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Preclinical Evaluation of Antiviral Activity and Toxicity of Abbott A77003, an Inhibitor of the Human Immunodeficiency Virus Type 1 Protease

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A synthetic, symmetry-based inhibitor of the human immunodeficiency virus type 1 (HIV-1) protease, A77003, was evaluated for antiviral activity and cytotoxicity in vitro in human peripheral blood lymphocytes or cell lines H9, CEM, and U937. Toxicity and antiviral activity of the HIV-1 protease inhibitor were compared with those of the reverse transcriptase inhibitors zidovudine and 2′,3′-dideoxy-2′,3′-didehydrothymidine and human recombinant alpha and beta interferons. Production of infectious virus particles, cell-free p24 antigen, and cell-associated viral proteins was reduced 50% by the HIV-1 protease inhibitor at concentrations of 0.12 to 0.26 μM (50% effective concentration [EC_{50}]) in acute infection and 0.2 to 1.7 μM (EC_{50}) in persistent infection. Fluorescence-activated cell sorter analysis of U937 cells persistently infected with HIV_{IIIB} using a monoclonal antibody to HIV also showed a reduction of cell-associated viral protein in A77003-treated cells. Furthermore, toxicity of A77003 assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay was not observed at greater than 100 times the EC_{50}. A77003 was more effective in persistent HIV-1 infection than alpha and beta interferons (1,000 U/ml), while zidovudine and 2′,3′-dideoxy-2′,3′-didehydrothymidine were not active.

Current treatment for human immunodeficiency virus (HIV) infection is dominated by inhibitors of the viral reverse transcriptase (RT) which act early in the virus life cycle prior to integration (5, 6, 23). Therapeutic strategies with antiretroviral agents targeting other steps in the life cycle of HIV are of major importance, considering the development of viral resistance against RT inhibitors, ineffectiveness of RT inhibitors in persistently infected cells, and toxicity of these agents (5, 8, 13, 14, 19, 20). HIV type 1 (HIV-1) has three major genes (*gag*, *pol*, and *env*) which are translated into polyprotein precursors and undergo post-translational proteolytic cleavage and modification (7). The *gag-pol* and *gag* polyproteins are specifically cleaved by a virally encoded aspartic protease, HIV protease, which is of interest as a target for the development of new antiviral agents (3). Earlier symmetry-based HIV protease inhibitors developed by Abbott Laboratories, Abbott Park, Ill., were found to inhibit the HIV protease at nanomolar concentrations but had poor water solubility (4, 11). The terminal residues of lead compounds were substituted to increase water solubility (10), and the most promising derivative, A77003, was chosen for further evaluation. While previous studies of HIV-1 protease inhibitors and A77003 in particular (10, 11) were based on the antiviral effect against acute HIV infection, we investigated the antiviral effect of A77003 in cultured lymphoid and monocyte cell lines persistently infected with HIV_{IIIB} and HIV_{MN} and in human peripheral blood lymphocytes (PBLs) with established HIV_{IIIB} infection. Our results provide further evidence that A77003 is effective against both acute and persistent infection of different cell types with HIV-1_{MN} and HIV-1_{IIIB} at similar concentrations.

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MATERIALS AND METHODS

Drugs and compounds. A77003 was provided by Abbott Laboratories. The compound was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO). For experiments, the compound was diluted in test media with a maximal final DMSO concentration of 0.1%, which was found to be not toxic to the cells used in this study. Zidovudine (AZT) was a gift from Philip Furman, Burroughs Wellcome Co. (Research Triangle Park, N.C.); 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) and 2′,3′-dideoxyinosine (ddI) were from Bristol Myers Squibb (Wallingford, Conn.). Human recombinant alpha interferon 2b (IFN-α) was kindly provided from Bill Tall, Schering Corp. (Bloomfield, N.J.). Human recombinant IFN-β was a gift from Gary Williams, Triton Biosciences (Alameda, Calif.).

