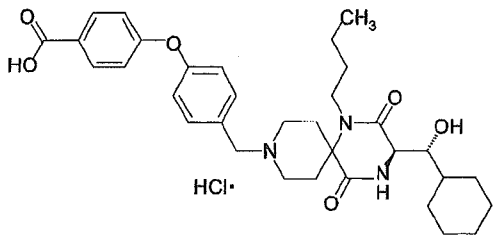

FIG. 1. Structure of AK602.

MATERIALS AND METHODS

Transplantation of human PBMC in NOG mice. NOD-SCID (NOG) mice (10, 33) were maintained in the Central Institute for Experimental Animals (Kawasaki, Japan). Mice were 4 to 6 weeks old at the time of transfer of human PBMC. The human PBMC-transplanted NOG (hu-PBMC-NOG) mice were generated by methods previously described (23, 24). Briefly, PBMC (10^7) were freshly prepared from heparinized blood of a single healthy HIV-1-seronegative donor by Ficoll-Hypaque density gradient centrifugation, resuspended in RPMI 1640-based culture medium (0.5 ml), and infused intraperitoneally to each mouse. The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of the participating institutions.

Assay for proliferation and CCR5 expression of transplanted human PBMC recovered from hu-PBMC-NOG mice. Freshly isolated human PBMC (2×10^7 cells/ml) were incubated in phosphate-buffered saline (PBS) containing 10 μM 5-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oreg.) for 15 min at 37°C for CFSE labeling as previously described by Lyons (16), washed, and resuspended in RPMI 1640. One part of the labeled PBMC preparation was intraperitoneally injected (10^7 PBMC) to each NOG mouse, and human PBMC were recovered from peritoneal lavages and spleen. The other part of the preparation was immediately stimulated with 10 μg of phytohemagglutinin (PHA)/ml, cultured, and harvested. PBMC samples thus obtained were labeled with phycoerythrin (PE)-conjugated anti-CCR5 monoclonal antibody 3A9 or peridinin chlorophyll protein-conjugated anti-HLA-DR antibody (BD Pharmingen, San Diego, Calif.) and subjected to flow cytometric analysis with a Becton Dickinson FACscan cytometer; the data were analyzed by Cell Quest software (Becton Dickinson, Franklin Lakes, N.J.). A quantitative fluorescence-activated cell sorting (FACS) assay that relies on a series of precalibrated beads that bind to a fixed number of mouse immunoglobulin G molecules (Quantum Simply Cellular Kit; Sigma, Saint Louis, Mo.) to determine the absolute number of CCR5s on the cell surface was also conducted according to the manufacturer's instructions (15).

Cells and viruses. The HeLa-CD4-LTR-β-gal indicator cell line expressing human CCR5 ($CCR5^+$ MAGI) (18), a kind gift from Yosuke Maeda, was used for the present study. 293T cells (a human embryonic kidney cell line) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) and antibiotics and used for transfection of DNA plasmid containing the R5 HIV-$1_{JR\text{-}FL}$ genome (13). PBMC isolated from HIV-1-seronegative individuals were cultured with 10% FCS and antibiotics with 10 μg of PHA/ml for 3 days prior to anti-HIV-1 activity assay in vitro (PHA-PBMC). A panel of HIV-1 strains was employed for the drug susceptibility attempt: HIV-$1_{Ba\text{-}L}$ (7), HIV-$1_{JR\text{-}FL}$ (13), HIV-$1_{NL4\text{-}3}$ (32), a wild-type HIV-1_{MOKW} isolated from a drug-naive AIDS patient (17), and MDR primary HIV-1 (HIV-1_{MDR}) strain (HIV-1_{JSL} and HIV-1_{MM}) (35). All primary HIV-1 strains were passaged once or twice in PHA-PBMC cultures and the culture supernatants were stored at −80°C until use. Antiviral assays using PHA-PBMC were conducted as previously reported (12, 17, 35).

Antiviral agents and assay for inhibition of R5 HIV-1 infectivity and replication. A series of different spirodiketopiperazine (SDP) derivatives were newly designed, synthesized, and tested for their activity against in vitro infectivity and replication of R5 HIV-1 as previously described (17). AK602 was chosen for this study based on its CCR5-specific, potent activity against R5 HIV-1. A method for the synthesis of AK602 will be published elsewhere. The structure of AK602 is illustrated in Fig. 1. An approved drug for therapy for HIV-1 infection, 2′,3′-dideoxyinosine (ddI) (20, 21), was kindly provided by Ajinomoto Co., Inc, Tokyo, Japan. TAK779 and SCH-C were synthesized according to previously published data (1, 30). The MAGI assay using $CCR5^+$ MAGI cells was conducted as previously described (17) with minor modifications. Briefly, $CCR5^+$ MAGI cells were seeded in 96-well, flat-bottomed microculture plates (10^4 cells/well) for 24 h, exposed to 0.1 or 1 μM AK602 for 30 min, washed three times, exposed to R5 HIV-1 (100 50% tissue culture infectious doses) at various time points after AK602 removal, and cultured in Dulbecco's modified Eagle medium containing 15% FCS for 48 h. Following the removal of supernatants and lysis of the cells with PBS (100 μl) containing 1% Triton X-100, a solution (100 μl) containing 10 mM chlorophenol red-β-D-galactopyranoside, 2 mM $MgCl_2$, and 0.1 M KH_2PO_4 was added to each well; the mixture was incubated at room temperature in the dark for 30 min; and the optical density (wavelength, 570 nm) was measured with a microplate reader (*V*max, Molecular Devices, Sunnyvale, Calif). All assays were performed in triplicate.

Pharmacokinetic analysis of AK602 in hu-PBMC-NOG mice. Pharmacokinetic analysis of AK602 in hu-PBMC-NOG mice was performed as previously described (28). In brief, plasma samples were collected periodically over 12 h, following a single AK602 administration at a dose of 60 mg/kg of body weight dissolved in 400 μl of 4% hydroxypropyl-β cyclodextrin (HPBC). Each plasma sample (150 μl) was centrifuged at 3,000 rpm for 10 min, and the supernatant was vacuum concentrated and injected into the high-performance liquid chromatography (HPLC) system. The eluent was monitored at 255 nm of UV, and the AK602 concentration in plasma was determined.

Determination of amounts of AK602 persistently bound to CCR5 in hu-PBMC-NOG mice. Blood samples were collected from the tail vein of each hu-PBMC-NOG mouse at various time points following a single intraperitoneal administration of AK602 at a dose of 60 mg/kg. PBMC were isolated by density gradient centrifugation and stained with fluorescein isothiocyanate-conjugated monoclonal antibody 45531 (R&D Systems, Minneapolis, Minn.) specific for the C-terminal half of the second extracellular loop (ECL2B) of CCR5 (15) known to be competitively replaced by SDP derivatives (17) or with PE-conjugated monoclonal antibody 3A9, which binds to the N-terminus extracellular domain of CCR5 (17). PBMC were then subjected to FACS analysis.

Treatment of R5 HIV-1-infected hu-PBMC-NOG mice with anti-HIV-1 agents. Sixteen days after PBMC infusion, the mice were bled from the tail vein, and three-color flow cytometric analysis was performed to confirm positive engraftment of human HLA, CD4, and CD8 antigens on the cells recovered. HIV-$1_{JR\text{-}FL}$ (2,000 50% tissue culture infectious doses) was intraperitoneally inoculated to each mouse in which PBMC engraftment was confirmed. Twenty-four hours after the R5 HIV-1 inoculation, administration of AK602 (120 mg in 4% HPBC/kg/day, twice a day), ddI (50 mg in 4% HPBC/kg/day, twice a day), or saline was implemented and continued by day 16. On days 5 and 9 after the R5 HIV-1 inoculation, blood samples were collected from mouse tail veins for immunologic and virological monitoring (see below). On day 16, blood samples were collected by cardiocentesis, and the mice were sacrificed. The experimental protocol for the treatment is illustrated in Fig. 2.

Immunologic and virological monitoring. Human PBMC recovered from mice were subjected to immunologic and virological monitoring as previously described (23, 24). The $CD4^+/CD8^+$ cell ratios were determined by FACS analysis with PE-conjugated mouse anti-CD4 and peridinin chlorophyll protein-conjugated mouse anti-CD8 (BD Pharmingen) monoclonal antibodies. Determination of HIV-1 DNA copy numbers in recovered human PBMC was performed by real-time PCR assay with Taqman Master mixture (PE Biosystems) and HIV long terminal repeat-specific primers M667 (5′-GGC TAA CTA GGG AAC CCA CTG-3′) and AA55 (5′-CTG CTA GAG ATT TTC CAC ACT GAC-3′). HIV-1-specific products were quantified with the ABI 7700 detection system (Applied Biosystems, Foster City, Calif.), and cell numbers were determined with the RAG-1 gene. The numbers of $CD4^+$ cells were calculated based on the percentage of $CD4^+$ values obtained from the FACS analysis of each test PBMC sample, and R5 HIV-1 proviral DNA copy numbers were expressed as copy numbers per 10^5 $CD4^+$ cells. In some experiments, $CD4^+$ and $CD4^-$ cells were separated before real-time PCR assay with the rapid immunomagnetic CD4-positive cell isolation kit (Dynabeads M-450 CD4; Dynal Biotech, Inc., Lake

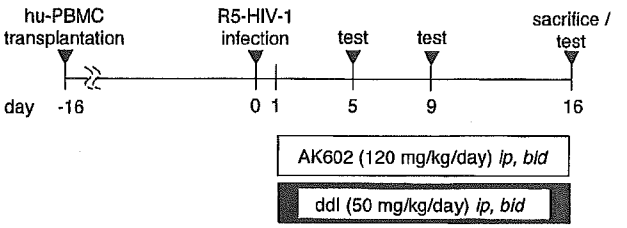

FIG. 2. Protocol for drug administration and immunological and virologic monitoring.

Success, N.Y.). The amounts of p24 antigen in murine sera were determined using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio, Inc., Tokyo, Japan) as previously described (12). Plasma viral load was quantified with the AMPLICOR HIV-1 monitor test kit, version 1.5 (Roche Diagnostics, Branchburg, N.J.).

Statistical analyses. Nonparametric statistical analyses were performed by using the Mann-Whitney U test (Statview, version 5.0; Abacus Concepts, Berkeley, Calif.). The difference between viremia levels in two groups of mice was determined by the Wilcoxon rank sum test. For each mouse, the value of $\log_{10}$ RNA copies was calculated, and the slope corresponding to the rate of increase per day was determined by simple linear regression for the days (5, 9, and 16) of blood collection. The resulting slopes for all mice in the untreated groups were compared to the slopes of mice in each of the other two groups.

RESULTS

Transplanted PBMC in hu-PBMC-NOG mice are intensely activated and express high levels of CCR5. When we examined the proliferation profile of PBMC stimulated with PHA in vitro by treatment with the vital dye CFSE, which allows the analysis of cell proliferation as the CFSE's fluorescence intensity is halved per each cell division, there was only a slight shift to the left in the flow cytometric profile on days 1 and 2 of culture (Fig. 3A). On day 4 of culture, a discrete shift to the left was identified, suggesting that the PHA-PBMC underwent up to four cycles of proliferation in vitro by day 4. In contrast, PBMC transplanted and recovered on day 2 had apparently undergone ~4 cycles of proliferation; by day 4, a majority of cells had undergone up to 10 cycles and beyond in proliferation (Fig. 3B). It was possible that the CFSE-negative and weakly CFSE-positive cells which accumulated on days 2 and 4 (Fig. 3B) were murine cells that engulfed and degraded CFSE. We therefore conducted experiments in which the cells with CFSE dilution were directly confirmed to be human CCR5-positive cells. As can be seen in Fig. 3C, when cells were recovered from the spleen of an NOG mouse into which CFSE-labeled PBMC had been transplanted and stained with monoclonal antibody 45531, which is specific for the C-terminal half of the second extracellular loop (ECL2B) of CCR5 (15), the majority of such human $CCR5^+$ cells proved to be CFSE negative. We also examined the levels of cellular activation by the expression of HLA-DR on cell surface. The levels of HLA-DR expression in PBMC recovered from uninfected NOG mice 3 days after transplantation were much greater than those in 3-day-cultured PBMC following PHA stimulation (Fig. 3D). The fluorescence intensity in the same donor's PHA-PBMC examined on three different occasions was 21 ± 4, while that of the PBMC recovered from mice was 91 ± 25 (Fig. 3D). When we further assessed the levels of CCR5 expression, the PBMC recovered from the mice on day 3 proved to be strongly positive for CCR5 (Fig. 3E). The CCR5-positive fraction in the PBMC recovered was 49.7%, while that in PHA-PBMC was 27.3%. The mean fluorescence intensity of the $CCR5^+$ cell population was 141, compared to the $CCR5^+$ cell population in PHA-PBMC with a mean fluorescence intensity of 51. The estimated number of CCR5 expressed on the PBMC recovered on day 3 was 25,348 (as antibody binding sites per cell) while that on PHA-PBMC on day 3 in culture was 8,981 antibody binding sites as examined by quantitative FACS assay. These data indicate that the transplanted human PBMC were intensely activated and rapidly proliferating and expressed high levels of CCR5 on their cell surfaces.

Potent activity of AK602 against R5 HIV-1 in vitro. Among SDP derivatives we designed and synthesized, AK602 was identified to be highly potent against a broad spectrum of R5 HIV-1 strains, including MDR clinical R5 HIV-1 isolates in vitro with 50% inhibitory concentration (IC_{50}) values of 0.3 to 0.6 nM, although two previously published CCR5 antagonists (TAK779 and SCH-C) were substantially less potent than AK602 (Table 1). AK602 and other CCR5 antagonists failed to inhibit the replication of an X4 HIV-1 strain, $HIV\text{-}1_{NL4\text{-}3}$.

Pharmacokinetics of AK602 in hu-PBMC-NOG mice. We examined the pharmacokinetics of AK602 in hu-PBMC-NOG mice by intraperitoneally administering the compound at a dose of 60 mg/kg. Plasma samples were collected periodically up to 12 h and subjected to HPLC analysis. As shown in Fig. 4A, the concentration of AK602 reached the maximal concentration immediately after intraperitoneal administration and decreased rapidly. The calculated plasma half-life in the α-phase of the concentration curve was as short as 29 min.

AK602 persists on cell surface CCR5. As shown above, the plasma half-life of AK602 turned out to be short; however, considering that AK602 possesses such a high affinity to CCR5 and potent activity against R5 HIV-1 in vitro, it was thought possible that AK602 would remain attached on cellular CCR5 for an extensive period of time and exert anti-R5 HIV-1 activity even when the compound was depleted from circulation. To examine this possibility, we used two monoclonal antibodies, 45531 and 3A9. When human PBMC were recovered from a hu-PBMC-NOG mouse 2 and 6 h after AK602 administration (60 mg/kg) and stained with 45531, AK602 proved to block the binding of 45531 to CCR5 (Fig. 4B), while AK602 failed to block 3A9 binding to CCR5 (Fig. 4C), suggesting that AK602 did not elicit CCR5 internalization or shedding at all at least for 6 h. We subsequently examined whether AK602 remained on cellular CCR5 with the 45531 monoclonal antibody. When the cells were recovered from mice 2, 6, and 14 h after the AK602 administration, the mean values of the percentage of AK602 occupancy were 85 (four mice), 54 (three mice), and 16 (three mice), respectively. It was calculated that it took about 9 h for AK602 occupancy to be reduced by 50% (Fig. 4D).

Anti-R5 HIV-1 activity of AK602 persistently seen after its removal from culture medium. In another depletion experiment, we exposed $CCR5^+$ MAGI cells to AK602 for 30 min, depleted the compound from the culture by thorough washing, incubated the cells for various lengths of time, exposed the cells to $HIV\text{-}1_{Ba\text{-}L}$, further cultured the cells for 48 h, and determined whether $HIV\text{-}1_{Ba\text{-}L}$ infection was blocked by AK602 exposure (Fig. 4E). When the $CCR5^+$ MAGI cells were exposed to 0.1 and 1 μM AK602 and exposed to $HIV\text{-}1_{Ba\text{-}L}$ immediately afterward, the values for protection were 68 and 85%, respectively. When the cells were exposed to $HIV\text{-}1_{Ba\text{-}L}$ 4 h after depletion, 49 and 72% of the cells were protected by 0.1 and 1 μM AK602. When the cells were exposed to $HIV\text{-}1_{Ba\text{-}L}$ 12 and 24 h after depletion, 57 and 45% of the cells were seen protected by 1 μM, respectively (Fig. 4E).

