ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1997, p. 965–971 Vol. 41, No. 5
0066-4804/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Antiviral Properties of Palinavir, a Potent Inhibitor of the Human Immunodeficiency Virus Type 1 Protease

D. LAMARRE,[1,2]* G. CROTEAU,[1] E. WARDROP,[1] L. BOURGON,[1] D. THIBEAULT,[1] C. CLOUETTE,[1] M. VAILLANCOURT,[2] E. COHEN,[2] C. PARGELLIS,[3] C. YOAKIM,[4] AND P. C. ANDERSON[4]

Departments of Biochemistry[1] and Chemistry,[4] Bio-Méga Research Division of Boehringer Ingelheim (Canada) Ltd., Laval, Québec, Canada H7S 2G5, Department of Microbiology-Immunology, Université de Montréal, Montréal, Québec, Canada H3C 3J7,[2] and Department of Inflammatory Diseases, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, Connecticut 06877[3]

Received 24 July 1996/Returned for modification 15 October 1996/Accepted 7 January 1997

Palinavir is a potent inhibitor of the human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) proteases. Replication of laboratory strains (HIV-1, HIV-2, and simian immunodeficiency virus) and HIV-1 clinical isolates is inhibited by palinavir with 50% effective concentrations ranging from 0.5 to 30 nM. The average cytotoxic concentration of palinavir (35 μM) in the various target cells indicates a favorable therapeutic index. Potent antiviral activity is retained with increased doses of virus and with clinical isolates resistant to zidovudine (AZT), didanosine (ddI), or nevirapine. Combinations of palinavir with either AZT, ddI, or nevirapine demonstrate synergy or additivity in the inhibition of HIV-1 replication. Palinavir retains anti-HIV-1 activity when administered postinfection until times subsequent to the reverse transcription step. In chronically infected CR-10 cells, palinavir blocks Gag precursor polyprotein processing completely, reducing greater than 99% of infectious particle production. The results indicate that the antiviral activity of palinavir is specific to inhibition of the viral protease and occurs at a late stage in the replicative cycle of HIV-1. On the basis of the potent in vitro activity, low-level cytotoxicity, and other data, palinavir was selected for in-depth preclinical evaluation.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS. The high replication rate of HIV-1 in seropositive patients has stressed the need for effective antiviral agents. Clinical benefits are expected from anti-HIV-1 agents that demonstrate a sustained activity preventing disease progression. Antiviral strategies against HIV-1 have concentrated on inhibiting the reverse transcriptase (RT) enzyme, leading to the first class of drugs approved for the treatment of AIDS. However, the clinical use of nucleoside analog RT inhibitors is limited by toxic effects and the emergence of viral resistance (23). Another critical enzyme required for HIV-1 replication is the protease (15). The virus-encoded aspartyl protease mediates the cleavage of Gag and Gag-Pol precursor polyproteins into mature proteins found in infectious virions. The maturation of precursor polyproteins is carried out in the latter stages of the virus life cycle. Protease activity is essential for HIV-1 replication as shown by the production of defective particles from cells with integrated proviral DNA containing a mutation at codon 25 of the protease (29). The viral protease is an obvious therapeutic target (15, 16) as is evident from the numerous inhibitors reaching clinical trials for the treatment of HIV-1 infection. Recently, HIV-1 protease inhibitors have received regulatory approval for use in monotherapy and/or in combination with approved nucleoside analogs in individuals with advanced HIV disease. These new drugs as a class will complement RT inhibitors and create new treatment options for HIV-1-infected patients.

The protease inhibitors are mainly substrate-based nonhydrolyzable peptide mimetics (15). The characterization of the cleavage sites and the three-dimensional structure of inhibitor-enzyme complexes have facilitated the design of inhibitors (52). Numerous inhibitors of HIV-1 protease exhibit powerful in vitro antiviral activity at low nanomolar levels (3, 7–9, 19, 27, 30, 47, 50). However, antiviral potency must be coupled with acceptable oral bioavailability and proper pharmacokinetic properties. Two HIV-1 protease inhibitors, indinavir (Crixivan; MK-639) and ritonavir (Norvir; ABT-538), were recently reported to have high oral bioavailability in humans (27, 50). The protease inhibitor saquinavir (Invirase; Ro 31-8959), however, showed poor oral bioavailability in humans and thus required a high dosage to maintain therapeutic levels in plasma (28).

Clinical trials with protease inhibitors in monotherapy have demonstrated profound declines in plasma viral RNA levels and concomitant increases in CD4 cells (14, 24, 28, 33, 51). A reduction in virus titer by as much as 10^4-fold was reported within 2 to 4 weeks of initiating drug therapy (51). The compounds used in vivo are generally well tolerated, although interactions with hepatic enzymes (P-450 isozymes) may lead to restrictive contraindicated medication, which may result in potentially serious adverse events or in decreased concentrations of the drugs in plasma (13, 26, 31, 38, 40). The efficacy of these compounds, however, seems limited due to the development of drug resistance by the virus. Patients exposed to prolonged treatment yielded viral variants with genotypic changes in the protease and diminishing inhibitor susceptibility in vitro (11, 25, 34). Upon in vivo development of resistance to indinavir, cross-resistance toward other protease inhibitors was observed (11). With higher dosages, ritonavir sustained antiviral activity for a longer duration (34), suggesting that higher suppressive drug concentrations may be critical in delaying the emergence of resistance. A more effective therapy is expected from antiviral drug combination. Combination therapy with saquinavir and zidovudine (AZT) resulted in a significant increase in sustained responders after 1 year compared to monotherapy (25). Combining protease inhibitors with no cross-

* Corresponding author. Mailing address: Department of Biochemistry, Bio-Méga Research Division of Boehringer Ingelheim (Canada) Ltd., 2100 Cunard St., Laval, Quebec, Canada H7S 2G5. Phone: (514) 682-4640. Fax: (514) 682-8434.

resistance in vitro may offer a therapy with substantial benefit. However, the emergence of in vivo resistance to protease inhibitors will necessitate further studies to determine resistance profiles and effective combinations.

In this report, results of in vitro studies with a novel protease inhibitor, palinavir (formerly BILA 2011 BS), are presented. Protease inhibition leading to antiviral activity against various clinical isolates and laboratory strains is described. The activity of palinavir was investigated in combination with either AZT, didanosine (ddI), or nevirapine and with increasing doses of HIV-1. Finally, the mode of action of palinavir was confirmed by time-addition experiments and by its ability to block Gag precursor polyprotein processing.