Cells and viruses. The $CD4^+$ HeLa clone 1022, CEM, H9, and U937 cell lines; human immunoglobulin G anti-HIV antibody; and HIV-1_{IIIB} and HIV-1_{MN} were obtained from the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Cell-free tissue culture fluid from H9 cells persistently infected with HIV-1_{MN} or HIV-1_{IIIB} containing approximately 10^4 or $10^{4.7}$ infectious units per ml, respectively, was used for acute infection experiments. An infectious unit was defined as the activity in a given sample which induced one syncytium in a HIV focal immunoassay. In studies of acute HIV infection, 2.5×10^3 H9, CEM, or U937 cells per well were preincubated in 200 μl of serum-free RPMI 1640 medium (Paragon, Baltimore, Md.) in 96-well plates for 4 h with the indicated concentrations of drugs prior to infection (0, 0.001, 0.01, 0.1, 1, 10, 25, 50, and 100

μM A77003; 1 μM AZT; 10 μM d4T; 1,000 U of IFN-α per ml; and 1,000 U of IFN-β per ml). Cells were infected with 25 U of infectious HIV (multiplicity of infection, 0.01), and 2 h postinfection cells were washed three times with medium and resuspended in medium containing the same concentrations of drugs as prior to infection plus 10% fetal calf serum (Hyclone, Logan, Utah). Persistently infected cell lines were established after infection of 10^6 CEM, H9, or U937 cells with 10^4 infectious units of HIV-1_{IIIB} or HIV-1_{MN}. Persistently infected cultures of the indicated cell lines produced relatively high titers of virus for more than 4 weeks as measured by focal immunoassay and p24 antigen enzyme-linked immunosorbent assay (ELISA), and greater than 85% of cells were positive for cellular p24 antigen by fluorescence-activated cell sorter (FACS) analysis. Prior to experiments, cells were washed twice with phosphate-buffered saline (PBS; Biofluids, Rockville, Md.) and seeded into 96-well plates at a density of 2.5×10^3 per well in RPMI 1640 medium plus 10% fetal calf serum, with or without drugs. PBLs were from a single leukophoresed donor and had been frozen at −90°C. PBLs were thawed and washed twice, and aliquots of 10^6 cells were induced with 0.5 μg of phytohemagglutinin (Sigma Chemical Co., St. Louis, Mo.) per ml and 50 U of interleukin 2 (Genzyme, Boston, Mass.) per ml and infected with HIV_{IIIB} (multiplicity of infection = 0.1) on day 3 postinduction. In acute infection studies PBLs were treated as described above. Studies using PBL cultures with an established HIV_{IIIB} infection were initiated 7 days postinfection when FACS analysis indicated that greater than 70% of the cells were p24 antigen positive. Treatment was initiated on day 0, and drugs were present over the entire time span of the experiments. Samples were taken at days 1, 3, 6, 9, and 12.

HIV antigen assay. HIV *gag* p24 protein in cell-free culture supernatants of cells acutely or persistently infected with HIV was measured by using the Coulter p24 ELISA according to the manufacturer's guidelines (Coulter Immunology, Hialeah, Fla.). Absorbance was measured and data were analyzed by using a computer-supported microplate reader (Molecular Devices, Menlo Park, Calif.) and DELTA-SOFT ELISA software (Biometalics Inc., Princeton, N.J.). Samples for p24 antigen assay were taken on days 0 and 3 and every other day thereafter until day 15 after the initiation of experiments. The baseline (day 0) p24 value was subtracted from values obtained on later days. Means ± standard deviations (SDS) of duplicate samples from two independent experiments were used for calculation of efficacy.

HIV focal immunoassay. Titration of infectious HIV produced by acutely and persistently infected cell cultures was performed as described by Chesebro and Wehrly (1) with the CD4 transfected HeLa cell line 1022.

Cell toxicity assay. Cytotoxicity and/or antiproliferative activity of A77003 was assessed by measuring the formation of formazan, a tetrazolium dye, in a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (12) modified as described before (17). Since DMSO was used to solubilize A77003, control studies were performed to determine a concentration of DMSO (0.1%) which had no effect on cell proliferation in the MTT assay. Day 5 cultures of uninfected and acutely or persistently infected cells in 96-well microtiter plates, treated with 0.0, 0.001, 0.01, 0.1, 1, 10, 50, and 100 μM A77003, were pulsed for 4 h with 1 mg of MTT per ml (final concentration) in RPMI 1640 medium plus 10% fetal calf serum. Medium was removed from the cells, and the intracellular colored formazan dye was solubilized by the addition of 200 μl of DMSO per well. The emission maximum for formazan (490 nm) in our system was determined by emission scans of representative samples using a Beckman DU 65 spectrophotometer. Subsequent test plates were measured at 490 nm with a reference filter at 410 nm by using an MR700 plate reader (Dynatech, Chantilly, Va.).