Effects of AK602 on $CD4^+$ and $CD8^+$ cell counts in R5 HIV-1-infected hu-PBMC-NOG mice. PBMC were recovered from murine blood samples collected on days 5, 9, and 16 after R5 HIV-1 inoculation and subjected to flow cytometric analysis for determination of $CD4^+/CD8^+$ cell ratios. As shown in Fig. 5A, in PBMC recovered on day 16 from a representative

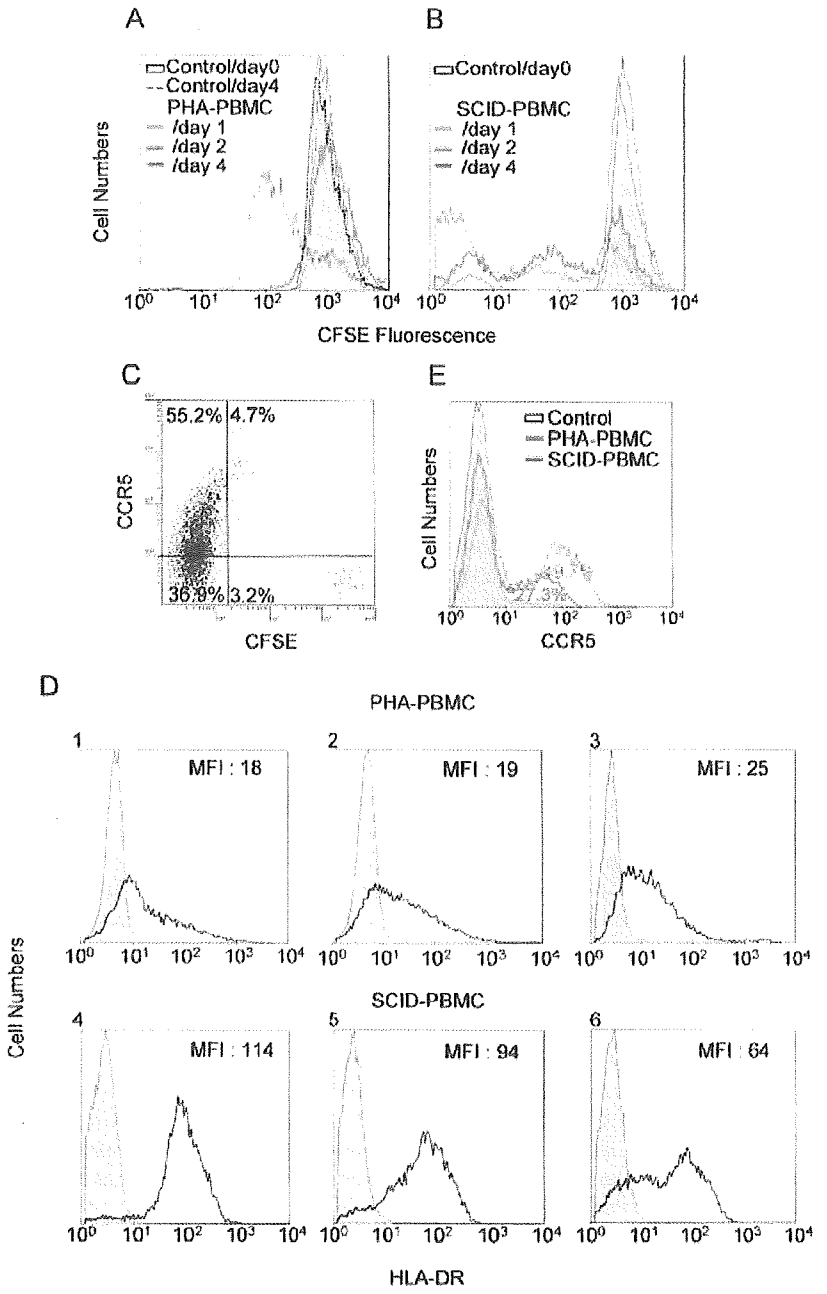

FIG. 3. Transplanted PBMC are intensely activated and express high levels of CCR5. (A and B) Proliferation profiles of PHA-PBMC and transplanted and recovered PBMC. Freshly prepared PBMC were incubated with the vital dye CFSE, and one part of such PBMC preparation was stimulated with PHA, while the other part was intraperitoneally transplanted to mice. On days 1, 2, and 4, the cells were harvested and the fluorescence intensity of CFSE was determined. Note that transplanted PBMC recovered on day 2 had undergone ~4 cycles of proliferation; by day 4, a majority of cells had undergone ~10 cycles and more of proliferation. (C) CCR5 expression level and CFSE intensity in human PBMC harvested from a spleen of hu-PBMC-NOG mouse on day 4. (D) Intense activation of PBMC after transplantation. PBMC stimulated with PHA and cultured for 4 days (panels 1 to 3) and transplanted PBMC recovered from the uninfected mice on day 4 (panels 4 to 6) were stained with an anti-HLA-DR monoclonal antibody. Note that HLA-DR expression levels in transplanted PBMC were much higher than those in PHA-PBMC. (E) CCR5 expression profiles of PHA-PBMC and transplanted PBMC. PBMC stimulated with PHA and cultured for 3 days and transplanted PBMC recovered from the uninfected mice on day 3 were stained with PE-conjugated anti-CCR5 monoclonal antibody 3A9 and subjected to flow cytometric analysis. SCID-PBMC, PBMC transplanted and recovered.

R5 HIV-1-infected, saline-treated mouse, there were only few $CD4^+$ cells (3.9% [1.4% + 2.5%]) resulting in a $CD4^+/CD8^+$ cell ratio of 0.05. However, a distinct $CD4^+$ cell population (55.1% [4.4% + 50.7%]) resulting in a $CD4^+/CD8^+$ ratio of 1.84 (Fig. 5B) was seen in PBMC recovered from an AK602-treated mouse, and the size of this $CD4^+$ cell population was comparable to that seen in a ddI-treated mouse (53.2% [3.8% + 49.4%]) and that in an uninfected mouse (48.9% [3.8% +

TABLE 1. Anti HIV-1 activity of novel SDP derivatives in PBMC[a]

Compound	IC_{50} value in p24 assay (nM)					
	HIV-1_{Ba-L} (R5)	HIV-1_{JRFL} (R5)	HIV-1_{MOKW} (R5)	HIV-1_{MM} ($R5_{MDR}$)	HIV-1_{JSL} ($R5_{MDR}$)	HIV-1_{NL4-3} (X4)
AK602	0.5 ± 0.3	0.2 ± 0.1	0.3 ± 0.2	0.7 ± 0.3	0.4 ± 0.2	>1,000
TAK779	14 ± 5	6 ± 2	9 ± 3	12 ± 4	10 ± 3	>1,000
SCH-C	3 ± 2	2 ± 1	2 ± 1.5	2.5 ± 1	2 ± 1	>1,000
ZDV	13 ± 5	7 ± 3	10 ± 6	520 ± 75	64 ± 13	9 ± 5
SQV	8 ± 3	6 ± 2	6 ± 3	212 ± 56	276 ± 44	10 ± 4

[a] IC_{50}s were determined by using PHA-PBMC isolated from three different donors, and the inhibition of p24 Gag protein production was used as an endpoint. All assays were conducted in triplicate. The results shown represent arithmetic means (±1 standard deviation) of three independently conducted assays. HIV-1_{MOKW} was isolated from a drug-naive AIDS patient, and HIV-1_{JSL} and HIV-1_{MM} were isolated from patients who received antiretroviral therapy for a long period of time and whose virus loads showed a number of RT and PR mutations. Two previously published CCR5 inhibitors, TAK779 and SCH-C, and zidovudine (ZDV) and saquinavar (SQV) were used as reference compounds.

45.1%]), resulting in the ratios of 1.43 and 1.40 (Fig. 5C and D), respectively. Figure 6A illustrates the overall profiles of $CD4^+/CD8^+$ cells ratios on day 16 in the four groups. The mean $CD4^+/CD8^+$ cell ratio in mice (n = 7) given saline was 0.1 (range, 0.06 to 0.20). In contrast, the ratios in AK602-treated mice (n = 8) were significantly higher with a mean value of 0.92 (range, 0.23 to 1.89; P = 0.001), which was comparable to that in ddI-treated mice (n = 9; mean, 1.29; range, 0.38 to 2.68; P = 0.001) and uninfected mice (n = 7; mean, 1.0; range, 0.50 to 1.49). The numbers of $CD4^+$ cells/μl

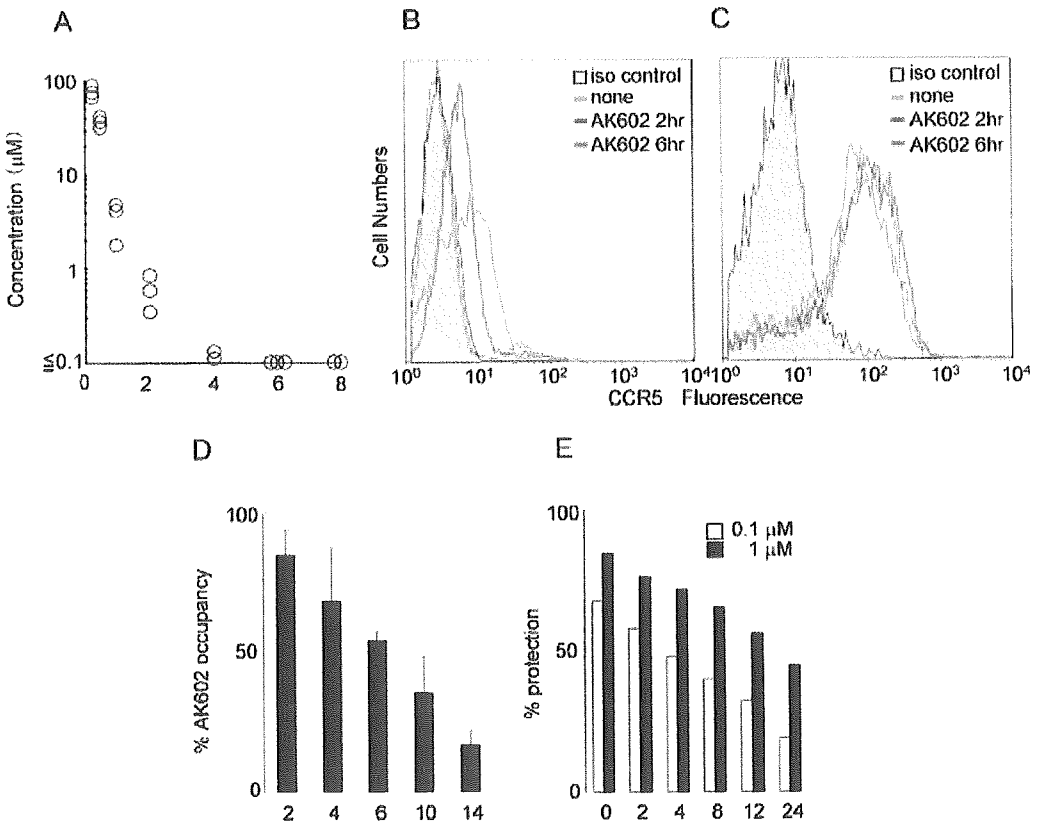

FIG. 4. Pharmacokinetics and persistence of anti-HIV-1 activity of AK602. (A) Pharmacokinetics of AK602. Each mouse was administered AK602 at a dose of 60 mg/kg, and blood samples were taken at 15, 30, 60, 120, 240, 480, and 720 min. Plasma concentrations of AK602 determined by HPLC analysis at 15, 30, 60, 120, and 240 min were 76.2, 36.1, 3.5, 0.6, and 0.13 μM, respectively. AK602 was not detected at later time points. (B and C) No CCR5 internalization or shedding was caused by AK602. Human PBMC were recovered 2 and 6 h after AK602 administration and stained with 45531 (B) or 3A9 (C). (D) Sustained AK602 occupancy on cell surfaces. At indicated periods of time after a bolus of AK-602 (60 mg/kg) was administered to hu-PBMC-NOG mice, PBMC were recovered and the percentages of AK602 occupancy on cellular CCR5 were determined with fluorescein isothiocyanate-conjugated monoclonal antibody 45531. (E) Persistence of in vitro activity of AK602 against R5 HIV-1 after AK602 depletion. $CCR5^+$ MAGI cells were exposed to 0.1 or 1 μM AK602 for 30 min and thoroughly washed to deplete AK602 from the medium. The cells were subsequently cultured for the indicated periods of time, exposed to HIV-1_{Ba-L}, and further cultured for 48 h, when the cells were harvested and lysed with Triton X-100-containing PBS. A solution containing chlorophenol red-β-D-galactopyranoside was added, the optical density was measured, and the percentage of protection was determined.

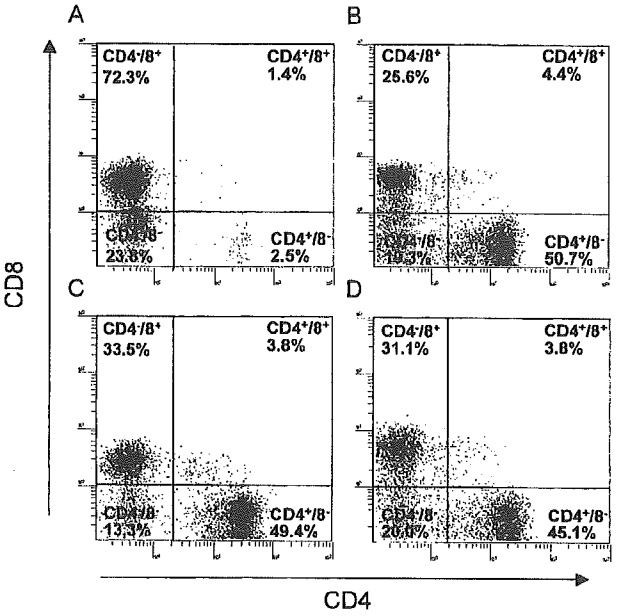

FIG. 5. Effects of AK602 on $CD4^+$ and $CD8^+$ cell counts in infected hu-PBMC-NOG mice. PBMC recovered on day 16 after R5 HIV-1 inoculation were subjected to flow cytometry. Shown are representative flow cytometric analysis profiles. Note that only 3.9% of $CD4^+$ cells were seen (A), resulting in a $CD4^+/CD8^+$ cell ratio of 0.05 in a mouse given saline, while distinct numbers of $CD4^+$ cells (55.1 and 53.2%) (B and C) were seen in AK602- and ddI-administered infected mice, resulting in $CD4^+/CD8^+$ cell ratios of 1.84 and 1.43, respectively. In an uninfected mouse (D), 48.9% of cells were positive for CD4, with a $CD4^+/CD8^+$ cell ratio of 1.40.

in saline-treated mice were significantly less than those of AK602-treated, ddI-treated, or uninfected mice (Fig. 6B).

Effects of AK602 on R5 HIV-1 proviral DNA copy numbers and serum p24 levels in R5 HIV-1-infected hu-PBMC-NOG mice. We next asked which population harbored proviral DNA in the cells recovered from R5 HIV-1-infected hu-PBMC-NOG mice, by purifying $CD4^+$ and $CD4^-$ cell populations and determining proviral DNA copy numbers in each population. As shown in Table 2, more than 99% of proviral DNA was found in $CD4^+$ cells and <0.3% of proviral DNA was detected in $CD4^-$ cells derived from saline-treated mice, indicating that R5 HIV-1 infection occurred in $CD4^+$ cells in the hu-PBMC-transplanted NOG environment. As illustrated in Fig. 6C, the mean number of R5 HIV-1 proviral DNA copies was 2.0×10^5 (range, 2.6×10^4 to 1.7×10^6) per 10^5 $CD4^+$ cells in R5 HIV-1-infected mice ($n = 7$) given saline. However, values for mice in groups given AK602 and ddI were 1.3×10^3 (range, 2.3×10^2 to 7.9×10^3; $P = 0.001$) and 1.8×10^2 (range, $<10^2$ to 7.9×10^2; $P = 0.001$), respectively.

