

Human Serum α_1 Acid Glycoprotein Reduces Uptake, Intracellular Concentration, and Antiviral Activity of A-80987, an Inhibitor of the Human Immunodeficiency Virus Type 1 Protease

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The therapeutic utility of a human immunodeficiency virus type 1 (HIV-1) protease inhibitor may depend on its intracellular concentration, which is a property of its uptake, metabolism, and/or efflux. Previous studies in our laboratory indicated that the addition of α_1 acid glycoprotein (α_1 AGP) to the medium markedly increased the amount of the drug required to limit infection in vitro. In this study, physiologically relevant concentrations of α_1 AGP and a radiolabeled inhibitor, A-80987, were used to determine both the uptake and activity of the agent in HIV-1-infected human peripheral blood mononuclear cells and cell lines. Both the uptake and efflux of ¹⁴C-labeled A-80987 were rapid ($t_{1/2}$, <5 min). Uptake of the drug was linearly dependent on the concentration but insensitive to the metabolic inhibitors KF, sodium cyanide, or CCCP (carbonyl cyanide *m*-chlorophenyl hydrazine). The amount of A-80987 which entered the cells was inversely proportional to the concentration of α_1 AGP (r^2 , 0.99) and directly proportional to the amount of extracellular non-protein-bound drug (r^2 , 0.99). Most importantly, the antiviral activity of the drug in HIV-1-infected peripheral blood mononuclear cells and MT-2 cells was directly related to the amount of intracellular A-80987. This study demonstrates that A-80987 binds to α_1 AGP, resulting in a free fraction below 10%. Cellular uptake of A-80987 is proportionally decreased in the presence of α_1 AGP, which results in less-than-expected antiviral activity. Importantly, we demonstrate for the first time that the inhibition of HIV protease is highly correlated with the amount of intracellular inhibitor.

Human immunodeficiency virus type 1 (HIV-1), like many viruses, utilizes a protease which posttranslationally proteolytically cleaves the viral polyprotein precursors to the mature viral proteins during the late stages of virion particle formation (18, 29). Genetic and biochemical evidence indicates that proteolysis of *gag* and *pol* gene products is carried out by the HIV aspartic protease. Site-specific mutagenesis of the HIV protease or inhibition of the enzyme with specific inhibitors results in production of noninfectious virions, which have the morphological features of immature particles (5, 9, 15, 20). The critical role of protease in virus maturation has established the HIV protease as an important target for therapeutic intervention at the postintegration step of HIV replication. The prospect of a highly potent agent which has activity against chronically infected cellular reservoirs of HIV with a target site distinct from the viral reverse transcriptase has heightened the interest in this class of compounds.

HIV protease was both cloned and chemically synthesized, and the resultant structural information (5, 21, 22, 24) helped to accelerate the development of potent transition state analogs with exquisite specificity for the enzyme (8, 11, 32). Earlier studies had indicated that A77003, a symmetric inhibitor of the HIV protease, had both antiviral activity at submicromolar levels against a spectrum of HIV strains and an excellent therapeutic index in primary and transformed human cell lines (14, 16). A structurally related and equally potent peptidomimetic protease inhibitor, A-80987, exhibited bioavailability and phar-

macokinetic properties which justified its study as an oral agent in clinical trials (13).

Equilibrium binding studies indicated that several peptidomimetic protease inhibitors were highly serum protein bound (>90%). Preliminary studies in this laboratory indicated that radiolabeled A-80987 was >94% protein bound in patients with advanced HIV disease. This finding raised the question of whether protein binding had an effect on antiviral activity. We previously demonstrated that a major human serum protein, α_1 acid glycoprotein (α_1 AGP, orosomucoid) markedly reduced the in vitro activity of A77003 (3). Kageyama et al. (12) reported that another peptide-based HIV protease inhibitor, KNI-272, was highly bound to purified human α_1 AGP. Their study also demonstrated that the antiviral activity of KNI-272 was markedly reduced in the presence of high concentrations (>50%) of fetal bovine serum (FBS). While acidic drugs commonly bind to albumin, α_1 AGP is the major serum binding protein for propranolol, progesterone, and a number of other basic drugs (19, 31). Human α_1 AGP is an acute-phase protein whose synthesis increases during acute inflammatory episodes, infections, injuries, and neoplastic disease. Studies in our laboratory and other laboratories indicated that α_1 AGP levels are increased by at least 1.5-fold in AIDS patients (1, 25).

While data for other classes of anti-infective agents indicate that protein binding adversely affects microbiologic activity (7, 27), one might conclude that this may not apply to peptidomimetic protease inhibitors. One could hypothesize that active or facilitated uptake could drive penetration of the inhibitor into cells. If this were the case, it would be possible that the protein-bound drug has a K_d value for a transporter in the cell membrane which was at least 10-fold higher than that of the drug for its binding protein. Under such circumstances, protein

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binding may not have an adverse effect on drug penetration into cells and, consequently, would not be expected to affect the antiviral activity. To further elucidate the effect of protein binding on antiviral activity, we undertook a series of experiments examining whether (i) there was any concentration dependency of penetration of an inhibitor of HIV protease into cells, (ii) drug penetration was affected by any of a series of metabolic poisons, (iii) the purified binding protein α_1 AGP added to the medium in physiologic concentrations altered the penetration of the drug into the cells, and (iv) the addition of the binding protein altered the anti-HIV activity of the compound.

