mice. The relationship between the dose of saquinavir and the resultant exposure (AUC) was described by a sigmoidal curve according to the formula $[AUC = AUC_{max}/1 + (dose/dose_{50})^{-P}]$ where $AUC_{max}$ is the exposure (35.3 μg · h/ml) at infinite dose, $dose_{50}$ is the dose (1,484 mg/kg/day) which gives 50% of $AUC_{max}$, and $P$ is a constant (1.42) which describes the degree of sigmoidicity.

those obtained in humans treated with the investigational regimen of 7,200 mg/day in six divided 1,200-mg doses (31). Analysis of plasma exposure indicated that mice treated with saquinavir by dietary admixture with 250 mg/kg/day (not shown) or with 2,500 mg/kg/day exhibited values comparable to that observed in humans treated with the standard dose of 1,800 mg/day or the investigational regimen of 7,200 mg/day, respectively (Fig. 1B). Therefore, we investigated the effect of saquinavir given by dietary admixture at either 250 or 2,500 mg/kg/day on acute HIV infection and subsequent dissemination into lymphoid tissues in our modified thy/liv-SCID-hu mice.

**Saquinavir suppresses HIV infection in thy/liv-SCID-hu mice.** The effect of saquinavir treatment on established HIV infection present in the lymphoid tissues of thy/liv-SCID-hu mice was examined by infecting the thy/liv-SCID-hu mice by direct injection of HIV-$1_{59}$ (300 $TCID_{50}$) into the left hu-thy/liv implant. We have previously demonstrated in our modified thy/liv-SCID-hu mice that 1 month after inoculation of HIV into the hu-thy/liv implant implanted under the left kidney capsule, the HIV infection disseminates into the hu-thy/liv implant under the right kidney capsule, and extensive infection of both hu-thy/liv-implants occurs (16, 17). The effect of HIV infection on thymopoiesis in the hu-thy/liv implant is dependent upon the strain of HIV used to infect the mice, and HIV-$1_{59}$, the monocytotropic primary patient isolate used in this study, causes only minimal suppression of thymopoiesis despite mediating a high degree of cellular infection (17). The mice were then divided into three groups: one group was not treated, and the other two groups were treated with saquinavir either at a dose of 250 mg/kg/day or at a dose of 2,500 mg/kg/day. The mice tolerated both doses of saquinavir without any evidence of toxicity. After 1 month of treatment, the thymocytes in the hu-thy/liv implants and the human T cells present in the mouse spleens were analyzed by flow cytometry (Table 1). Comparable levels of immature CD4/CD8 double-positive, CD4 single-positive, and CD8 single-positive thymocytes were observed in the untreated and the saquinavir-treated mice. In addition, a distinct population of human T cells was present in the spleens of all of the thy/liv-SCID-hu mice examined except for thy/liv-SCID-hu mice 182b5 and 178-1.

The extent of HIV infection in the lymphoid tissues of the mice was evaluated by quantitative coculture to determine the number of HIV-infected cells present in the left and right hu-thy/liv implants and spleens. In contrast to the untreated thy/liv-SCID-hu mice, in which extensive HIV infection was detected in the hu-thy/liv implants (>3,125 $TCID/10^6$ cells), the thy/liv-SCID-hu mice treated with saquinavir doses of 250 and 2,500 mg/kg/day exhibited a lower viral load in the thy/liv implants, with means of 475 and 17.5 $TCID/10^6$ cells, respectively (Table 2). Thus, treatment with saquinavir at a dose of 250 or 2,500 mg/kg/day significantly decreased ($P < 0.01$) the level of HIV infection in the hu-thy/liv implants of the thy/liv-SCID-hu mice in a dose-related manner compared to the level of HIV infection in the hu-thy/liv implants of the untreated thy/liv-SCID-hu mice. Similar results were observed for the spleens of thy/liv-SCID-hu mice treated with saquinavir at 250 or 2,500 mg/kg/day, in which the mean viral loads of 15 and 2 $TCID/10^6$ cells, respectively, were significantly smaller ($P < 0.03$) than the mean viral load of 500 $TCID/10^6$ cells detected in the spleens from the untreated mice. When spleens from two thy/liv-SCID-hu mice that contained similar levels of human $CD4^+$ cells were compared, a markedly lower viral load of 5 $TCID/10^6$ cells was observed in the saquinavir-treated thy/liv-SCID-hu mouse (mouse 183-1) spleen compared to the viral load of 625 $TCID/10^6$ cells in untreated thy/liv-SCID-hu mouse (mouse 182b4) spleen. This suggested that the effects of saquinavir on the viral load in the spleens were independent of the numbers of human T cells present in thy/liv-SCID-hu mouse spleens.