The amounts of R5 HIV-1 p24 in serum were also found to be very high in saline-treated mice, with a mean amount of 1.1×10^5 pg/ml (range, 3.1×10^4 to 2.8×10^5 pg/ml). AK602 and ddI were found to significantly suppress the serum p24 amounts as examined on day 16 with a mean amount of 5.6×10^3 pg/ml (range, 8.1×10^2 to 2.1×10^4 pg/ml; $P = 0.001$) and 7.1×10^2 pg/ml (range, 1.3×10^2 to 1.1×10^4 pg/ml; $P = 0.001$), respectively (Fig. 6D).

AK602 suppressed R5 HIV-1 viremia in hu-PBMC-NOG mice. As described above, the PBMC transplanted to NOG mice were intensely activated in the xenogeneic environment and had undergone ~4 cycles of proliferation by day 2; a majority of the cells had undergone ≥10 cycles of proliferation by day 4 (Fig. 3B). These data suggested that R5 HIV-1 might extensively replicate in the hu-PBMC-NOG mice immediately after R5 HIV-1 inoculation. When we collected blood samples on days 5, 9, and 16 following the inoculation and determined R5 HIV-1 RNA copy numbers in infected, saline-treated mice ($n = 7$), the geometric mean copy number was 8.6×10^3/ml (range, 1.7×10^3 to 1.0×10^5) on day 5 and rapidly increased to 1.9×10^5/ml (range, 2.2×10^4 to 3.0×10^6) on day 9; by day 16, the mean copy number had reached 7.7×10^5/ml (range, 2.6×10^5 to 3.0×10^6/ml). However, AK602 significantly suppressed viremia by ~1.1 log, as examined on day 5; the mean numbers of R5 HIV-1 RNA copies in AK602-administered mice were 1.6 and 1.8 logs lower than those in saline-treated mice examined on days 9 and 16, respectively (Fig. 7). Comparable viremia suppression was seen in the mice receiving ddI (Fig. 7). It was noted that although AK602 did not completely prevent the viremia from further increasing after day 5, there was a clear reduction in the viremia increase rates. The mean slopes (change in RNA copies per day over the range of data from 5 to 16 days) for the group receiving saline was 0.167 ± 0.042, whereas those for the AK602 and ddI groups were 0.102 ± 0.041 and 0.091 ± 0.037, respectively. Thus, the rates of increase in the AK602 ($P = 0.0057$) and ddI ($P = 0.0023$) mice were significantly lower than that for the mice given saline, indicating that both of the agents significantly inhibited R5 HIV-1 replication in this mouse model over the range of days evaluated. No apparent AK602- or ddI-associated adverse effects were seen throughout the study period.

DISCUSSION

In the present hu-PBMC-NOG mouse model, human $CD4^+/CD8^+$ cell ratios went down to 0.1 by 16 days after R5 HIV-1 inoculation, the amounts of proviral DNA and p24 gag antigen reached 10^5 to 10^6 copies/10^5 $CD4^+$ cells and 10^5 pg/ml, respectively (Fig. 6), and no mice failed to be infected with R5 HIV-1. It is noteworthy that the use of NOG mice provides a higher engraftment rate than with other SCID mice such as NOD/Shi-SCID mice treated with anti-NK cell antibody or the β_2-microglobulin-deficient NOD-SCID mice (10). With NOG mice, the chimeric rate of 30 to 40% is achieved, and cord blood $CD34^+$ cells have been shown to "take" with as few as 100 cells (10). Moreover, all infected mice developed high levels of R5 HIV-1 viremia by day 16, reaching as high as 10^6 copies/ml (Fig. 7). It is worth noting that the notably high levels of HIV-1 viremia seen in the present mouse model by 16 days after R5 HIV-1 exposure can be seen only on acute infection or up to 10 years after HIV infection in humans (3, 4).

In the present study, we found that the conspicuous susceptibility to the infectivity and replication of R5 HIV-1 in these mice appeared to stem from the hyperactivation of the implanted human PBMC. The implanted PBMC were highly activated in the xenogeneic environment, expressed quite high

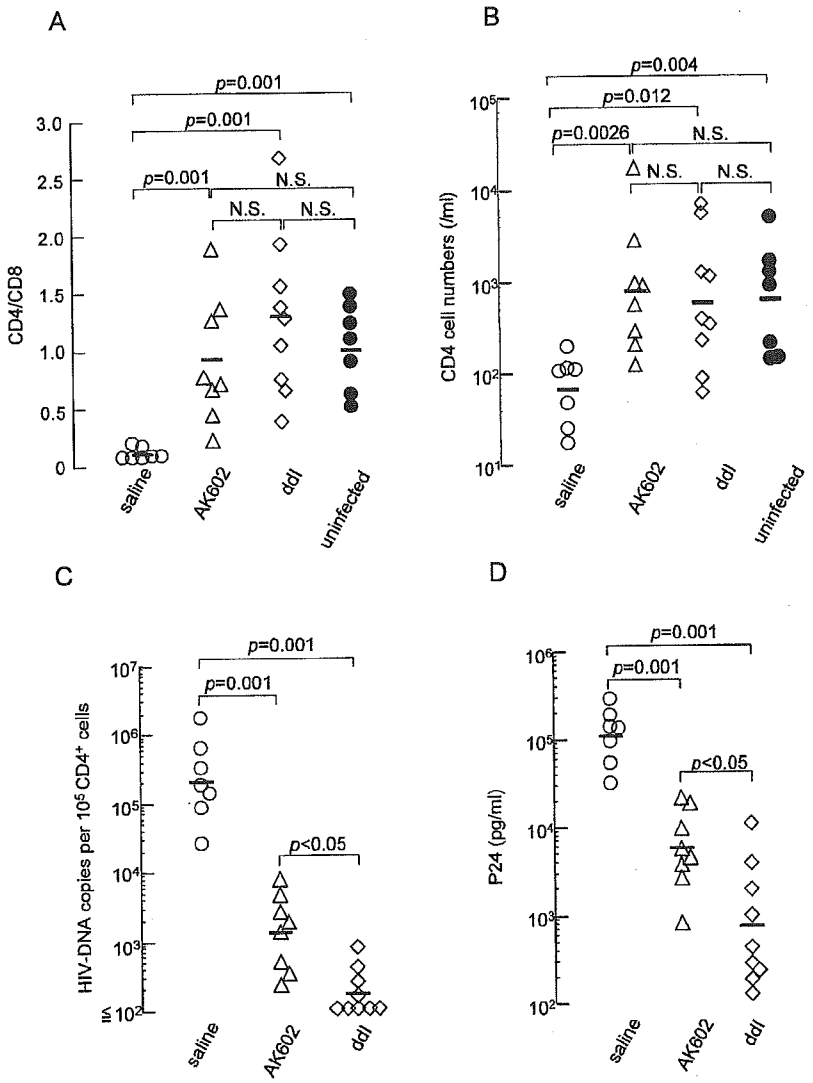

FIG. 6. Effects of AK602 on $CD4^+/CD8^+$ ratios and the amounts of proviral DNA and HIV-1 p24 in infected hu-PBMC-NOG mice. (A) Overall profiles of $CD4^+/CD8^+$ cell ratios. Note that the mean $CD4^+/CD8^+$ cell ratio in mice given saline ($n = 7$) was 0.1, while those in mice given AK602 or ddI were 0.92 and 1.29, respectively. The mean ratio in uninfected mice was 1.0. (B) Numbers of $CD4^+$ cells per microliter in each mouse group. (C) HIV-1 proviral DNA copy numbers in $CD4^+$ cells from each mouse group were determined by real-time PCR assay. Values are shown per 10^5 $CD4^+$ cells, as described in Materials and Methods. Note that the mean number of HIV-1 proviral DNA copies was 2.0×10^5 per 10^5 $CD4^+$ cells in mice given saline, while those in AK602- and ddI-treated groups were 1.3×10^3 and 1.8×10^2 per 10^5 $CD4^+$ cells (both, $P = 0.001$), respectively. (D) Amounts of plasma p24 antigen. Note that the amounts of p24 in plasma were high in saline-treated mice while AK602 and ddI significantly suppressed the serum p24 amounts as examined on day 16 after $HIV\text{-}1_{Ba\text{-}L}$ inoculation. The short bars indicate the arithmetic (A) and geometric (B, C, and D) means obtained.

levels of HLA-DR, and rapidly and continuously proliferated immediately after intraperitoneal infusion (Fig. 3A, B, and D). Moreover, the implanted PBMC expressed as much as 2.8-fold-higher levels of CCR5 on day 3 following implantation compared to PHA-PBMC on day 3 in culture (Fig. 3E). The combination of rapid proliferation and high levels of CCR5 expression of the implanted PBMC should explain the reason R5 HIV-1 rapidly replicated in the hu-PBMC-NOG mice and presented such high levels of R5 HIV-1 viremia. In this regard, only a few groups to date have documented the levels of viremia in the scientific literature. Among them are those by Garaci et al. (8) and Koyanagi et al. (14). The former documented high levels of viremia with a peak of 2.67×10^6 copies/ml in hu-PBL-NOD-SCID mice in which HIV-1-infected macrophages were inoculated, unlike our NOG mouse model where HIV-1 was directly inoculated. The latter report by Koyanagi et al. does not have viremia data but has data on p24 levels with a geometric mean of 11,092 pg/ml on day 14 after HIV-1 inoculation. However, the variation was much greater (178 to 1,434,444 pg/ml). Thus, one can say that the present model provides a greater reproducibility of high viremia levels than the mouse system reported by Koyanagi (14). It should be noted that the high levels of viremia and high engraftment rate achieved in this mouse model made it possible to monitor the

TABLE 2. Comparison of HIV-1 proviral DNA in human $CD4^+$ and $CD4^-$ cell fractions[a]

Sample	HIV-1 DNA copies (10^5 cells)		
	SCID-PBMC	$CD4^+$ cells	$CD4^-$ cells
Saline 1	138,858	162,193	461
Saline 2	135,967	117,949	<100
Saline 3	83,863	94,590	<100
AK602 1	3,390	2,300	<100
AK602 2	5,575	4,606	<100
AK602 3	1,925	1,398	<100
ddI 1	301	516	<100
ddI 2	793	1,317	<100
ddI 3	<100	118	<100

[a] HIV-1 proviral DNA copy numbers were determined by real-time PCR assay of unseparated human PBMC and purified $CD4^+$ and $CD4^-$ cells, following recovery from hu-PBMC-NOG mice. Values are shown per 10^5 cells, as described in Materials and Methods.

changes in the viremia levels periodically in the same set of mice without sacrificing them, while most of the previously described SCID mouse models required mice to be sacrificed at each time point of testing (25, 29, 30) or needed further in vitro coculture of the PBMC recovered from the mice with freshly prepared uninfected target cells for an additional period of days (9, 34).

We demonstrated in this study that a novel SDP derivative, AK602, exerted highly potent activity against laboratory and primary R5 HIV-1 strains as well as MDR R5 HIV-1 variant with IC_{50} values of subnanomolar concentrations (Table 1). It should be noted that AK602 represents a novel SDP derivative, which binds to human CCR5 but not to human CXCR4, CCR1, CCR2, CCR3, CCR4 or murine CCR5; blocks the binding of MIP-1α to CCR5 with an extremely high affinity (K_d values of ~3 nM); potently blocks HIV-1-gp120/CCR5 binding; and exerts potent activity against a wide spectrum of laboratory and primary R5 HIV-1 isolates including MDR HIV-1 and HIV-1 strains of various clades with IC_{50} values of 0.2 to 0.6 nM in vitro (K. Maeda, H. Ogata, S. Harada, Y. Tojo, T. Miyakawa, H. Nakata, Y. Takaoka, S. Shibayama, D. Fukushima, J. Moravek, E. Arnold, and H. Mitsuya, 11th Conf. Retrovir. Opp. Infect., abstr. 540, 2004; J. Demarest et al., XV Int. AIDS Conf., abstr. WeOrA1231, 2004). The plasma half-life of AK602 in the hu-PBMC-NOG mice, however, proved to be as short as 29 min when the agent was administered intraperitoneally (Fig. 4A). Considering that AK602 possesses such a high binding affinity to CCR5, we presumed that AK602 could remain on CCR5 for an extended period of time even after the agent was removed from the bloodstream in mice. The high and extensive level of AK602 occupancy observed in PBMC recovered from mice receiving AK602 substantiated this presumption (Fig. 4D). The subsequent in vitro experiment in which $CCR5^+$ MAGI cells were incubated with AK602 but exposed to R5 HIV-1 after the removal of the compound from the culture medium showed that AK602's anti-R5 HIV-1 activity can persist for an extensive period of time even if AK602 is no longer present in the culture (Fig. 4E). It is of note that unlike certain reports of in vivo anti-HIV-1 activity of chemokine antagonists which were administered before HIV-1 inoculation, thus demonstrating prophylactic effects of such agents (9, 30), the present system demonstrates anti-HIV-1 treatment after the establishment of HIV-1 infection, analogous to antiviral therapy in clinical settings.

When highly active antiretroviral therapy exerts its potent antiviral effects in clinical settings, a decrease in HIV-1 viremia is seen often within weeks, ultimately resulting in undetectable viremia; however in the present study, the viremia levels in mice receiving AK602 or ddI continued to increase although the rate of increment significantly declined (Fig. 7). The failure of AK602 and ddI to decrease viremia levels could be due in part to such a rapid viral replication in hyperactivated and proliferating $CD4^+$ cells. As discussed earlier, PBMC recovered from the hu-PBMC-NOG mice were highly positive for CCR5 and HLA-DR (Fig. 3D and E), compared to the levels of activation seen in the same donor's PHA-PBMC. It should be noted, however, that the mean numbers of proviral DNA copies on day 16 in mice receiving AK602 and ddI were 1.3×10^3 and 1.8×10^2 per 10^5 $CD4^+$ cells, respectively (Fig. 6C), suggesting that most $CD4^+$ cells (98.7 and 99.8% on average, respectively) were free of HIV-1 and proliferating in those

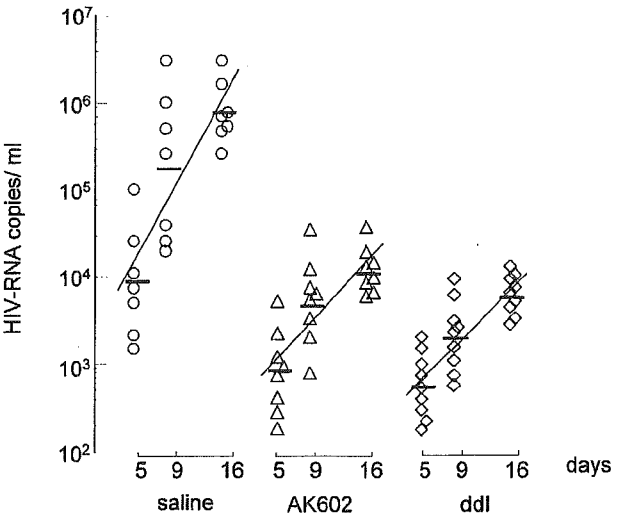

FIG. 7. AK602 suppresses R5 HIV-1 viremia in hu-PBMC-NOG mice. Blood samples were collected on days 5, 9, and 16 after inoculation and were subjected to the determination of R5 HIV-1 RNA copy numbers. Note that the copy numbers in saline-treated mice rapidly increased and reached ~10^6/ml by day 16, while AK602 significantly suppressed the viremia by 1.6 and 1.8 logs as examined on day 9 ($P = 0.001$ compared to saline-treated mice) and day 16 ($P = 0.001$), respectively. Comparable viremia suppression was seen in ddI-treated mice, except on day 16, when ddI activity was greater than that of AK602 ($P = 0.027$). Note that there was a clear reduction in the rate of increase of viremia as well. When the values of $\log_{10}$ HIV-1 RNA copies were calculated and the slopes corresponding to the rates of increase per day were determined, the resulting mean slope (solid line) for the saline-treated mice was 0.167 ± 0.042, whereas those for the AK602- and ddI-treated mice were 0.102 ± 0.041 and 0.091 ± 0.037, respectively. The increase rate for saline-treated mice was significantly higher than those of AK602-treated mice ($P = 0.0057$) and ddI-treated mice ($P = 0.0023$), respectively. The horizontal bars and solid lines represent the geometric means of HIV-1 RNA copy numbers and the slopes calculated, respectively.

mice on day 16 after the virus inoculation, if one copy of proviral DNA was postulated to reside in one $CD4^+$ cell.