MATERIALS AND METHODS

Antiviral agents. The chemical name for palinavir is *N*-{1(*S*)-[[[3-[2(*S*)-{[(1,1-dimethylethyl)amino] carbonyl}-4(*R*)-[(4-pyridinylmethyl)oxy]-1-piperidinyl]-2(*R*)-hydroxy-1(*S*)-(phenylmethyl)propyl]amino] carbonyl]-2-methylpropyl}-2-quinolinecarboxamide. Palinavir was synthesized at Bio-Méga Research Division of Boehringer Ingelheim (Canada) Ltd. Nevirapine was obtained from Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, Conn.). AZT was graciously donated by Glaxo Wellcome (Montréal, Québec, Canada), and ddI was purchased from ICN (catalog no. 150881).

Viruses and cells. Wg3522 and Wg3523 are HIV-1 clinical isolates (Montréal) obtained from M. Wainberg. VR949 (Haitian) and VR1372 (Zaire) are foreign HIV-1 clinical isolates obtained from the Laboratoire de Santé Publique du Québec. UHC-1 virus, produced by U937 cells chronically infected with HIV-1 IIIB, and ddI-resistant virus were obtained from M. Wainberg. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: HIV-1 IIIB from R. Gallo (43–45); HXB2 from B. Hahn and G. M. Shaw (48); HIV-2 ROD and SIVmac251 from R. Desrosiers (13, 39); AZT-resistant HIV-1 strain (AO18 G 910-6), predrug strain (AO18 H 112-2), and nevirapine-resistant strain (no. 1392) from D. Richman (32, 46); HIV-1 ADA (no. 416) from H. Gendelman (21, 22); and the U1 cell line from T. Folks (18). The chronically infected CR-10 cell line was produced at Bio-Méga Research Division of Boehringer Ingelheim (Canada) Ltd. through persistent infection with HIV-1 IIIB and isolation of clonal HIV-1-positive cells. Peripheral blood mononuclear cells (PBMCs) were obtained from a healthy male donor who tested negative for HIV, cytomegalovirus, and Epstein-Barr virus. The PBMCs were separated by leukophoresis performed at the Maisonneuve-Rosemont Hospital (Montréal, Canada). The monocyte/macrophage (M/M) preparation was isolated from PBMCs by an adherence separation protocol (20). H9 cells were obtained from R. Gallo (American Type Culture Collection [ATCC] no. HTB176), CEMx174 cells were obtained from P. Cresswell (ATCC no. 274), U937 cells were from C. Sundstrom and K. Nilsson (ATCC no. 1593), and CR-10 cells were from D. J. Volsky (ATCC no. 391). The C8166 cell line was obtained from J. Sullivan. All cells were maintained in RPMI 1640 medium with 10 or 20% fetal calf serum (FCS), 2 mM L-glutamine, 10 μg of gentamicin per ml, and 10 μM β-mercaptoethanol (complete medium).

Inhibition of proteases and K_i determination. Recombinant HIV-1 and HIV-2 proteases were expressed from *Escherichia coli* and purified as previously described (41, 42). Inhibition of HIV-1 and HIV-2 proteases was evaluated by a high-pressure liquid chromatography-based peptide substrate cleavage assay (4). A nonlinear curve fit with the Hill model was applied to the inhibition-concentration data, and the 50% effective concentration (EC_{50}) was calculated by the use of SAS software (Statistical Software System; SAS Institute, Inc., Cary, N.C.). Inhibition constants (K_is) were determined by a steady-state velocity method (36) and a novel paired progress curve method (41). Briefly, enzyme activity was determined by monitoring the fluorescence change (excitation at 320 nm and emission at 417 nm) associated with the cleavage of the fluorogenic substrate 2-aminobenzoyl-Thr-Ile-Nle-Phe(p-NO_2)-Gln-Arg-NH_2 (Bachem, Torrance, Calif.). Reactions were performed at 23°C in assay buffer (100 mM Na acetate, 4 mM EDTA, 0.025% NaN_3, 0.1% bovine serum albumin, 1% dimethyl sulfoxide, 1.5 M NaCl, pH 5.5) containing 1 nM HIV-1 protease or 20 nM HIV-2 protease and 10 μM substrate. The onset of inhibition was determined by varying the inhibitor concentration from 0.25 to 25 nM. The steady-state velocity was estimated by fitting the data to the integrated rate equation describing competitive binding. The apparent K_i [$K_{i(app)}$] was obtained by fitting steady-state velocity and inhibitor concentrations to the quadratic equation describing tight-binding inhibition (35). Under similar assay conditions, the Michaelis-Menten constant (K_m) was determined for HIV-1 protease (0.58 μM) and for HIV-2 protease (15.6 μM). K_i was obtained by the given equation $K_{i(app)} = K_i [1 + (S)/K_m]$ for a competitive mode of inhibition. For K_i determination by the paired progress curve method, reactions were performed as described above and progress curves for the association and dissociation of the enzyme-inhibitor complex were generated at a single concentration of inhibitor. Data were collected and analyzed for the determination of the association and dissociation constants for the binding of palinavir to HIV-1 protease.

Virus production and titration. Virus production and infectivity assays were performed based on standard protocols (1). Clinical isolates Wg3522, Wg3523, VR949, and VR1372 were produced with PBMCs stimulated with 5 μg of phytohemagglutinin per ml and 10% interleukin-2-conditioned medium. The monocytotropic ADA strain was produced with adherent M/M preparation. Laboratory strains, UHC-1, and resistant isolates (AZT^r, ddI^r, and Nev^r) were produced with the appropriate cell lines. Viruses were sedimented by ultracentrifugation at 40,000 × *g* for 2 h at 4°C. Virus pellets were resuspended in RPMI 1640 medium–50% FCS and kept at −80°C until used. The 50% tissue culture infective dose ($TCID_{50}$) of viral stocks was determined by monitoring the macroscopic formation of syncytia by using the C8166 cell line. Titers were usually between 10^6 and 10^8 $TCID_{50}$/ml. A multiplicity of infection (MOI) of 1 was defined as 1 $TCID_{50}$ per cell.

Inhibition of virus replication. The extracellular core protein level, determined by enzyme-linked immunosorbent assay with the HIV-1 p24 antigen (Ag) or simian immunodeficiency virus (SIV) p27 Ag Coulter kit, was used to monitor the replication of clinical isolates and laboratory strains. Cells were infected at an MOI of 0.001 or 0.01 in complete medium. In experiments using increased virus dosages, C8166 and H9 cells were infected at an MOI of 0.001 to 1.0 with HIV-1 IIIB. Before infection, PBMCs were incubated for 3 days in complete medium containing 5 μg of phytohemagglutinin per ml. Infected cells were incubated with serial dilutions of inhibitors for periods of 3 days (C8166), 7 days (H9, CEMx174), or 14 days (PBMCs). For the 7- and 14-day incubation periods, half of the medium was replaced every 2 to 3 days with complete medium containing the corresponding dilution of inhibitor. The percentage of inhibition was obtained by the core Ag levels from supernatant pools (eight replicas). A nonlinear curve fit with the Hill model was applied to the inhibition-concentration data, and 50% (EC_{50}) or 90% (EC_{90}) concentrations were obtained through the use of the SAS software.