This study demonstrates that A-80987 binds to α_1 AGP, resulting in a free fraction below 10%. Cellular uptake of A-80987 is proportionally decreased in the presence of α_1 AGP, which results in a decrease in antiviral activity. Importantly, we demonstrate for the first time that the inhibition of HIV protease is highly correlated with the amount of the intracellular inhibitor.

MATERIALS AND METHODS

Cell culture. Human T-lymphoblastoid cell lines CEM, MT-2, and H9 cells infected with HIV-1 strain IIB (HIV-1_{IIB}) and other strains were originally obtained from the AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Cell lines were cultured in RPMI 1640 medium containing 10% (vol/vol) FBS, 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), and 50 μ g of gentamicin (Paragon Biotech, Baltimore, Md.) per ml. Peripheral blood lymphocytes from HIV-negative donors, obtained from the American Red Cross (Albany, N.Y.), were stimulated in RPMI 1640 containing 5 μ g phytohemagglutinin-P (Difco, Detroit, Mich.), interleukin-2 IL-2 (Advanced Biotechnologies, Inc., Columbia, Md.), 20% FBS, and penicillin (100 U/ml) and streptomycin (100 μ g/ml) (Gibco-BRL, Grand Island, N.Y.) for 2 to 3 days. Dose response assays were performed by incubating 10^6 cells with HIV at a multiplicity of infection of approximately 0.1 infectious unit per ml (3). Polybrene (4 μ g/ml; Sigma Chemical Co., St. Louis, Mo.) was present in the incubation medium (RPMI with 0.1% FBS) for cell lines but not for peripheral blood lymphocytes. After the 1-h incubation, the cells were centrifuged, and the medium was replaced with the appropriate growth medium.

Drugs and other compounds. A-80987 (molecular weight, 652.87) was provided by Abbott Laboratories (Abbott Park, Ill.). The solubility of A-80987 was <0.01 mg/ml in 0.1 M phosphate buffer (pH 6.8) and 445 mg/ml in dimethyl sulfoxide at room temperature. Therefore, the A-80987 compound was prepared as a 10 mM stock solution in dimethyl sulfoxide. For experiments, the compound was diluted in test media with a maximal final dimethyl sulfoxide concentration no higher than 0.1%, which was not toxic to the cells used in this study. 14 C-A-80987 (36.7 Ci per mmol) (purity, >97%) was prepared by the radiochemistry group, (Pharmaceutical Products Division, Abbott Laboratories). Potassium fluoride (catalog no. P-6642) and carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP; catalog no. C-2759) were obtained from Sigma Chemical Co. Sodium cyanide (38097) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Human serum α_1 acid glycoprotein (catalog no. G-9885; purity, 99%) were from Sigma Chemical Co. Human serum proteins were prepared as sterile solutions in RPMI 1640 with 10% FBS, prior to the assay.

Protein-binding assay. The extent of radiolabeled A-80987 bound in the presence of protein was determined with Amicon (Beverly, Mass.) Centrifree micro-partition units according to the manufacturer's recommendations. Radiolabeled A-80987 was added to 600 μ l of undiluted human plasma or tissue culture medium supplemented with 10% FBS and the indicated concentrations of purified human α_1 AGP. The drug was incubated with protein for 15 min at room temperature prior to centrifugation. The amount of free drug (unbound drug) was measured by liquid scintillation spectrometry of samples from the ultrafiltrate.

HIV antigen assay. Samples for the p24 antigen assay were taken at the indicated time points after initiation of the experiments and processed as described previously (1a). Absorbance was determined in a computer-supported microplate reader, and the levels of the p24 protein were calculated with Soft-Max software (Molecular Devices, Menlo Park, Calif.).

Uptake assay. The uptake of radiolabeled A-80987 was determined in uninfected and HIV-infected cells by a modification of an "oil-stop" microtube assay (7, 30, 33). Briefly, for uptake and efflux kinetics, PBMC or MT-2 cells (2×10^6 /ml) were mock infected or infected with HIV-1_{IIB} at a multiplicity of infection of 1. The cells were incubated for 1 h at 37°C, centrifuged, and suspended at 2×10^6 cells per ml in the medium. After 10 min, 0.1 ml of 100 μ M 14 C-A-80987 was added per each 0.9 ml of cell suspension. At the indicated time points, 500- μ l aliquots were pipetted onto 500- μ l aliquots of an oil mixture (1 part corn oil [Best Foods, Englewood Cliffs, N.J.] to 5 parts dibutyl phthalate