One of us (Y.K.) previously attempted to investigate the mechanism of $CD4^+$ cell depletion seen in individuals with HIV-1 infection by employing a PBMC-transplanted NOD (NOD/Shi) *scid/scid* mouse system (24). Massive apoptosis was observed in HIV-1-uninfected $CD4^+$ cells in the spleens of the HIV-1-infected NOD-*scid/scid* mice. A combination of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and immunostaining for death-inducing tumor necrosis factor (TNF) family molecules showed that apoptotic cells were frequently found in conjugation with TNF-related apoptosis-inducing ligand (TRAIL)-expressing $CD3^+$ $CD4^+$ human T cells. Further observation that a neutralizing anti-TRAIL antibody inhibited the development of $CD4^+$ cell apoptosis suggested that a large number of HIV-1-uninfected $CD4^+$ cells undergo TRAIL-mediated apoptosis, contributing to the marked depletion of $CD4^+$ cells (24). The observation by Miura and his colleagues that the number of TRAIL-positive cells was consistently higher in HIV-1-infected mice than in uninfected ones makes it apparent that TRAIL expression is induced upon HIV-1 infection (23, 24). In this regard, the present observation that AK602 and ddI potently blocked the decrease in $CD4^+$ cells in spite of the rather increasing HIV-1 viremia in the face of AK602 or ddI (Fig. 7) suggests that the mere presence of viremia might not be sufficient for the HIV-induced apoptosis in $CD4^+$ cells. Our observation that most surviving $CD4^+$ cells in mice receiving AK602 or ddI were free of HIV-1 (see above) suggests that these anti-HIV-1 agents might block not only de novo HIV-1 infection, but also bystander killing of uninfected $CD4^+$ cells. The present data also suggest that a certain factor(s) such as cytokines produced by the freshly HIV-1-infected cells might mediate the apoptosis of bystander $CD4^+$ cells through the upregulation of TRAIL expression, death receptors (e.g., DR4 and DR5), and/or downregulation of decoy receptors (e.g., DcR1 and DcR2) (26, 27). However, experiments with a combination of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and TNF family molecules have to be conducted for better understanding of the bystander killing in regard to AK602's effects.

It is of note that several CCR5 antagonists are currently in various stages of development. AK602 has recently been administered to healthy adult subjects in a phase I clinical trial and shown to bind to CCR5 for an extended period of time, suggesting that an oral formulation with fewer administrations and lower dosage is possible for AK602 as a therapeutic agent for HIV-1 infection (J. Demarest, K. Adkison, S. Sparks, A. Shachoy-Clark, K. Schell, S. Reddy, L. Fang, K. O'Mara, S. Shibayama, and S. Piscitelli, 11th Conf. Retrovir. Opp. Infect., abstr. 139, 2004). Taken together, our observations that plasma viral load reached $\sim 10^6$ RNA copies/ml and that AK602 potently inhibited the replication of R5 HIV-1 strongly suggest that the present hu-PBMC-NOG mouse AIDS model could serve as a useful instrument for analyzing the pathogenesis of HIV-1 infection and testing the efficacy of antiviral agents.

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Structure-Based Design: Synthesis and Biological Evaluation of a Series of Novel Cycloamide-Derived HIV-1 Protease Inhibitors

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The structure-based design and synthesis of a series of novel nonpeptide HIV protease inhibitors are described. The inhibitors were designed based upon the X-ray crystal structure of inhibitor **1** (UIC-94017)-bound HIV-1 protease. The inhibitors incorporated 3-hydroxysalicyclic acid-derived acyclic and cyclic P2 ligand into the (*R*)-(hydroxyethylamino)sulfonamide isostere. The inhibitors contain only two chiral centers and are readily synthesized in optically active form utilizing Sharpless asymmetric epoxidation, regioselective epoxide opening, and ring-closing olefin metathesis using Grubbs' catalyst as the key steps. We have synthesized 13−15-membered cycloamides and evaluated their HIV-1 protease enzyme inhibitory and antiviral activities in MT-2 cells. Interestingly, all cycloamide-derived inhibitors are noticeably more potent than the corresponding acyclic compounds. The ring size and substituent effects were investigated. It turned out that the 14-membered saturated ring is preferred by the S_1-S_2 active sites of HIV-1 protease. Macrocycle **26** showed excellent enzyme inhibitory potency with a K_i value of 0.7 nM and an antiviral IC_{50} value of 0.3 μM. In view of their structural simplicity and preliminary interesting results, further optimization of these inhibitors is underway.

Introduction

The global pandemic of HIV/AIDS is one of the most extraordinary humanitarian and medical challenges of our time. Combination therapy or highly active antiretroviral therapy with HIV protease inhibitors and reverse transcriptase inhibitors has become the major current treatment regimen for AIDS.[1] Our recent structure-based designed strategies enhancing backbone binding led to the discovery of a nonpeptidyl HIV protease inhibitor **1** (UIC-94017, now renamed as TMC-114; Figure 1), which is exceedingly potent against wild-type ($K_i = 15 \pm 1$ pM, $n = 4$ and $ID_{50} = 1.4 \pm 0.25$ nM, $n = 5$) and resistant viruses.[2,3] It is currently undergoing advanced clinical trials.[4] In inhibitor **1**, we incorporated a structure-based designed bis-tetrahydrofuran as the P_2 ligand in (*R*)-(hydroxyethylamino)sulfonamide isostere.[5] A high-resolution X-ray crystal structure of this inhibitor-bound HIV-1 protease structure revealed critical ligand-binding site interaction in the HIV protease active site.[6] Upon the basis of this structure, we subsequently speculated that various 13−15-membered macrocycles involving P_1-P_2 ligands would effectively hydrogen bond with the critical Asp-29 and Asp-30 backbone residues and tight-bound water molecule as well as fill in the hydrophobic pockets of S_1-S_2 binding sites. In an effort to introduce structural diversity, we have designed and explored 2,3-dihydroxybenzoic acid-derived 13−15-membered cycloamides as P_1-P_2 ligands, which could conceivably make critical interactions in the S_1-S_2 binding sites. Herein, we report our preliminary results of these investigations. The inhibitors were synthesized stereoselectively by using Sharpless asymmetric epoxidation and subsequent regioselective opening of epoxide rings as the key steps. Cycloamide

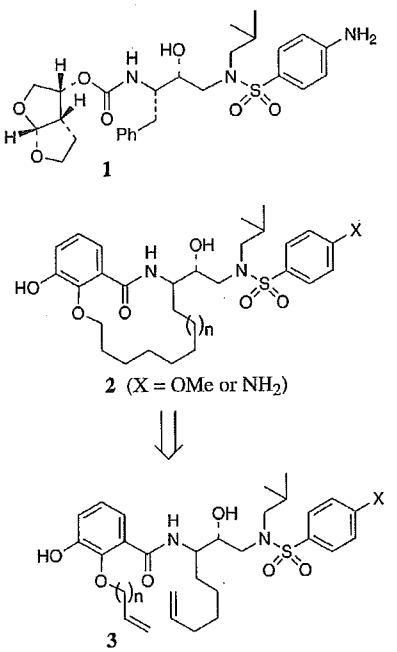

Figure 1. Structures of inhibitors **1** and cyclic and acyclic inhibitors.

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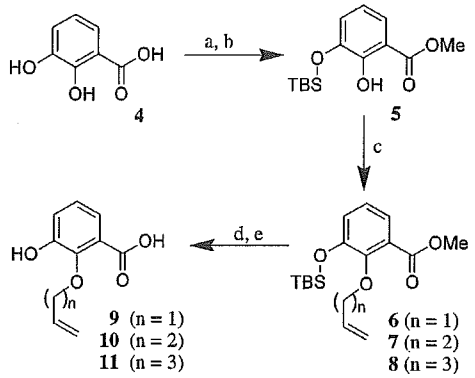
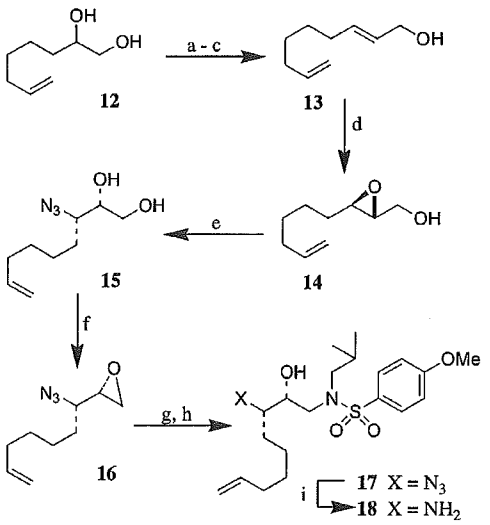
Scheme 1[a]



[a] Reagents and conditions: (a) Cs_2CO_3, MeOH–H_2O, 23 °C; then MeI, DMF, 0–23 °C. (b) TBSCl, *I*-Pr_2NEt, DMF, 0 °C. (c) Allylic alcohol, DEAD, PPh_3, THF, 23 °C. (d) TBAF, THF, 0 °C. (e) LiOH, THF, H_2O, 55 °C.

Scheme 2[a]



[a] Reagents and conditions: (a) $NaIO_4$, THF, H_2O, 23 °C. (b) $(EtO)_2POCH_2CO_2Et$, NaH, THF, 0–23 °C. (c) DIBAL-H, CH_2Cl_2, −78 °C. (d) (−)-DET, 4 Å MS, $Ti(i\text{-}OPr)_4$, TBHP, CH_2Cl_2, −20 °C. (e) $Ti(i\text{-}OPr)_4$, $TMSN_3$, benzene, 90 °C. (f) $AcOCMe_2COCl$, $CHCl_3$, 0 °C; then NaOMe, THF, 23 °C. (g) $(CH_3)_2CHCH_2NH_2$, *i*-PrOH, 90 °C. (h) 4-MeO-Ph-SO_2Cl, 1:1 (CH_2Cl_2, aqueous $NaHCO_3$), 23 °C. (i) $LiAlH_4$, THF, 23 °C.

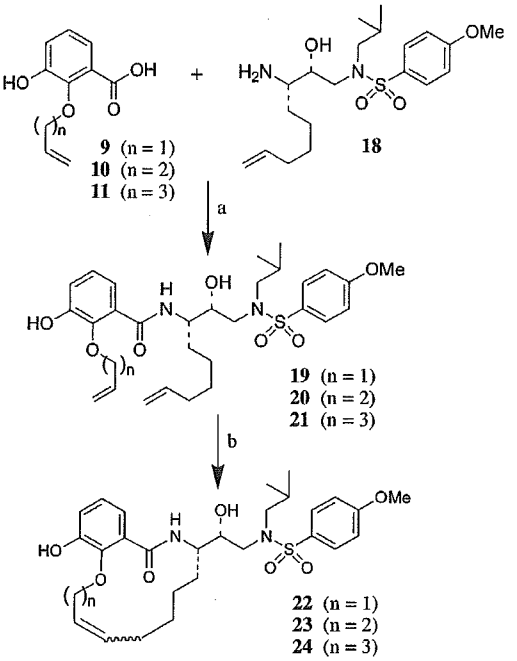
derivatives were prepared efficiently by using ring-closing olefin metathesis.

Chemistry

The synthesis of 3-hydroxysalicyclic acid-based non-peptidyl P_2 ligands is illustrated in Scheme 1. Esterification of commercially available 3-hydroxysalicyclic acid with Cs_2CO_3 in MeOH followed by selective protection of the 3-hydroxyl group as the TBS ether provided **5** in 78% yield. Ester **5** was then converted to allyl ethers and its homologues (**6**–**8**) using Mitsunobu conditions[7] in 58–89% yields. Removal of the TBS ether followed by saponification of the ester afforded various acids **9**–**11** in 83–94% yields.

Enantioselective synthesis of requisite hydroxyethylamine sulfonamide isostere is outlined in Scheme 2. Commercially available diol **12** was converetd to allylic alcohol **13** in a three-step sequence involving (i) oxidative cleavage of diol **12** with $NaIO_4$; (ii) Horner–Emmons olefination of the resulting aldehyde to α,β-unsaturated ester; and (iii) DIBAL-H reduction of ester to alcohol **13**. The allylic alcohol **13** was subjected to the Sharpless asymmetric epoxidation[8] condition with (−)-diethyl-D-tartrate to furnish the epoxide **14** in 78% yield. Regioselective ring opening of epoxide **14** with diisopropoxytitanium diazide in benzene at 90 °C, as described by Sharpless and co-workers,[9] provided the azidodiol **15** in near quantitative yield. The diol was converted to the azidoepoxide **16** by treatment with 2-acetoxyisobutyryl chloride in chloroform at 23 °C followed by exposure of the resulting chloroacetate derivative to NaOMe to afford **16**.[10] Sulfonamide **17** was synthesized in a two-step sequence by first opening the epoxide with isobutylamine and subsequent reaction of the amine with *para*-methoxybenzenesulfonyl chloride to afford sulfonamide **17** in 58% yield. Reduction of the azides by $LiAlH_4$ furnished the amine **18**, which was used directly for the next reaction without further purification.

Scheme 3[a]

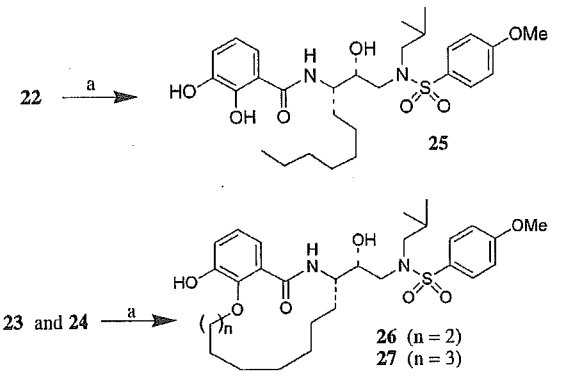


[a] Reagents and conditions: (a) EDC, HOBt, Et_3N, DMF, 0–23 °C. (b) Grubbs' cat. (first generation), CH_2Cl_2, 23 °C.

Synthesis of various acyclic inhibitors and subsequent preparation of cyclic derivatives is carried out according to Scheme 3. Using a standard peptide coupling procedure,[11] amine **18** was reacted with carboxylic acids **9**–**11** in the presence of N-ethyl-N′-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC), triethylamine, and 1-hydroxybenzotriazole hydrate (HOBt) in DMF to furnish various acyclic inhibitors **19**–**21** in 50–76% yields. Ring-closing olefin metathesis of these acyclic derivatives with commercial first generation Grubbs' catalyst[12] (10 mol %) in CH_2Cl_2 (0.003 M solution) afforded 13–15-membered unsaturated cycloamides **22**–**24** in excellent yield (89–96%).

As shown in Scheme 4, catalytic hydrogenation of 13-membered unsatuarated inhibitor **22** in the presence

Scheme 4[a]



[a] Reagents and conditions: (a) H_2, 10% Pd–C, MeOH, 23 °C.

of 10% Pd–C did not provide the corresponding saturated cycloamides. Instead, hydrogenation resulted in cleavage of the phenolic ether to give phenol **25** in 63% yield. Bergmann and Heimhold[13] have previously reported similar cleavage of allylic ethers of phenol during hydrogenation reaction. Attempts to reduce the allylic double bond of **22** under different hydrogenation conditions (Lindlar catalyst, 5% Pd on $BaSO_4$, Rh on alumina, and Wilkinson's catalyst) failed to give the desired product, resulting in either the cleaved ether or the recovery of starting material. Besides hydrogenation, reduction with $NaBH_4$ and $CoCl_2$[14] also resulted in the ether cleavage. Catalytic hydrogenation of the unsaturated cycloamides **23** and **24**, however, furnished the saturated macrocycles **26** and **27** in good yields (69–78%).

