Identification and Characterization of UK-201844, a Novel Inhibitor That Interferes with Human Immunodeficiency Virus Type 1 gp160 Processing[▽]

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More than 10⁶ compounds were evaluated in a human immunodeficiency virus type 1 (HIV-1) high-throughput antiviral screen, resulting in the identification of a novel HIV-1 inhibitor (UK-201844). UK-201844 exhibited antiviral activity against HIV-1 NL4-3 in MT-2 and PM1 cells, with 50% effective concentrations of 1.3 and 2.7 μ M, respectively, but did not exhibit measurable antiviral activity against the closely related HIV-1 IIB laboratory strain. UK-201844 specifically inhibited the production of infectious virions packaged with an HIV-1 envelope (Env), but not HIV virions packaged with a heterologous Env (i.e., the vesicular stomatitis virus glycoprotein), suggesting that the compound targets HIV-1 Env late in infection. Subsequent antiviral assays using HIV-1 NL4-3/IIB chimeric viruses showed that HIV-1 Env sequences were critical determinants of UK-201844 susceptibility. Consistent with this, in vitro resistant-virus studies revealed that amino acid substitutions in HIV-1 Env are sufficient to confer resistance to UK-201844. Western analysis of HIV Env proteins expressed in transfected cells or in isolated virions showed that UK-201844 inhibited HIV-1 gp160 processing, resulting in the production of virions with nonfunctional Env glycoproteins. Our results demonstrate that UK-201844 represents the prototype for a unique HIV-1 inhibitor class that directly or indirectly interferes with HIV-1 gp160 processing.

Therapeutic options are often limited in treatment-experienced human immunodeficiency virus (HIV) patients, due in large part to drug resistance (reviewed in reference 26). The discovery of compounds targeting new mechanisms in the HIV type 1 (HIV-1) replication cycle is, in theory, the most effective strategy to generate drugs that are active against HIV-1 variants resistant to all current therapies. This is based on the premise that viral mutations conferring resistance to existing drug classes would not confer cross-resistance to drugs targeting new mechanisms. Although the discovery of novel mechanism inhibitors is not trivial, several antiviral compounds that target new mechanisms are in development, including CCR5 inhibitors (6), HIV-1 integrase (IN) inhibitors (4, 15, 18), a CXCR4 inhibitor (9), a gp120/CD4 inhibitor (8), and a virion maturation inhibitor (16). Each of these compounds was originally discovered in screening efforts focused on viral (e.g., HIV-1 IN) or cellular (e.g., CCR5) targets known to be required for HIV-1 replication in culture or by randomly screening for antiviral activity in HIV-1 replication assays (e.g., CXCR4, gp120/CD4, and virion maturation inhibitors) (3, 23, 25). As part of our effort to discover novel target inhibitors, we developed an HIV-1 high-throughput full-replication screen (HIV Rep) that incorporates all of the HIV-1 targets required for replication in cell culture (2). This afforded us the opportunity to screen for multiple targets in the context of a full

replication cycle and to identify compounds directed against new HIV-1 mechanisms.

As alluded to above, the production and packaging of functional HIV-1 envelope (Env) glycoproteins into infectious virions is included as a target in the HIV Rep screen. The HIV-1 Env glycoprotein is synthesized as a gp160 precursor that is proteolytically processed into the mature Env proteins gp120 and gp41 (17). Both gp120 (the external glycoprotein) and gp41 (the transmembrane protein) remain noncovalently associated within oligomeric structures and are routed to the cell surface, where they are packaged into budding virions, by the constitutive secretory pathway. Cleavage of the gp160 precursor is mediated by one or more cellular proprotein convertases (PC) and is required for the production of infectious virions (reviewed in reference 19). Cellular coexpression and other in vitro studies have implicated furin; paired basic amino acid-cleaving enzyme 4 (PACE4); and PC1, PC2, PC5/6, PC7, and possibly other convertases in gp160 processing (7, 19). Cleavage of gp160 occurs immediately after a cluster of basic amino acids (Arg-Glu-Lys-Arg), a PC consensus motif that is highly conserved across HIV-1 strains (12, 17).

Inhibitors targeting the production of a functional HIV-1 Env have previously been reported (19). Multiple peptides that mimic the gp160 cleavage site and inhibit gp160 processing have been described; however, clinical development of such molecules is limited by the challenges associated with developing drugs from peptide-based compounds. A second class of compounds that inhibits gp160 processing indirectly by disrupting gp160 intracellular trafficking has also been described. Such inhibitors target important host cell functions; thus, their clin-

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ical potential is limited due to safety concerns. In this study, we describe a highly specific small-molecule inhibitor (UK-201844) of HIV-1 Env processing that was identified in our HIV Rep screen. We demonstrate that UK-201844 inhibits the production of infectious virions in an HIV-1 Env-dependent manner. Consistent with this, we show that HIV-1 Env gene sequences are critical determinants of UK-201844 susceptibility. Further mechanism-of-action studies demonstrated that UK-201844 directly or indirectly interferes with gp160 processing, resulting in the production of virions lacking functional Env glycoproteins. Our data show that UK-201844 represents the prototype of a unique class of HIV-1 Env inhibitors. This compound could be used to further investigate the process of HIV-1 Env expression and incorporation into infectious virions and may also serve as the starting point for the development of a new therapeutic class for HIV-1 infection.

MATERIALS AND METHODS

Cells and virus. HeLa CD4 LTR/beta-Gal (catalog no. 1470), MT-2 (catalog no. 237), PM1 (catalog no. 3038), and HEK 293 (catalog no. 103) cells were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Bethesda, MD. HeLa CD4 LTR/beta-Gal cells and HEK 293 cells were propagated in Dulbecco's modified Eagle medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT). MT-2 and PM1 cells were propagated in RPMI 1640 medium (Invitrogen Life Technologies) containing 10% FBS (HyClone). HIV-1 IIB (catalog no. 398) and HIV-1Ba-L (catalog no. 510) and the pNL4-3 HIV-1 infectious molecular clone (catalog no. 114) were also obtained through the National Institutes of Health AIDS Research and Reference Reagent Program.

Compounds. Efavirenz (EFV) was kindly provided by DuPont Merck Pharmaceutical Company (Wilmington, DE). Nelfinavir (NFV) and UK-201844 were synthesized by Pfizer Inc. (San Diego, CA). Aurintricarboxylic acid (ATA) was obtained from Sigma-Aldrich (St Louis, MO).