[Sigma]) in 1.7-ml Eppendorf tubes. Samples were centrifuged for 5 min at 9,500 rpm in a microcentrifuge. Tubes are frozen at -80°C after centrifugation. The tips of the tubes containing the cell pellet were removed with a scalpel and transferred to a vial. Cell pellets were solubilized with 1% sodium dodecyl sulfate, and Ecocint-H (National Diagnostics, Manville, N.J.) was added. The radioactivity in each sample was determined by liquid scintillation spectrometry. Other studies with different radiolabeled tracers in aqueous solution showed that cell pellets prepared by this method were essentially free of contaminating extracellular radiolabel (<0.2%). For uptake-efflux studies, samples were taken at 5, 15, 30, 45, and 60 min. The remaining cell suspension was centrifuged at low speed, and the pellet was suspended in medium without radiolabel to determine the efflux of labeled A-80987 from the cells at 0, 5, 15, 30, 45, and 60 min after suspension. For studies in which both uptake and the antiviral activity of the drug were determined in the presence and absence of binding proteins, the cell suspensions were labeled for 10 min. Samples (500 μ l) were layered on the oil mixture and processed as described above. The remaining cell suspension was further diluted in medium containing the appropriate amount of radioactive A-80987 and/or α_1 AGP and distributed to microtiter plates for a subsequent assay of p24 antigen or cytopathology. For inhibitor studies, cells are distributed to sample tubes containing either 1 mM KF, 1 mM sodium cyanide, 20 μ M CCCP, or the indicated concentrations of α_1 AGP. Radiolabeled A-80987 (final concentration, 10 μ M) was added to each tube, and at time zero and at 15 min, triplicate 0.5-ml samples were layered onto 0.5 ml of the oil mix and processed as described above.

RNA PCR. Total RNA extraction was performed by a modification of the procedure described by Mulder et al. (23). Briefly, 150 μ l of tissue culture medium from HIV-infected and treated cultures was lysed in four volumes of 5.75 M guanidinium thiocyanate-50 mM Tris (pH 7.5)-100 mM β -mercaptoethanol-1 μ g of poly(A) (obtained from Pharmacia, Piscataway, N.J.). Lysates were incubated at 65°C for 10 min, isopropanol precipitated, and suspended in 60 μ l of diethylpyrocarbonate-treated water containing 200 U of RNasin (Promega, Madison, Wis.) per ml and 1 mM dithiothreitol. Samples were serially diluted 10-fold four times.

Reverse transcription (RT) PCR, with SK38 and SK39 primers for the HIV-1 *gag* gene region (26), was performed as single-tube reactions. An unlabeled SK38 primer, a biotinylated SK19 primer, and a horseradish peroxidase-conjugated HIV-1 *gag* gene probe, SK39, were obtained from Synthetic Genetics (San Diego, Calif.). A dilution series consisting of 2,500, 10,000, 40,000, 160,000, and 640,000 copies of RNA transcribed from a plasmid containing a portion of the HIV-1 *gag* gene was also subjected to RT-PCR. RT of diluted RNA extracts or RNA standards was carried out in 20- μ l reaction mixtures containing a 500 μ M deoxynucleoside triphosphate mixture, 10 mM Tris, 50 mM KCl, 5 mM MgCl₂, 100 pmol of antisense primer, 50 U of Moloney murine leukemia virus RT (Gibco-BRL), and AmpliMax PCR Gem 50 (Perkin-Elmer, Norwalk, Conn.). Subsequent hot-start amplification of cDNA was achieved by adjusting the RT reaction to 50- μ l mixtures containing 10 mM Tris, 50 mM KCl, 2.5 mM MgCl₂, 200 μ M deoxynucleoside triphosphates, 50 pmol of SK38, and 1.25 U of Ampli-Taq (Perkin-Elmer). All reactions were carried out in a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer) with thin-walled MicroAmp reaction tubes with the following programs: for RT, 60 min at 42°C followed by 10 min at 95°C; for PCR, 28 two-step cycles of 95°C for 15 s and 60°C for 60 s, followed by 5 min at 72°C.

Amplified products were quantitated by an enzyme-linked oligosorbent assay (ELISA) as described by Holodniy et al. (10). Briefly, 5 μ l of heat-denatured PCR product was added to 100 μ l of hybridization solution containing 1 pmol of SK19-horseradish peroxidase, 7.5 \times Denhardt's solution, and 3.5 \times SSPE (1 \times SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 1 mM EDTA [pH 7.7]) in Reacti-Bind streptavidin-coated plates (Pierce, Rockford, Ill.). The plates were incubated at 42°C for 60 min and washed six times with phosphate-buffered saline containing 0.05% Tween 20 in a Wellwash 4 Microtiter Plate Washer (Denley Instruments, Durham, N.C.). A colorigenic substrate, σ -phenylenediamine (Abbott Diagnostics; Abbott Park, Ill.) was added to each well and incubated at room temperature for 30 min before substrate development was stopped with 1 M H₂SO₄. The A₄₉₀ was measured, and the data were analyzed with a Molecular Devices microplate reader.

MTT virus infectivity assay. The cytotoxicity of HIV-1 in the presence and absence of A-80987 was correlated to the formation of formazan in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (16). Day 5 cultures of MT-2 cells treated without or with A-80987 and/or other additives and acutely infected with HIV_{IIB} in 96-well microtiter plates were pulsed for 4 h with 1 mg of MTT per ml in RPMI 1640 plus 10% FBS. The supernatant was removed from the cells, and the formazan was solubilized with 85 μ l of acid isopropanol per well. The A₅₄₀ was read in a Biotech plate reader. The results (means \pm standard deviations) for quintuplicate wells were used in calculations to determine the cytotoxicity or inhibition of HIV-1-mediated apoptosis.