Flow cytometric analysis. Cell-associated HIV p24 protein expressed in untreated and A77003-treated (10 μM) acutely HIV_{IIIB}-infected U937 cells was measured by flow cytometry. One million viable cells were fixed with methanol, washed twice with PBS, incubated with 0.1% Triton X-100 in PBS, washed twice again, and fixed with 0.5% paraformaldehyde in PBS. Cells were stained with fluorescein isothiocyanate-labeled anti-p24 (KC57; Coulter Immunology), washed, and analyzed on an EPICS flow cytometer (Coulter). Negative, positive, and irrelevant antibody controls were included in each experiment, as were relevant HIV p24-positive and -negative cell lines.

Statistics and calculations of efficacy and toxicity. The 50% cytotoxic concentration was the concentration of protease inhibitor at which 50% of the cells show a cytotoxic or antiproliferative effect in an MTT assay. The 50% effective concentration (EC_{50}) was the concentration of protease inhibitor that gave a 50% reduction in HIV core protein in cell-free culture supernatant as determined by a p24 ELISA. The percent cytotoxicity at a particular drug concentration was calculated according to the following formula: [1 − (optical density with a concentration of A77003/optical density with no treatment)] × 100. The percent efficacy at a particular drug concentration was calculated as {1 − [(p24 with a concentration of A77003 − baseline p24)/(p24 with no treatment − baseline p24)]} × 100. Values were entered in a nonlinear regression analysis program, ADAPT II (2). A sigmoid-Emax model was used to fit the data through the use of nonlinear least-squares regression employing a weight of 1.

RESULTS

Activity and toxicity of A77003 in acutely HIV-1-infected cells. The antiviral activity of A77003 in CEM, H9, and U937 cell lines challenged with HIV-1 strains MN and IIIB was evaluated in parallel to its cytotoxicity. On the basis of reduction of supernatant p24 from acutely infected cells, the EC_{50}s (0.12 to 0.26 μM) were similar in all the cell lines and HIV-1 strains tested (Table 1). A77003 also decreased the titers of supernatant HIV in a concentration-dependent manner (Table 2). Results obtained with human peripheral lymphocytes treated with A77003 and challenged with HIV_{IIIB} were similar to those obtained with cell lines (Table 1 and 2).

Additional experiments using FACS analysis and the Western blot (immunoblot) technique were performed to investigate the effect of A77003 on cell-associated HIV-1. Analysis of mean fluorescence intensity (in arbitrary units) 5 days postinfection indicated that continuous exposure to A77003 at a concentration of 1 μM when started 4 h prior to infection with HIV_{IIIB} resulted in a dose-dependent decrease in cell-associated virus p24 antigen per cell from 107 ± 21 to 64 ± 31 (mean ± SD; the uninfected control had a mean fluorescence intensity of 63 ± 24) and a decrease in the total number of HIV-infected cells from 37.4 to 18.5%. Concentrations of A77003 above 1 μM reduced cell-associated p24 to levels undetectable by both FACS and Western blot analysis. When cultures of U937 cells and PBLs exposed to 1 μM A77003 for 7 days were washed free of the drug and cultured in drug-free media, virus production was observed

TABLE 1. Antiviral activity of the HIV protease inhibitor A77003

Cell line	HIV strain	Infection	EC_{50} (μM) fitted[a]	Therapeutic index[b]
CEM	MN	Acute	0.26 ± 0.11	>380
		Persistent	0.46 ± 0.20	>217
	IIIB	Acute	0.12 ± 0.10	>833
		Persistent	0.50 ± 0.13	>200
H9	MN	Persistent	1.77 ± 0.83	>56
	IIIB	Persistent	0.57 ± 0.31	>175
U937	MN	Acute	0.14 ± 0.07	>714
		Persistent	0.19 ± 0.19	>526
	IIIB	Acute	0.12 ± 0.06	>833
		Persistent	0.68 ± 0.45	>147
PBL	IIIB	Acute	0.16 ± 0.12	ND
		Established	0.25 ± 0.15	ND

[a] Shown are means ± SDS of duplicates calculated from two independent experiments as described in Materials and Methods.
[b] The therapeutic index is estimated as the minimal therapeutic range of $100/EC_{50}$. The highest concentration of A77003 tested was 100 μM. ND, not determined.

by infectivity and p24 assay as early as 36 h after A77003 treatment was discontinued (data not shown).