CPE assays. In cytopathic effect (CPE) assays, host cells were infected with HIV-1 NL4-3 or NL4-3 variants at a multiplicity of infection (MOI) of 0.08 or mock infected with medium only and added at 2×10^4 cells per well to 96-well plates containing half-logarithmic dilutions of the test compounds. Six days later, 50 μ l of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) (1 mg/ml XTT tetrazolium, 0.02 nM phenazine methosulfate) was added to the wells, and the plate was reincubated for 4 hours. Viability, as determined by the amount of XTT formazan produced, was quantified spectrophotometrically by absorbance at 450 nm (24). Data from CPE assays were expressed as the percentage of formazan produced in compound-treated cells compared to the formazan produced in the wells of uninfected, compound-free cells. The 50% effective concentration (EC_{50}) was calculated as the concentration of compound that effected an increase in the percentage of formazan production in infected, compound-treated cells to 50% of that produced by uninfected, compound-free cells. The 50% cytotoxicity concentration (CC_{50}) was calculated as the concentration of compound that decreased the percentage of formazan produced in uninfected, compound-treated cells to 50% of that produced in uninfected, compound-free cells. The therapeutic index (TI) was calculated by dividing the CC_{50} by the EC_{50} . The antiviral-activity values measured for UK-201844 against chimeric HIV-1 variants were compared to that of wild-type NL4-3 using Student's *t* test to determine if the observed differences were significant.

HIV-1 p24 assays. PM1 cells were infected with HIV-1 NL4-3 or HIV-1 Ba-L using an MOI of 0.16 for 2 h. Infected cultures were then washed with RPMI and resuspended in 5 ml of RPMI medium at a final cell density of 2×10^5 cells/ml and added at 1×10^4 cells per well into 96-well plates containing half-logarithmic dilutions of the test compounds. Five days after infection, virus replication was measured by quantifying HIV-1 p24 antigen present in the supernatants of infected cell cultures using the COULTER HIV-1 p24 antigen assay kit (Beckman Coulter, Miami, FL) according to the manufacturer's protocol. The EC_{50} was calculated as the concentration of compound that effected a decrease in p24 production in the supernatants of infected, compound-treated cells to 50% of that produced in the supernatants of infected, compound-free cells. The CC_{50}

was measured in PM1 cells using the XTT dye reduction method described above.

Single-cycle infection assays. The single-cycle infectious HIV-1 reporter viruses packaged with a vesicular stomatitis virus (VSV) Env (VSV/HIVLuc) or the HIV-1 NL4-3 Env (NL4/HIVLuc) were generated by cotransfecting HEK 293 cells with an HIV-1 NL4-3 single-cycle infectious reporter virus cDNA encoding firefly luciferase (pNL4-3 Δ Env) (previously described in reference 1) and either a VSV envelope expression vector (Stratagene, La Jolla, CA) or an NL4-3 envelope expression vector using LipofectAMINE Plus according to the manufacturer's protocol (Invitrogen Life Technologies). Half-logarithmic dilutions of the test compounds were added to HeLa CD4 LTR/beta-Gal or MT-2 target cells seeded in 96-well plates at a cell density of 1×10^4 cells per well in DMEM containing 10% FBS or 1.6×10^4 cells per well in RPMI containing 10% FBS. Compound-treated or compound-free target cells were then infected with VSV/HIVLuc or NL4/HIVLuc at an MOI of 0.03. Seventy-two hours after infection of the HeLa CD4 LTR/beta-Gal cells, viral infection was monitored by measuring the induction of the beta-galactosidase reporter gene present in the HeLa CD4 LTR/beta-Gal target cells using the Dual-Light System according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Data from the reporter gene measurements were expressed as the percentage of reporter gene activity in infected compound-treated cells relative to that of infected, compound-free cells. EC_{50} s were calculated as the concentrations of compound that effected a decrease in the percentage of the virally encoded reporter gene activity in infected, compound-treated cells to 50% of that produced in infected, compound-free cells.

Virus production assays. In the virus production assay, an envelope-deleted NL4-3 reporter virus cDNA (pNL4-3 Δ Env) was cotransfected into HEK 293 cells with either a VSV-G expression vector or an HIV envelope expression vector using LipofectAMINE Plus according to the manufacturer's protocol (Invitrogen Life Technologies). Compounds (UK-201844 or NFV) were then added to transfected cell cultures at two times the EC_{90} , which corresponded to concentrations of 15 μ M and 0.12 μ M for UK-201844 and NFV, respectively, 3 h after transfection. The supernatants of the transfected cells were then harvested 72 h after transfection. Infectious-virus production was subsequently measured by quantifying luciferase reporter gene activity after various dilutions of the supernatants of transfected cells were incubated in the presence of MT-2 cells for 72 h. The results are presented as percent inhibition of the luciferase reporter signal in infected MT-2 cells in the presence of compound relative to that observed for the no-compound control.

To measure the effects of amino acid substitutions in HIV-1 Env sequences on UK-201844 susceptibility, recombinant NL4-3 infectious cDNAs were transfected into HEK 293 cells. Twenty-four hours after transfection, the transfected cells were rinsed with phosphate-buffered saline, treated with 0.05% trypsin-EDTA, resuspended in DMEM, and then added at 5×10^4 cells per well to 96-well plates containing half-logarithmic dilutions of the test compound. Microtiter plates containing the transfected cells were then incubated for 48 h at 37°C. The supernatants of transfected cells were subsequently harvested from the microtiter plates, and infectious-virus production was measured by quantifying beta-galactosidase reporter gene activity after various dilutions of the supernatants of transfected cells were incubated in the presence of MT-2 cells and HeLa CD4 LTR/beta-Gal indicator cells for 72 h (2). The EC_{50} s measured for UK-201844 against NL4-3 recombinants containing mutations in Env were compared to that measured against wild-type NL4-3 using Student's *t* test to determine if the observed differences were significant.

Construction of HIV-1 NL4-3/IIB recombinants. Total DNA was isolated from HIV-1 IIB-infected cells, and HIV-1 IIB sequences corresponding to nucleotides (nt) 5729 to 8487 of HXB2 (accession number AF033819) were amplified by PCR using the oligonucleotide primers 5'-GGAAGCCATAATAA GAATTCTGCAACAACACTGC-3' and 5'-GTGCCAAGGATCCGTTCACTAA TCGAATGG-3'. The PCR products were digested with the BamHI and EcoRI restriction endonucleases and ligated to pNL4-3 digested with the same restriction enzymes to generate the pNL4/IIBFL chimeric cDNA, which contains IIB nucleotide sequences 5743 to 8480. To construct additional NL4-3/IIB Env chimeras, pNL4/IIBFL was digested with either EcoRI and NheI or BamHI and NheI. The N-terminal (EcoRI and NheI) or C-terminal (BamHI and NheI) IIB Env-containing fragments were isolated and ligated to pNL4-3 digested with either EcoRI and NheI or BamHI and NheI to generate the plasmids pNL4/IIBNt (containing IIB nucleotide sequences 5743 to 7264) and pNL4/IIBCT (containing IIB nucleotide sequences 7264 to 8480), respectively. Plasmid pNL4/IIB5'Env was constructed after three different PCRs (see below) using two plasmid templates (pNL4-3 and pNL4/IIBFL) and four oligonucleotide primers: (i) 5'-AATAAGAATTCTGCAACAACACTGCTGTTTA-3', (ii) 5'-GAA GACAGTGGCAATGAGAGTGAAGGAGA-3', (iii) 5'-CACTCTCATTGCC

FIG. 1. Structure of UK-201844, 2-[(3R)-1-[2-(2,3-dihydro-1-benzofuran-5-yl)ethyl]piperidin-3-yl]-2,2-diphenylacetamide, MW 440.6.