Cytotoxicity assay. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] metabolic assay (37) was used to determine the cytotoxicity in the various target cells. CC_{50} corresponds to the concentration of inhibitor that decreased the percentage of formazan production by 50% of that produced by untreated cells after 4 days of incubation. MTT was added, and cells were incubated for 3 h. The formazan product was solubilized with 10% Triton X-100 in 0.01 N HCl and quantitated by measuring the optical density at 570 nm. For PBMCs, cell viability was also determined by the trypan blue dye exclusion method at day 14. For the 14-day incubation period, half of the medium was replaced every 3 days with complete medium containing the corresponding dilution of inhibitor.

Antiviral synergy analysis. C8166 cells infected at an MOI of 0.001 with HIV-1 IIIB were incubated for 3 days with serial dilutions of palinavir, AZT, ddI, and nevirapine and fixed-ratio combinations of inhibitors. Inhibition of viral replication was determined by extracellular p24 Ag levels from supernatant pools (four replicas). Antiviral activities of the various fixed-ratio combinations of inhibitors were analyzed. The degree of synergy was evaluated by the median-effect principle with the combination index (CI) calculation and the mutually nonexclusive drug interaction condition (10). A computer program based on the median-effect plot and the CI equation were used for data analysis (Elsevier-Biosoft, Cambridge, England, 1987). CIs at both 50 and 90% inhibition were obtained from the program. CIs of <1, 1, and >1 are indicative of synergy, additivity, and antagonism, respectively. MTT metabolic assays were performed with combinations of inhibitors with the C8166 cells.

Time-addition experiments. C8166 cells infected at an MOI of 1.0 with HIV-1 IIIB were incubated with 10 μM AZT, nevirapine, and palinavir added at various times from 0 to 22 h postinfection. After 24 h of incubation, the extracellular p24 Ag levels were determined from supernatant pools (eight replicates). The percentage of p24 Ag inhibition was expressed relative to a 100% inhibition control in which inhibitor was added immediately after virus absorption.

Inhibition of virus replication in chronically infected cells. The extracellular p24 Ag level was used to monitor the virus maturation in chronically infected cells. CR-10 and U1 cells, chronically infected with HIV-1 IIIB and LAV, respectively, were washed and incubated with serial dilutions of inhibitors for 48 h. U1 cells were cultured in the presence of 50 nM phorbol 12-myristate 13-acetate. Levels of extracellular p24 Ag were determined from supernatant pools (four replicates). Infectious particle numbers were determined by viral titration of the supernatant of inhibitor-treated CR-10 cells. A nonlinear curve fit with the Hill model was applied to the inhibition-concentration data, and EC_{50}s were obtained through the use of the SAS software. Gag precursor polyprotein processing was analyzed by Western blotting in CR-10 cells treated with 0.1 and 1 μM palinavir. After 48 h of incubation, cells and cell-free virions pelleted by high-speed centrifugation (40,000 × *g*) for 120 min were solubilized in Laemmli buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Viral proteins, after transfer to nitrocellulose, were visualized with anti-HIV-1 p17 (NEN Dupont no. 9282) or anti-HIV-1 p24 (NEN Dupont no. 9283) monoclonal antibodies and ^{125}I-labelled goat anti-mouse antibodies (Amersham).

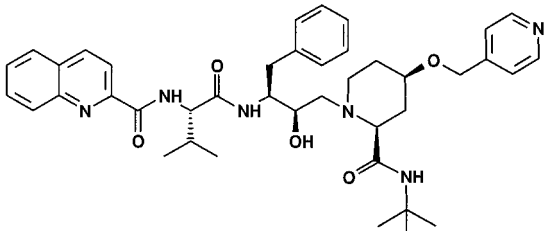

FIG. 1. Chemical structure of the protease inhibitor palinavir.

RESULTS

Inhibition of HIV-1 and HIV-2 proteases by palinavir and activity against human aspartyl proteases. Palinavir was selected from a collection of hydroxyethylamine-based HIV protease inhibitors containing a substituted pipecolinic amide moiety (Fig. 1). The bound conformation of palinavir complexed with HIV-2 ROD protease was previously described (49). In a peptide substrate high-pressure liquid chromatography-based assay, palinavir inhibits the proteases of HIV-1 IIIB and HIV-2 ROD with mean EC_{50}s of 4 and 10 nM, respectively (Table 1). K_i was determined with both proteases by a steady-state velocity method (Table 1). Preliminary experiments have demonstrated a competitive mechanism for this class of inhibitors. Mean K_is of 31 and 134 pM were obtained with HIV-1 and HIV-2 proteases, respectively. Paired progress curves were generated at a single concentration of inhibitor and analyzed simultaneously to determine the kinetic rate constants for the binding of palinavir to HIV-1 protease. Palinavir exhibited an association constant of 1.1×10^8 M^{-1} s^{-1} and a dissociation constant of 6.5×10^{-4} s^{-1}, corresponding to a K_i of 6 pM. The specificity of palinavir was assessed by evaluating its ability to inhibit a variety of human aspartyl proteases, including renin, cathepsin D, pepsin, and gastricsin (Table 1). In the different enzymatic assays, palinavir showed a weak affinity to all aspartyl proteases tested as indicated by EC_{50}s greater than 33 μM.

Inhibition of virus replication and cytotoxicity of palinavir. The antiviral activity of palinavir was determined with laboratory strains and clinical isolates by using cell lines, activated PBMCs, or M/M preparations (Table 2). Palinavir is a potent inhibitor of the replication of different HIV-1 strains (lymphotropic and monocytotropic) and HIV-1 clinical isolates from several geographic locations. Mean EC_{50}s and EC_{90}s varied from 0.5 to 28 nM and from 5 to 91 nM, respectively. Palinavir is also a potent inhibitor of the replication of HIV-2 and SIV, with EC_{50}s and EC_{90}s ranging from 4 to 30 nM and from 14 to 158 nM, respectively. The antiviral activity of palinavir was further demonstrated with HIV-1 clinical isolates resistant to AZT, ddI, and nevirapine. Mean EC_{50}s and EC_{90}s of 3.6 to 71 nM and 26 to 235 nM, respectively, were obtained with infected PBMCs. Similar potency was observed upon infection of cell lines with resistant isolates. The cytotoxicity of palinavir was also evaluated in the different cell lines and PBMCs (Table 2). CC_{50}s of palinavir ranged from 30 to 45 μM as determined by the MTT assay and the trypan blue exclusion method. With mean EC_{50}s from 0.5 to 30 nM for the inhibition of HIV, the therapeutic index of palinavir exceeds 1,000.

