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Antiviral Properties of Aminodiol Inhibitors against Human Immunodeficiency Virus and Protease

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A series of aminodiol inhibitors of human immunodeficiency virus type 1 (HIV-1) protease were identified by using an in vitro peptide cleavage assay. BMS 182,193, BMS 186,318, and BMS 187,071 protected cells against HIV-1, HIV-2, and simian immunodeficiency virus infections, with 50% effective doses ranging from 0.05 to 0.33 μM, while having no inhibitory effect on cells infected with unrelated viruses. These compounds were also effective in inhibiting p24 production in peripheral blood mononuclear cells infected with HIV-1 IIIB and against the zidovudine-resistant HIV-1 strain A018C. Time-of-addition studies indicated that BMS 182,193 could be added as late as 27 h after infection and still retain its antiviral activity. To directly show that the activity of these compounds in culture was due to inhibition of proteolytic cleavage, the levels of HIV-1 gag processing in chronically infected cells were monitored by Western blot (immunoblot) analysis. All compounds blocked the processing of p55 in a dose-dependent manner, with 50% effective doses of 0.4 to 2.4 μM. To examine the reversibility of BMS 186,318, chronically infected CEM-SS cells were treated with drug and virions purified from the culture medium. Incubation of HIV-1 particles in drug-free medium indicated that inhibition of p55 proteolysis was slowly reversible. The potent inhibition of HIV-1 during both acute and chronic infections indicates that these aminodiol compounds are effective anti-HIV-1 compounds.

The expanding AIDS epidemic has created a desperate need for effective antiviral therapies to control the replication of human immunodeficiency virus type 1 (HIV-1) in the population. Although a variety of nucleoside and nonnucleoside reverse transcriptase inhibitors have been described, the adverse side effects of these compounds (5, 38) and the development of drug-resistant strains following clinical use (10, 19, 20, 30, 34) remain serious problems. A promising new target for chemotherapeutic intervention in HIV-1 infection is the HIV-1-encoded protease. This enzyme is responsible for the cleavage of both gag and gag-pol precursor polyproteins at specific sites to yield essential enzymes and structural proteins which are required for the maturation of newly assembled virus particles into infectious virions (4, 13, 16, 27). Mutations within the HIV-1 protease-coding region or chemical inhibition of HIV-1 protease generates viral particles which are morphologically immature and noninfectious (9, 13, 16, 21, 27, 32). Since the HIV-1 protease is functional during the later stages of the infectious cycle, inhibitors of this enzyme are also effective in chronically infected cells. To date, a variety of HIV-1 protease inhibitors have been described, including Ro 31-8959 (saquinavir), A-77003, A-80987, SC-52151, L-735,524, P9941, and XM323 (3, 7, 8, 12, 14, 15, 17, 18, 23, 25, 31, 35, 37). These peptidomimetic inhibitors are characterized by the replacement of the normal amide bond usually cleaved by HIV-1 protease with a nonhydrolyzable isostere, such as hydroxyethylene analogs (35), hydroxyethylamine isosteres (8, 29, 31), and dihydroxyethyl isosteres (15, 18, 25). In this report, we describe the anti-HIV-1 activity and mode of action of a select group of aminodiol inhibitors (2) which are representative of a novel structural class of peptide mimetics.

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MATERIALS AND METHODS

Cells and viruses. The CEM-SS human T-cell line, the chronically infected 8E5/HIV-1 LAV cell line, HIV-1 RF, HIV-1 IIIB, HIV-1 A018A, HIV-1 A018C, HIV-2 CBL-20, and simian immunodeficiency virus (SIV) SIVmac251 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. The chronically infected cell line CEM-SS/HIV-1 RF was generated in our laboratory by propagation of CEM-SS cells that survived acute infection with HIV-1 RF. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Sigma, St. Louis, Mo.) and stimulated with phytohemagglutinin (Sigma) for 3 days before use.

Antiviral agents. Zidovudine (AZT) was purchased from Burroughs Wellcome (Research Triangle Park, N.C.). Monoclonal antibody OKT4-A was obtained from Coulter (Hialeah, Fla.), while didanosine and the protease inhibitors BMS 182,193, BMS 186,318, BMS 187,071, Ro 31-8959, and A-77003 were synthesized at Bristol-Myers Squibb.