Toxicity assessed by MTT assay was not observed at the highest concentration of A77003 tested (100 μM, continuous exposure for 4 consecutive days) for any of the four cell types. Similarly, cell viability assessed by trypan blue dye exclusion was not significantly reduced when cells were treated for 4 days with 100 μM A77003.

Antiviral activity and toxicity of A77003 in persistently HIV-1-infected cells. The effect of A77003 at different concentrations was compared with those of single concentrations of AZT, d4T, and IFN-α and -β known to be effective against HIV infection in U937 cells (Fig. 1A) and in PBLs with established HIV_{IIIB} infection (Fig. 1B). While RT inhibitors (AZT and d4T) were not active (as expected) in cells with established HIV-1 infection, A77003 inhibited p24 release at concentrations of 0.19 to 1.77 μM (Table 1). In general, A77003 appeared more active in acute infection than in persistent infection. A77003 also reduced the amount of infectious virions in supernatant of persistently HIV-infected cell lines and PBLs with established HIV infection (Table 2). Western blots of persistently HIV_{IIIB}-infected U937 cells showed a dose-dependent reduction of cell-associated virus *gag* proteins $pr55^{gag}$ and p24 when cells were treated with A77003 (Fig. 2). No cell-associated HIV p24 was detected with above 1 μM A77003 in Western blot studies, while the RT inhibitors, AZT, d4T, and ddI, did not significantly reduce cell-associated viral *gag* protein in persistently infected U937 cells.

Importantly, the protease inhibitor was not cytotoxic in MTT assays at a concentration of 100 μM. In contrast, concentrations of IFN-α and -β (1,000 U/ml), which decreased virus release from persistently infected cells (Fig. 1) (17, 18) by 20 to 40%, reduced cell growth and inhibited cellular protein synthesis by 10 to 20% compared with that in the control.

DISCUSSION

The HIV protease is responsible for posttranslational processing of the *gag-pol* and *gag* gene products. Inhibition of this processing prevents the release of infectious virus. This process is essential for virus maturation in both acutely and persistently infected cells. Since the crystal structure of the HIV-1 protease alone and in the presence of inhibitors was elucidated (4, 15, 16, 22), computer-assisted design of these drugs from X-ray diffraction crystallography data allows production of compounds with high specificity. Inhibition of the HIV-1 protease results in a marked suppression of acute virus infection, as was shown recently in other laboratories with A77003 and other C_2 symmetric HIV protease inhibitors (9, 10). The results presented in this study confirm those findings, although A77003 was less efficacious in our experimental setting when compared with findings of Kempf et al. (10), who reported an EC_{50} for $HIV\text{-}1_{MN}$ of 0.03 μM for acute infection of EM cells. This disparity may be explained by the larger amount of DMSO vehicle used by those colleagues, which could influence antiviral activity, i.e., by altering the permeability of the cells for A77003.

Here we supply evidence that A77003 was an effective inhibitor of infectious virus production in persistently infected cells while, as expected, nucleoside analogs were not.

TABLE 2. Inhibition of production of cell-free infectious virus by A77003 and other antiretroviral agents

Treatment	% of syncytia[a] in supernatant from:					
	PBLs with:		CEM cells with:		U937 cells with:	
	Acute infection	Established infection	Acute infection	Persistent infection	Acute infection	Persistent infection
A77003 (μM)						
100	ND	ND	0	0	0	0
50	ND	ND	0	1	0	0
10	3	6	4	6	4	7
1	6	9	8	19	12	23
0.1	46	55	66	81	63	71
0.01	86	91	94	96	84	85
0.001	ND	ND	102	95	98	99
AZT (1.0 μM)	3	92	5	94	6	91
d4T (10 μM)	5	95	7	93	6	93
IFN-α (1,000 U/ml)	ND	ND	28	57	35	65
IFN-β (1,000 U/ml)	ND	ND	24	53	30	62

[a] Infectious HIV_{IIIB} in cell-free supernatant from day 5 cultures was measured by the focal immunoassay described in Materials and Methods. The number of foci of multinucleated giant cells stained with anti-HIV sera was counted under a dissecting microscope. Values are expressed as percentages, with the value for supernatant from untreated cultures set at 100%, and are means from duplicate dishes. ND, not determined.