**Prevention of HIV infection in thy/liv-SCID-hu mice by treatment with saquinavir.** To investigate whether acute HIV infection and subsequent dissemination into lymphoid tissues could be prevented by treatment with saquinavir, our modified thy/liv-SCID-hu mice were infected with HIV by direct injection into the left hu-thy/liv implant with a dose of HIV-$1_{59}$ (300

TABLE 1. Flow cytometric analysis of the hu-thy/liv implants and spleens of HIV-infected thy/liv-SCID-hu mice that were either untreated or treated with the indicated dose of saquinavir[a]

| Mouse | Dose of saquinavir (mg/kg/day) | % of cells positive for CD4 or CD8 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | R-hu-thy/liv implant | | | L-hu-thy/liv implant | | | Spleen | |
| | | CD4 | CD8 | DP | CD4 | CD8 | DP | CD4 | CD8 |
| 181a6 | 0 | 23 | 16 | 59 | 19 | 12 | 67 | 16 | 14 |
| 182b4 | | 11 | 5 | 83 | 15 | 7 | 77 | 6 | 3 |
| 183-5 | | 19 | 8 | 71 | 22 | 11 | 66 | 13 | 3 |
| 186-4 | | 10 | 7 | 82 | 10 | 8 | 81 | 19 | 12 |
| Avg ± SD | | 15.7 ± 6.3 | 9.0 ± 4.8 | 73.7 ± 11.2 | 16.5 ± 5.2 | 9.5 ± 2.4 | 72.7 ± 7.4 | 13.5 ± 5.6 | 8 ± 5.8 |
| 182b3 | 250 | 14 | 5 | 78 | 16 | 9 | 72 | 2 | 1 |
| 183-1 | | 18 | 9 | 71 | 14 | 10 | 76 | 8 | 3 |
| Avg ± SD | | 16 ± 2.8 | 7 ± 2.8 | 74.5 ± 4.9 | 15 ± 1.4 | 9.5 ± 0.7 | 74.0 ± 2.8 | 5.0 ± 4.2 | 2.0 ± 1.4 |
| 182b5 | 2,500 | 12 | 5 | 83 | 20 | 17 | 39 | 0 | 0 |
| 182b2 | | 10 | 5 | 85 | 11 | 6 | 82 | 3 | 1 |
| 178-1 | | 4 | 11 | 20 | 10 | 4 | 85 | 0 | 0 |
| Avg ± SD | | 8.7 ± 4.1 | 7.0 ± 3.5 | 62.7 ± 36.9 | 13.7 ± 5.5 | 9.0 ± 7.0 | 68.7 ± 25.7 | 1.0 ± 1.7 | 0.3 ± 0.6 |

[a] Thy/liv-SCID-hu mice were infected with a primary patient isolate, HIV-$1_{59}$, by injection of 300 $TCID_{50}$ into the left thy/liv implant. One month later, the mice were either not treated or started on saquinavir treatment (250 or 2,500 mg/kg/day). One month later, the mice were sacrificed, and the lymphocytes isolated from the right (R) and left (L) hu-thy/liv implants and spleens of the indicated thy/liv-SCID-hu mice were then analyzed by three-color flow cytometry for the expression of human CD4 and CD8 as described in Materials and Methods. The percentages of thymocytes in the hu-thy/liv implant single positive for CD4 or CD8 or double positive (DP) for CD4 and CD8 and lymphocytes in the spleen positive for CD4 or CD8 are indicated.

$TCID_{50}$) that is infectious 100% of the time (16). The mice were then divided into three groups: one group of mice was not treated, and the other two groups were treated with saquinavir at a dose of either 250 or 2,500 mg/kg/day. One month later, the mice were killed, and the population of human T cells in the hu-thy/liv implants and the mouse spleens was analyzed by flow cytometry (Table 3). Similar levels of immature CD4/CD8 double-positive, CD4 single-positive, and CD8 single-positive thymocytes were detected in the hu-thy/liv implants, and comparable numbers of human T cells were observed in the spleens of the untreated and saquinavir-treated thy/liv-SCID-hu mice.