The potency of palinavir was also assessed and compared to that of AZT by increasing doses of HIV-1 in acutely infected cells (Table 3). The antiviral activities of palinavir and AZT were retained when the MOI was increased from 0.01 to 1.0 in H9 cells. EC_{50}s of 6 to 12 nM and 5 to 10 nM were obtained for palinavir and AZT, respectively. Increasing the MOI from 0.001 to 1.0 in C8166 cells resulted in a loss of antiviral activity for both inhibitors. However, a more dramatic loss of activity was seen with AZT as EC_{50}s increased from 5 to 270 nM for palinavir and from 8 to 10,000 nM for AZT.

Previous studies indicated that the addition of human serum alpha-1 acid glycoprotein (AAG) to the medium markedly increased the concentration of protease inhibitor required to

TABLE 1. Inhibition of HIV-1 and HIV-2 proteases by palinavir and activity against human aspartyl proteases

Aspartyl protease	EC_{50} (nM)
HIV-1	4 (0.031[a])
HIV-2	10 (0.134[a])
Renin	>50,000
Cathepsin D	100,000
Pepsin	33,000
Gastricsin	45,000

[a] K_i was determined by a steady-state velocity method.

TABLE 2. Inhibition of virus replication and cytotoxicity of palinavir

Virus	Strain or isolate[a]	Cell type	Cytotoxicity[b] (CC_{50} [μM])	EC_{50} (nM)	EC_{90} (nM)
HIV-1	IIIB	C8166	32	5	25
	HXB2	C8166		0.5	5
	IIIB	H9	30	2	9
	HXB2	H9		1	14
	Wg3522	PBMCs	33	15	50
	Wg3523	PBMCs		24	91
	VR949	PBMCs		11	25
	VR1372	PBMCs		4	14
	UHC-1	PBMCs		4	31
	ADA	M/M		4	12
	AZT^r postdrug	PBMCs		71	235
	AZT^r predrug	PBMCs		28	55
	AZT^r	C8166		15	75
	ddI^r	PBMCs		5.0	29
	ddI^r	C8166		6.2	34
	Nev^r	PBMCs		3.6	26
	Nev^r	C8166		1.4	29
HIV-2	ROD	PBMCs		8	158
	ROD	C8166		30	152
	ROD	CEMx174	30	4	14
SIV	MAC251	CEMx174		4	14

[a] Wg3522 and Wg3523 (Montréal), VR949 (Haitian), and VR1372 (Zaire) are HIV-1 clinical isolates. ADA is a characterized HIV-1 monocytotropic strain. EC_{50}s of 1.1, 9, and 1 μM were determined for AZT, ddI, and nevirapine and with AZT^r, ddI^r, and Nev^r isolates, respectively.

[b] CC_{50} of 45 μM was obtained for palinavir with the CR-10 and U937 cell lines.

TABLE 3. Antiviral activity of palinavir and AZT with increasing doses of HIV-1 IIIB in H9 and C8166 cells

Cell line and drug	EC_{50} (nM) at MOI:					
	1.0	0.5	0.1	0.05	0.01	0.001
H9						
Palinavir	7	9	12	6	10	ND[a]
AZT	7	6	6	5	10	ND
C8166						
Palinavir	270	117	60	35	8	5
AZT	10,000	10,000	300	141	13	8

[a] ND, not determined.

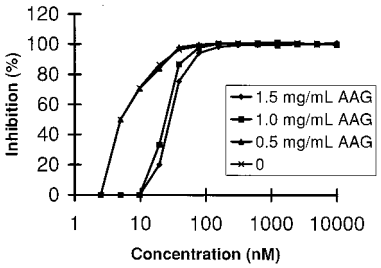

FIG. 2. Effect of human AAG on antiviral activity of palinavir for C8166 cells acutely infected with HIV-1 IIIB (MOI of 0.001). Cells were incubated for 3 days with serial dilutions of palinavir in 10% FCS and various concentrations of AAG (Sigma G-9885) from 0.5 to 1.5 mg/ml. The percent inhibition was determined by measuring extracellular p24 antigen, and EC_{50}s and EC_{90}s were obtained through the use of the SAS software. In the absence and the presence of 1.5 mg of AAG per ml, EC_{50}s and EC_{90}s of 5.5 and 23 nM and 27 and 70 nM, respectively, were obtained.

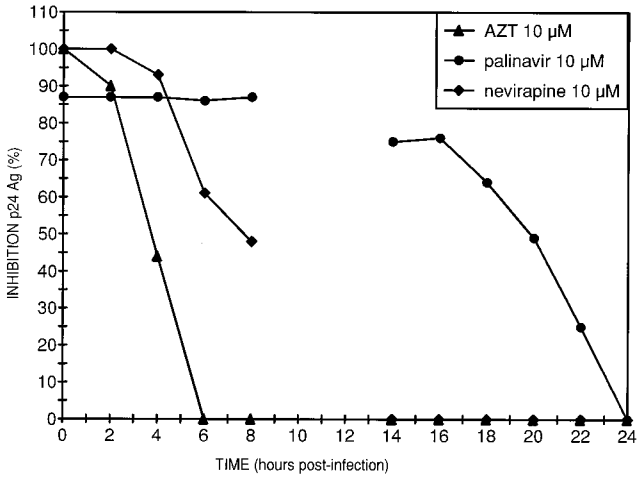

FIG. 3. Effect of postinfection additions of palinavir, AZT, and nevirapine to C8166 cells acutely infected with HIV-1 IIIB. Cells were infected at an MOI of 1.0 and incubated for 24 h with HIV-1 inhibitors (10 μM) added at various times postinfection. The effects of inhibitors added from 0 to 8 h and from 14 to 24 h were obtained from independent infections. The percent inhibition of p24 Ag, from a representative experiment, is expressed relative to a 100% inhibition control in which inhibitor was added immediately after virus absorption.

limit infection in vitro (5, 6). Therefore, the effect of AAG on the antiviral activity of palinavir was evaluated in acutely infected C8166 cells (Fig. 2). Cells were incubated for 3 days with serial dilutions of palinavir in RPMI–10% FCS containing physiologically relevant concentrations of AAG. In the presence of AAG, at concentrations of 0.5 to 1.5 mg/ml, a three- to fivefold reduction in the antiviral activity of palinavir was observed. This was indicated by the increase in EC_{50}s and EC_{90}s from 5.5 to 27 nM and from 23 to 70 nM, respectively.