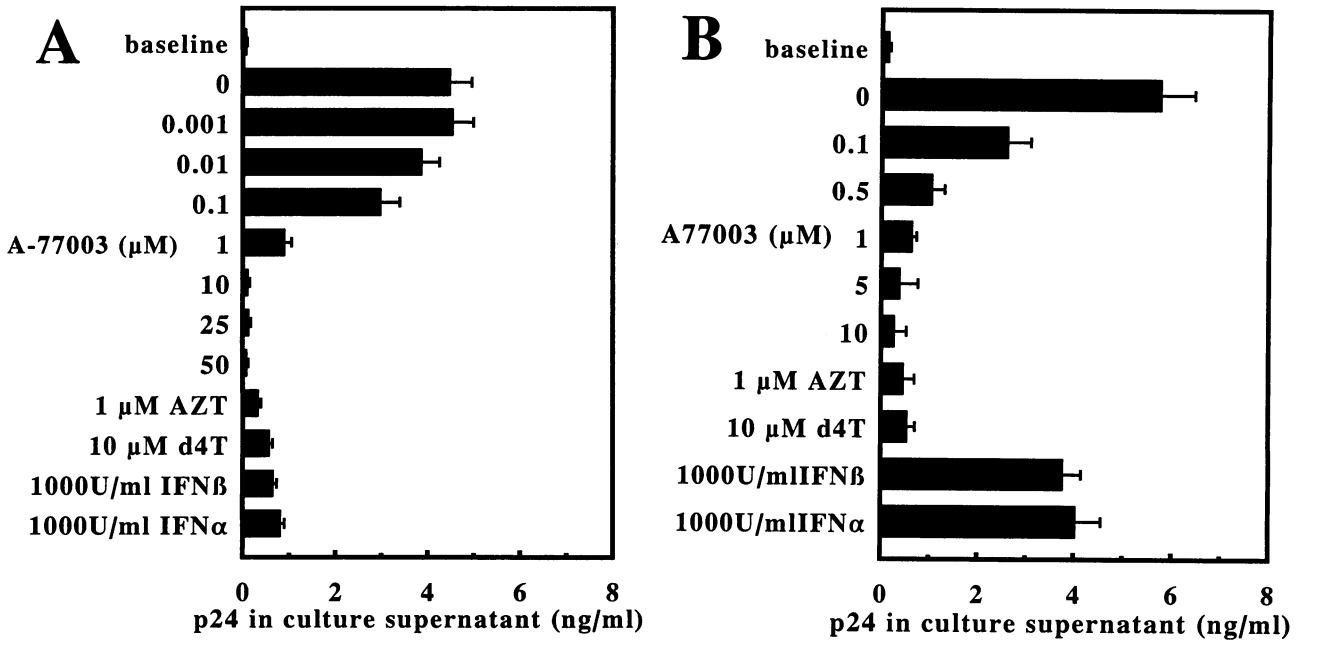

FIG. 1. Effect of A77003 and other antiviral agents on the replication of HIV_{IIIB} in U937 cells and PBLs. HIV p24 of day 5 supernatants of treated cultures was detected by Coulter p24 antigen ELISA. (A) Persistently HIV_{IIIB}-infected U937 cells; (B) PBLs with established HIV_{IIIB} infection. Bars indicate means ± SDS from duplicate cultures in two independent experiments.