The degree of HIV infection in the lymphoid tissues in the mice was evaluated by quantitative coculture of mononuclear cells isolated from both the left and right hu-thy/liv implants and from the mouse spleens. The degree of HIV infection in these mice was lower than that of the thy/liv-SCID-hu mice presented above (Table 2), because these thy/liv-SCID-hu mice were evaluated only 1 month after infection. As shown in Table 4, whereas a high degree of HIV infection was detected in the thy/liv implants (mean = 1,292 $TCID/10^6$ cells) and spleens (mean = 32 $TCID/10^6$ cells) of untreated mice, significantly less infection ($P < 0.01$) was observed in the thy/liv implants (mean = 2 $TCID/10^6$ cells), and less infection ($P < 0.18$) was detected in the spleens (mean = 1.8 $TCID/10^6$ cells) of mice treated with 2,500 mg of saquinavir per kg. In one mouse treated with 2,500 mg of saquinavir per kg (mouse 187a2), no HIV could be cultured either from the hu-thy/liv implants or from the spleen. To further evaluate these mice for HIV infection, lymphoid tissue samples from this mouse (187a2) and representative thy/liv-SCID-hu mice that were either untreated (mouse 187b5) or treated with 250 mg of saquinavir per kg per day (mouse 189a4) were further evaluated by investigating the hu-thy/liv implants and spleens for integrated HIV by *gag*-specific DNA PCR and for active viral replication by RT-PCR detection of spliced *tat-rev* RNA sequences (Fig. 2A). HIV *gag* DNA and *tat-rev* RNA sequences were detected in both of the hu-thy/liv implants and the spleens from the untreated thy/liv-SCID-hu mouse and the thy/liv-SCID-hu mouse treated with 250 mg of saquinavir per kg per day. In contrast, the hu-thy/liv mouse treated with 2,500 mg of saquinavir per kg per day had detectable HIV *gag* DNA only in the left hu-thy/liv implant that had been injected with HIV and not in the right thy/liv implant and the spleen. However, treatment with 2,500 mg of saquinavir per kg per day did not completely prevent dissemination in this mouse, as evidenced by the presence of a weak *tat-rev* RNA band in the right thy/liv implant and the spleen. Detection of human $\beta_2$-microglobulin DNA and RNA in the hu-thy/liv implants and spleens from the three mice evaluated confirmed the presence of human cells and the integrity of the DNA PCR and RT PCR (Fig. 2B).

TABLE 2. Effect of saquinavir on established HIV infection in thy/liv-SCID-hu mice[a]

| Mouse | Dose of saquinavir (mg/kg/day) | Virus titer ($TCID/10^6$ cells) | | |
|---|---|---|---|---|
| | | R-hu-thy/liv implant | L-hu-thy/liv implant | Spleen |
| 181a6 | 0 | >3,125 | >3,125 | 625 |
| 182b4 | | >3,125 | >3,125 | 625 |
| 183-5 | | >3,125 | >3,125 | 125 |
| 186-4 | | >3,125 | >3,125 | 625 |
| 182b3 | 250 | 625 | 25 | 25 |
| 183-1 | | 625 | 625 | 5 |
| 182b5 | 2,500 | 25 | 25 | 0 |
| 182b2 | | 25 | 25 | 5 |
| 178-1 | | 0 | 5 | 1 |

[a] Thy/liv-SCID-hu mice were infected with a primary patient isolate, HIV-$1_{59}$, by injection of 300 $TCID_{50}$ into the left thy/liv implant. One month later, the mice were either not treated, or were started on saquinavir treatment (250 or 2,500 mg/kg/day). One month later, the mice were sacrificed, and the viral loads in the right (R) and left (L) thy/liv implants and spleens were assayed by quantitative coculture.