ACTGCTCTTCTGCTCTTCT-3', and (iv) 5'-ATTGTTCTCTTAATTGCTA GCTATCTGT-3'. For PCR A, primers i and iii were used to amplify a 491-bp fragment from pNL4-3 corresponding to pNL4-3 nucleotide sequences 5738 to 6229 (accession number AF324493) by PCR. For PCR B, primers ii and iv were used to amplify a 1,069-bp fragment from pNL4/IIIBFL corresponding to HXB2 nucleotide sequences 6212 to 7281 by PCR. For PCR C, the two products from PCRs A and B were combined and amplified using primers i and iv, yielding a 1,560-bp product by PCR. The product of PCR C was digested with EcoRI and NheI and ligated to pNL4/IIIBFL digested with the same restriction enzymes to generate plasmid pNL4/IIIB5'Env, which contained IIIB sequences 6225 to 8480. To generate virus, pNL4/IIIBFL, pNL4/IIIBNt, pNL4/IIIBCt, or pNL4/IIIB5'Env was transfected into HEK 293 cells using LipofectAMINE Plus according to the manufacturer's protocol (Invitrogen Life Technologies). Seventy-two hours after transfection, infectious HIV-1 was harvested from the supernatants of the transfected cells, and titers (50% tissue culture infective doses) of the resulting viral stocks were determined after infecting MT-2 T-cell lines with serial dilutions of the viral stocks (10) and monitoring the CPE.

Selection and characterization of resistant virus. MT-2 cells (10^6) were infected with HIV-1 NL4-3 at an MOI of 0.01 and then cultured in RPMI medium containing 10% FBS and UK-201844 at an initial concentration of 0.56 μ M. The cultures were monitored daily by microscopic observation for viral replication, and when 50% of the cells in the culture displayed CPE, the supernatants from the infected cultures were removed by centrifugation and 0.2 ml was transferred to fresh MT-2 cell cultures containing UK-201844 compound concentrations that were twofold higher than the concentration in the previous culture. The cell pellets were washed once in phosphate-buffered saline (PBS) (Invitrogen Life Technologies) and stored at -70°C for DNA sequence analysis. This process was repeated (14 serial passages) until viral replication was observed in MT-2 cell cultures containing UK-201844 concentrations of 22.4 μ M (>17 -fold higher than the mean EC_{50}). Total DNA was isolated from the cell pellets using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA), and HIV Env sequences corresponding to nt 5729 to 8487 of NL4-3 were amplified by PCR using the oligonucleotide primers 5'-GGAAGCCATAATAAGAAATTCTGCAACAAC GC-3' and 5'-GTGCCAAGGATCCGTTCACTAATCGAATGG-3'. The amplified cDNAs were ligated to pCR-XL-TOPO according to the manufacturer's protocol (Invitrogen Life Technologies), and 15 individual clones were isolated and subjected to sequence analysis. Compound-selected mutations were identified by comparing the sequences of the HIV Env clones to that of virus propagated in parallel in the absence of compound.

To construct NL4-3 recombinant virus containing HIV-1 Env amino acid substitutions identified in the serial-passage studies, a pCR-XL-TOPO clone containing all five substitutions identified (S162N, N302Y, G354E, V372E, and D474N) was digested with the restriction endonucleases EcoRI and NheI (Env fragment 1) or NheI and BamHI (Env fragment 2), and Env cDNA fragment 1 or 2 was ligated to full-length NL4-3 infectious cDNAs digested with either EcoRI and NheI or NheI and BamHI to construct pNL4 S162N/N302Y or pNL4 G354E/V372E/D474N, respectively. The infectious cDNA encoding all five amino acid substitutions (pNL4 S162N/N302Y/G354E/V372E/D474N) was constructed by ligating Env fragment 2 to the pNL4 S162N/N302Y cDNA digested with the NheI and BamHI restriction endonucleases. Recombinant pNL4-3 infectious cDNAs encoding single amino acid substitutions (G354E, V372E, or

D474N) or additional combinations of amino acid substitutions (G354E/V372E, G354E/D474N, or V372E/D474N) were generated by site-directed mutagenesis using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol.

HIV gp120 Western analyses. For Western analyses, HEK 293 cells were transfected with pNL4-3 or pNL4/IIIBFL or mock transfected, and the supernatants were harvested 72 h later. Infectious-virus production was measured using a portion of the supernatants of transfected cells in an HIV Rep assay as described above. In addition, the transfected cells were washed with PBS (Invitrogen Life Technologies) and lysed with Western lysis buffer (50 mM Tris-Cl, pH 7.2, 0.15 M sodium chloride [Sigma-Aldrich], 0.1% sodium dodecyl sulfate [Sigma-Aldrich], and 1% Triton X-100 [Fisher Scientific, Fairlawn, NJ]). The supernatants of the transfected cells were first clarified by centrifugation in a Sorval RC-7 (Kendro Laboratory Products, Newtown, CT) for 5 min at 1,000 rpm ($200 \times g$) at 4°C . One-milliliter aliquots were then layered on a 0.25-ml 20% sucrose (Sigma-Aldrich)-PBS cushion and centrifuged in an Eppendorf 5417C microcentrifuge (Brinkman Instruments, Westbury, NY) at 4°C and 14,000 rpm ($20,000 \times g$) for 3 h to pellet the virions. The supernatant was removed, and the virus pellet was resuspended in 0.050 ml Western lysis buffer. Proteins from cell lysates or isolated virions were separated on a 4 to 12% NuPAGE Bis-Tris gel using the MOPS (morpholinepropanesulfonic acid) buffer system (Invitrogen Life Technologies) and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The blots were probed with either a 1:1,000 dilution of a mouse monoclonal anti-HIV-1 p24 antibody (Ab) (ICN Pharmaceuticals, Inc., Aurora, OH) or a 1:2,500 dilution of rabbit polyclonal antiserum directed against HIV-1 gp120 (Advanced Biotechnologies, Columbia, MD). Proteins were subsequently detected using the Tropix Western-Star chemiluminescence kit (Applied Biosystems) and visualized by exposure to Kodak BioMax MR-1 film (Kodak Scientific Imaging Systems, New Haven, CT). The Western analyses were quantified by densitometer scanning using an Alpha Innotech Chemi-Imager (Alpha Innotech Corporation, San Leandro, CA) and the 1D Multidensitometry software provided by the manufacturer.