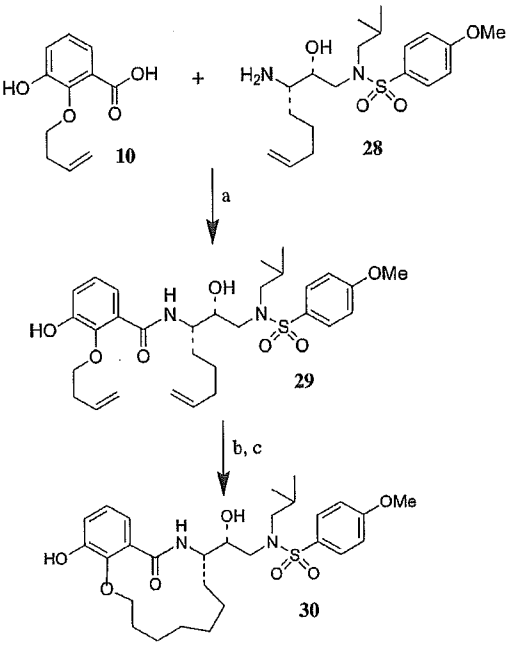
The synthesis of saturated 13-membered cycloamide was then carried out by an alternative route. Amino alcohol **28** was prepared enantioselectively following the route outlined in Scheme 2 from 5-hexenal, which was obtained by PCC oxidation of commercial 5-hexene-1-ol. Coupling of carboxylic acid **10** and amine **28** in the presence of EDC, HOBt, and Et_3N in DMF afforded the amide derivative **29**. It was converted to saturated 13-membered cycloamide derivative **30** in a two-step sequence by exposure of **29** to commercial first generation Grubbs' catalyst[12] (10 mol %) in CH_2Cl_2 followed by catalytic hydrogenation of the resulting unsaturated cycloamide in the presence of 10% Pd–C in methanol to provide **30** in 71% yield for two steps.

The synthesis of 14-membered inhibitor **33** was carried out as described in Scheme 6. Opening of epoxide **16** with isobutylamine and reaction of the resulting amine with 3,4-methylenedioxybenzene sulfonyl chloride[15] followed by LAH reduction of the azide afforded the amine **31** in 61% yield. It was coupled with acid **10** under standard reaction conditions to furnish the acyclic inhibitor **32** in 57% yield. Exposure of **32** to Grubbs' catalyst[12] under dilute conditions (0.002 M CH_2Cl_2) afforded the corresponding unsaturated macrocycle, which upon hydrogenation furnished the saturated inhibitor **33** in 82% yield for two steps.

Biological Evaluation and Discussion

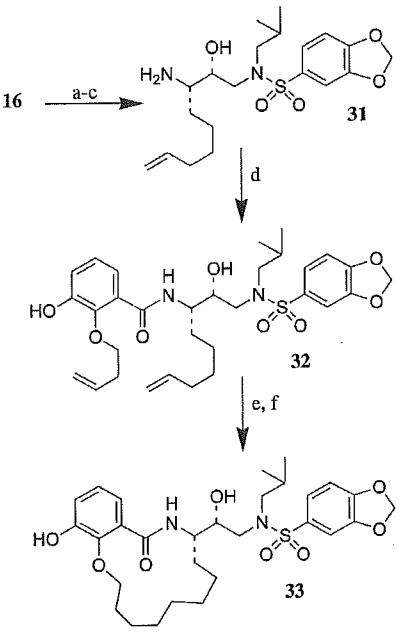
The inhibitory potencies of various acyclic and cyclic inhibitors in Table 1 were measured by the assay protocol of Toth and Marshall.[17] The values with standard deviation denote the mean values from three

Scheme 5[a]



[a] Reagents and conditions: (a) EDC, HOBt, Et_3N, DMF, 0–23 °C. (b) Grubbs' cat. (first generation), CH_2Cl_2,23 °C. (c) H_2, 10% Pd–C, MeOH, 23 °C.

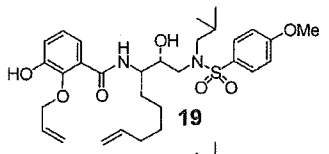
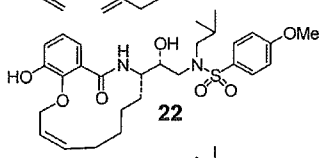
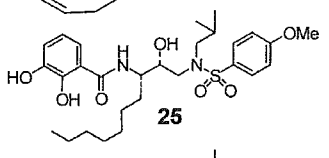
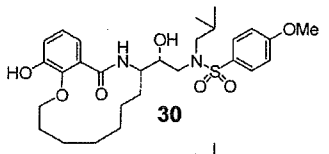
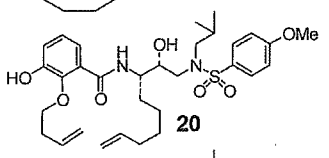
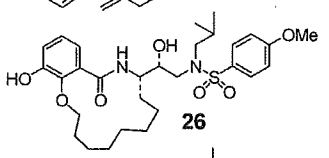
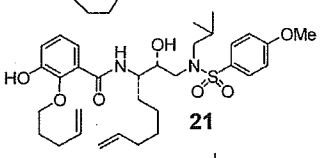
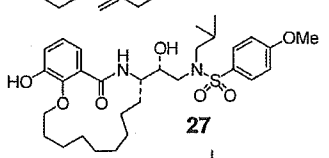
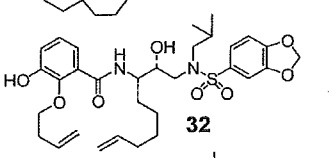
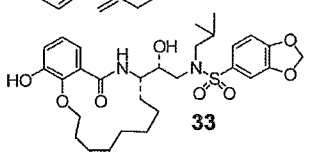
Scheme 6[a]



[a] Reagents and conditions: (a) $(CH_3)_2CHCH_2NH_2$, *i*PrOH, 90 °C. (b) 3,4-Methylenedioxybenzene sulfonyl chloride, CH_2Cl_2, aqueous $NaHCO_3$, 23 °C. (c) $LiAlH_4$, THF, 23 °C. (d) Acid **10**, EDC, HOBt, Et_3N, DMF, 23 °C. (e) Grubbs' cat. CH_2Cl_2, 23 °C. (f) H_2, 10% Pd–C, MeOH, 23 °C.

determinations. As can be seen, various saturated cycloamide-derived inhibitors have shown very impressive HIV-1 protease inhibitory activities. In general, the acyclic inhibitors were much less active than the corresponding macrocycles. Both 13-membered unsaturated inhibitor **22** and saturated inhibitor **30** with respective K_i values of 2 and 1 nM are significantly more

Table 1. Structure and Inhibitory Potencies of Various Acyclic and Cyclic Inhibitors

Entry No.	Compd	K_i (nM)
1.	 **19**	12
2.	 **22**	2 ± 0.15
3.	 **25**	270
4.	 **30**	1 ± 0.16
5.	 **20**	2 ± 0.13
6.	 **26**	0.7 ± 0.1
7.	 **21**	9 ± 1.2
8.	 **27**	2 ± 0.28
9.	 **32**	20
10.	 **33**	3 ± 0.33

potent as compared to acyclic inhibitor **19** with a K_i value of 12 nM. It is interesting to note the substantial loss in biological activity in the acyclic inhibior **25** (K_i value of 270 nM) that resulted after cleavage of allylic ether. The 14-membered saturated macrocycle **26** with the *para*-methoxybenzenesulfonamide as the P_1' ligand is the most potent inhibitor in this series with a K_i value of 0.7 nM. The corresponding monounsaturated (inseparable 5:1 *cis–trans* olefins) compound mixture displayed a K_i value of 1 nM. The 15-membered inhibitor **27** has shown nearly a 3-fold attenuation in potency as compared to the 14-membered inhibitor **26**. Because the 14-membered saturated cycloamide **26** was the most potent, we have investigated the substituent effects on the P_1' aromatic sulfonamide ring. In this context, we incorporated a 1,3-[benzo]dioxolane sulfonamide for the *para*-methoxybenzenesulfonamide. The inhibitory potency of the resulting saturated inhibitor **33** did not improve. The K_i value of the corresponding unsaturated macrocycle (inseparable 9:1 *cis–trans olefins*) was 4 nM, and the acyclic inhibitor **32** was significantly less potent.

Table 2. Antiviral Activity of Selected Compounds against HIV-1_{LAI}

inhibitor	IC_{50} (μM)	IC_{75} (μM)
20	>1	>1
26	0.30 ± 0.11	0.74 ± 0.15
27	0.56 ± 0.33	>1
33	>1	>1
saquinavir	0.012 ± 0.006	0.047 ± 0.011
amprenavir	0.028 ± 0.009	0.079 ± 0.011

We determined the antiviral activity of selected inhibitors. The results are summarized in Table 2. The IC_{50} and IC_{75} values shown were determined based on the inhibition of HIV-induced cytopathogenicity in MT-2 cells. All assays were conducted in duplicate, and the values with standard deviations denote the mean values from two or three. As can be seen, acyclic inhibitor **20** showed no antiviral activity. Inhibitors **26** and **27** (15-membered cycloamide) showed a moderate activity with antiviral IC_{50} values of about 0.3 and 0.55 μM, respectively. The antiviral activity of these compounds was substantially limited as compared to saquinavir[18] or amprenavir.[19] To improve antivitral potency, further modifications including incorporation of basic amine functionalities are in progress.

To gain insight into specific ligand-binding site interactions, an energy-minimized model structure of **26** was created (Figure 2). The structure was modeled in the active site of HIV-1 protease using the Molecular Operating Environment program (Chemical Computing Group, Montreal). The structure was built into the active site, based upon our published crystal structure of **1** complexed with HIV-1 protease (Protein Data Bank entry 1S6G) as a template.[6] The conformation and placement of the inhibitor was manually adjusted, and then, minimization was carried out using the force field[20] with the protease structure fixed. Finally, minimization was carried out with all atom positions relaxed.

Upon the basis of this model, it appears that the 3-hydroxyl group of P2-salicylic acid derivative is within an effective hydrogen-bonding distance to Asp-29 (3.3 Å) in the S_2 region of the active site. Also, the P2-carbonyl can effectively hydrogen bond to the tight-bound water molecule. The flexible macrocyclic carbon chain fills the S1–S2 hydrophobic pockets. Furthermore, the key hydrogen-bonding interaction of the P_2'-OMe group and the Asp-29′ is also quite apparent (distance 3.3 Å) as shown in Figure 2. However, the proper understanding of these active site interactions should await the solution of the X-ray crystal structure of a protein–ligand complex of **26**.[21]

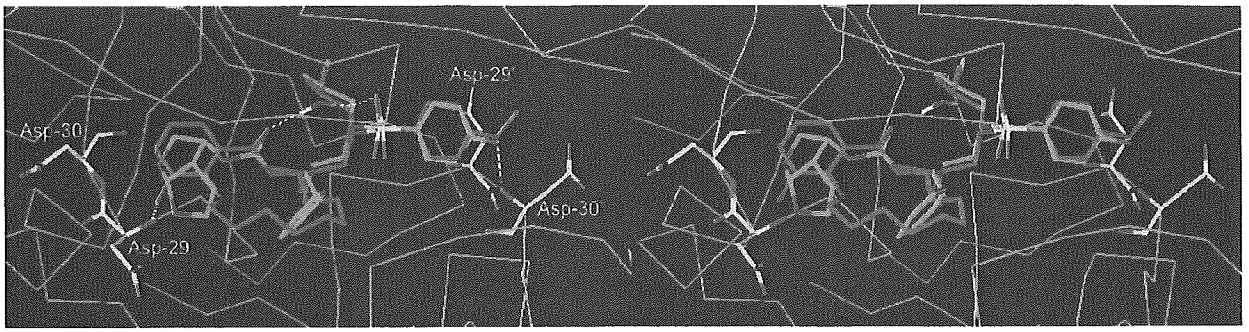


Figure 2. Stereoview of an energy minimized model structure of inhibitor **26** (magenta) overlayed with the inhibitor **1** (green)-bound crystal structure of HIV-1 protease.

Conclusion

A series of novel HIV protease inhibitors incorporating 3-hydroxysalicyclic acid-derived acyclic and cyclic P2 ligand has been designed, synthesized, and evaluated. The cyclic inhibitors incorporated 13–15-membered macrocycles. In general, cyclic inhibitors are considerably more potent than their acyclic counterparts both in enzyme inhibitory and in antiviral assays. Inhibitors with 13–15-membered macrocyclic ligands have shown subnanomolar enzyme inhibitory potencies. The inhibitors contain only two stereocenters, and the cyclic inhibitors can be readily prepared by using a ring-closing olefin metathesis. The saturated 14-membered macrocycle **26** with the *para*-methoxybenzenesulfonamide P_2' ligand displayed the highest enzyme inhibitory potency with a K_i value of 0.7 nM. This compound has also shown the best antiviral IC_{50} value of 0.3 μM. Substitution of a 1,3[benzo]dioxolane sulfonamide for the *para*-methoxybenzenesulfonamide resulted in a noticeable attenuation of biological activity of the 14-membered macrocycle. Further design and chemical modifications of these inhibitors utizing our structure-based design strategies are currently underway.

Experimental Section

General. ^{1}H and ^{13}C NMR spectra were obtained in $CDCl_3$ with an AM or Avance 400 Bruker instrument operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shifts are reported in ppm, and coupling constants (J) are reported in Hertz. Optical rotations were measured using a Perkin-Elmer 341 polarimeter using a sodium lamp (589 nm) in chloroform unless otherwise stated. Infrared spectra were measured using a Genesis II FTIR in chloroform as thin films using sodium chloride plates. Thin-layer chromatography (TLC) was performed on E. Merck silica gel 60-F-254 plates. Mass spectra were recorded on a Finnegan LCQ mass spectrometer as the value m/z. Flash chromatography was performed using 230–400 mesh silica gel. Tetrahydrofuran was distilled from sodium/benzophenone, and benzene, methylene chloride, N,N-dimethylformamide, and toluene were distilled from CaH_2 under N_2.

3-(*tert*-Butyl-dimethyl-silanyloxy)-2-hydroxy-benzoic Acid Methyl Ester (5). To a stirring solution of 1.51 g (9.8 mmol) of 2,3 dihydroxybenzoic acid **4** in 35 mL of MeOH and 7 mL of water was added 1.60 g (4.9 mmol) of $CsCO_3$. The solution was stirred at 23 °C for 30 min and then concentrated. Absolute ethanol (3 × 4 mL) was added, the solution was reconcentrated to remove water, and the crude product was dried under vacuum. The resulting brown solid was dissolved in 10 mL of DMF, and the solution was cooled to 0 °C. Methyl iodide (0.64 mL, 10.3 mmol) was added, and after the solution was warmed to 23 °C, stirring was continued in the dark for 13 h. Water (40 mL) and EtOAc (70 mL) were added, and the layers were separated. The organic layer was then washed with water (3 × 40 mL) and then 40 mL of brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash silica gel chromatography (5–20% EtOAc/hexanes) to provide the methyl ester as a pale yellow solid (1.33 g, 81%); mp 78–78.5 °C. IR (neat): 3463, 1675 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.90 (s, 1 H), 7.36 (dd, 1 H, J = 1.7, 8.2 Hz), 7.11 (dd, 1 H, J = 1.3, 8.0 Hz), 6.80 (t, 1 H, J = 7.9 Hz), 5.71 (s, 1 H), 3.95 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.8, 148.8, 145.0, 120.6, 119.8, 119.2, 112.4, 52.5.