Statistical analysis. For calculation of the 50% effective concentration (EC₅₀), values for viral inhibition were entered into a nonlinear regression analysis program, ADAPT II (4). A sigmoid E_{max} model was used to fit the data through the use of nonlinear least-squares regression. Likewise, for calculation of the relationship between both the external free drug concentration and intracellular

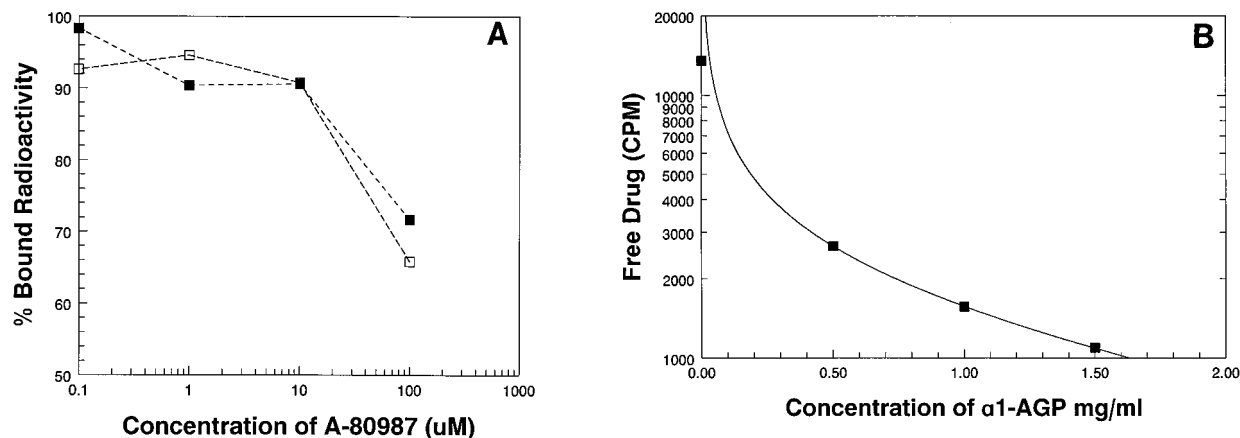


FIG. 1. Protein binding of A-80987. The free fraction of radiolabeled A-80987 was determined by a micropartition assay. (A) The protein-bound fraction of A-80987 in plasma (0.62 mg of α_1 -AGP per ml) was determined by the addition of increasing concentrations of radiolabeled drug to duplicate 600 μ l of undiluted plasma (□). The binding of A-80987 was assayed in duplicate 600- μ l samples of RPMI 1640 with 10% FBS and 1 mg of α_1 AGP per ml (■). (B) The percentage of 1 μ M A-80987 bound by the indicated concentration of α_1 AGP was determined by micropartition assay as described for panel A.

A-80987 (independent variables) and viral suppression (dependent variable), an inhibitory sigmoid E_{max} model was employed.

RESULTS

Effect of α_1 acid glycoprotein on the in vitro activity of A-80987. Figure 1A shows that >90% of 1 μ M A-80987 is protein bound in undiluted human plasma or purified α_1 AGP (1 mg/ml). The α_1 AGP binding is saturable since increasing concentrations of the drug increase the free (unbound) fraction of A-80987. Similarly, at a fixed concentration of the drug, increasing amounts of α_1 AGP reduce the free fraction to less than 10% (Fig. 1b). The exposure of HIV-infected MT-2 cells to A-80987 in medium with 10% FBS results in a concentration-dependent reduction in the replication of HIV, with an EC_{50} value of 0.244 μ M. The addition of 1 mg of α_1 AGP per ml, the mean of the normal range of drug concentration in serum, increases the EC_{50} by approximately 10-fold (Fig. 2). Other experiments showed that the α_1 AGP-mediated reduction in A-80987 activity can be determined by assay of viral infectivity, by RNA PCR, or by p24 production from acute or chronically infected cells (data not shown). By way of example, the activity of A-80987 was determined from the amount of HIV-1 RNA in tissue culture medium from HIV-1_{IIIB}-infected CEM cell cultures; there was greater than a 100-fold reduction in HIV RNA in treated cells which was reversed with the addition of 1 mg of AGP per ml. We obtained similar results for acute infection of PBMC with HIV-1_{IIIB} in the presence and absence of α_1 AGP (Fig. 3). For HIV-1_{IIIB}-infected PBMC, the average EC_{50} s were 0.23 μ M and 2.83 in the absence and presence of α_1 AGP, respectively, as determined by p24 assay of five replicate wells.

Uptake and efflux of radiolabeled A-80987. 14 C-labeled A-80987 was incubated with uninfected and HIV-infected peripheral blood lymphocytes or CD_4^+ cell lines. At the indicated time points, cells were pipetted over a layer of the dibutyl phthalate-corn oil mixture and rapidly centrifuged. The cells were pelleted through the oil, while the labeled medium remained above the oil layer. In all cases studied, the uptake of the drug from the medium was rapid, reaching a plateau within 15 min after exposure. An A-80987 uptake curve for HIV-infected MT-2 cells is shown in Fig. 4. Half-maximal uptake occurs within 1 min after the addition of the radiolabeled drug. Similar curves were obtained for uninfected CEM and MT-2

cells and PBMC and HIV-infected PBMC and CEM cells. Maximal uptake of A-80987 after a 15-min incubation was dependent on the concentration of the drug used, which ranged between 1 and 10 μ M, but the response was linear. Figure 5 shows that the amount of labeled A-80987 in PBMC after a 15-min incubation was directly proportional to the amount of extracellular free drug. Higher concentrations of the drug, >25 μ M, were precipitated and resulted in an artifactual response. The results with concentrations of radiolabeled A-80987 below 1 μ M were difficult to quantify because of the small number of counts in the cell pellets. Studies with the metabolic inhibitors sodium cyanide, potassium fluoride, or CCCP (the mitochondrial uncoupler) indicated that energy was not required for efficient uptake of A-80987 (Table 1).