RESULTS

In vitro antiviral activity of UK-201844. The antiviral activity of UK-201844 (Fig. 1), originally identified in an antiviral high-throughput screen (see reference 2 for a description of the screening assay), was confirmed in different antiviral assay formats. In CPE assays using the HIV-1 NL4-3 strain and MT-2 T-cell lines, UK-201844 exhibited an EC_{50} of 1.3 μ M (Table 1) and a CC_{50} of 53 μ M, yielding a TI of 41. Similarly, the compound demonstrated an EC_{50} of 2.7 μ M in antiviral assays using HIV-1 NL4-3, PM-1 cells, and a p24 endpoint (Table 1). UK-201844 also exhibited comparable EC_{50} s against HIV-1 NL4-3 in antiviral assays utilizing different host cell lines (e.g., CEM-SS, C8166, CEM-T4, and 174XCEM T-cell lines) (data not shown). In contrast, UK-201844 exhibited reduced activity in antiviral assays using the HIV-1 IIIB or HIV-1 Ba-L with EC_{50} s of >32 and 24 μ M, respectively (Table 1), and

TABLE 1. In vitro antiviral activities of UK-201844 against different HIV-1 laboratory strains

Virus	Cell line	Assay	Antiviral activity ^a		
			EC_{50} (μ M)	CC_{50} (μ M)	TI
HIV-1 NL4-3	MT-2	XTT	1.3 $\pm$ 0.65	53 $\pm$ 32	41
HIV-1 NL4-3	PM1	P24	2.7 (2.6, 2.7)	47 (60, 33)	17
HIV-1 IIIB	MT-2	XTT	>32	53 $\pm$ 32	ND
HIV-1 Ba-L	PM1	P24	24	47	2

^a Antiviral activity was determined in CPE assays after infection of MT-2 cells with HIV-1 NL4-3 or HIV-1 IIIB or by measuring p24 production 6 days after infection of PM1 cells with HIV-1 NL4-3 or HIV-1 Ba-L as described. Cytotoxicity was determined by measuring cell viability using the XTT dye reduction method 6 days after compound addition to MT-2 or PM1 cells. The results represent the means $\pm$ standard deviations (4 to 10 experiments) or means and individual values (2 experiments). ND, not determined.

TABLE 2. In vitro antiviral activities of UK-201844 in HIV-1 single-cycle infection assays^a

Virus	EC ₅₀ (μM)			
	UK-201844	EFV	ATA	NFV
VSV/HIVLuc	>32	0.0011 ± 0.0001	>76	>1
NL4/HIVLuc	>32	0.0020 ± 0.0008	0.076 (0.069; 0.083)	>1

^a Antiviral activity was determined after infection of HeLa CD4 LTR/beta-Gal cells with HIV or VSV enveloped HIV reporter viruses as described in Materials and Methods. The results represent the means ± standard deviations (three experiments) or means and individual values (two experiments).

UK-201844 did not exhibit measurable antiviral activity against 15 HIV-1 clade B clinical isolates up to concentrations that were cytotoxic (data not shown).

HIV-1 single-cycle infection assays. To initially characterize the target of UK-201844, the activity of the compound was evaluated in HIV-1 single-cycle infection assays. Three control compounds (ATA, EFV, and NFV), each targeting a different step in the HIV-1 replication cycle, were included for comparison. In these assays, reporter gene expression is dependent on viral entry, reverse transcription, integration of viral cDNA, and Tat-mediated gene expression; therefore, the assay is sensitive to inhibitors targeting early events in the HIV-1 replication cycle. The assay can also be used to distinguish entry inhibitors from inhibitors targeting other early events in the HIV-1 replication cycle based on differential activities against HIV-1 reporter viruses packaged with an HIV-1 Env versus the VSV-G Env protein. In this study, ATA (an HIV-1 entry inhibitor control) inhibited infection of HeLa CD4 LTR/beta-Gal cells by the HIV enveloped reporter virus (NL4/HIVLuc) with an EC₅₀ of 0.076 μM but did not inhibit infection by the VSV-G enveloped reporter virus (VSV/HIVLuc) (Table 2). EFV (a nonnucleoside reverse transcriptase inhibitor control) inhibited infection by both reporter viruses with EC₅₀s of 1 to 2 nM, while the protease inhibitor control NFV was not active against either the VSV or HIV enveloped HIV-1 reporter virus (Table 2). Similar to NFV, UK-201844 was not active against either single-cycle reporter virus. These data demonstrate that UK-201844 does not target HIV-1 entry or other early events in the replication cycle. Rather, the data suggest that UK-201844 targets a late event in the HIV-1 replication cycle. Consistent with these results, UK-201844 did not inhibit HIV-1 Env-mediated membrane fusion in cell-based fusion assays (data not shown).

UK-201844 inhibits the production of infectious virus in an HIV-1 Env-dependent manner. To confirm that UK-201844 targets a late event in the HIV-1 replication cycle, the compound was evaluated in virus production assays. In these assays, transiently transfected cells producing HIV-1 reporter viruses packaged with either HIV-1 (NL4-3) or VSV-G Env protein were treated with two times the EC₉₀ of compound, and infectious-virus production was measured as described in Materials and Methods. The results showed that UK-201844 inhibited the production of infectious viruses packaged with an HIV-1 Env glycoprotein (99.5% inhibition) (Table 3) but did not inhibit the production of infectious HIV-1 virions packaged with a heterologous Env glycoprotein (VSV-G). In contrast, the protease inhibitor control (NFV) inhibited the pro-

TABLE 3. In vitro antiviral activities of UK-201844 in HIV-1 production assays^a

Env	Reporter	% Inhibition	
		UK-201844	NFV
NL4-3	HIVLuc	99.5 (99; 100)	88 (83; 93)
VSV-G	HIVLuc	−6 (−13; 1)	97 (94; 99)

^a The production of HIV-1 infectious virions packaged with either an HIV-1 envelope (NL4-3) or the VSV-G envelope protein (VSV-G) was measured as described in Materials and Methods. The results are presented as percent inhibition of infectious-virus production in the presence of twice the EC₉₀ (UK-201844, 15 μM; NFV, 0.12 μM) of compound relative to the no-compound control and represent the mean of two independent experiments (individual values are shown).

duction of infectious virions packaged with either HIV-1 or VSV Env protein (88 and 97%, respectively). These data demonstrate that UK-201844 inhibits virus production in an HIV-1 Env-dependent manner.