Enzyme preparation. HIV-1 protease was expressed in *Escherichia coli* as a maltose-binding protein–protease fusion protein and purified as previously described (22).

Protease assays. The sensitivity of HIV-1 protease to protease inhibitors was determined by a peptide substrate cleavage assay (11). Reaction mixtures consisted of 50 mM sodium acetate (pH 5.5), 1 mg of bovine serum albumin per ml, 0.284 mM peptide substrate H_2N-Val-Ser-Gln-Asn-(β-naphthylalanine)-Pro-Ile-Val-COOH, and 1 to 10 nM purified HIV protease. After 30 min at 37°C, reactions were quenched with 150 μl of 5% aqueous phosphoric acid. Protease products were analyzed by reverse-phase high-pressure liquid chromatography by a variation of the method of Heimbach et al. (11). The K_m for the substrate used was 1,009 μM, and the K_{cat} for this peptide was determined to be 6,400 min^{-1}. Inhibition constants (K_is) were determined by measuring rates of conversion of substrate to product at six inhibitor concentrations (33).

Inhibition of cathepsin D (human liver; Calbiochem) and pepsin (porcine stomach mucosa; Sigma) was measured by a spectrophotometric assay with 0.1 formate (pH 3), 0.32 mM substrate H-Pro-Thr-Glu-Phe-*p*-nitro-Phe-Arg-Leu-OH (Protogen), and inhibitor diluted with reaction buffer at 37°C. Assays were started by the addition of enzyme (0.36 U for cathepsin D and 0.03 U for pepsin). The A_{310} was measured for 20 min.

Cell culture assays. The inhibitory effect of compounds on HIV-1 replication was measured by the XTT dye reduction method (36). The 50% effective dose (ED_{50}) was calculated as the concentration of drug that increased the percentage of formazan production in virus-infected cells to 50% of that in uninfected cells. Cytotoxicity (CC_{50}) was calculated as the concentration of drug that decreased the percentage of formazan produced in uninfected cells to 50% of that produced in untreated cells.

Alternatively, MT-2 cells (5×10^5 cells per ml) were infected with either HIV-1 RF or SIVmac251 at a multiplicity of infection of 0.001. After a 1- to 2-h adsorption period, cells were washed and dispensed in duplicate into 96-well plates containing half-log-unit dilutions of the appropriate drug. Three days after infection, culture supernatants were collected and the levels of p24 or p27 were determined by HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) (NEN Research Products, Dupont, Wilmington, Del.) or p27 ELISA (Coulter). Data are expressed as (amount of p24 or p27 produced in the presence of drug divided by that in the absence of drug) $\times$ 100 and are and plotted against log drug concentration (micromolar). The ED_{50} was calculated by interpolation from the curve.

Phytohemagglutinin-stimulated PBMCs were infected with viruses at a multiplicity of infection of 0.001 to 0.02. Infected PBMCs were incubated in the presence of various concentrations of test compounds. Three days after infection, half of the volume of each well was removed and replaced with fresh medium containing compound. Levels of p24 in culture supernatants 7 days after infection were determined by p24 ELISA. The ED_{50} was calculated as the dose of compound that resulted in a 50% reduction in p24 levels compared with those in control wells.

Time-of-addition assay. CEM-SS cells (5×10^5 cells per ml) were infected with HIV-1 RF at a multiplicity of infection of >1. Following a 1-h adsorption period, cells were washed three times and incubated at 37°C. Compound or medium alone was added to duplicate cultures at various times after infection. Forty-eight hours after infection, culture supernatants were collected and the levels of p24 were determined by HIV-1 p24 ELISA.

Western blot (immunoblot) analysis. Expression of viral precursor p55 and its proteolytic cleavage product p24 were determined by using cellular lysates. Cell pellets were lysed in radioimmunoprecipitation assay buffer, diluted with sample buffer (0.065 M Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue), and boiled for 5 min in a water bath before equal concentrations of proteins (determined by using the BCA Protein Assay [Pierce, Rockford, Ill.] in the presence of SDS) were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% gels (ISS, Natick, Mass.). Proteins separated by electrophoresis were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) and immunostained with a mouse monoclonal antibody specific for p55 and p24 (NEN Research Products). Antibody-protein complexes were identified by using anti-mouse antibody conjugated to horseradish peroxidase.