The efflux of radiolabeled A-80987 from uninfected and HIV-infected cells preincubated with the drug for 30 min to 4 h was also rapid, with the half-maximal efflux occurring within

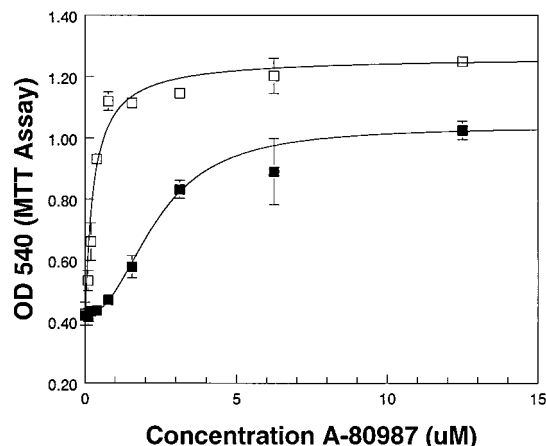


FIG. 2. In vitro efficacy of A-80987 in the presence and absence of α_1 AGP. The in vitro efficacy of the indicated doses of A-80987 was determined in medium containing 10% FBS with and without the addition of 1 mg of α_1 AGP per ml. The antiviral activity of A-80987 was determined after de novo infection of MT-2 cells with HIV-1_{IIIB} by the MTT virus infectivity assay described in Materials and Methods. The EC_{50} values were 0.267 and 2.373 μ M for A-80987 without (□) and with (■) 1 mg of α_1 -AGP per ml, respectively. Error bars indicate standard deviations. OD 540, optical density at 540 nm.

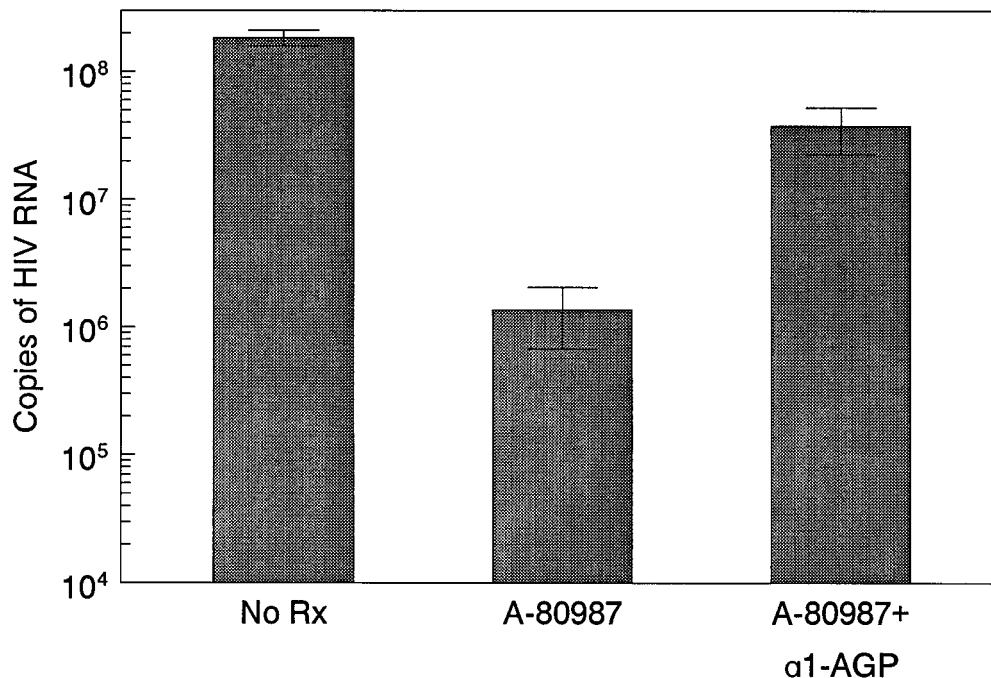


FIG. 3. The effect of A-80987 on release of HIV-1 RNA into supernatant of HIV-1-infected PBMC. PBMC were infected with HIV-1_{IIIB} and treated with 1 μ M A-80987 with and without the addition of α_1 AGP at 1 mg/ml. The number of copies of HIV RNA in 50 μ l of tissue culture supernate at day 7 postinfection was determined by RNA PCR as described in Materials and Methods. Error bars indicate standard deviations.

5 min of suspension in medium without A-80987 (Fig. 6). The rapid efflux kinetics of A-80987 were similar for HIV-infected and uninfected primary PBMC or cell lines (data not shown). The inhibitors KF, sodium cyanide, and CCCP had no effect on the efflux of A-80987 from cells preincubated with the drug.