To a stirring solution of above ester (1.28 g, 7.6 mmol) in 6 mL of DMF at 0 °C was added 1.25 g (8.3 mmol) of TBDMSCl and 1.8 mL (10.3 mmol) of *N,N*-diisopropylethylamine. Stirring was continued at 0 °C for 20 min, and then, 35 mL of water and 45 mL of EtOAc were added. The layers were separated, and the organic layer was washed with 35 mL of water and 20 mL of brine, dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica gel chromatography (hexanes to 5% EtOAc/hexanes) provided **5** as a yellow oil (2.07 g) 96%. IR (neat): 3173, 1679 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.76 (s, 1 H), 7.45 (dd, 1 H, J = 1.6, 8.1 Hz), 7.03 (dd, 1 H, J = 1.5, 8.0 Hz), 6.73 (t, 1 H, J = 8.0 Hz), 3.94 (s, 3 H), 1.01 (s, 9 H), 0.20 (s, 6 H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.3, 154.3, 144.9, 126.7, 122.7. 118.9, 113.6, 52.7, 26.1, 18.9, −4.2.

2-Allyloxy-3-(*tert*-butyl-dimethyl-silanyloxy)benzoic Acid Methyl Ester (6). To a stirring solution of **5** (101 mg, 0.36 mmol) in 4 mL of THF was added 60 μL (0.381 mmol) of DEAD, 26 μL (0.382 mmol) of allyl alcohol, and 99.5 mg (0.379 mmol) of PPh_3. The solution was stirred at 23 °C for 3 h and then concentrated. Purification by flash silica gel chromatography (0.3–5% EtOAc/hexanes) provided the product **6** as a yellow oil in 58% yield. IR (neat): 2952, 1733, 1470 1299, 1020, 934, 836 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.37 (m, 1 H), 7.00 (m, 2 H), 6.11 (dddd, 1 H, J = 5.9, 5.9, 10.5, 17.2 Hz), 5.35 (ddd, 1 H, J = 1.6, 3.2, 17.2 Hz), 5.23 (ddd, 1 H, J = 1.2, 2.7, 10.4 Hz), 4.53 (ddd, 2 H, J = 1.2, 1.3, 5.9 Hz), 3.88 (s, 3 H), 1.01 (s, 9 H), 0.19 (s, 6 H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.3, 150.4, 150.3, 134.5, 127.2, 125.3, 124.3, 124.0, 118.1, 74.8, 52.6, 26.1, 18.7, −4.1.

2-But-3-enyloxy-3-(*tert*-butyl-dimethyl-silanyloxy)benzoic Acid Methyl Ester (7). To a stirred solution of **5** (201 mg, 0.71 mmol) in 3 mL of THF with stirring was added 0.13 mL (0.83 mmol) of DEAD, 74 μL (0.86 mmol) of 3-buten-1-ol, and 226 mg (0.86 mmol) of PPh_3. The solution was stirred for 5 h and then concentrated. The crude product was purified by flash silica gel chromatography (0.5–4% EtOAc/hexanes) to give pure **7** as a yellow oil in 63% yield. IR (neat): 1734 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.34 (m, 1 H), 6.99 (m, 2 H), 5.88 (tdd, 1 H, J = 10.3, 13.6, 17.2 Hz), 5.13 (ddd, 1 H, J = 1.6, 3.2, 17.2 Hz), 5.06 (dd, 1 H, J = 1.5, 10.3 Hz), 4.05 (t, 2 H, J = 7.2 Hz), 3.89 (s, 3 H), 2.55 (m, 2 H), 1.01 (s, 9 H), 0.2 (s, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.3, 150.5, 150.2, 135.1, 127.1, 125.1, 124.1, 123.9, 117.1, 73.2, 52.5, 34.9, 26.1, 18.7, −4.0.

3-(*tert*-Butyl-dimethyl-silanyloxy)-2-pent-4-enyloxy-benzoic Acid Methyl Ester (8). Etherification of **5** with

4-penten-1-ol following the procedure for **6** provided 160 mg of **8** in 89% yield as a colorless oil. IR (neat): 1733 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 7.33 (dd, 1 H, J = 2.9, 6.6 Hz), 6.98 (m, 2 H), 5.86 (tdd, 1 H, J = 6.6, 10.3, 17.2 Hz), 5.04 (ddd, 1 H, J = 1.6, 3.4, 17.1 Hz), 4.97 (dd, 1 H, J = 1.6, 10.2 Hz), 4.01 (t, 2 H, J = 7.0 Hz), 3.89 (s, 3 H), 2.19 (m, 2 H), 1.89 (m, 2 H), 1.01 (s, 9 H), 0.20 (s, 6 H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.4, 150.7, 150.2, 138.7, 127.1, 125.1, 124.0, 123.8, 115.1, 73.8, 52.5, 30.6, 29.7, 26.1, 18.7, −4.0.

2-Allyloxy-3-hydroxy-benzoic Acid (9). To a stirring solution of 113 mg of **6** in 4 mL of THF at 0 °C was added 0.56 mL of TBAF (1 M THF) dropwise under N_2. Stirring was continued at 0 °C for about 10 min after which time 10 mL of saturated NH_4Cl solution, 4 mL of water, and 15 mL of EtOAc. The layers were separated, and the organic layer was washed with 15 mL of water and then 15 mL of brine, dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica gel chromatography (2−5% EtOAc/hexanes) resulted in 56.2 mg (0.349 mmol) of the corresponding methyl ester in 77% yield as a colorless oil. IR (neat): 3420, 2952, 1722, 1466, 1284, 988, 755 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 7.41 (dd, 1 H, J = 1.7, 7.9 Hz), 7.15 (dd, 1 H, J = 1.7, 8.1 Hz), 7.06 (t, 1 H, J = 8.0 Hz), 6.10 (qdd, 1 H, J = 6.0, 6.1, 10.4 Hz), 5.96 (s, 1 H), 5.43 (m, 1 H), 5.32 (dd, 1 H, J = 0.69, 10.3 Hz), 4.53 (d, 2 H, J = 6.1 Hz), 3.91 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.3, 150.4, 146.2, 133.6, 124.9, 124.2, 123.2, 119.9, 119.8, 76.6, 52.7.

A mixture of the above ester (30.7 mg, 0.15 mmols) and 32.7 mg (0.78 mmols) of LiOH in 2 mL of THF and 2 mL of water was stirred at 55 °C for 2.5 h. Heating was stopped, and 2 mL of 1 N HCl, 2 mL of water, and 8 mL of EtOAc were added. After the layers were separated, the organic layer was washed with 5 mL of brine, dried over sodium sulfate, filtered, and concentrated. Purification by flash silica gel chromatography of the crude product resulted in 23.6 mg of acid **9** in 83% yield as a colorless solid; mp 101.5−103.5 °C. IR (neat): 3162, 1703, 1417, 1295, 1210, 944, 747 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 7.62 (dd, 1 H, J = 1.7, 7.9 Hz), 7.20 (dd, 1 H, J = 1.7, 8.0 Hz), 7.13 (t, 1 H, J = 8.0), 6.13 (tdd, 1 H, J = 6.2, 10.5, 16.9 Hz), 5.46 (m, 1 H), 5.38 (dd, 1 H, J = 0.6, 9.7 Hz), 4.64 (d, 2 H, J = 6.2 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.5, 149.8, 146.2, 132.6, 125.6, 124.5, 123.1, 121.7, 121.2, 76.9.

2-But-3-enyloxy-3-hydroxy-benzoic Acid (10). To 129 mg (0.38 mmol) of **7** in 3 mL of THF at 0 °C with stirring was added slowly 0.6 mL of TBAF (1 M THF). After 20 min, 10 mL of saturated NH_4Cl solution, 2 mL of water, and 15 mL of EtOAc were added. The organic layer was washed with 10 mL of brine, dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica gel chromatography (5−10% EtOAc/hexanes) gave 65 mg of the corresponding diol in 77% yield as a colorless oil. IR (neat): 3443, 1726 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 7.39 (dd, 1 H, J = 1.7, 7.7 Hz), 7.13 (dd, 1 H, J = 1.7, 8.1 Hz), 7.04 (t, 1 H, J = 8.0 Hz), 6.08 (s, 1 H), 5.95 (tdd, 1 H, J = 6.9, 10.2, 17.2 Hz), 5.30 (ddd, 1 H, J = 1.5, 2.8, 16.9 Hz), 5.24 (dd, 1 H, J = 1.1, 9.7 Hz), 4.08 (t, 2 H, J = 6.1 Hz), 3.92 (s, 3 H), 2.55 (m, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.4, 150.4, 146.5, 135.3, 124.8, 124.0, 123.1, 119.9, 119.0, 74.5, 52.6, 35.0.

A mixture of above diol (61 mg, 0.28 mmol) and 79 mg (1.9 mmols) of LiOH in 2 mL of THF and 2 mL of water was heated at 55 °C. After 2 h, 2 mL of 1 N HCl and 15 mL of EtOAc. The layers were separated, and the organic layer was washed with 10 mL of brine, dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica gel chromatography (10% MeOH/CH_2Cl_2) provided **10** in 94% yield (54 mg) as a white solid; mp 70−73 °C. IR (neat): 3177, 1716 cm^{-1}. ^1H NMR (400 MHz, $CHCl_3$): δ 7.58 (d, 1 H, J = 7.7 Hz), 7.19 (d, 1 H, J = 7.9 Hz), 7.11 (t, 1 H, J = 7.9 Hz), 5.95 (m, 1 H), 5.29 (m, 2 H), 4.17 (t, 2 H, J = 6.1 Hz), 2.58 (m, 2 H). ^{13}C NMR (100 MHz, $CHCl_3$) δ 168.9, 149.7, 146.4, 134.4, 124.9, 123.8, 122.4, 121.1, 119.0, 74.5, 34.4.

3-Hydroxy-2-pent-4-enyloxy-benzoic Acid (11). A mixture of 160 mg (0.45 mmol) of **8** and 111 mg (2.64 mmol) of LiOH in 6 mL of THF and 2 mL of water was stirred at 23 °C for about 2 h and then at 50 °C for 12.5 h. Heating was stopped, and 1 N HCl was added to a pH of 1 followed by 12 mL of EtOAc. The layers were separated, and the organic layer was washed wih 5−6 mL of brine, dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica gel chromatography (20−50% EtOAc/hexanes) gave pure product **11** as a white amorphous solid; 89 mg in 88% yield; mp 70−74 °C. IR (neat): 3082, 1702 cm^{-1}. ^1H NMR (500 MHz, $CHCl_3$): δ 7.58 (dd, 1 H, J = 1.6, 7.9 Hz), 7.21 (dd, 1 H, J = 1.5, 8.1 Hz), 7.09 (t, 1 H, J = 8.0 Hz), 5.87 (tdd, 1 H, J = 6.7, 10.3, 17.1 Hz), 5.04 (dd, 2 H, J = 1.4, 10.2 Hz), 4.13 (t, 2 H, J = 6.6 Hz), 2.27 (q, 2 H, J = 7.1 Hz), 1.97 (quint, 2 H, J = 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.9, 150.1, 146.9, 138.1, 125.2, 124.2, 122.8, 121.8, 116.1, 75.6, 30.5, 29.5.

***(E)*-Nona-2,8-dien-1-ol (13).** To a stirring solution of diol **12** (1.36 g, 9.5 mmol) in 10 mL of THF and 1 mL of water was added 5.29 g (24.7 mmol) of $NaIO_4$. The resulting white mixture was stirred at 23 °C for about 30 min. The mixture was dried with Na_2SO_4, filtered through Celite, and concentrated. The resulting aldehyde was again dried with Na_2SO_4, concentrated, and used without further purification for the next reaction.

To a stirring solution of triethylphosphonoacetate (2.43 g, 10.8 mmols) in 6 mL of THF at 0 °C was added 436 mg of NaH (60% dispersion in mineral oil). After 20 min, the above aldehyde was added in 6 mL of THF, and the solution was allowed to warm to 23 °C. After 15 min, the reaction was quenched with about 2 mL of saturated NH_4Cl solution and 1 mL of water. Diethyl ether (15 mL) was added, and the layers were separated. The organic layer was washed with 10 mL of brine, dried over Na_2SO_4, filtered, and concentrated. Purification (flash silica gel chromatography-hexanes to 2% EtOAc/hexanes) gave the corresponding α,β-unsaturated ester (1.36 g, 79%) as a pale yellow liquid. IR (neat): 1722, 1654 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 6.96 (ddd, 1 H, J = 6.9, 7.0, 15.6 Hz), 5.80 (m, 2 H), 5.00 (ddd, 1 H, J = 1.6, 3.2, 17.0 Hz), 4.95 (dd, 1 H, J = 1.1, 9.7 Hz), 4.18 (q, 2 H, J = 7.1 Hz), 2.20 (m, 2 H), 2.06 (q, 2 H, J = 7.0 Hz), 1.45 (m, 4 H), 1.29 (t, 3 H, J = 7.2 Hz). ^{13}C NMR (400 MHz, $CDCl_3$): δ 166.8, 149.2, 138.6, 121.3, 114.6, 60.1, 33.5, 32.0, 28.3, 27.4, 14.3.

To a stirred solution of the above ester (1.32 g, 7.2 mmol) in 20 mL of CH_2Cl_2 at −78 °C was added 17 mL of DIBAL-H (1 M hexanes). After it was stirred at −78 °C for 20 min, 20 mL of EtOAc and 40 mL of 1 N HCl were added, and after this mixture was warmed to 23 °C, 140 mL of 1 N HCl and 20 mL of CH_2Cl_2 were added. The layers were separated, dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica gel chromatograghy (10% EtOAc/hexanes) provided **13** as a pale yellow liquid in 90% yield (910 mg). IR (neat): 3336 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 5.81 (tdd, 1 H, J = 6.7, 10.3, 17.3), 5.66 (dtd, 2 H, J = 5.8, 15.3, 20.2), 5.00 (ddd, 1 H, J = 1.7, 3.4, 16.9 Hz), 4.93 (m, 1 H), 4.08 (d, 2 H, J = 5.1 Hz), 2.05 (m, 4 H), 1.39 (m, 4 H), 1.31 (s, 1 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.9, 133.3, 129.0, 114.4, 63.8, 33.6, 32.0, 28.6, 28.4.

(2*R*,3*R*)-(3-Hex-5-enyl-oxiranyl)methanol (14). A 125 mL reaction flask with 183 mg of 4 Å molecular sieves powder was flame-dried and cooled under vacuum. To the flask was added 20 mL of CH_2Cl_2, followed by 0.10 mL (0.34 mmol) of $Ti(O^iPr)_4$ and 60 μL (0.055 mmol) of (−)-DET. The resulting solution was cooled to −20 °C, and 2.0 mL of *tert*-butyl hydroperoxide (5−6 M decane) was added dropwise. After 45 min, 0.887 g (6.3 mmol) of **13** in 10 mL of CH_2Cl_2 was added and stirring was continued at −20 °C for 3 h and was aged in a refrigerator for 11 h at about −20 °C. The solution mixture was warmed to 0 °C, and 3 mL of water was added. After 30 min, a 30% NaOH/brine solution was added, and the mixture was warmed to 23 °C. After 1 h, 20 mL of CH_2Cl_2 was added, and the layers were separated. The aqueous layer was extracted with 10 mL of CH_2Cl_2, and the combined extracts were dried over Na_2SO_4. Purification by flash silica gel chromatography (20−25% EtOAc/hexanes) gave 0.798 g of **14** in 78% yield as a pale yellow liquid: $[\alpha]_D^{23}$ +34.2 (*c* 0.30, $CHCl_3$). IR (neat): 3400, 1026 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 5.80 (tdd, 1 H, J = 6.8, 10.3, 17.1 Hz), 5.00 (ddd, 1 H, J = 1.7, 3.5, 17.2 Hz), 4.95 (dd, 1 H, J = 1.0, 9.5 Hz), 3.91

(ddd, 1 H, J = 2.6, 5.6, 12.5 Hz), 3.63 (m, 1 H), 2.96 (ddd, 1 H, J = 2.4, 5.5, 5.7 Hz), 2.92 (ddd, 1 H, J = 2.1, 2.5, 4.5 Hz), 2.06 (m, 2 H), 1.8 (m,1 H), 1.57 (m, 2 H), 1.46 (m, 4 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.6, 114.6, 61.6, 58.4, 55.9 33.6, 31.4, 28.6, 25.4.