Antiviral activity of palinavir in combination with AZT, ddI, or nevirapine. Several studies assessing in vitro interactions between protease inhibitors and RT inhibitors have demonstrated additive and synergistic antiviral interactions (38, 40). The antiviral activity of palinavir in two-drug combinations with the RT inhibitors AZT, ddI, and nevirapine was investigated for possible synergy in acutely infected C8166 cells (Table 4). For all fixed-ratio combinations of inhibitors, analysis of the antiviral activity gave CIs predominantly of ≤1, at both 50 and 90% inhibition, indicative of synergy or additivity. The combination of AZT and palinavir demonstrated synergy at ratios of 10:1, 3:1, and 1:1 at both 50 and 90% inhibition of HIV-1 replication, despite variation in CIs from independent experiments. Ratios of AZT to palinavir of 1:3 and 1:10 exhibited predominantly additive effects with CIs of ~1. The antiviral activity of palinavir in combination with nevirapine demonstrated synergy or additivity at the various ratios. The combination of ddI and palinavir at ratios of 30:1 and 10:1 showed synergy at 90% inhibition as illustrated by the CIs of 0.4 and 0.3, respectively. All fixed-ratio combinations of drugs showed no cytotoxicity when tested at the highest concentration in the MTT metabolic assay (data not shown).

TABLE 4. CIs for two-drug combinations with RT inhibitors AZT, ddI, nevirapine and palinavir

Drug combination	Molar ratio	CI at the following levels of HIV inhibition (%)[a]		Overall result
		50	90	
AZT-palinavir	10:1	0.4–0.9	0.5–0.8	Synergy
	3:1	0.2–1.0	0.2–1.0	Synergy
	1:1	0.7–1.4	0.3–0.9	Synergy
	1:3	1.0–1.3	0.4–1.6	Additive
	1:10	1.0–1.6	1.3–1.5	Additive
Nevirapine-palinavir	10:1	0.7–1.2	0.6–1.0	Synergy
	3:1	0.4–1.0	0.6–1.1	Synergy
	1:1	0.9	1.1	Synergy
	1:3	0.2–1.0	0.2–0.8	Synergy
	1:10	1.2	0.8	Synergy
ddI-palinavir	30:1	1.3	0.4	Synergy
	10:1	0.8	0.3	Synergy

[a] The range in CIs from independent experiments is illustrated. CIs of <1, 1, and >1 are indicative of synergy, additivity, and antagonism, respectively. EC_{50}s for palinavir, AZT, nevirapine, and ddI were 5, 10, 10, and 580 nM, respectively.

Antiviral activity of HIV-1 inhibitors added at various times postinfection. The viral protease is believed to play its major role at a late stage in the virus life cycle subsequent to the reverse transcription. The antiviral effect of palinavir, AZT, and nevirapine was investigated when inhibitors were added at various times between 2 and 22 h postinfection (Fig. 3). Conditions that favor a synchronous and unique HIV-1 replication cycle were used in these time-addition experiments, that is, C8166 cells, highly permissive for HIV-1 infection, an MOI of 1.0, and a 24-h incubation period. In these cells, antiviral activity of palinavir was retained for a longer period after infection than that of RT inhibitors. Delaying the addition of palinavir from 0 to 8 h postinfection did not affect its activity. A 50% inhibition in extracellular p24 Ag level was observed with addition of palinavir at 20 h postinfection. Significant activity (25% inhibition of p24 Ag) was still retained at 22 h postinfection. AZT and nevirapine demonstrated a more rapid loss of activity. Delaying the addition of AZT and nevirapine for 4 and 6 h postinfection, respectively, resulted in a 50% inhibition of the p24 Ag level. At 8 h postinfection, AZT had already lost all of its activity whereas palinavir retained complete activity at this same time. The addition of nevirapine at 14 h postinfection resulted in a complete loss of antiviral activity.

Inhibition of virus replication in chronically infected cell lines. The virus maturation was assayed by the p24 Ag levels of chronically infected CR-10 and U1 cell lines after inhibitor treatment for 48 h (data not shown). The levels of extracellular p24 Ag are inhibited by palinavir in both cell lines. EC_{50}s of 130 and 90 nM were obtained for the CR-10 and U1 cell lines, respectively. Incubation with AZT, however, had no effect on

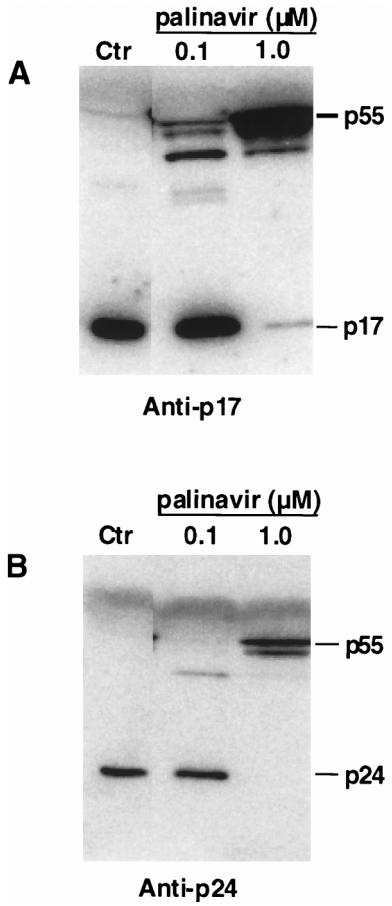

FIG. 4. Western blot analysis of pelleted virions from palinavir-treated CR-10 cells. Cells were incubated for 48 h with 0.1 and 1 μM palinavir. Viral proteins were transferred to nitrocellulose following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized with anti-HIV-1 p17 (A) and anti-HIV-1 p24 (B) monoclonal antibodies and labelled goat anti-mouse antibodies. Viral proteins from untreated cells are shown in the control lanes (Ctr). The positions of Gag precursor polyproteins (p55), matrix proteins (p17), and core proteins (p24) are indicated.

the extracellular p24 Ag levels from CR-10 and U1 cell lines. Treatment of CR-10 cells with 1 to 10 μM palinavir showed a 99 to 99.5% reduction of infectious particle numbers, as determined by virus titration. The virus maturation was also assessed by Western blot analysis of pelleted virions from palinavir-treated CR-10 cells (Fig. 4). The incubation of cells with 0.1 and 1 μM palinavir resulted in partial to almost complete blockage of processing as demonstrated by the presence of intermediate and/or Gag precursor polyproteins in virions. Proteins of similar molecular weight were not detected in virions from untreated cells. An almost complete disappearance of mature p24 and p17 proteins is also observed for the virions with 1 μM palinavir treatment.