HIV-1 Env sequences determine susceptibility to UK-201844. Results from the virus production experiments suggest that UK-201844 interferes with HIV-1 Env expression during virion maturation, resulting in the production of non-infectious virions. To explore this hypothesis, we amplified Env-containing sequences from our HIV-1 IIIB, which was not susceptible to the activity of UK-210844. An NL4-3/IIIB recombinant virus (NL4/IIIBFL) that contains HIV-1 IIIB sequences (nt 5743 to 7264), including the complete coding regions for gp120, Vpu, and Tat, as well as portions of the Vpr, Rev, and gp41 coding regions, was constructed (Fig. 2). The antiviral activity of UK-201844 was measured against the NL4/IIIBFL recombinant virus and compared to that of the parental NL4-3 virus. The results showed that the NL4/IIIBFL

FIG. 2. HIV-1 Env sequences are critical determinants of UK-201844 susceptibility. The antiviral activity of UK-201844 (EC₅₀) was measured against a panel of NL4-3/IIIB chimeric viruses containing HIV-1 IIIB sequences (i.e., NL4/IIIBFL, NL4/IIIBNt, NL4/IIIBCt, and NL4/IIIB5'Env) and compared to that observed for the NL4-3 parental virus. The open bars represent NL4-3 sequences, while the gray bars denote IIIB sequences. The numbers above the diagram correspond to HIV-1 NL4-3 nucleotide sequences, and HIV-1 gene products encoded by the viral genomic region highlighted are represented at the top. Fold change (FC) values represent the EC₅₀ measured for the NL4-3 recombinant divided by the EC₅₀ measured for the NL4-3 parental virus. The results represent the mean of two independent experiments or means ± standard deviations determined from three or four experiments. nd, not determined.

recombinant virus exhibited a >25-fold reduction in susceptibility to UK-201844 compared to NL4-3 (Fig. 2). These results are similar to that observed for HIV-1 IIIB (Table 1) and demonstrate that HIV-1 IIIB sequences corresponding to nt 5743 to 8480 contain critical determinants of UK-201844 susceptibility.

In an attempt to localize the IIIB sequences that confer resistance to UK-201844, three additional NL4-3/IIIB chimeric viruses were constructed and evaluated for susceptibility to UK-201844 as described in Materials and Methods (Fig. 2). The first chimera (NL4/IIIBNt) contained IIIB sequences (nt 5743 to 7264) that included the N-terminal half of the gp120 coding region, the complete Vpu coding region, and partial coding regions for Vpr, Tat, and Rev. The second chimera (NL4/IIIBCt) contained IIIB sequences (nt 7264 to 8480) corresponding to the C-terminal half of the gp120 coding region and partial coding regions for Tat, Rev, and gp41. The third chimera (NL4/IIIB5'Env) contained IIIB sequences (nt 6225 to 8480) that included the complete gp120 coding region, as well as portions of the Vpu, Tat, Rev, and gp41 coding regions. UK-201844 exhibited antiviral activity against NL4/IIIBNt with an EC_{50} (1.2 μ M) comparable to that observed for the NL4-3 parent (1.3 μ M) (Fig. 2). Alternatively, UK-201844 exhibited a modest yet significant fourfold reduction in activity against NL4/IIIBCt compared to NL4-3 (Fig. 2) ($P = 0.005$). In addition, the NL4/IIIB5'Env chimeric virus exhibited a >25-fold reduction in susceptibility to UK-201844, which was comparable to that observed for the NL4/IIIBFL recombinant and HIV-1 IIIB (Fig. 2 and Table 1). These data demonstrate that HIV-1 IIIB sequences corresponding to nt 6225 to 8480 are sufficient to recapitulate the resistant phenotype observed for HIV-1 IIIB.

Selection of UK-201844-resistant virus. To identify specific amino acid residues that affect UK-201844 susceptibility, NL4-3 variants resistant to UK-201844 were selected in vitro serial-passage experiments as described in Materials and Methods. HIV Env sequences derived from the resistant variants were amplified by PCR, and 15 individual HIV Env clones were subjected to sequence analysis. Five amino acid substitutions (S162N, N302Y, G354E, V372E, and D474N) were identified in the Env clones derived from the UK-201844-resistant virus population. All 15 clones contained the S162N substitution, 13 clones contained the N302Y and/or D474N substitution, 8 clones contained the V372E substitution, and 5 clones contained the G354E substitution. NL4-3 infectious cDNAs encoding all five substitutions were generated as described in Materials and Methods and evaluated in virus production assays to determine UK-201844 susceptibility (Fig. 3). The results showed that NL4-3 recombinants encoding all five amino acid substitutions exhibited a >31-fold reduction in susceptibility to UK-201844 (Fig. 3), which was comparable to that observed for the resistant-virus population selected in the in vitro serial-passage studies (data not shown). These data demonstrate that amino acid substitutions in HIV-1 Env are sufficient to confer resistance to UK-2901844. As part of an initial effort to better understand the contributions of individual amino acid substitutions to the resistant phenotype, NL4-3 infectious cDNAs encoding either the S162N/N302Y or the G354E/V372E/D474N substitution were generated and tested in virus production assays. As shown in Fig. 3, recombinant

FIG. 3. Amino acid substitutions in HIV-1 Env sequences confer resistance to UK-201844. The activity of UK-201844 (EC_{50}) was measured in virus production assays using HIV-1 NL4-3 recombinant infectious cDNAs containing amino acid substitutions in HIV-1 Env sequences. The substitution(s) present in each construct is denoted by a black bar and labeled at the top of the diagram. Fold change (FC) values represent the EC_{50} measured for the NL4-3 recombinant divided by the EC_{50} measured for the NL4-3 parental virus, which was determined to be 1.03 μ M (data not shown). The results represent the means $\pm$ standard deviations determined from four to nine experiments.

NL4-3 cDNAs encoding the S162N/N302Y substitutions exhibited a modest yet significant 8.3-fold reduction in susceptibility to the compound ($P = 0.004$). Alternatively, NL4-3 cDNAs encoding the G354E/V372E/D474N substitutions showed a >31-fold reduction in susceptibility to UK-201844, which was comparable to that observed for cDNAs containing all five mutations. Similar results were observed when NL4 recombinant viruses encoding S162N/N302Y, G354E/V372E/D474N, or all five amino acid substitutions (S162N, N302Y, G354E, V372E, and D474N) were tested in CPE assays (data not shown).

Based on the observation that NL4-3 cDNAs encoding the G354E/V372E/D474N substitutions were associated with high levels of UK-201844 resistance, we examined the individual contributions of the three substitutions to compound resistance. To achieve this, additional NL4-3 recombinant cDNAs were constructed that encoded each of the three single-amino-acid substitutions (G354E, V372E, or D474N) or pairwise combinations of the three substitutions (G354E/V372E, G354E/D474N, or V372E/D474N). The recombinant cDNAs were then evaluated in virus production assays as described in Materials and Methods to determine susceptibility to UK-201844. NL4-3 recombinants containing the G354E/D474N or V372E/D474N substitutions showed a >31-fold reduction in susceptibility to UK-201844, while the NL4-3 recombinant encoding the D474N single-amino-acid substitution showed a sixfold reduction in susceptibility ($P = 0.003$). Alternatively, UK-201844 exhibited activity against NL4-3 recombinants containing the G354E, V372E, or G354E/V372E substitution that was comparable to that observed for wt NL4-3.