(2*S*,3*S*)-3-Azido-non-8-ene-1,2-diol (15). A solution of 1.5 mL (5.1 mmol) of $Ti(O^iPr)_4$ and 1.2 mL (9.0 mmol) of $TMSN_3$ in 20 mL of benzene was heated at 90 °C for 4 h. To the heating yellow solution was added 0.77 g (4.9 mmol) of **14** in 5 mL of benzene, and reflux was continued for 20 min. The solution was then cooled to 0 °C, 40 mL of 15% H_2SO_4 solution was added, and the mixture was stirred vigorously for 1 h. The layers were separated, and the organic layer was washed with 20 mL of saturated $NaHCO_3$ solution, then with 20 mL of brine, dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica gel chromatography (40% EtOAc/hexanes) provided **15** as a yellow oil in 100% yield (0.97 g): $[\alpha]_D^{23}$ +13.2 (c 0.49, $CHCl_3$). IR (neat): 3077, 2101 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.80 (ddd, 1 H, J = 6.6, 10.1, 16.8 Hz), 4.99 (m, 2 H), 3.72 (m, 3 H), 3.46 (m, 1 H), 2.80 (s, 1 H), 2.09 (m, 3 H), 1.54 (m, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.5, 114.7, 73.5, 64.6, 63.1, 33.5, 30.4, 28.6, 25.7. MS-ESI (m/z): 222.2 $(M + Na)^+$.

(1*S*,2′*R*)-2′-(1-Azido-hept-6-enyl)oxirane (16). A clear solution of (960 mg, 4.8 mmol) of **15** and 1-chlorocarbonyl-methylethyl acetate (0.9 mL, 0.62 mmol) in 10 mL of $CHCl_3$ was stirred at 23 °C for 15 min. Saturated $NaHCO_3$ solution was added, and the mixture was stirred for 10 min. The layers were separated, and the organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude product was used directly for the next reaction without further purification.

A mixture of the above crude product and NaOMe (0.37 g, 6.8 mmol) in 10 mL of THF was stirred at 23 °C for 3 h. To the mixture was added 6 mL of saturated NH_4Cl solution, 2 mL of water, and 20 mL of EtOAc. The layers were separated, and the organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash silica gel chromatography (5% EtOAc/hexanes) to give 2.60 g and 54% yield of **16** as a yellow oil: $[\alpha]_D^{23}$ +5.9 (c 0.32, $CHCl_3$). IR (neat): 2101 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.80 (tdd, 1 H, J = 6.7, 10.3, 17.2 Hz), 4.99 (m, 2 H), 3.32 (m, 1 H), 3.01 (m, 1 H), 2.80 (m, 2 H), 2.08 (m, 2 H), 1.80–1.41 (m, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.5, 114.7, 62.5, 53.4, 44.9, 33.5, 31.7, 28.6, 25.2.

(2*R*,3*S*)-*N*-(3-Azido-2-hydroxy-non-8-enyl)-*N*-isobutyl-4-methoxy-benzenesUlfonamide (17). A solution of **16** (0.45 g, 2.5 mmol) and isopropylamine (0.41 mL, 4.1 mmol) in 5 mL of ^{i}PrOH was stirred at 90 °C for 80 min. After this period, the reaction mixture was concentrated. The residue was purified by flash silica gel chromatography (60–80% EtOAc/hexanes) to provide the corresponding amine as a yellow oil (0.38 g, 62%): $[\alpha]_D^{23}$ +2.0 (c 0.45, $CHCl_3$). IR (neat): 3323, 2100 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.80 (tdd, 1 H, J = 6.7, 10.3, 17.1 Hz), 5.01 (ddd, 1 H, J = 1.6, 3.4, 17.1 Hz), 4.95 (dd, 1 H, J = 1.8, 10.1 Hz), 3.58 (ddd, 1 H, J = 3.7, 5.3, 12.6 Hz), 3.39 (m, 1 H), 2.77 (dd, 1 H, J = 3.6, 12.1 Hz), 2.64 (dd, 1 H, J = 8.9, 12.1 Hz), 2.43 (ddd, 3 H, J = 6.8, 9.0, 11.7 Hz), 2.07 (dd, 2 H, J = 7.0 Hz), 1.72 (octet, 1 H, J = 6.7 Hz), 1.63–1.51 (m, 2 H), 1.51–1.40 (m, 5 H), 0.92 (d, 3 H, J = 2.1 Hz), 0.91 (d, 3 H, J = 2.1 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 139.0, 115.0, 71.3, 66.1, 58.0, 50.8, 34.0, 30.8, 29.0, 28.9, 26.3, 20.9.

A mixture of above amine (88 mg, 0.34 mmol) and 4-methoxybenzenesulfonyl chloride (70.9 mg, 0.34 mmol) in a mixture (1:1) of CH_2Cl_2 and saturated $NaHCO_3$ solution (10 mL) was stirred at 23 °C. After 12.5 h, 10 mL of CH_2Cl_2 was added, and the layers were separated. The aqueous layer was extracted with 10 mL of CH_2Cl_2. The combined extracts were dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash silica gel chromatography to give **17** (132 mg, 90%) as a clear oil: $[\alpha]_D^{23}$ −19.6 (c 0.5, $CHCl_3$). IR (neat): 3498, 2101, 1597, 1335 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (dd, 1 H, J = 3.0, 9.9 Hz), 7.75 (dd, 1 H, J = 2.0, 7.0 Hz), 7.02 (dd, 1 H, J = 2.9, 10.1 Hz), 7.01 (dd, 1 H, J = 1.7, 7.2 Hz), 5.80 (tdd, 1 H, J = 6.7, 10.3, 17.0 Hz), 5.01 (dd, 1 H, J = 1.6, 17.2 Hz), 4.96 (d, 1 H, J = 10.4 Hz), 3.88 (s, 3 H), 3.78 (m, 1 H), 3.47 (d, 1 H, J = 3.1 Hz), 3.34 (ddd, 1 H, J = 3.7, 5.5, 8.7 Hz), 3.21 (dd, 1 H, J = 9.4, 15.3 Hz), 3.02 (m, 2 H), 2.81 (dd, 1 H, J = 6.5, 13.3 Hz), 2.08 (q, 2 H, J = 6.8 Hz), 1.85 (m, 1 H), 1.65 (m, 1 H), 1.60–1.42 (m, 5 H), 0.96 (d, 3 H, J = 6.6 Hz), 0.90 (d, 3 H, J = 6.6 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.1, 138.5, 129.7, 129.5, 114.7, 114.4, 72.4, 65.4, 58.9, 55.6, 52.7, 33.5, 30.3, 28.6, 27.2, 25.6, 20.2, 19.8. MS-ESI (m/z): 425.2 $(M + H)^+$.

(2*R*,3*S*)-*N*-(3-Amino-2-hydroxy-non-8-enyl)-*N*-isobutyl-4-methoxy-benzeneSulfonamide (18). To a stirred suspension of $LiAlH_4$ (20 mg, 0.25 mmol) in 5 mL of THF was added **17** (110 mg, 0.24 mmol) in 5 mL of THF. Stirring was continued for 50 min, and the reaction was quenched by adding successively 20 μL of water, 20 μL of 15% NaOH, and 60 μL of brine. The mixture was filtered through Celite and concentrated. The resulting product **18** was used in the next reaction without further purification. IR (neat): 3351, 1596, 1333 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (dd, 1 H, J = 2.9, 11.7 Hz), 7.74 (dd, 1 H, J = 1.7, 7.1 Hz), 6.98 (dd, 1 H, J = 2.9, 11.8 Hz), 6.97 (dd, 1 H, J = 1.9, 7.1 Hz), 5.79 (tdd, 1 H, J = 6.6, 10.2, 17.2 Hz), 5.02 (ddd, 1 H, J = 1.6, 3.3, 17.0 Hz), 4.94 (m, 1 H), 3.87 (s, 3 H), 3.70 (m, 1 H), 3.25 (dd, 1 H, J = 9.3, 15.0 Hz), 3.04 (m, 2 H), 2.85 (m, 2 H), 2.06 (dd, 2 H, J = 6.7, 13.5 Hz), 1.90 (m, 2 H), 1.50–1.30 (m, 5 H), 1.43 (m, 3 H), 0.94 (d, 3 H, J = 6.5 Hz), 0.90 (d, 3 H, J = 6.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 163.3, 139.1, 130.7, 129.9, 115.0, 114.7, 73.6, 58.9, 56.0, 54.8, 52.4, 34.1, 33.1, 29.3, 27.6, 26.3, 20.6, 20.3.

(1′*S*,1″*R*)-2-Allyloxy-3-hydroxy-*N*-(1′-{1″-hydroxy-2″-[isobutyl-(4-methoxy-benzenesulfonyl)amino]ethyl}hept-6′-enyl)benzamide (19). To a stirring solution of acid **9** (23.9 mg, 0.12 mmol) in 2 mL of DMF at 0 °C was added EDC (30.1 mg, 0.16 mmol), HOBt (22.2 mg, 0.16 mmol), 50.8 mg (0.127 mmol) of amine **18** in 2 mL of DMF, and 86 μL (0.62 mmol) of Et_3N. The solution was allowed to warm to 23 °C, and stirring was continued for 19 h. To the solution was then added 4 mL of water and 20 mL of EtOAc. The layers were separated, and the organic layer was washed with 10 mL of water and 10 mL of brine, dried over Na_2SO_4, filtered, and concentrated. Flash silica gel chromatography yielded **19** (35.1 mg, 50%) as an oil: $[\alpha]_D^{23}$ −12.5 (c 0.12, $CHCl_3$). IR (neat): 3347, 2926, 1638, 1577, 1534, 1497, 1463, 1334, 1260, 1151, 1091, 807, 757 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.70 (m, 3 H), 7.52 (dd, 1 H, J = 2.8, 6.7 Hz), 7.12 (m, 2 H), 6.96 (d, 2 H, J = 8.9 Hz), 6.11 (m, 1 H), 5.77 (tdd, 1 H, J = 6.6, 10.3, 17.1 Hz), 5.64 (s, 1 H), 5.48 (d, 1 H, J = 17.1 Hz), 5.37 (d, 1 H, J = 10.5 Hz), 4.97 (dd, 1 H, J = 1.6, 15.5 Hz), 4.92 (d, 1 H, J = 10.4 Hz), 4.55 (dd, 1 H, J = 5.8, 12.4 Hz), 4.45 (dd, 1 H, J = 5.8, 12.4 Hz), 4.10 (m, 1 H), 3.93 (dd, 1 H, J = 3.2, 9.1 Hz), 3.87 (s, 3 H), 3.78 (d, 1 H, J = 3.4 Hz), 3.18 (dd, 1 H, J = 9.0, 15.2 Hz), 3.04 (d, 1 H, J = 2.4 Hz), 2.98 (dd, 1 H, J = 8.4, 13.4 Hz), 2.84 (dd, 1 H, J = 6.8, 13.3 Hz), 2.04 (m, 2 H), 1.86 (m, 1 H), 1.63 (m, 2 H), 1.44 (m, 2 H), 1.38 (m, 2 H), 0.91 (d, 3 H, J = 6.6 Hz), 0.86 (d, 3 H, J = 6.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.8, 163.4, 149.6, 144.0, 139.1, 132.7, 130.4, 129.9, 127.3, 125.8, 122.9, 120.2, 119.6, 115.0, 114.7, 76.3, 73.5, 58.9, 56.0, 54.0, 53.4, 34.0, 30.1, 29.5, 29.3, 27.6, 26.1, 20.5, 20.3. HRMS-ESI (m/z): $(M + Na)^+$ calcd for $C_{30}H_{42}N_2O_7NaS$, 574.2713; found, 574.2792.

(1″*S*,1‴*R*)-2-But-3′-enyloxy-3-hydroxy-N-(1″-{1‴-hydroxy-2‴-[isobutyl-(4-methoxy-benzenesulfonyl)amino]-ethyl}hept-6″-enyl)benzamide (20). To a stirring solution of 52 mg (0.12 mmol) of amine **18** in 2 mL of DMF at 0 °C was added 25 mg (0.13 mmol) of EDC, 16 mg (0.12 mmol) of HOBt, 20 mg (0.096 mmol) of acid **10** in 2 mL of DMF, and finally 66 μL (0.47 mmol) of triethylamine. The mixture was allowed to warm to 23 °C, and stirring was continued for 9 h. Water (5 mL) and EtOAc (20 mL) were added, and the layers were separated. The organic layer was washed with 5 mL of water and 6 mL of brine, dried over Na_2SO_4, filtered, and concentrated. Purification by flash silica gel chromatography (40% EtOAc/hexanes) provided **20** (34.1 mg) as an oil (61%): $[\alpha]_D^{23}$ −12.9 (c 0.19, $CHCl_3$). IR (neat): 3339, 1638, 1577, 1535, 1334 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.73 (m, 3 H), 7.48 (dd, 1

H, J = 2.3, 7.3 Hz), 7.10 (m, 2 H), 6.98 (dd, 1 H, J = 2.9, 11.9 Hz), 6.97 (dd, 1 H, J = 2.0, 6.9 Hz), 5.96 (tdd, 1 H, J = 6.9, 10.2, 17.2 Hz), 5.83 (s, 1 H) 5.77 (tdd, 1 H, J = 6.7, 10.3, 17.1, Hz), 5.33 (m, 1 H), 5.29 (d, 1 H, J = 10.2 Hz), 4.97 (ddd, 1 H, J = 1.7, 3.5, 17.0 Hz), 4.91 (m, 1 H), 4.09 (m, 2 H), 3.95 (m, 2 H), 3.87 (s, 3 H), 3.80 (d, 1 H, J = 3.2, Hz), 3.21 (dd, 1 H, J = 9.1, 15.1 Hz), 3.01 (m, 1 H), 2.98 (dd, 1 H, J = 2.9, 5.6 Hz), 2.83 (dd, 1 H, J = 6.6, 13.3 Hz), 2.57 (m 2 H), 2.04 (m, 2 H), 1.86 (m, 1 H), 1.60 (m, 2 H), 1.45 (m, 2 H), 1.38 (m, 2 H), 0.91 (d, 3 H, J = 6.6 Hz), 0.86 (d, 3 H, J = 6.6 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.9, 163.5, 149.6, 144.3, 139.1, 134.6, 130.3, 129.9, 127.3, 125.8, 122.6, 119.7, 119.5, 115.0, 114.8, 74.8, 73.5, 59.1, 56.0, 54.1, 53.2, 34.6, 34.0, 30.1, 29.3, 27.6, 26.1, 20.5, 20.3. MS-ESI (m/z): 589.2 (M + H)$^+$. HRMS-ESI (m/z) [M + Na]$^+$ calcd for $C_{31}H_{44}N_2O_7NaS$, 611.2769; found, 611.2767.

(1″*S*,1‴*R*)-3-Hydroxy-N-(1″-{1‴-hydroxy-2‴-[isobutyl-(4-methoxy-benzenesulfonyl)amino]ethyl}hept-6″-enyl)-2-pent-4‴″-enyloxy-benzamide (21). Acid **11** was coupled with amine **18** according to the general procedure for **20** to give **21** (39 mg, 76%) as an oil: $[\alpha]_D^{23}$ −15.3 (c 0.15, $CHCl_3$). IR (neat): 3347, 1638, 1577, 1535, 1335 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.77 (d, 1 H, J = 8.6 Hz), 7.73 (dd, 1 H, J = 2.9, 11.9 Hz), 7.71 (dd, 1 H, J = 2.0, 6.9 Hz), 7.51 (dd, 1 H, J = 2.9, 6.7 Hz), 7.10 (m, 2 H), 6.98 (dd, 1 H, J = 2.9, 11.8 Hz), 6.97 (dd, 1 H, J = 2.0, 6.9 Hz), 5.89 (tdd, 1 H, J = 6.7, 10.2, 17.1 Hz), 5.77 (tdd, 1 H, J = 6.7, 10.3, 17.0 Hz), 5.71 (s, 1 H), 5.15 (ddd, 1 H, J = 1.6, 3.2, 17.2 Hz), 5.08 (dd, 1 H, J = 1.5, 10.1 Hz), 4.98 (ddd, 1 H, J = 1.6, 3.4, 17.1 Hz), 4.92 (m, 1 H), 4.09 (m, 1 H), 4.01 (m, 1 H), 3.93 (m, 2 H), 3.87 (s, 3 H), 3.78 (d, 1 H, J = 3.2 Hz), 3.21 (dd, 1 H, J = 9.1, 15.1 Hz), 3.01 (d, 1 H, J = 1.9 Hz), 2.98 (m, 1 H), 2.83 (dd, 1 H, J = 6.6, 13.2 Hz), 2.29 (q, 2 H, J = 7.0 Hz), 2.03 (m, 2 H), 1.98 (quint., 2 H, J = 7.0 Hz), 1.85 (m, 1 H), 1.63 (m, 2 H), 1.45 (m, 2 H), 1.38 (m, 2 H), 0.91 (d, 3 H, J = 6.6 Hz), 0.86 (d, 3 H, J = 6.6 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.8, 163.4, 149.6, 144.4, 139.1, 138.3, 130.3, 129.9, 127.1, 125.7, 122.9, 119.6, 116.3, 115.0, 114.8, 75.5, 73.5, 59.0, 56.0, 54.1, 53.2, 34.0, 30.7, 30.1, 29.4, 29.3, 27.6, 26.1, 20.5, 20.3. HRMS-ESI (m/z): (M + Na)$^+$ calcd for $C_{32}H_{46}N_2O_7NaS$, 625.2934; found, 625.2923.