DISCUSSION

Palinavir is a potent inhibitor of HIV-1 and HIV-2 proteases, leading to inhibition of viral replication in cell culture. On the basis of potency and oral bioavailability in various animal models (2, 26), palinavir was isolated from a collection of hydroxyethylamine-based inhibitors containing a substituted pipecolinic amide. By using two different methods for K_i determination, values in the low picomolar range were obtained for palinavir (Table 1). The inhibited enzyme has a half-life of approximately 18 min when given a dissociation constant of 6.5×10^{-4} s^{-1} in the assay condition used. Palinavir has a weaker affinity for the HIV-2 ROD protease as demonstrated by the increase in the K_i (134 pM). The specificity of palinavir was demonstrated by its lack of significant inhibitory activity against a variety of human aspartyl proteases including renin, cathepsin D, pepsin, and gastricsin. The present data also illustrate the potent antiviral activity of the compound in cell culture. Palinavir inhibits the replication of various divergent HIV-1 clinical isolates and laboratory strains (HIV-1, HIV-2, SIV, and HIV-1 monocytotropic strain) at concentrations in the low nanomolar range with primary cultures and cell lines. As high plasma protein binding can adversely affect drug activity (5, 6), the effect of AAG on the activity of palinavir was investigated. Potent antiviral activity was maintained with only a threefold reduction in EC_{90} under physiological concentrations of AAG. Palinavir is also active against the replication of AZT-, ddI-, and nevirapine-resistant isolates. This supports its potential use as a therapeutic alternative to nucleoside or non-nucleoside RT inhibitors.

Palinavir retained its potency with increased doses of HIV-1 in H9 cells. There was, however, a reduction in the antiviral activity of palinavir and other protease inhibitors tested (data not shown) at high MOIs in C8166 cells. A 50-fold increase in the EC_{50} (270 versus 5 nM) was observed with palinavir compared to a 1,250-fold increase in EC_{50} with AZT (10,000 versus 8 nM). The reduced antiviral activity of palinavir and AZT could be due to superinfection (higher levels of intracellular viral enzyme) as a consequence of multiple HIV-1 replication cycles in C8166 cells. After 24 h of incubation in C8166 cells, however, 10 μM AZT added immediately after infection was shown to inhibit p24 Ag to an undetectable level (Fig. 3). This time period corresponds to approximately one HIV-1 replication cycle in C8166 cells (see below). With the high replication rate of HIV-1 in infected patients, these results may be relevant to setting therapeutically effective levels of HIV-1 inhibitors in plasma. Two-drug combinations of palinavir with either AZT, ddI, or nevirapine displayed synergy or additivity in the inhibition of HIV-1 replication of acutely infected cells. There was no antagonism observed in HIV-1-infected C8166 cells or H9 cells and HIV-2-infected C8166 cells (Table 4 and data not shown). Additive or synergistic antiviral interactions observed between various protease inhibitors and RT inhibitors, without increasing toxicity, suggested that many protease inhibitors may be useful partners in combination regimens. In addition, significant three-way synergy which includes protease inhibitors and other agents in selected three-drug combinations has been described elsewhere (38). In vitro interactions between palinavir and the RT inhibitors tested, all approved for HIV disease, also indicate a potential for combination therapy in vivo.

Palinavir inhibits the replication of HIV-1 subsequent to the reverse transcription step. This is consistent with its expected mode of action at a late stage in the virus life cycle. Antiviral activity was retained with the addition of palinavir at 14 h postinfection while similar time-addition of RT inhibitors showed a complete loss of activity in C8166 cells. These results indicate that the reverse transcription step is completed within 14 h in these infected cells, not accounting for the time needed for the RT inhibitor to access the intracellular viral enzyme. The earlier loss of activity for AZT compared to nevirapine possibly reflects the metabolism required for AZT and/or a cell penetration difference. The loss of antiviral activity of palinavir (25% inhibition of p24 Ag) observed at 22 h postinfection also indicates that the HIV-1 replication cycle is approximately 22 h

in the majority of C8166 cells. Finally, the antiviral activity of palinavir due to protease inhibition was demonstrated in chronically infected cells. This is first suggested by the dose-dependent reduction of extracellular p24 Ag levels with palinavir treatment in both CR-10 and U1 cell lines. A 48-h incubation with 10 μM palinavir resulted in undetectable levels of p24 Ag in supernatants of both cell lines. This is associated with a 99.5% reduction in infectious particle numbers observed with 10 μM palinavir-treated CR-10 cells. In contrast, p24 Ag levels were not affected by AZT treatment in both cell lines, demonstrating that virus production is due mainly to integrated proviral DNA and not to reinfection cycles. The mode of action of palinavir was confirmed by its ability to block Gag precursor polyprotein processing. Western blot analysis of pelleted virions and cell extracts (data not shown) from 1 μM palinavir-treated cells showed a complete disappearance of p24 and p17 mature proteins. Treatment of chronically infected CR-10 cells with palinavir reduced p24 Ag levels and the number of infectious particles but generated immature particles containing mainly Gag precursor polyproteins (Fig. 4). The antiviral activity of palinavir is thus mediated by the inhibition of the viral protease.

The clinical emergence of drug-resistant viruses has limited the long-term effectiveness of antiviral drugs, a phenomenon that is exacerbated in HIV infection. In order to evaluate the potential of palinavir to develop resistance, HIV-1 variants with reduced susceptibility to palinavir were generated in vitro by sequential passages of the virus in the presence of the drug for up to 6 months (31). HIV-1 variants recovered at various passages showed moderate to high-level resistance to palinavir which was associated with a stepwise accumulation of mutations in the protease locus (active-site mutations V32I and I84A) and in the protease substrate (Gag precursor p1/p6). Sets of protease mutations required for high-level resistance to palinavir, when engineered into HIV-1 clones, conferred a significant growth reduction of these clones in peripheral blood lymphocytes and T-cell lines. The second-locus cleavage site mutations were previously described as compensatory mutations in highly resistant viruses with impaired viral fitness (12, 17). The requirement for a stepwise accumulation of mutations in HIV and the impaired viral fitness of HIV variants showing high-levels of resistance to palinavir highlighted its potential to delay the emergence of resistance in vivo.

In summary, these results demonstrate that palinavir is a selective and potent inhibitor of HIV-1 protease with antiviral activity against laboratory strains, clinical isolates, and RT inhibitor-resistant isolates. Palinavir retains potent antiviral activity with increasing doses of virus. The antiviral activity of palinavir in combination with either AZT, nevirapine, or ddI results in synergy or additivity in the inhibition of HIV-1 infection. Palinavir inhibits the replication of HIV-1 subsequent to the reverse transcription step, consistent with its expected mode of action at a late stage in the infectious cycle. In chronically infected cells treated with palinavir, the reduction of extracellular p24 Ag levels and infectious particle numbers is associated with the inhibitor-mediated prevention of the virus maturation. Palinavir has good oral bioavailability in rats (2), dogs, and chimpanzees, with a positive deviation from dose proportionality (26). In view of the potent antiviral activity in vitro, low-level cytotoxicity, and excellent pharmacokinetic data in animal models, palinavir was considered to be a competitive compound and was selected for in-depth preclinical evaluation.

ACKNOWLEDGMENTS

We thank F. Soucy, P. Beaulieu, and P. Lavallée for the synthesis of palinavir. We also thank G. McKercher, L. Pilote, G. Bolger, and S. Larouche for technical assistance. We thank M. G. Cordingley and L. Doyon for critical review of the manuscript.