UK-201844 inhibits HIV-1 gp160 processing. To characterize the mechanism of action of UK-201844, we examined HIV

FIG. 4. UK-201844 inhibits gp160 processing in HIV-1-transfected cells. HEK 293 cells were transfected with pNL4-3 or pNL4/IIIBFL or mock transfected (cell control) in the presence of various concentrations of UK-201844 or in the absence of compound. HIV-1 Env (A) or p24 (B) expression was measured in transfected cells by Western analysis using monoclonal Abs directed against gp120 or p24, respectively. In addition, inhibition of infectious-virus production (AV % Inhibition) was measured in the supernatants of transfected cells as described in Materials and Methods.

Env protein expression in cells transfected with either the HIV-1 NL4-3 infectious cDNA or an infectious cDNA encoding the HIV-1 NL4/IIIBFL chimera by Western analysis using a polyclonal Ab directed against HIV-1 gp120 (Fig. 4A). Antiviral activity was also determined in parallel virus production assays as described in Materials and Methods. In the Western analysis using an Ab directed against HIV-1 gp120, two distinct protein molecular weights that were consistent with the molecular weights expected for the gp120 and the gp160 Env proteins were detected in the lysates of infected cells. As shown in Fig. 4A, a concentration-dependent inhibition of gp160 processing was observed in cells transfected with NL4-3 in the presence of UK-201844, which correlated with inhibition of infectious-virus production. Quantification of the Western experiments in Fig. 4A using densitometer scanning showed that gp160 represented 76% of the total Env protein present in cells transfected with NL4-3 in the presence of 15 μ M UK-201844 (data not shown). This corresponded to complete inhibition (100%) of infectious-virus production (Fig. 4A). Alternatively, the gp160/gp120 ratio was not altered in cells transfected with the NL4/IIIBFL cDNA in the presence of UK-201844, which was consistent with the observation that NL4/IIIBFL chimeric-virus replication was not inhibited by UK-201844 (Fig. 2 and 4A). A separate Western analysis was performed on the same samples using a monoclonal Ab directed against the HIV-1 p24 capsid protein (Fig. 4B). The data showed that HIV-1 Gag processing was not affected by UK-201844 in cells transfected with either the HIV-1 NL4-3 or NL4/IIIBFL cDNA. These data strongly suggest that UK-201844 specifically interferes with gp160 processing, resulting in a defect in infectious-HIV production.

To determine if UK-201844 affects the incorporation of HIV-1 Env proteins into virions, a Western analysis was performed using a gp120 Ab and isolated virions produced in the presence of twice the EC₉₀ of UK-201844 (15 μ M) or NFV (0.12 μ M) or in the absence of compound (Fig. 5). As with the previous experiment, infectious-virus production was measured in the supernatants of transfected cells in parallel. Con-

sistent with the data in Table 3, UK-201844 and NFV inhibited infectious-virus production by 100 and 94%, respectively, when present at twice the EC₉₀ (data not shown). The Western analysis showed that the majority of the HIV-1 Env protein present in virions in the absence of compound or in the presence of NFV is in the form of gp120 (Fig. 5). These data are consistent with previous reports (5) and show that although both gp160 and gp120 are present in transfected cells (Fig. 4A), gp120 represents the predominant Env protein incorporated into virions (Fig. 5). Alternatively, virions produced in the presence of twice the EC₉₀ of UK-201844 contain almost

FIG. 5. UK-201844 affects the incorporation of HIV-1 Env proteins in virions. HEK 293 cells were transfected with pNL4-3 in the presence of twice the EC₉₀ of UK-201844 or NFV or in the absence of compound. Virions were isolated from the supernatants of transfected cells and subjected to Western analyses using monoclonal Abs directed against gp120 or p24 as described in Materials and Methods.

exclusively unprocessed gp160 Env protein or aberrant Env protein products (Fig. 5). In addition, the relative quantities of total HIV-1 Env protein (i.e., gp160) appear to be reduced in UK-201844-treated virions compared to untreated virions. These data demonstrate that UK-201844 directly or indirectly inhibits gp160 processing, resulting in the incorporation of nonfunctional (i.e., unprocessed) Env proteins in HIV virions.

HIV-1 gp160 processing is mediated by host cell proteases belonging to the PC family (e.g., furin). Previous reports have demonstrated that inhibition of furin and other convertases using peptide-based inhibitors resulted in the inhibition of gp160 processing (7, 19). To address the possibility that UK-201844 inhibits gp160 processing by directly targeting cellular convertases, UK-201844 was evaluated in biochemical assays using recombinant human furin or recombinant rat PACE4 and peptide substrates. The results showed that UK-201844 was not active against human furin or rat PACE4 up to the highest concentration tested (100 μ M) (data not shown).

DISCUSSION

In this study, we describe a novel inhibitor, UK-201844, which directly or indirectly targets HIV-1 Env. Our data strongly suggest that UK-201844 interferes with gp160 processing, resulting in the inhibition of infectious-virion production. Given that the compound is active against HIV-1 NL4-3 but not against the closely related HIV-1 IIIB strain, it is likely that the compound targets some variable region of HIV Env, rather than a host factor involved in gp160 processing (e.g., furin). In support of this, UK-201844 did not exhibit activity against the human furin enzyme or the related rat PACE4 enzyme in peptide cleavage assays. Alternatively, UK-201844 may bind HIV-1 Env and prevent intracellular trafficking of gp160, affecting HIV-1 Env processing indirectly. Several compounds (megalomycin, castanospermine, dexynojirmycin, and monensin) that inhibit gp160 intracellular trafficking and subsequent processing have been described (11, 20, 21, 22). Although inhibition of gp160 processing is the most likely antiviral mechanism, our data cannot definitively rule out all other mechanisms, such as specific compound effects on HIV envelope protein (i.e., gp120) stability. Further study of UK-201844 is warranted to determine the molecular target and precise mechanism of action.