Reversibility studies. Chronically infected CEM-SS/HIV-1 RF cells were initially washed to remove extracellular virus and incubated at 5×10^5 cells per ml in the presence of compound or medium alone for 24 h. Cells were then washed and resuspended in fresh compound. On the following day, cells were refed with fresh medium containing drug in the original volume, and 24 h later, cells were harvested. Culture supernatant was harvested by centrifugation at $600 \times g$ (2,500 rpm) at 4°C for 5 min, and cell debris was removed by filtration through a 0.22-μm μStar disc (Costar, Cambridge, Mass.). Virions were pelleted by ultracentrifugation through 3.5 ml of a 20% (wt/vol) sucrose cushion in an SW41 rotor for 2 h at $120,000 \times g$ (26,000 rpm). Virions were then resuspended in phosphate-buffered saline (PBS). At appropriate times after incubation, samples were removed and assayed by p24 ELISA or solubilized and analyzed by SDS-PAGE.

RESULTS

Effect of aminodiol inhibitors on HIV-1 protease. A new structural class of C_2-symmetric inhibitors has been recently described (2). The prototype of this novel group of inhibitors, BMS 182,193, is a C_2-symmetric compound containing an aminodiol isostere (Fig. 1). Analogs of BMS 182,193 include BMS 187,071, which contains a hydroxyamide instead of a carbamate group at the P2 position, and BMS 186,318, which contains a phenyl group at the P1 position with an attached morpholino amide moiety at P1′ (Fig. 1). All three compounds are potent inhibitors of HIV-1 protease when assayed in an in vitro peptide cleavage assay, having 50% inhibitory concentrations (IC_{50}s) ranging from 50 to 136 nM (Table 1). However, these compounds were significantly less potent against purified HIV-1 protease than the A-77003 (IC_{50} = 1.3 nM) and Ro 31-8959 (IC_{50} = 1 nM) inhibitors (data not shown). The selectivity of the aminodiols was shown by the absence of activity against other aspartyl proteases, including human and bovine cathepsin D as well as porcine pepsin (Table 1). In addition, no inhibitory activity was found when these compounds were tested against several serine proteases and a metalloprotease (Table 1). From further biochemical analysis it was determined that the K_is of BMS 182,193, BMS 187,071, and BMS 186,318 are 100, 18, and 34 nM, respectively. All three compounds were also shown to be competitive inhibitors of HIV-1 protease (data not shown).

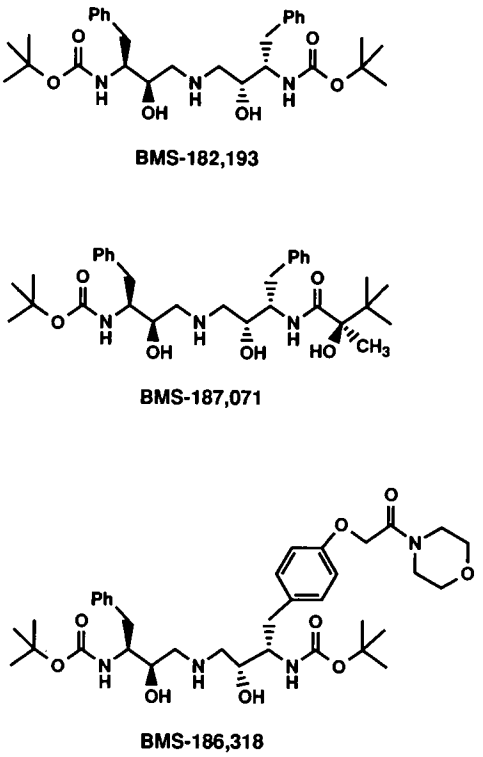

FIG. 1. Structures of aminodiol HIV-1 protease inhibitors. Ph, phenyl group.