Effect of α_1 AGP on the uptake and activity of A-80987. In order to determine whether there was a strong association between inhibition of A-80987 uptake and activity, we designed experiments to determine the effect of α_1 AGP on uptake and antiviral activity in the same samples. We infected PBMC with HIV-1_{IIIB} and after a 1-h incubation centrifuged the cells free of virus. α_1 AGP was added at concentrations of 0, 0.5, 1, and 1.5 mg/ml to aliquots of infected cells. We rapidly adjusted the concentration of radioactive A-80987 to 1 μ M. At 15 min after the addition of ¹⁴C-A-80987, four 0.5-ml aliquots of treated cells were layered onto dibutyl phthalate-oil and centrifuged to

determine the amount of the cell-associated radioactivity. The remaining radioactive cell suspension was cultivated for 5 days, and samples were removed for a p24 assay. As can be seen in Fig. 7A, the uptake of 1 μ M ¹⁴C-A-80987 by HIV-1_{IIIB}-infected PBMC was reduced with increasing amounts of α_1 AGP. In contrast, the p24 produced by cells exposed to 1 μ M ¹⁴C-A-80987 increased with increasing amounts of α_1 AGP (Fig. 7B). We generated similar curves with HIV-infected MT-2 cells. In these studies, uptake was determined as described for PBMC. However, antiviral activity was determined by the MTT cytotoxic assay for the same sample of MT-2 cells treated with 1 μ M ¹⁴C-A-80987 and concentrations of α_1 AGP from 0 to 1.5 mg/ml. Figure 8 shows the correlation between antiviral activity and the intracellular drug concentration as described by inhibitory sigmoid E_{max} modeling of data from Fig. 7.

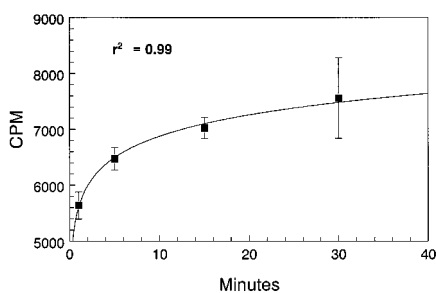


FIG. 4. Uptake of A-80987 by HIV-1-infected MT-2 cells. ¹⁴C-A-80987 was added to 2×10^6 HIV-1-infected MT-2 cells at a concentration of 10 μ M. At the indicated time points, 500- μ l samples were taken in duplicate and the counts associated with the cell pellets were determined as described in Materials and Methods. The curve was fitted by using the curve-fitting programs in Slide Write Plus (Advanced Graphics Software, Carlsbad, Calif.). Error bars indicate standard deviations.

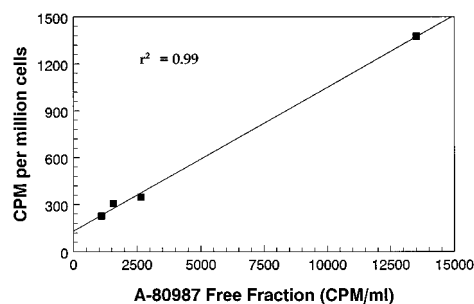


FIG. 5. The intracellular concentration of A-80987 is determined by the level of extracellular free drug. The uptake of A-80987 was measured by exposure of 2×10^6 HIV-infected PBMC treated with 0, 0.5, 1, or 1.5 mg of α_1 AGP per ml to 1 μ M ¹⁴C-A-80987 for 15 min. At that time, 500- μ l samples were taken in quadruplicate and centrifuged by the stop-oil method. Cell-associated ¹⁴C-A-80987 was measured as described in Materials and Methods. The fraction of free drug was determined by micropartition assay of the radiolabeled media.

TABLE 1. Effect of inhibitors on anti-HIV activity of A-80987

Cell ^a	A-80987 (μ M)	cpm (SD from the mean)			
		Control (no treatment)	+ NaCN ^b (1 mM)	+ KF (1 mM)	+ CCCP (20 μ M)
HIV infected					
PBMC	10	7,262 (1,052)	7,637 (946)	6,822 (347)	
	1	652 (79)	731 (113)	721 (23)	672 (129)
MT-2	10	6,461 (250)	6,507 (61)	6,648 (1,187)	
	10	7,121 (130)			7,562 (234)
PBMC	1	546 (21)	489 (56)	578 (57)	504 (34)
	2	1,145 (14)	1,341 (123)	1,095 (254)	1,201 (56)
	10	13,217 (459)	13,456 (234)	14,213 (673)	12,976 (297)

^a HIV- or mock-infected MT-2 cells or PBMC were labeled with the indicated concentrations of radiolabeled A-80987 and either α_1 AGP or the indicated metabolic inhibitor(s). Cell-associated counts after a 15-min incubation were determined as described in Materials and Methods.

^b NaCN, sodium cyanide.

DISCUSSION

We have previously shown that the anti-HIV activity of A77003 appears to be markedly reduced in the presence of human α_1 AGP, its binding protein (3). Similarly, the inhibitory effect of human serum or α_1 AGP on the virological activity of A-80987 in both acute and chronic infections was concentration dependent. We have shown that the EC_{50} for A-80987 increased more than 10-fold in the presence of 1 mg of α_1 AGP per ml. This reduction of antiviral efficacy was demonstrated in cell lines and in primary cultures of HIV-infected lymphocytes, by assays in which viral protein production or infectivity is determined directly. The α_1 AGP-mediated loss of activity is consistent with protein binding that reduces antiviral efficacy. Both equilibrium dialysis (6) and ultrafiltration indicated that A-80987 is more than 90% protein bound in human plasma. One would then predict that in plasma the free fraction would be less than 10% of the total drug concentration (assuming that the protein binding is linear over the concentration range examined), which should increase the amount of drug necessary for any degree of viral suppression by 10-fold or more, as was seen in our data.