HIV-1 NL4-3/IIIB chimera experiments demonstrated that HIV-1 Env sequences were key determinants of UK-201844 susceptibility. Our data showed that NL4-3/IIIB recombinants containing the entire IIIB gp120 coding region exhibited reductions in UK-201844 susceptibility similar to that observed for HIV-1 IIIB. Alternatively, NL4-3 chimeric viruses containing the N-terminal portion of the HIV-1 IIIB Env protein did not exhibit a reduction in susceptibility to UK-201844, while chimeric viruses containing the C-terminal portion of IIIB Env exhibited only a modest reduction in susceptibility to UK-201844. These data suggest that multiple IIIB Env sequences may be required to confer maximal levels of UK-201844 resistance. Sequence analysis of the IIIB Env region used in these experiments revealed 22 amino acid differences compared to the NL4-3 Env, with 13 differences in the N-terminal portion (6225 to 7264) and 9 differences in the C-terminal portion (7264 to 8480) of the encoded protein. Based on the number of

amino acid differences observed in the IIIB Env sequences, we concluded that determining the precise combination(s) of IIIB Env-specific amino acid residues required for maximal levels of resistance to UK-201844 would be highly labor-intensive.

Instead, to pinpoint determinants in HIV-1 Env that confer resistance to UK-201844, we performed in vitro serial-passage studies and identified five amino acid substitutions in HIV Env that were selected in the presence of compound. Our initial experiments showed that high levels of UK-201844 resistance were associated with the G354E, V372E, and D474N substitutions. Subsequent experiments showed that D474N combined with G354E or V372E was sufficient to recapitulate maximal levels of UK-201844 resistance (i.e., >31-fold). In addition, significant levels of UK-201844 resistance were observed for the NL4-3 recombinant containing the D474N single-amino-acid substitution. These data suggest that the D474N substitution is important for UK-201844 resistance and that high levels of resistance can be achieved when D474N is combined with at least one other amino acid substitution identified in the in vitro serial-passage experiments (i.e., G354E or V372E). However, our data do not preclude the possibility that high levels of resistance could be achieved with other combinations of mutations that were not tested, such as S162N or N302Y in combination with G354E, V372E, or D474N. It is interesting to note that three (S162N, N302Y, and D474N) of the five substitutions identified in the in vitro serial-passage experiments either disrupt or introduce an Asn residue in HIV-1 Env. In addition, the S162N, N302Y, and G354E substitutions are located proximal to predicted N-linked glycosylation sites in HIV-1 Env (14). These observations suggest the possibility that UK-201844 susceptibility may be affected by the glycosylation state of the HIV-1 Env protein. It should also be noted that none of the substitutions identified in our resistant-virus studies were present in Env sequences derived from our HIV-1 IIIB stock or present in the published envelope sequence for HIV-1 Ba-L (<http://www.hiv.lanl.gov/content/hiv-db/mainpage.html>). These results demonstrate that UK-201844 targets HIV-1 Env directly or indirectly and suggest that a variety of HIV-1 Env sequence variations could result in a reduction in UK-201844 susceptibility.

The Asp residue at position 474 of gp120 has been implicated in the gp120-CD4 interaction (13); however, it is unlikely that this is relevant to UK-201844 susceptibility, given that the compound has no effect on viral entry. Consistent with this, UK-201844 exhibits activity in virus production assays in HEK 293 cells which do not express CD4. A scan of HIV-1 gp120 sequences in the HIV sequence database (<http://www.hiv.lanl.gov/content/hiv-db/mainpage.html>) revealed that an Asn residue is commonly observed at position 474 of HIV-1 gp120 sequences derived from HIV-1 clinical isolates. Based on our data showing that an Asn residue at position 474 of gp120 is associated with UK-201844 resistance, combined with the suggestion that a variety of Env sequence variations may affect UK-201844 susceptibility, it is not surprising that UK-201844 exhibits a poor spectrum of activity against HIV-1 clinical isolates and laboratory strains.

As mentioned above, several inhibitors (megalomycin, castanospermine, dexynojirmycin, and monensin) that inhibit gp160 processing indirectly by disrupting gp160 intracellular trafficking have been previously described (11, 20, 21, 22).

Castanospermine and dexynojirmycin inhibit alpha-glucosidase I and prevent the early glycosylation trimming steps. Monensin is a monovalent carboxylic ionophore that inhibits the transport and expression of membrane glycoproteins and some secretory proteins, while megalomicin is a macrolide antibiotic that inhibits vesicular transport between the medial- and trans-Golgi. All such inhibitors mediate their antiviral effects by targeting important host cell functions; therefore, toxicity may limit the clinical utility of these compounds. In addition, many peptide-based inhibitors derived from convertase cleavage sequences that inhibit gp160 processing through the inhibition of convertase (e.g., furin) activity have been described. However, cytotoxicity has been reported for some of these compounds, as well (19). In addition, peptide-based inhibitors have high molecular masses (>500 Da) and do not exhibit traditional small-molecule drug-like properties. In this study, we report the identification of a highly selective small-molecule inhibitor of gp160 processing. The highly selective activity of UK-201844 suggests that the compound's mechanism of action is distinct from those of previous gp160 inhibitors and that cytotoxicity may not be inextricably linked with antiviral activity. However, the reduced antiviral activity observed against several HIV-1 laboratory strains (e.g., HIV-1 IIB and Ba-L) and HIV-1 clinical isolates suggests that UK-201844 would have limited therapeutic utility in its current form. Additional studies are required to determine if the poor spectrum of activity is a property of the compound or inherent in the mechanism of action. If the former is the case, then UK-201844 may be used as a starting point for developing a new HIV-1 therapeutic approach. In either case, UK-210844 should be a useful tool for interrogating events in the HIV Env maturation pathway.