Antiviral activity against acute HIV-1 and SIV infections. The antiviral activity of the aminodiol protease inhibitors was determined with different HIV-1 strains and host cells, and the results are summarized in Table 2. The compounds were effective at protecting CEM-SS cells against HIV-1 RF infections, with ED_{50}s ranging from 0.05 to 0.10 μM as measured by the XTT dye reduction method. Similar activity was observed against the HIV-2 isolate CBL-20 (ED_{50}s, 0.07 to 0.33 μM). Both BMS 186,318 and BMS 187,071 were also effective in reducing the level of p24 released from MT-2 cells infected with HIV-1 RF (ED_{50}s, 0.01 to 0.03 μM), while BMS 186,318 had comparable antiviral activity against SIV replication in MT-2 cells. The aminodiols showed similar potencies when assayed in PBMCs (Table 2). In addition, BMS 186,318 and

TABLE 1. Inhibition of proteases by aminodiol inhibitors

Protease	Class	IC_{50} (nM)[a] of:		
		BMS 182,193	BMS 187,071	BMS 186,318
HIV-1	Aspartate	136 ± 35	50 ± 18	75 ± 20
Bovine cathepsin D	Aspartate	>31,000	>31,000	>31,000
Human cathepsin D	Aspartate	>31,000	ND[b]	>31,000
Porcine pepsin	Aspartate	ND	>31,000	>31,000
Trypsin	Serine	ND	>50,000	>50,000
Chymotrypsin	Serine	ND	>50,000	>50,000
Elastase	Serine	ND	>50,000	>50,000
Thermolysin	Metallo	ND	>50,000	>50,000
Papain	Thio	ND	>50,000	>50,000

[a] Results for HIV protease (means ± standard deviations) are derived from a minimum of four assays.
[b] ND, not done.

TABLE 2. Antiviral activities of aminodiol inhibitors against acute HIV and SIV infections

Virus	Cells	Assay	ED_{50} (μM)[a] of:					
			BMS 182,193	BMS 187,071	BMS 186,318	A-77003	Ro 31-8959	AZT
HIV-1 RF	CEM-SS	XTT	0.05 ± 0.03	0.06 ± 0.02	0.1 ± 0.04	0.17 ± 0.04	0.004 ± 0.002	0.003 ± 0.001
HIV-2 CBL-20	CEM-SS	XTT	0.33 ± 0.08	0.16 ± 0.03	0.07 ± 0.04	ND[b]	0.004 ± 0.001	ND
HIV-1 RF	MT-2	p24	0.06 ± 0.01	0.01 ± 0.009	0.03 ± 0.03	0.04 ± 0.03	0.002 ± 0.001	0.003 ± 0.001
SIVmac251	MT-2	p27	ND	ND	0.09 ± 0.03	0.04 ± 0.015	0.003 ± 0.002	ND
HIV-1 IIIB	PBMC	p24	0.025 ± 0.008	0.04 ± 0.01	0.02 ± 0.014	0.14 ± 0.02	0.002 ± 0.003	0.002 ± 0.0005
HIV-1 A018A	PBMC	p24	ND	0.21 ± 0.05	0.04 ± 0.008	0.22 ± 0.04	ND	0.01 ± 0.006
HIV-1 A018C	PBMC	p24	ND	0.11 ± 0.06	0.11 ± 0.04	0.11 ± 0.04	ND	4.8 ± 1.6

[a] Data (means ± standard deviations) are derived from a minimum of three assays.
[b] ND, not done.

BMS 187,071 were equally effective against HIV-1 clinical isolates A018A and the AZT-resistant strain A018C (Table 2). Direct comparison with other reported protease inhibitors indicated that the aminodiols are equivalent in potency to A-77003 and an order of magnitude less potent than Ro 31-8959 and AZT (Table 2). The AZT results also confirmed the resistance phenotype of clinical isolate A018C, showing a 48-fold change in ED_{50}. The CC_{50}s for BMS 182,193 and BMS 187,071 in CEM-SS cells were 6 and 38 μM, respectively, yielding therapeutic indices of 18 to 120 and 240 to 630, respectively. Less cytotoxicity was associated with BMS 186,318 (CC_{50} = 100 μM), yielding a therapeutic index of 1,000 to 1,400.