TABLE 3. Flow cytometric analysis of hu-thy/liv implants and spleens of thy/liv-SCID-hu mice in whom saquinavir treatment was started immediately after infection with HIV[a]

| Mouse | Dose of saquinavir (mg/kg/day) | % of cells positive for CD4 or CD8 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | R-hu-thy/liv implant | | | L-hu-thy/liv implant | | | Spleen | |
| | | CD4 | CD8 | DP | CD4 | CD8 | DP | CD4 | CD8 |
| 187a1 | 0 | 11 | 3 | 85 | 15 | 4 | 81 | 10 | 2 |
| 187b3 | | 18 | 4 | 78 | 15 | 5 | 80 | 5 | 1 |
| 187b5 | | ND[b] | ND | ND | 12 | 5 | 83 | 3 | 1 |
| 187b6 | | 13 | 4 | 82 | 11 | 3 | 85 | 2 | 1 |
| 189a2 | | 10 | 3 | 86 | 13 | 4 | 83 | 7 | 0 |
| 189b1 | | 7 | 3 | 90 | 6 | 3 | 91 | 11 | 1 |
| Avg ± SD | | 11.8 ± 4.1 | 3.4 ± 0.5 | 84.2 ± 4.5 | 12.0 ± 3.3 | 4.0 ± 0.9 | 83.8 ± 3.9 | 6.3 ± 3.7 | 1.0 ± 0.6 |
| 187a5 | 250 | 14 | 5 | 81 | 14 | 4 | 83 | 20 | 4 |
| 187b4 | | 15 | 3 | 82 | 11 | 3 | 86 | 5 | 1 |
| 189a4 | | 10 | 5 | 84 | 9 | 4 | 86 | 14 | 2 |
| Avg ± SD | | 13.0 ± 2.6 | 4.3 ± 1.1 | 82.3 ± 1.5 | 11.3 ± 2.5 | 3.7 ± 0.6 | 85.0 ± 1.7 | 13.0 ± 7.5 | 2.3 ± 1.5 |
| 187a2 | 2,500 | 10 | 3 | 86 | 13 | 4 | 83 | 10 | 2 |
| 189a1 | | 8 | 4 | 88 | 8 | 4 | 88 | 13 | 3 |
| 189a5 | | 9 | 4 | 87 | 9 | 5 | 86 | 15 | 5 |
| 189b3 | | 10 | 4 | 85 | 8 | 4 | 88 | 10 | 1 |
| Avg ± SD | | 9.2 ± 0.9 | 3.7 ± 0.5 | 86.5 ± 1.3 | 9.5 ± 2.4 | 4.2 ± 0.5 | 86.2 ± 2.4 | 12.0 ± 2.4 | 2.7 ± 1.7 |

[a] thy/liv-SCID-hu mice were infected with a primary patient isolate, HIV-$1_{59}$, by injection of 300 $TCID_{50}$ into the left thy/liv implant and then were either not treated, or were immediately started on saquinavir treatment (250 or 2,500 mg/kg/day). One month later, the mice were sacrificed, and lymphocytes isolated from the right (R) and left (L) hu-thy/liv implants and spleens of the indicated thy/liv-SCID-hu mice were analyzed by three-color flow cytometry for the expression of human CD4 and CD8. The percentages of thymocytes in the hu-thy/liv implant single positive for CD4 or CD8 or double positive (DP) for CD4 and CD8 and lymphocytes in the spleen positive for CD4 or CD8 are indicated.

[b] ND, not determined.

To evaluate if the HIV infection in the saquinavir-treated mice was mediated by saquinavir-resistant isolates, the sensitivity to saquinavir of HIV isolated by coculture from the hu-thy/liv implants from thy/liv-SCID-hu mice was assessed. HIV isolated by coculture from the left and right hu-thy/liv implants of thy/liv-SCID-hu mice (Table 4) that were either untreated (mouse 187b5), treated with 250 mg of saquinavir per kg per day (mouse 189a4), or treated with 2,500 mg of saquinavir per kg per day (mouse 189a5) was harvested. The sensitivity of the isolated HIV to saquinavir was determined with an in vitro assay (13) by culturing an aliquot of virus isolated from the indicated thy/liv implant with activated PBMCs and saquinavir added at doses ranging from 0 to 1,800 nM. The saquinavir concentration required to inhibit approximately 50% of p24 antigen production by PBMCs ($IC_{50}$) after infection with the initial inoculum of HIV-$1_{59}$ (22 nM) and the HIV isolated from the untreated thy/liv-SCID-hu mouse 187b5 (20 nM) was comparable to the $IC_{50}$ of the HIV isolated from the saquinavir-treated thy/liv-SCID-hu mice 189a4 and 189a5 (40 and 12 nM, respectively) (Fig. 3). In contrast, the saquinavir-resistant isolate p11 had an $IC_{50}$ of about 200 nM.