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REFERENCES

- Blair, W. S., J. S. Isaacson, X. Li, J. Q. Cao, Q. Peng, G. F. Z. Kong, and A. K. Patick. 2005. A novel HIV-1 antiviral high throughput screening approach for the discovery of HIV-1 inhibitors. *Antivir. Res.* **65**:107–117.
- Cao, J., J. S. Isaacson, A. K. Patick, and W. S. Blair. 2005. A high-throughput human immunodeficiency virus type 1 (HIV-1) full-replication assay that includes HIV-1 Vif as an antiviral target. *Antimicrob. Agents Chemother.* **49**:3833–3841.
- De Clercq, E., N. Yamamoto, R. Pauwels, M. Baba, D. Schols, H. Nakashima, J. Balzarini, Z. Debyser, B. A. Murrer, D. Schwartz, D. Thornton, G. Bridger, S. Fricker, G. Henson, M. Abrams, and D. Picker. 1992. Potent and selective inhibition of human immunodeficiency virus (HIV)-1 and HIV-2 replication by a class of bicyclams interacting with a viral uncoating event. *Proc. Natl. Acad. Sci. USA* **89**:5286–5290.
- DeJesus, E., D. Berger, M. Markowitz, C. Cohen, T. Hawkins, P. Ruane, R. Elion, C. Farthing, A. Cheng, B. Kearney, and the 183-0101 Study Team. 2006. The HIV integrase inhibitor GS-9137 (JTK-303) exhibits potent antiviral activity in treatment-naïve and experienced patients, abstr. 160LB. Thirteenth Conf. Retrovir. Oppor. Infect.
- Dubay, J. W., S. R. Dubay, H.-J. Shin, and E. Hunter. 1995. Analysis of the cleavage site of the human immunodeficiency virus type 1 glycoprotein: requirement of precursor cleavage for glycoprotein incorporation. *J. Virol.* **69**:4675–4682.
- Fätkenheuer, G., A. L. Pozniak, M. Johnson, M. A. Plettenberg, S. Staszewski, I. M. Hoepelman, M. Saag, F. Goebel, J. Rockstroh, B. Dezube, T. M. Jenkins, C. Medhurst, J. F. Sullivan, C. Ridgway, S. Abel, M. Youle, and E. van der Ryst. 2004. Evaluation of dosing frequency and food effect on viral load reduction during short-term monotherapy with UK-427,857 a novel CCR5 antagonist, abstr. TuPeB4489. Fifteenth Int. AIDS Conf.
- Hallenberger, S., V. Bosch, H. Angliker, E. Shaw, H.-D. Klenk, and W. Garten. 1992. Inhibition of furin-mediated cleavage activation of HIV-1 gp160. *Nature* **360**:358–361.
- Hanna, G., J. Lalezari, J. Hellinger, D. Wohl, T. Masterson, W. Fiske, J. Kadow, P. Lin, M. Giordano, R. Colonna, and D. Grasela. 2004. Antiviral activity, safety, and tolerability of a novel, oral small-molecule HIV-1 attachment inhibitor, BMS-488043, in HIV-1-infected subjects, abstr. 141. Eleventh Conf. Retrovir. Oppor. Infect.
- Hendrix, C. 2004. Safety, pharmacokinetics and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. *J. Acquir. Immune Defic. Syndr.* **37**:1253–1262.
- Johnson, V. A., and R. E. Barrington. 1990. Infectivity assay, p. 71–76. *In* A. Aldovini and B. D. Walker (ed.), *Techniques in HIV research*. Stockton Press, New York, NY.
- Jose, E. S., M. A. Munoz-Fernandez, and B. Alarcon. 1997. Megalomicin inhibits HIV-1 replication and interferes with gp160 processing. *Virology* **239**:303–314.
- Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski. 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science* **237**:1351–1355.
- Kwong, P. D., R. Wyatt, J. Robinson, R. Sweet, W. J. Sodroski, and W. A. Hendrickson. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**:648–659.
- Leonard, C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J. Biol. Chem.* **265**:10373–10382.
- Little, S., G. Drusano, R. Schooley, H. Haas, P. Kumar, S. Hammer, D. McMahon, K. Squires, R. Asfour, D. Richman, J. Chen, A. Saah, R. Leavitt, D. Hazuda, B. Y. Nguyen, and the Protocol 004 Study Team. 2005. Antiretroviral effect of L-000870810, a novel HIV-1 integrase inhibitor, in HIV-1-infected patients, abstr. 161. Twelfth Conf. Retrovir. Oppor. Infect.
- Martin, D., J. Jacobson, D. Schurmann, E. Osswald, J. Doto, C. Wild, and G. Allaway. 2005. PA-457, the first-in-class maturation inhibitor, exhibits antiviral activity following a single oral dose in HIV-1-infected patients, abstr. 159. Twelfth Conf. Retrovir. Oppor. Infect.
- McCune, J. M., L. B. Rabin, M. B. Feinberg, M. Lieberman, J. C. Kosek, G. R. Reyes, and I. L. Weissman. 1988. Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. *Cell* **53**:55–67.
- Morales-Ramirez, J. O., H. Teppler, C. Kovacs, R. T. Steigbigel, D. Cooper, R. L. Liporace, R. Schwartz, L. Wenning, J. Zhao, L. Gilde, R. Isaacs, and B.-Y. Nguyen. 2005. Antiretroviral effect of MK-0518, a novel HIV-1 integrase inhibitor, in ART-naïve HIV-infected patients, abstr. LBPS1/6. 10th Eur. AIDS Conf.
- Moulard, M., and E. Decroly. 2000. Maturation of HIV envelope glycoprotein precursors by cellular endoproteases. *Biochim. Biophys. Acta* **1469**:121–132.
- Pal, R., R. C. Gallo, and M. G. Sarngadharan. 1988. Processing of the structural proteins of human immunodeficiency virus type 1 in the presence of monensin and cerulenin. *Proc. Natl. Acad. Sci. USA* **85**:9283–9286.
- Pal, R., G. M. Hoke, and M. G. Sarngadharan. 1989. Role of oligosaccharides in the processing and maturation of envelope glycoproteins of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **86**:3384–3388.
- Walker, B. D., M. Kowalski, W. C. Goh, K. Kozarsky, M. Krieger, C. Rosen, L. Rohrschneider, W. A. Haseltine, and J. Sodroski. 1987. Inhibition of human immunodeficiency virus syncytium formation and virus replication by castanospermine. *Proc. Natl. Acad. Sci. USA* **84**:8120–8124.
- Wang, T., Z. Zhang, O. B. Wallace, M. Deshpande, H. Fang, Z. Yang, L. M. Zadjura, D. L. Tweedie, S. Huang, F. Zhao, S. Ranadive, B. S. Robinson, Y.-F. Gong, K. Ricarri, T. P. Spicer, C. Deminie, R. Rose, H. Wang, W. S. Blair, P.-Y. Shi, P.-F. Lin, R. J. Colonna, and N. A. Meanwell. 2003. Discovery of 4-benzoyl-1-[(4-methoxy-1H-pyrrolo[2,3-b]pyridine-3-yl)oxoacetyl]-2-(R)-methylpiperazine (BMS-378806): a novel HIV-1 attachment inhibitor that interferes with CD4-gp120 interactions. *J. Med. Chem.* **46**:4236–4239.
- Weislow, O. S., R. Kiser, D. L. Fine, J. Bader, R. H. Shoemaker, and M. R. Boyd. 1989. New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. *J. Natl. Cancer Inst.* **81**:577–586.
- Wild, C., N. Kilgore, M. Reddick, F. Li, K. Salzwedel, C. Matallana, G. Allaway, and D. Martin. 2003. PA-457 is a small molecule inhibitor of HIV-1 budding/maturation that potentially inhibits replication of virus isolates resistant to all classes of approved drugs, abstr. 14. Tenth Conf. Retrovir. Oppor. Infect.
- Yeni, P. G., S. M. Hammer, M. S. Hirsch, M. S. Saag, M. Schechter, C. C. J. Carpenter, M. A. Fischl, J. M. Gatell, B. G. Gazzard, D. M. Jacobsen, D. A. Katzenstein, J. S. G. Montaner, D. D. Richman, R. T. Schooley, M. A. Thompson, S. Vella, and P. A. Volberding. 2004. Treatment for adult HIV infection: 2004 recommendations of the international AIDS society-USA panel. *JAMA* **292**:251–265.