Mechanism of action. A review of the data in Tables 1 and 2 indicates that the aminodiol inhibitors have activities in cell culture equivalent to their activities in in vitro protease assays. This was of interest since many protease inhibitors have been shown to be more active against purified HIV-1 protease than they are in protecting cells from HIV-1 infection (2). To confirm that the observed inhibition in cell culture resulted from inhibition of viral proteolytic processing, a time-of-addition experiment was performed. In this assay, CEM-SS cells are infected with HIV-1 RF at a high multiplicity of infection to achieve a single cycle of infection, and compounds are added at various times after virus infection. The results (Fig. 2) show that BMS 182,193 could be added as late as 27 h postinfection and maintain its full antiviral effect, consistent with inhibition of a late-stage event. In contrast, monoclonal antibody OKT4-A, an inhibitor of HIV-1 binding to the CD4 receptor, had to be added within 1 h of infection, and the reverse transcriptase inhibitor didanosine was most effective in reducing p24 levels when it was added by 6 h postinfection (Fig. 2).

Antiviral activity in chronically infected cells. In contrast to reverse transcriptase inhibitors, which are effective only against acute HIV-1 infections, protease inhibitors are also effective against chronic HIV-1 infections because of their ability to block a late stage of viral maturation (4, 28). As further evidence that the aminodiol protease inhibitors inhibit HIV-1 infection at a postintegration stage, the antiviral effect of these compounds was examined in two cell systems. In the first, the chronically infected cell line 8E5 was treated with BMS 182,193 for 7 days. The 8E5 cell line (6) contains a single integrated copy of HIV-1 LAV and provides an excellent model to ascertain the effects of inhibitors active upon events subsequent to integration. As illustrated in Fig. 3, levels of p24 were reduced when cells were treated with BMS 182,193 for 7 days compared with the untreated control. As expected, the reverse transcriptase inhibitor AZT showed no inhibitory effect on p24 production compared with the untreated culture. In a second cell system, CEM-SS cells chronically infected with HIV-1 RF were treated for 3 days with BMS 186,318 or BMS 187,071. Both compounds were effective in reducing the levels of p24 in culture supernatants from treated cells, with ED_{50}s of 2.4 μM (BMS 186,318) and 2.0 μM (BMS 187,071) (data not shown).

Inhibition of gag (p55) processing. Inhibition of HIV-1 proteolytic cleavage results in the accumulation of gag precursor

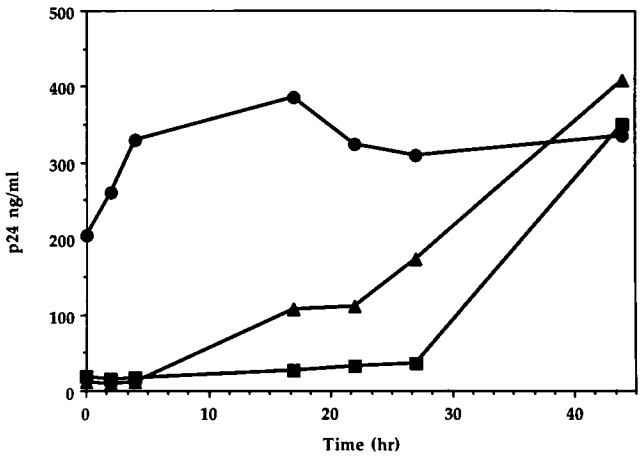

FIG. 2. Time-of-addition assay. CEM-SS cells were infected with HIV-1 RF at high multiplicity of infection. At various times after infection, 12.5 μg of monoclonal antibody OKT4-A per ml (circles), 200 μM didanosine (triangles), or 5 μM BMS 182,193 (squares) was added. Forty-eight hours after infection, levels of p24 in culture supernatants were measured.