The uptake of radiolabeled A-80987 was shown to be rapid and linearly dependent on concentration. Experiments in which metabolic inhibitors were used indicate that energy is not required and suggest that A-80987 enters the cell by passive diffusion. No differences in the kinetics of uptake of A-80987 by uninfected or HIV-infected PBMC or cell lines were observed. Similarly, the efflux of A-80987 was rapid when the concentration gradient was removed and there was no

evidence for accumulation of the radiolabeled drug in infected cells. The rapid efflux of A-80987 suggests that the amount of the protease inhibitor available to inhibit viral maturation at a site of infection is dependent on the concentration-time profile of the drug at that site. These data suggest that the circulating level of A-80987 or related protease inhibitors must be maintained above some threshold concentration in order to prevent the replication and spread of the virus. Indeed, we have previously shown that the effect of a related peptidomimetic protease inhibitor, A77003, on p24 release from mixtures of chronically infected H9IIIB and uninfected CEM cells was rapidly reversible (2). However, the infectivity of the virus released from treated cells after removal of the drug was neg-

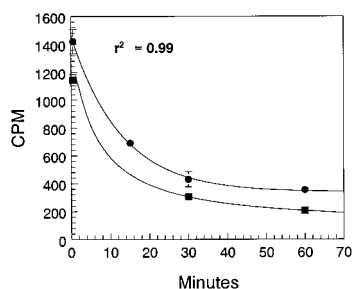


FIG. 6. Efflux of A-80987 from preloaded HIV-1-infected PBMC. PBMC infected with HIV-1 were preincubated with 10 μ M ¹⁴C-A-80987 for 90 min were centrifuged and suspended in medium without A-80987 at 2×10^6 cells per ml. At the indicated time points, duplicate 500- μ l samples were layered onto an oil mixture, centrifuged, and processed as described in Materials and Methods. Curves were fitted by the curve-fitting programs in Slide Write Plus. Error bars indicate standard deviations. ■, acute infection; ●, established infection.

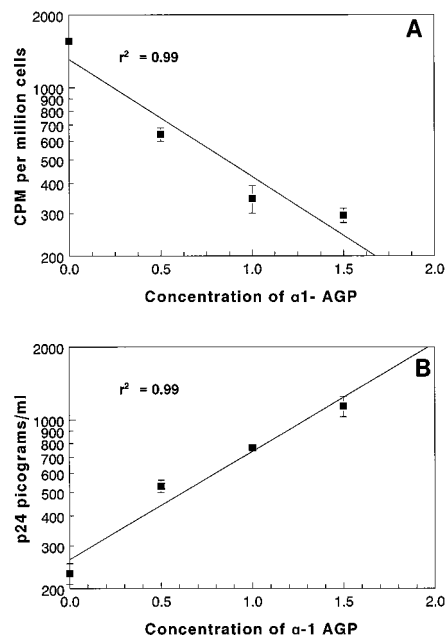


FIG. 7. Uptake and antiviral activity of A-80987 by HIV-infected PBMC in the presence of α_1 AGP. (A) The uptake of 1 μ M ¹⁴C-labeled A-80987 by HIV-infected PBMC was determined after a 15-min exposure to labeled drug in the presence of the indicated concentrations of α_1 AGP. (B) The antiviral activity of 1 μ M ¹⁴C-labeled A-80987 was determined by incubating the radiolabeled samples of HIV-infected PBMC for 7 days. The amount of p24 produced by infected cells treated with the indicated amount of α_1 AGP was measured by ELISA as described in Materials and Methods. The data was log transformed prior to curve fitting to emphasize the reciprocity of the uptake activity curves. Error bars indicate standard deviations.