## DISCUSSION

The dramatic suppression of HIV RNA levels in plasma of HIV-infected individuals treated with protease inhibitors alone or in combination with inhibitors of reverse transcriptase has indicated that chemotherapy may be able to potently suppress HIV replication and thereby significantly delay the progression of HIV-mediated disease (29). Although lymphoid tissues are the primary location of HIV replication in HIV-infected individuals, the limited ability to sample them has focused the evaluation of the efficacy of these new therapeutic approaches upon measuring their effect on the plasma HIV RNA levels. The investigation of protease inhibitor-mediated inhibition of HIV replication would be greatly facilitated by being able to

TABLE 4. Effect of saquinavir treatment on HIV infection when saquinavir treatment was started immediately after infection with HIV[a]

| Mouse | Dose of saquinavir (mg/kg/day) | Virus titer ($TCID/10^6$ cells) | | |
|---|---|---|---|---|
| | | R-thy/liv implant | L-thy/liv implant | Spleen |
| 187a1 | 0 | 625 | 625 | 125 |
| 187b3 | | 125 | 625 | 5 |
| 187b5 | | 3125 | 3125 | 5 |
| 187b6 | | 125 | 125 | 25 |
| 189a2 | | 625 | 625 | 5 |
| 189b1 | | 3125 | 625 | 25 |
| 187a5 | 250 | 25 | 25 | 5 |
| 187b4 | | 125 | 125 | 1 |
| 189a4 | | 125 | 25 | 5 |
| 187a2 | 2,500 | 0 | 0 | 0 |
| 189a1 | | 5 | 1 | 5 |
| 189a5 | | 1 | 5 | 1 |
| 189b3 | | 5 | 25 | 1 |

[a] thy/liv-SCID-hu mice were infected with a primary patient isolate, HIV-$1_{59}$, by injection of 300 $TCID_{50}$ into the left thy/liv implant. The mice were then either not treated or immediately treated with saquinavir at either 250 or 2,500 mg/kg/day. One month later, the mice were sacrificed, and the viral load in the right (R) and left (L) thy/liv implants and spleens was assayed by quantitative coculture. The data are presented as $TCID/10^6$ cells.

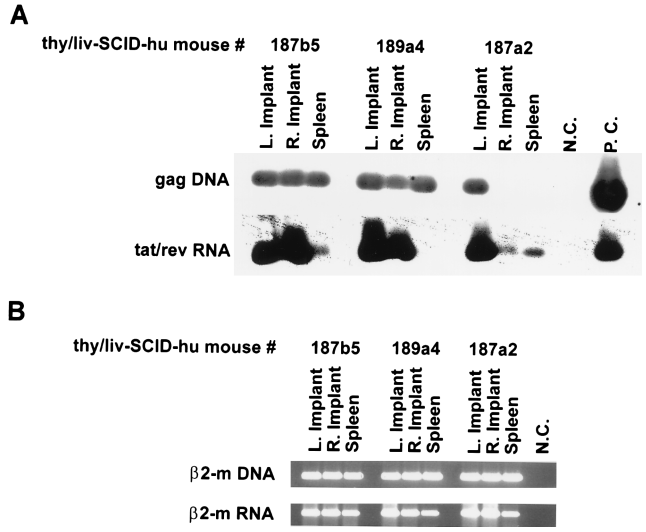


FIG. 2. Detection of *gag* DNA and *tat-rev* RNA in the hu-thy/liv implants and spleens of acutely treated, HIV-1-infected thy/liv-SCID-hu mice. (A) The presence of *gag* DNA or *tat-rev* RNA in the left (L.) and right (R.) hu-thy/liv implants and spleens of thy/liv-SCID-hu mice infected with HIV-1 by intraimplant injection and then either not treated (mouse 187b5), or treated with saquinavir at a dose of 250 mg/kg/day (mouse 189a4) or 2,500 mg/kg/day (mouse 187a2) for 1 month was detected by Southern blotting of PCR-amplified DNA or cDNA as described in Materials and Methods. (B) The presence of human cells and integrity of the DNA PCR and RT-PCR for each sample are indicated by the detection of human $\beta_2$-microglobulin ($\beta$2-m) DNA and RNA by PCR and RT-PCR with a primer pair specific for human $\beta_2$-microglobulin cDNA as described previously (18). The amplification products of the predicted size were visualized in ethidium bromide-stained gels by UV radiation. P.C. and N.C., positive and negative controls, respectively.