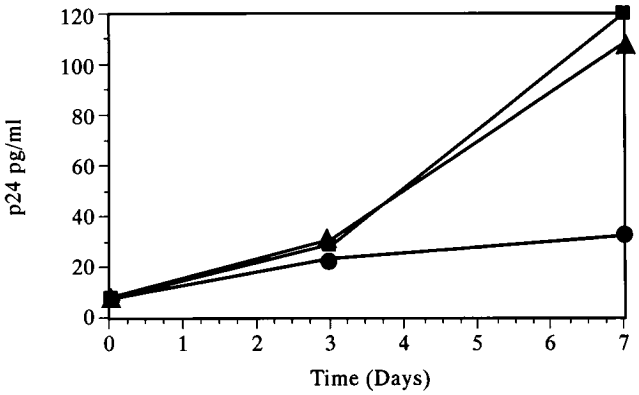

FIG. 3. Inhibition of p24 in 8E5/HIV-1 LAV cells. Chronically infected 8E5 cells were untreated (squares) or treated with 1 μM AZT (triangles) or 1 μM BMS 182,193 (circles). Levels of p24 were measured at 3 and 7 days following treatment.

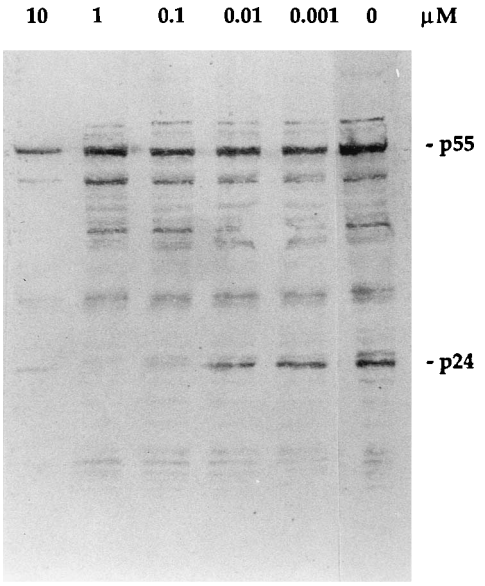

FIG. 4. Inhibition of HIV-1 coat processing by aminodiol inhibitors. Chronically infected 8E5/LAV cells were treated with various concentrations (listed across the top) of BMS 182,193 or with medium alone for 5 days. Cell lysates were solubilized in SDS and analyzed for p24 and p55 by Western blotting as described in the text.

p55 protein and a reduction in its processed product p24 (4, 9, 13). To directly show that the antiviral activity of the aminodiols results from inhibition of viral processing, chronically infected 8E5/HIV-1 LAV cells were treated with various concentrations of BMS 182,193. The polyproteins present in SDS-solubilized cell lysates from 8E5/HIV-1 LAV-treated cells were analyzed by Western blotting with a monoclonal antibody that recognizes p24 and p55. As illustrated in Fig. 4, BMS 182,193 inhibited the proteolytic cleavage of viral gag precursor p55 to mature p24 protein in a dose-dependent manner, with an ED_{50} of between 10 and 100 nM. These results, in combination with the results described above, clearly demonstrate that the aminodiols act as selective protease inhibitors in HIV-1-infected cells.

Reversibility of inhibition of gag (p55) processing. It has been shown that defective HIV-1 particles produced from protease-treated cells are able to resume proteolysis when the drug concentration is reduced or the drug is removed (24). Experiments were designed to examine the kinetics of reversibility following inhibition of gag processing by BMS 186,318. Chronically infected CEM-SS/HIV-1 RF cells were treated with BMS 186,318 for 3 days at a concentration (20 μM) that resulted in complete inhibition of p55 processing. Virions were pelleted from culture medium by ultracentrifugation, resuspended in PBS without drug, and incubated at 37°C for 0 to 37 h. Nearly 90% of the immunoreactive protein prepared from cultures treated with medium alone was present as processed p24 (data not shown). In contrast, in drug-treated cultures, HIV-1 particles were composed primarily of unprocessed precursor protein p55 (Fig. 5). Reversal of p55 proteolytic inhibition by BMS 186,318 was evident within hours following removal of the compound, with nearly complete processing observed by 12 h (Fig. 5). These results suggest that the aminodiols inhibit HIV-1 protease through a noncovalent interaction and do not irreversibly modify the viral enzyme.