Relatively high concentrations (1,000 U/ml) of IFN-α or -β inducing a significant antiproliferative effect in treated cells were unable to completely suppress production of infectious virions. In contrast, in the face of a high viral burden (>85% infected cells) A77003 was able to completely inhibit production of infectious particles in acute and established HIV infection. Surprisingly, no accumulation of the 55-kDa precursor protein was observed in Western blots of A77003-treated cells, while reduction of the $p24^{gag}$ protein occurred as expected. Theoretically, A77003 would inhibit cleavage of $pr55^{gag}$, which would accumulate, and p24 would be decreased. We do not have an explanation for this finding at the molecular level, and further experiments are in progress to investigate this observation.

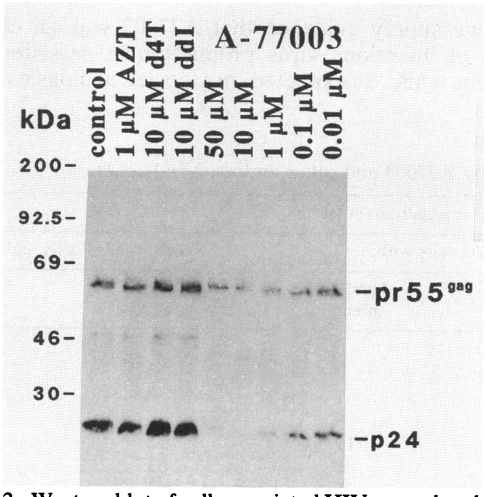

FIG. 2. Western blot of cell-associated HIV *gag*-related polyproteins in persistently HIV_{IIIB}-infected U937 cells. Cell extracts prepared from 7-day cultures were applied to sodium dodecyl sulfate-polyacrylamide gels for electrophoresis, and separated proteins were transferred to nitrocellulose. The blot was probed with a cocktail of anti-p24 antibodies (mouse) and reacted with horseradish peroxidase-labeled anti-mouse immunoglobulin G, and detection was done with a chemiluminescent substrate. Bands of a 55-kDa *gag* precursor polyprotein and mature *gag* p24 are recognized.

The antiviral effect of A77003 occurred at concentrations well below (>100-fold) those which induced cytotoxicity in parallel cultures of uninfected and infected cells. Results from MTT assays and 50% cytotoxic concentrations were similar to previous findings for which different techniques were employed to measure cytotoxicity (9, 10). The EC_{50} for suppression of virus release from persistently infected cells was in general 0.2 to 0.6 μM, whereas the EC_{50} range in acutely infected cells was from 0.1 to 0.3 μM. This may indicate a difference in the ability of the drug to inhibit viral replication in acute or persistent infection or may be related to the detection of a reduced amount of p24 produced by the small number of infected cells in the acute infection model. Changes in membrane permeability brought by the vehicle (DMSO) and in persistently infected cells may account for differences in uptake of the compound. Further studies which address the uptake and intracellular concentration of this compound may be important for the development of progenitor compounds with improved water solubility and bioavailability. Concentrations required to limit production of virus in persistently infected cells should be approachable in vivo (9). However, the moderate solubility (197 μg/ml at pH 7.4) and low oral bioavailability (<3%) of A77003 known from animal experiments (9, 10) suggest that it may be limited to intravenous administration for clinical use against HIV infection.

A77003 is a promising inhibitor of the HIV protease which should be investigated in controlled clinical trials. However, since viral integration in acute infection of cells in the

presence of A77003 was expected and observed (unpublished data), a combination of a protease inhibitor and a nucleoside analog may be optimal therapy for intervention early in HIV infection. Indeed, a very recent report suggests synergistic drug interaction in vitro with a combination of AZT and A77003 and additive efficacy with a combination of ddI and A77003 (9). Initial experiments using acute infection of human PBLs with HIV_{IIIB} in our laboratory indicate similar drug interaction of a combination of AZT and A77003. AZT (0.1 μM) and A77003 (0.1 μM) in combination decreased supernatant p24 in acutely HIV_{IIIB}-infected PBLs by 62.5% ± 5.5% (mean ± SD) compared to 42% ± 4.4% after exposure to single agents.

These observations may have important clinical implications from the standpoint of both reducing virus burden and limiting the emergence of resistance to the therapeutic regimen. The possibility that ongoing replication is necessary to generate mutants which can be selected by sustained exposure to antiviral pressure may provide a rationale for using concentrations of protease inhibitors which will markedly suppress the release of infectious virus.

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