REFERENCES

1. **Aldovini, A., and B. D. Walker.** 1990. Virus production in stable cell lines, quantitative assays for virus infectivity, p. 66–69 and 71–76. *In* A. Aldovini and B. D. Walker (ed.), Techniques in HIV research. Stockton Press, New York, N.Y.
2. **Anderson, P. C., and C. Yoakim.** Unpublished data.
3. **Bechtold, C. M., A. K. Patick, M. Alam, J. Greytok, J. A. Tino, P. Chen, E. Gordon, S. Ahmad, J. C. Barrish, R. Zahler, P.-F. Lin, and R. Colonno.** 1995. Antiviral properties of aminodiol inhibitors against human immunodeficiency virus and protease. Antimicrob. Agents Chemother. **39:**374–379.
4. **Betageri, R., J. L. Hopkins, D. Thibeault, M. J. Emmanuel, G. C. Chow, M. T. Skoog, P. deDreu, and K. A. Cohen.** 1993. Rapid, sensitive and efficient HPLC assays for HIV-1 proteinase. Biochem. Biophys. Methods **27:**191–197.
5. **Bilello, J. A., P. A. Bilello, K. Stellrecht, J. Leonard, D. W. Norbeck, D. J. Kempf, T. Robins, and G. L. Drusano.** 1996. 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. Antimicrob. Agents Chemother. **40:**1491–1497.
6. **Bilello, J. A., P. A. Bilello, M. Prichard, T. Robins, and G. L. Drusano.** 1995. Reduction of the *in vitro* activity of A77003, an inhibitor of human immunodeficiency virus protease, by human serum α1 acid glycoprotein. J. Infect. Dis. **171:**546–551.
7. **Billich, A., G. Fricker, I. Müller, P. Donatsch, P. Ettmayer, H. Gstach, P. Lehr, P. Peichl, D. Scholz, and B. Rosenwirth.** 1995. SDZ PRI 053, an orally bioavailable human immunodeficiency virus type 1 proteinase inhibitor containing the 2-aminobenzylstatine moiety. Antimicrob. Agents Chemother. **39:**1406–1413.
8. **Bryant, M., D. Getman, M. Smidt, J. Marr, M. Clare, R. Dillard, D. Lansky, G. DeCrescenzo, R. Heintz, K. Houseman, K. Reed, J. Stolzenbach, J. Talley, M. Vazquez, and R. Mueller.** 1995. SC-52151, a novel inhibitor of the human immunodeficiency virus protease. Antimicrob. Agents Chemother. **39:**2229–2234.
9. **Chokekijchai, S., T. Shirasaka, J. N. Weinstein, and H. Mitsuya.** 1995. *In vitro* anti-HIV-1 activity of HIV protease inhibitor KNI-272 in resting and activated cells: implications for its combined use with AZT or ddI. Antivir. Res. **28:**25–38.
10. **Chou, T. C., and P. Talalay.** 1984. Quantitative analysis of dose-effect relationships: the combined effect of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. **22:**27–55.
11. **Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, D. Titus, T. Yang, H. Teppler, K. E. Squires, P. J. Deutsch, and E. A. Emini.** 1995. *In vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature **374:**569–571.
12. **Croteau, G., L. Doyon, D. Thibeault, G. McKercher, L. Pilote, and D. Lamarre.** 1997. Impaired fitness of human immunodeficiency virus type 1 variants with high-level resistance to protease inhibitors. J. Virol. **71:**1089–1096.
13. **Daniel, M. D., N. L. Letvin, N. W. King, M. Kannagi, P. K. Sehgal, R. D. Hunt, P. J. Kanki, M. Essex, and R. C. Desrosiers.** 1985. Isolation of T-cell tropic HTLF-III-like retrovirus from macaques. Science **228:**1201–1204.
14. **Danner, S. A., A. Carr, J. M. Leonard, L. M. Lehman, F. Gudiol, J. Gonzales, A. Raventos, R. Rubio, E. Bouza, V. Pintado, A. Gil Aguado, J. Garcia de Lomas, R. Delgado, J. C. C. Borleffs, A. Hsu, J. M. Valdes, C. A. B. Boucher, and D. A. Cooper.** 1995. A short-term study of the safety, pharmacokinetics, and efficacy of Ritonavir, an inhibitor of HIV-1 protease. N. Engl. J. Med. **333:**1528–1533.
15. **Darke, P. A., and J. R. Huff.** 1994. HIV protease as an inhibitor target for the treatment of AIDS. Adv. Pharmacol. **25:**399–454.
16. **Debouck, C.** 1992. The HIV-1 protease as a therapeutic target for AIDS. AIDS Res. Hum. Retroviruses **8:**153–164.
17. **Doyon, L., G. Croteau, D. Thibeault, F. Poulin, L. Pilote, and D. Lamarre.** 1996. Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. J. Virol. **70:**3763–3769.
18. **Folks, T. M., J. Justement, A. Kinter, C. A. Dinarello, and A. S. Fauci.** 1987. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science **238:**800–802.
19. **Galpin, S., N. A. Roberts, T. O'Connor, D. J. Jeffries, and D. Kinchington.** 1994. Antiviral properties of the HIV-1 proteinase inhibitor Ro 31-8959. Antivir. Chem. Chemother. **5:**43–45.
20. **Geleziunas, R., E. J. Arts, F. Boulerice, H. Goldman, and M. A. Wainberg.** 1993. Effect of 3′-azido-3′-deoxythymidine on human immunodeficiency virus type 1 replication in human fetal brain macrophages. Antimicrob. Agents Chemother. **37:**1305–1312.
21. **Gendelman, H. E., J. M. Orenstein, L. M. Baca, B. Weiser, H. Burger, D. C.**