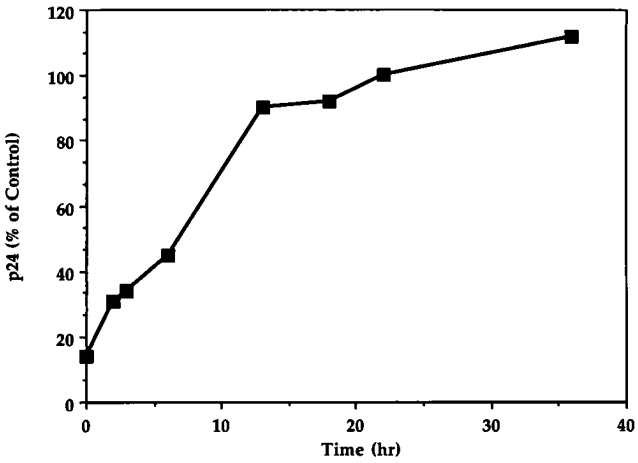

FIG. 5. Reversibility of inhibition of HIV-1 coat processing by BMS 186,318. Chronically infected CEM-SS/HIV-1 RF cells were treated with 20 μM BMS 186,318 or with medium alone for 3 days. Virions were separated from drug by ultracentrifugation and incubated in PBS at 37°C. Samples were removed at various times after incubation and analyzed for p24 by ELISA.

DISCUSSION

We have previously described the design and synthesis of a novel series of aminodiol HIV-1 protease inhibitors (2). In the present study, we have evaluated the antiviral efficacies of these compounds against both the purified HIV-1 protease and several different virus-host cell systems. Our data indicate that aminodiol compounds are potent and selective inhibitors of HIV-1 protease, with IC_{50}s ranging from 50 to 136 nM. BMS 182,193, BMS 186,318, and BMS 187,071 have a broad spectrum of activity against several different strains of HIV-1 and HIV-2, as well as SIV, in different host cell systems, with ED_{50}s ranging from 0.01 to 0.33 μM. Although the prototype HIV-1 protease inhibitor, BMS 182,193, had a CC_{50} of 6 μM, modification of its structure produced a compound (BMS 186,318) with nearly equivalent antiviral efficacy but which was better tolerated by cells (CC_{50} of 100 μM). Moreover, BMS 186,318 was effective against clinical strains of HIV-1, including the AZT-resistant strain A018C. This is important since the development of drug-resistant strains following clinical use of nucleoside and nonnucleoside reverse transcriptase inhibitors remains a serious problem (10, 19, 20, 30, 34).

The aminodiol compounds possessed the unique characteristic of equivalent potency in vitro and in cell culture. This observation suggests that these compounds either are preferentially taken up by cells or are capable of localizing at the site of proteolytic processing. This characteristic coupled with their relatively high bioavailability (40%) and long elimination half-life (4 h) in rats (2) may enhance their efficacy in vivo.

A series of experiments were performed that clearly demonstrated that the aminodiols function as protease inhibitors in HIV-1-infected cells. BMS 182,193 reduced p24 levels in a single-cycle viral growth experiment when added up to 27 h postinfection and blocked viral production in HIV-1 chronic infection. More conclusive data confirming the mode of action of the aminodiols as protease inhibitors came from immunoblot analyses, which demonstrated the inhibition of processing of the gag precursor protein p55 to its processed protein p24 by all three compounds in both chronically infected 8E5/HIV-1 LAV and CEM-SS/HIV-1 RF cell systems (Fig. 4 and unpublished data).

The potential of defective HIV-1 particles resulting from

drug treatment to subsequently process p55 (1, 24) and reactivate to an infectious form when appropriate pharmacokinetic levels of drug are not maintained is of clinical concern. Therefore, information regarding the reversibility of protease inhibition should be important in establishing appropriate dosing schedules in vivo. Treatment of chronically infected cells with BMS 186,318 resulted in the production of defective, noninfectious virus containing unprocessed precursor protein p55. Our results indicate that when these particles were purified away from inhibitor, the inhibition of p55 proteolysis was slowly but almost completely reversible by 12 h. Since the aminodiols appear to have a long half-life in animal studies, steady-state levels in plasma above those necessary to maintain an antiviral effect should be obtainable in humans.

In summary, we have shown that BMS aminodiol compounds are specific and potent inhibitors of HIV-1 protease and consequently of viral replication. The spectrum of antiviral activity, good selectivity index, and distinct resistance profile (26), together with their relative ease of synthesis, bioavailability, and distribution in tissue (2), suggest that they should be considered as new clinical candidates for treatment of HIV-1 infection.

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