easily assess their effect on HIV infection in the lymphoid tissues. If drug therapy can completely suppress HIV infection in the lymphoid tissues, it will be important to delineate whether there are potential reservoirs of quiescent HIV infection such as in long-lived T cells, monocytes, dendritic cells, or stem cells that can be reactivated after withdrawal of therapy. An additional potential reservoir for HIV infection is the central nervous system, in which adequate therapeutic levels of saquinavir may not be obtained. These studies should be greatly facilitated by the availability of an animal model that develops disseminated HIV infection in lymphoid tissues and that can be treated orally with drugs. SCID-hu mice have been used to demonstrate the in vivo effects of zidovudine treatment on HIV infection by PCR analysis of the human thymic implants 2 weeks after they were directly injected with HIV (21). However, in that model, no evidence of dissemination of HIV infection from the injected thymic implant was observed in the untreated SCID-hu mice (21). To enhance the utility of this mouse model for studying in vivo HIV infection, we developed a modified thy/liv-SCID-hu mouse model whose peripheral blood and lymphoid tissues were populated with large numbers of human T cells that could become infected with HIV after inoculation of HIV into the hu-thy/liv implant (16).

In the current study, we used these modified thy/liv-SCID-hu mice to investigate the in vivo effect of oral drug therapy with the potent protease inhibitor saquinavir on HIV infection in lymphoid tissues. Pharmacokinetic studies demonstrated that dosing the mice by mixing the saquinavir with powdered feed provided therapeutic levels of drug exposure. Treatment with 250 or 2,500 mg/kg/day provided the mice with plasma drug levels that were comparable to those observed in patients treated with saquinavir at a dose of 1,800 or 7,200 mg/kg/day, respectively. We also demonstrated that oral treatment of thy/liv-SCID-hu mice with established HIV infection with saquinavir for 1 month had a potent inhibitory effect on HIV infection present in the hu-thy/liv implants and the spleen. The effectiveness of saquinavir was dose related, with a moderate effect observed when given at 250 mg/kg/day and a potent effect observed when given at 2,500 mg/kg/day. It is likely that because of the poor oral absorption of saquinavir (9, 33), greater doses are required to achieve the higher plasma drug levels necessary to obtain the optimal saquinavir-mediated suppressive effect on HIV replication. Our observation that treatment with the higher dose of saquinavir of 2,500 mg/kg/day was more effective than therapy with the lower dose of 250 mg/kg/day in suppressing HIV load in the lymphoid tissues is compatible with the recent observation that monotherapy of HIV-infected individuals with 7,200 mg of saquinavir per kg per day produced a more durable suppression of viral load and elevation of $CD4^+$ T-cell counts than did treatment with lower doses (31). Thus, our data demonstrated that the anti-HIV effect of saquinavir on HIV infection in lymphoid tissues could be markedly enhanced by increasing the dose and furthermore indicated that this model should be useful in correlating in vivo drug levels with their in vivo suppressive effect on HIV replication.

Investigation of the capacity of saquinavir to treat acute HIV infection demonstrated that saquinavir alone had a significant but incomplete effect on inhibiting HIV infection. HIV could be cultured from the hu-thy/liv implants and spleens from three of four of the thy/liv-SCID-hu mice treated soon after infection with 2,500 mg of saquinavir per kg per day. In the other thy/liv-SCID-hu mouse (mouse 187a2) immediately treated with 2,500 mg of saquinavir per kg per day, who had levels of human thymocytes in the hu-thy/liv implants and human T cells in the spleen comparable to those of the untreated thy/liv-SCID-hu mice, no evidence of HIV infection of lymphoid tissues could be demonstrated by coculture. However, further analysis of lymphoid tissues from this mouse