Kalter, and M. S. Meltzer. 1989. The macrophage in the persistence and pathogenesis of HIV infection. AIDS **3:**475–495.
22. **Gendelman, H. E., J. M. Orenstein, M. A. Martin, C. Ferrua, R. Mitra, T. Phipps, L. A. Wahl, H. C. Lane, A. S. Fauci, D. S. Burke, D. Skillman, and M. S. Meltzer.** 1988. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. J. Exp. Med. **167:**1428–1441.
23. **Hirsch, M. S., and R. T. D'Aquila.** 1993. Therapy for human immunodeficiency virus infection. N. Engl. J. Med. **328:**1686–1695.
24. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature **373:**123–126.
25. **Jacobsen, H.** 1995. Resistance to protease inhibitors *in vivo*. Int. Antivir. News **3:**S9.
26. **Keirns, J., J. Jaramillo, and P. C. Anderson.** Unpublished data.
27. **Kempf, D. J., K. C. Marsh, E. Mcdonald, S. Vasavanonda, C. A. Flentge, B. E. Green, L. Fino, C. H. Park, X. P. Kong, N. E. Wideburg, A. Saldivar, L. Ruiz, W. M. Kati, H. L. Sham, T. Robins, K. D. Stewart, A. Hsu, J. J. Plattner, J. M. Leonard, and D. W. Norbeck.** 1995. ABT-538 is a potent inhibitor of human immunodeficiency virus protease and has high oral bioavailability in humans. Proc. Natl. Acad. Sci. USA **92:**2484–2488.
28. **Kitchen, V. S., C. Skinner, K. Ariyoshi, E. A. Lane, I. B. Duncan, J. Burckhardt, H. U. Burger, K. Bragman, A. J. Pinching, and J. N. Weber.** 1995. Safety and activity of saquinavir in HIV infection. Lancet **345:**952–955.
29. **Kohl, N. E., E. A. Emini, W. A. Schleiff, L. J. Davis, J. C. Heimbach, R. A. F. Dixon, E. M. Scolnick, and I. S. Sigal.** 1988. Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. USA **85:**4686–4690.
30. **Lam, P. Y. S., P. K. Jadhav, C. J. Eyeremann, C. N. Hodge, Y. Ru, L. T. Bacheler, J. L. Meek, M. J. Otto, M. M. Rayner, Y. N. Wong, C. H. Chang, P. C. Weber, D. A. Jackson, T. R. Sharpe, and S. Erickson-Viitanen.** 1994. Rational design of potent, bioavailable, nonpeptide cyclic ureas as HIV protease inhibitors. Science **263:**380–384.
31. **Lamarre, D., D. Doyon, G. Croteau, E. Wardrop, L. Pilote, G. McKercher, and D. Thibeault.** 1995. Molecular basis of HIV-1 resistance to protease inhibitors. Structural flexibility of the protease and second-site compensatory mutations in cleavage sites, abstr. 62. *In* Fourth International Workshop on HIV Drug Resistance, Sardinia, Italy, 6 to 9 July.
32. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243:**1731–1734.
33. **Markowitz, M., M. Saag, W. G. Powderly, A. M. Hurley, A. Hsu, J. M. Valdes, D. Henry, F. Sattler, A. La Marca, J. M. Leonard, and D. D. Ho.** 1995. A preliminary study of Ritonavir, an inhibitor of HIV-1 protease, to treat HIV-1 infection. N. Engl. J. Med. **333:**1534–1539.
34. **Molla, A., M. Korneyeva, Q. Gao, S. Vasavanonda, P. J. Schipper, H.-M. Mo, M. Markowitz, T. Chernyavskiy, P. Niu, N. Lyons, A. Hsu, G. R. Granneman, D. D. Ho, C. A. B. Boucher, J. M. Leonard, D. W. Norbeck, and D. J. Kempf.** 1996. Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. Nature Med. **2:**760–766.
35. **Morrison, J. F., and C. T. Walsh.** 1988. The behavior and significance of slow-binding enzyme inhibitors. Adv. Enzymol. **61:**201–301.
36. **Morrison, J. F., and S. R. Stone.** 1985. Approaches to the study and analysis of the inhibition of enzymes by slow- and tight-binding inhibitors. Comments Mol. Cell. Biophys. **2:**347–368.
37. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
38. **Moyle, G., and B. Gazzard.** 1996. Current knowledge and future prospects for the use of HIV protease inhibitors. Drugs **51:**701–712.
39. **Naidu, Y. M., H. W. Kestler III, Y. Li, C. V. Butler, D. P. Silva, D. K. Schmidt, C. D. Troup, P. K. Sehgal, P. Sonigo, M. D. Daniel, and R. C. Desrosiers.** 1988. Characterization of infectious molecular clones of simian immunodeficiency virus (SIV_{mac}) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIV_{mac}. J. Virol. **62:**4691–4696.
40. **Noble, S., and D. Faulds.** 1996. Saquinavir: a review of its pharmacology and clinical potential in the management of HIV infection. Drugs **52:**93–112.
41. **Pargellis, C. A., M. M. Morelock, E. T. Graham, P. Kinkade, S. Pav, K. Lubbe, D. Lamarre, and P. C. Anderson.** 1994. Determination of kinetic rate constants for the binding of inhibitors to HIV-1 protease and for the association and dissociation of active homodimer. Biochemistry **33:**12527–12534.
42. **Pav, S., K. Lubbe, F. Dô, D. Lamarre, C. A. Pargellis, and L. Tong.** 1994. Microtube batch protein crystallization: applications to human immunodeficiency virus type 2 (HIV-2) protease and human renin. Proteins Struct. Funct. Genet. **20:**98–102.
43. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224:**497–500.
44. **Popovic, M., E. Read-Connole, and R. C. Gallo.** 1984. T4 positive human neoplastic cell lines susceptible to and permissive for HTLV-III. Lancet **ii:**1472–1473.
45. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature **313:**277–284.
46. **Richman, D., C.-K. Shih, I. Lowy, J. Rose, P. Prodanovich, S. Goff, and J. Griffin.** 1991. Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. Proc. Natl. Acad. Sci. USA **88:**11241–11245.
47. **Roberts, N. A., J. A. Martin, D. Kinchington, A. V. Broadhurst, J. C. Craig, I. B. Duncan, S. A. Galpin, B. K. Handa, J. Kay, A. Krohn, R. W. Lambert, J. H. Merrett, J. S. Mills, K. E. B. Parks, S. Redshaw, A. J. Ritchie, D. L. Taylor, G. J. Thomas, and P. Machin.** 1990. Rational design of peptide-based HIV proteinase inhibitors. Science **248:**358–361.
48. **Shaw, G. M., B. H. Hahn, S. K. Arya, J. E. Groopman, R. C. Gallo, and F. Wong-Staal.** 1984. Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. Science **226:**1165–1170.
49. **Tong, L., S. Pav, S. Mui, D. Lamarre, C. Yoakim, P. Beaulieu, and P. C. Anderson.** 1995. Crystal structures of HIV-2 protease in complex with inhibitors containing the hydroxyethylamine dipeptide isostere. Structure **3:**33–40.
50. **Vacca, J. P., B. D. Dorsey, W. A. Schleif, R. B. Levin, S. L. McDaniel, P. L. Darke, J. Zugay, J. C. Quintero, O. M. Blahy, E. Roth, V. V. Sardana, A. J. Schlabach, P. I. Graham, J. H. Condra, L. Gotlib, M. K. Holloway, J. Lin, I.-W. Chen, K. Vastag, D. Ostovic, P. S. Anderson, E. A. Emini, and J. R. Huff.** 1994. L-735,524: an orally bioavailable human immunodeficiency virus type 1 protease inhibitor. Proc. Natl. Acad. Sci. USA **91:**4096–4100.
51. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373:**117–122.
52. **Wlodawer, A., and J. Erickson.** 1993. Structure-based inhibitors of HIV-1 protease. Annu. Rev. Biochem. **62:**543–585.