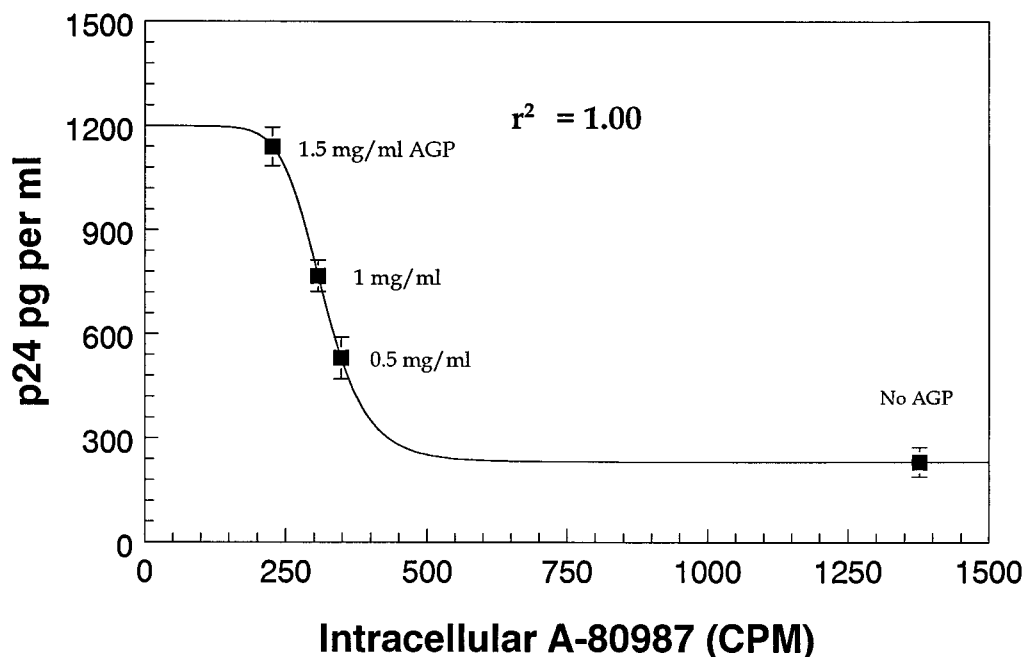


FIG. 8. Dose-effect modeling of data from HIV-infected PBMC in the presence of A-80987 and its binding protein α_1 AGP. The level of p24 released by HIV-infected PBMC at day 5 was plotted against intracellular free drug counts. The curve $p24 = 1,199 - [967.6 \times \text{free drug}^{8.242} / (\text{free drug}^{8.242} + 315.2^{8.242})]$ describes relationship between intracellular drug and antiviral activity. The p24 antigen level in the absence of drug was $1,199 \pm 40$ pg of p24 per ml. Error bars indicate standard deviations.

ligible for a period of 72 h. If such a post-antiviral effect exists in vivo, it might permit the time between doses to be extended.

We have shown that A-80987, like most drugs and many small molecules, passes through membranes by nonfacilitated diffusion. Uptake of A-80987 was not saturable since the amount in cells increases linearly with concentration (Table 1). If A-80987 enters cells by carrier mediated transport or passive facilitated diffusion, we would have observed saturation at high concentrations of the drug. Since active transport requires metabolic energy, it can be affected by metabolic inhibitors, but this effect was shown not to be the case for A-80987. Zimmerman et al. (33) reported that zidovudine, unlike other nucleosides and nucleoside analogs, also penetrates lymphocytes by nonfacilitated diffusion. The lack of active or facilitated uptake is important, as it provides a theoretical underpinning for the impact of protein binding on antiviral activity. Because of the molecular size and complexity of the drug-binding protein complex, only the free drug would be able to partition across the membrane. While binding is rapidly reversible on a thermodynamic basis, only unbound molecules can begin to partition through the membrane. At all drug concentrations, the amount of a protein-bound drug depends on the affinity constant and the protein concentration. With the number of binding sites per molecule remaining constant, the amount of A-80987 available to inhibit virus replication depends on its concentration and the plasma concentration of α_1 -AGP.

If A-80987 were transported by active or facilitated diffusion, α_1 AGP binding may not have an adverse effect on activity. One scenario in which protein binding may potentiate activity in vivo is if α_1 AGP binding leads to increased tissue penetration and/or reduced clearance. A77003, a closely structurally related, highly protein-bound protease inhibitor, was studied in humans in a phase I/II clinical trial (28). The fact that A77003 was extensively protein bound would have led one to believe that clearance rate for this drug would be quite low. However,

the drug clearance rate was quite high and was accomplished by hepatic extraction. The binding was nonrestrictive, that is, there was a high-affinity receptor in the liver which efficiently extracted the drug, in spite of its binding to α_1 AGP. This lack of a receptor-driven system for cellular uptake prevents this type of mechanism from overcoming the impact of binding on antiviral activity.

Lastly, it appears that binding of A-80987 by α_1 AGP limits the cellular uptake of the protease inhibitor. As the number of binding sites present in the medium is increased by higher concentrations of α_1 AGP, the equilibrium concentration of the drug in the cell diminishes. Attenuation of antiviral activity was highly correlated with this decrease in cellular penetration. This correlation provides convincing evidence that the protein binding is causative for the observed decrease in antiviral activity.

This observation has important implications. In the discovery process of protease inhibitors, it is important to examine the protein binding. Obviously, the apparent potency of the compound will be affected by high percentages of binding. Consequently, much higher than anticipated concentrations of the drug may be necessary to effect viral inhibition in vivo than would be predicted on the basis of in vitro data generated without binding proteins in the assay. Consequently, in the setting of phase I and II clinical trials, a lack of antiviral effect becomes understandable if the free drug does not exceed the threshold necessary for viral inhibition for a defined period of time. While, in principle, this threshold might be exceeded by increasing the dose (Fig. 1A), both toxicity and the cost of HIV-1 protease inhibitors may limit the use of such an approach.

In summary, we have demonstrated that uptake into cells is passive and is not affected by metabolic poisons. We have further demonstrated that the addition of purified binding proteins to the medium results in decreased cellular penetra-

tion by the drug. This decreased penetration appears to be directly linked to a reduced antiviral effect. Analogous attenuation of antiviral activity in vivo should be anticipated for other highly bound HIV protease inhibitors and deliberately overcome by appropriate increases in the drug dose.

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