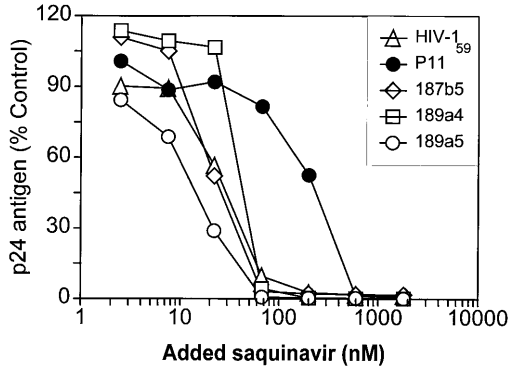


FIG. 3. Sensitivity of HIV isolated from saquinavir-treated thy/liv-SCID-hu mice to saquinavir. HIV was isolated by coculture from the hu-thy/liv implants obtained 1 month after thy/liv-SCID-hu mice were inoculated with HIV and then either not treated (mouse 187b5), or started on treatment with saquinavir at a dose of 250 mg/kg/day (mouse 189a4) or 2,500 mg/kg/day (mouse 189a5). An aliquot of the coculture supernatant containing equivalent levels of virus was harvested and added to new cultures containing activated PBMCs and the indicated concentration of saquinavir. In addition, equivalent doses of HIV-$1_{59}$ and p11, a saquinavir-resistant isolate, were also cultured with activated PBMCs and the indicated concentration of saquinavir. After 7 days of culture, culture supernatants were harvested, and the mean p24 concentration (picograms per milliliter) of duplicate culture supernatants of the left and right hu-thy/liv implant isolates from the indicated thy/liv-SCID-hu mouse was determined. The data are presented as the percentage of the control of the mean p24 concentration obtained from culture of the indicated isolate in the absence of added saquinavir.

demonstrated that although the hu-thy/liv implants and spleen were negative by coculture, evidence of infection and dissemination was detected by PCR. The left hu-thy/liv implant that had been injected with virus was positive for both HIV *gag* DNA and *tat-rev* RNA, and the right hu-thy/liv implant and the spleen were negative for HIV DNA but positive for *tat-rev* RNA. This suggested that although saquinavir treatment was potently suppressing HIV infection, subclinical dissemination had occurred that involved the infection of only small numbers of cells. These data raise the possibility that after withdrawal of therapy, virus present in these lymphoid tissues may serve as a reservoir to initiate recurrence of active HIV infection. This has major implications for the evaluation and management of individuals treated with potent anti-HIV therapy soon after acute infection. It is likely that other protease inhibitors such as ritonavir and indinivir with higher bioavailabilities and more potent antiviral effects than saquinavir (8, 9, 20) will be even more effective in inhibiting HIV infection in our modified thy/liv-SCID-hu mice. In addition, treatment with the combination of protease inhibitors and reverse transcriptase inhibitors may be more effective than monotherapy to prevent HIV infection, especially when started acutely after exposure to HIV. Thus, our modified thy/liv-SCID-hu mice should prove to be a useful model for evaluating whether drug combinations that include the newer protease inhibitors can eradicate or just suppress HIV infection in the major location for HIV replication, the lymphoid tissue. The availability of an animal model will be especially important for experiments designed to observe whether HIV infection becomes reactivated after the termination of therapy. Since these mice can be observed for up to a year after infection, they can be used for long-term studies.

Although protease inhibitors are potent suppressers of HIV replication, their clinical use may be limited by the development of HIV mutants that are resistant to their action (30). Saquinavir-resistant isolates of HIV can be generated in vitro by culturing HIV with increasing concentrations of saquinavir (6, 13). In vivo development of protease resistance variants has also been observed in patients treated with the protease inhibitor MK-639 who became populated with HIV isolates that exhibited resistance to MK-639 and cross-resistance to six structurally diverse protease inhibitors, including saquinavir (4). Therefore, it is unlikely that protease inhibitors will be effective as single agents for the long-term treatment of HIV-infected individuals. After 4 weeks of saquinavir treatment, we did not see the in vivo development of saquinavir-resistant isolates. Because protease-resistant HIV was first isolated in patients after 24 weeks of therapy with the protease inhibitor MK-639 (4), it is likely that 1 month of treatment may be too short a period of time to detect the development of saquinavir-resistant isolates. Therefore, we plan to observe the mice for at least 6 months after starting therapy for the development of resistant isolates.

Taken together, the data presented in this paper demonstrate that the effect of antiviral therapy on HIV infection in the major site of HIV replication, the lymphoid tissues, can be readily evaluated in our thy/liv-SCID-hu mice and that the HIV isolated from this tissue can be easily analyzed for drug resistance. Because these thy/liv-SCID-hu mice can be infected with a broad range of HIV isolates, they should prove to be a useful model with which to determine which drug therapy targeted to HIV exhibits the most potent antiviral effect while leading to the least development of resistant isolates. These studies should facilitate the in vivo evaluation of the effectiveness of different therapeutic interventions on acute and chronic HIV infection.

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