(2″*R*,13*S*)-N-[2″Hydroxy-2″-(4-hydroxy-15-oxo-6,9,10,-11,12,13,14,15-octahydro-5-oxa-14-aza-benzocyclotridecen-13-yl)ethyl]-N-isobutyl-4-methoxy-benzenesulfonamide (22). To a stirred solution of **19** (29.3 mg, 0.05 mmol) in 20 mL of CH_2Cl_2 was added 4.2 mg (0.0051 mmol) of Grubbs' catalyst. The solution was allowed to stir for 45 min under Ar and then concentrated. Flash silica gel chromatography (40 to 45% EtOAc/ hexanes) resulted in **22** (26.8 mg, 96%) as a pale yellow foam as a mixture (5.5:1). IR (neat): 3341, 2953, 1635, 1575, 1538, 1463, 1333, 1260, 1152, 1091, 756 cm^{-1}. ^{1}H NMR of trans diastereomer (400 MHz, $CDCl_3$): δ 8.13 (d, 1 H, J = 9.2 Hz), 7.66 (m, 2 H), 7.09 (d, 2 H, J = 4.8 Hz), 6.90 (d, 2 H, J = 8.8 Hz), 5.75 (m, 2 H), 5.35 (s, 1 H), 4.99 (dd, 1 H, J = 6.4, 11.2 Hz), 4.27 (dd, 1 H, J = 7.2, 11.2 Hz), 4.00 (m, 1 H), 3.84 (s, 3 H), 3.81 (m, 1 H), 3.61 (d, 1 H, J = 3.1 Hz), 3.11 (dd, 1 H, J = 8.7, 15.2 Hz), 3.00 (m, 1 H), 2.89 (dd, 1 H, J = 8.2, 13.4 Hz), 2.80 (dd, 1 H, J = 6.9, 13.4 Hz), 2.25 (m, 1 H), 2.15 (m, 1 H), 1.79 (m, 1 H), 1.57−1.7 (m, 2 H), 1.56 (m, 3 H), 1.42 (m, 2 H), 0.87 (d, 3 H, J = 6.6 Hz), 0.81 (d, 3 H, J = 6.7 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.4, 162.9,148.8, 145.3, 137.7, 129.7, 129.5, 126.3, 125.7, 124.6, 123.2, 120.0, 114.3, 73.4, 58.6, 55.6, 53.9, 52.9, 30.5, 27.1, 26.9, 24.0, 22.3, 20.0, 19.9. MS-ESI (m/z): 547.2 (M + H)$^+$. HRMS-ESI (m/z) [M + Na]$^+$ calcd for $C_{28}H_{38}N_2O_7NaS$, 569.2299; found, 569.2297.

(2″*R*,14*S*)-*N*-[2″-Hydroxy-2″-(4-hydroxy-16-oxo-7,10,11,-12,13,14,15,16-octahydro-6*H*-5-oxa-15-aza-benzocyclotetradecen-14-yl)ethyl]-N-isobutyl-4-methoxy-benzenesulfonamide (23). To a stirring solution of 29 mg (0.046 mmol) of **20** in 25 mL of CH_2Cl_2 at 23 °C was added 3.6 mg (0.0044 mmol) of Grubbs' catalyst. The solution was stirred at 23 °C for 13.5 h and then concentrated. Purification by flash silica gel chromatography (35−40% EtOAc/hexanes) resulted in **23** (25 mg) as a white solid (89%) as a mixture of isomers (10:1). IR (neat): 3340, 1634, 1577, 1530, 1332 cm^{-1}. ^{1}H NMR of major isomer (500 MHz, $CDCl_3$): δ 7.69 (d, 2 H, J = 8.9 Hz), 7.51 (m, 2 H), 7.10 (m, 2 H), 6.94 (d, 2 H, J = 8.9 Hz), 5.78 (ddd, 1 H, J = 5.5, 10.9, 14.9 Hz), 5.50 (m, 1 H), 5.47 (s, 1 H), 4.16 (d, 1 H, J = 4.7 Hz), 3.99 (m, 2 H), 3.86 (s, 3 H), 3.81 (m, 1 H), 3.18 (dd, 1 H, J = 2.8, 15.1 Hz), 3.07 (dd, 1 H, J = 8.4, 15.1 Hz), 2.91 (d, 2 H, J = 7.6 Hz), 2.59 (m, 1 H), 2.44 (m, 1 H), 2.19 (m, 2 H), 1.85 (m, 1 H), 1.74 (m, 1 H), 1.65−1.42 (m, 5 H), 1.36 (m, 1 H), 0.86 (d, 3 H, J = 6.6 Hz), 0.84 (d, 3 H, J = 6.7 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.8, 163.3, 149.4, 144.8, 133.2, 130.8, 129.8, 128.5, 127.2, 125.6, 123.3, 120.0, 114.7, 75.9, 74.4, 58.4, 56.0, 55.1, 53.4, 34.2, 29.5, 27.3, 26.8, 25.3, 22.7, 20.5, 20.4.

(2″*R*,15*S*)-*N*-[2″-Hydroxy-2″-(4-hydroxy-17-oxo-6,7,8,-11,12,13,14,15,16,17-decahydro-5-oxa-16-aza-benzocyclopentadecen-15-yl)ethyl]isobutyl-4-methoxy-benzenesulfonamide (24). Acyclic diene **21** was subjected to the conditions of olefin metathesis as described for **20** resulting in **24** (29 mg, 89%) as a white solid (2:1 mixture). IR (neat): 3338, 1640, 1596, 1530 cm^{-1}. ^{1}H NMR of major isomer (400 MHz, $CDCl_3$): δ 7.71 (d, 2 H, J = 8.9 Hz), 7.37 (m, 1 H), 7.06 (m, 2 H), 6.95 (d, 2 H J = 8.8 Hz), 5.78 (s, 1 H), 5.42 (m, 1 H), 5.25 (m, 1 H), 4.11 (m, 1H), 4.04 (m, 1 H), 3.86 (m, 4 H), 2.9 (d, 1 H, J = 2.9 Hz), 3.2 (dd, 1 H, J = 9.0, 15.2 Hz), 2.97 (m, 2 H), 2.81 (dd, 1 H, J = 6.8, 15.0 Hz), 2.30−2.00 (m, 3 H), 2.00−1.80 (m, 3 H), 1.70 (m, 1 H), 1.57 (m, 4 H), 1.4 (m, 2 H), 1.3 (m, 2 H), 0.91 (d, 3 H, J = 6.7 Hz), 0.85 (d, 3 H, J = 6.7 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.6, 163.0, 149.1, 144.4, 131.6, 130.5, 129.5, 126.6, 124.8, 121.8, 118.5, 114.4, 75.4, 74.4, 73.2, 58.7, 55.6, 53.9, 52.7, 30.9, 29.0, 27.6, 27.5, 27.2, 26.7, 24.0, 20.1, 19.9.

(1′*S*,1″*R*)-2,3-Dihydroxy-N-(1′-{1″-hydroxy-2″-[isobutyl-(4-methoxy-benzenesulfonyl)amino]ethyl}octyl)-benzamide (25). To a stirred solution of **22** (20.4 mg, 0.03 mmol), 2.8 mg of 10% Pd−C in 6 mL of MeOH was added, and the resulting suspension was stirred at 23 °C under H_2 balloon for 3 h. The mixture was filtered through Celite and concentrated. Flash silica gel chromatography (35% EtOAc/ hexanes) provided **25** (12.9 mg, 63%); mp 44−46 °C; $[\alpha]_D^{23}$ +3.6 (c 0.30, $CHCl_3$). IR (neat): 3381, 2926, 1640, 1595, 1540, 1459, 1331, 1264, 1150, 1091, 1025, 806, 760 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.70 (dd, 1 H, J = 3.0, 11.9 Hz), 7.69 (dd, 1 H, J = 2.0, 6.9 Hz), 7.07 (d, 1 H, J = 7.2 Hz), 7.02 (dd, 1 H, J = 1.1, 8.2 Hz), 6.96 (dd, 1 H, J = 1.6, 5.6 Hz), 6.95 (dd, 1 H, J = 2.3, 11.8 Hz), 6.80 (m, 2 H), 5.80 (s, 1 H), 4.14 (qd, 1 H, J = 4.3, 13.7 Hz), 3.94 (td, 1 H, J = 4.1, 11.6 Hz), 3.86 (s, 3 H), 3.54 (d, 1 H, J = 3.7 Hz), 3.09 (m, 2 H), 2.92 (dd, 1 H, J = 7.8, 13.4 Hz), 2.87 (dd, 1 H, J = 7.3, 13.4 Hz), 1.86 (septet, 1 H, J = 6.8 Hz), 1.69 (m, 2 H), 1.25−1.30 (m, 9 H), 0.90 (d, 3 H, J = 6.3 Hz), 0.89 (d, 3 H, J = 6.3 Hz), 0.86 (t, 3 H, J = 6.9 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.6, 163.5, 149.6, 146.3, 130.0, 129.9, 119.2, 118.7, 116.6, 114.8, 114.1, 73.5, 59.3, 56.0, 53.9, 53.3, 32.2, 29.8, 29.7, 29.6, 27.7, 26.6, 23.0, 20.5, 20.4, 14.5. MS-ESI (m/z): 573.3 (M + Na)$^+$. HRMS-ESI (m/z) [M + Na]$^+$ calcd for $C_{28}H_{42}N_2O_7NaS$, 573.2613; found, 573.2610.

(2″*R*,14*S*)-*N*-[2″-Hydroxy-2″-(4-hydroxy-16-oxo-7,8,9,-10,11,12,13,14,15,16-decahydro-6*H*-5-oxa-15-aza-benzocyclotetradecen-14-yl)ethyl]-*N*-isobutyl-4-methoxy-benzenesulfonamide (26). A mixture of **23** (21 mg, 0.037 mmol) and 2.7 mg of 10% Pd−C in 10 mL of MeOH was stirred at 23 °C under H_2 balloon for 4 h. The mixture was then filtered through Celite and concentrated. Purification by flash silica gel chromatography (40% EtOAc/hexanes) provided **26** (14 mg, 69%) as a white solid; mp 63−65 °C. $[\alpha]_D^{23}$ +12.9 (c 0.35, $CHCl_3$). IR (neat): 3350, 1637, 1596, 1531, 1331 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.69 (dd, 1 H, J = 2.9, 11.7 Hz), 7.68 (dd, 1 H, J = 1.8, 7.0 Hz), 7.38 (dd, 1 H, J = 3.6, 5.9 Hz), 7.28 (d, 1 H, J = 7.3 Hz), 7.09 (m, 2 H), 6.94 (dd, 2 H, J = 2.9, 11.7 Hz), 5.79 (m, 1 H), 4.16−4.10 (m, 2 H), 3.86 (m, 4 H), 3.72 (d, 1 H, J = 3.2 Hz), 3.14 (dd, 1 H, J = 8.7, 15.2 Hz), 3.04 (dd, 1 H, J = 3.0, 15.2 Hz), 2.91 (dd, 1 H, J = 8.1, 13.4 Hz), 2.83 (dd, 1 H, J = 7.0, 13.4 Hz), 1.92 (m, 2 H), 1.82 (septet, 1 H, J = 6.9 Hz), 1.68 (m, 2 H), 1.57−1.37 (m, 9 H), 1.25 (m, 2 H), 0.87 (d, 3 H, J = 7.1 Hz), 0.83 (d, 3 H, J = 6.6 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.7, 163.4, 149.6, 144.5, 130.3, 129.9, 128.1, 125.5, 122.6, 119.4, 114.7, 75.6, 74.1, 59.0, 56.0, 54.0, 53.2, 28.5,

28.3, 27.5, 26.7, 24.4, 24.2, 23.6, 23.2, 20.5, 20.3. HRMS-ESI (*m/z*) [M + Na]$^+$ calcd for $C_{30}H_{44}N_2O_7NaS$, 599.2767; found, 599.2792.

(2″*R*,15*S*)-*N*-[2″-Hydroxy-2″-(4-hydroxy-17-oxo-6,7,8,9,-10,11,12,13,14,15,16,17-dodecahydro-5-oxa-16-aza-benzocyclopentadecen-15-yl)ethyl]-*N*-isobutyl-4-methoxy-benzenesulfonamide (27). Hydrogenation of **24** following the procedure for **26** resulted in **27** (13 mg, 78%) as a white solid; mp 69−72.5 °C. $[\alpha]_D^{23}$ +9.3 (*c* 0.49, $CHCl_3$). IR (neat): 3343, 1638, 1596, 1577, 1531, 1332 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.69 (m, 2 H), 7.28 (d, 1 H, J = 2.2 Hz), 7.18 (dd, 1 H, J = 8.9 Hz), 7.06 (m, 2 H), 6.94 (d, 2 H, J = 8.9 Hz), 5.90 (s, 1 H), 4.13 (m, 2 H), 3.96−3.87 (m, 2 H), 3.85 (s, 3 H), 3.84 (d, 1 H, J = 3.3 Hz), 3.12 (dd, 1 H, J = 8.4, 15.2 Hz), 3.05 (dd, 1 H, J = 3.1, 15.2 Hz), 2.92 (dd, 1 H, J = 8.1, 13.4 Hz), 2.86 (dd, 1 H, J = 7.0, 13.3 Hz), 1.90 (m, 2 H), 1.84 (m, 1 H), 1.73−1.66 (m, 3 H), 1.50−1.36 (m, 11 H), 0.89 (d, 3 H, J = 6.6 Hz), 0.85 (d, 3 H, J = 6.6 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.1, 163.4, 149.7, 144.4, 130.3, 129.9, 128.6, 125.2, 121.8, 119.0, 114.7, 75.1, 73.7, 59.1, 56.0, 54.1, 53.1, 29.4, 28.1, 27.6, 26.6, 26.0, 25.3, 24.6, 24.1, 23.5, 20.5, 20.3. MS-ESI (*m/z*): 599.3 [M + Na]$^+$. HRMS-ESI (*m/z*) [M + Na]$^+$ calcd for $C_{30}H_{44}N_2O_7$-NaS, 599.2769; found, 599.2767.

(1″*S*,1‴*R*)-2-But-3′-enyloxy-3-hydroxy-*N*-(1″-{1‴-hydroxy-2‴-[isobutyl-(4-methoxy-benzenesulfonyl)amino]-ethyl}hex-5″-enyl)benzamide (29). Acid **10** was coupled with amine **28** according to the general procedure for **19** to give **29** in 45% yield as an oil: $[\alpha]_D^{23}$ −4.4 (*c* 3.1, $CHCl_3$). IR (neat): 3344, 2958, 1632, 1577, 1534, 1497, 1462, 1333, 1260, 1152, 1091, 1023, 995, 807, 757 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (d, 2 H, J = 8.8 Hz), 7.48 (dd, 1 H, J = 2.2, 7.3 Hz), 7.10 (m, 2 H), 6.97 (d, 2 H, J = 8.8 Hz), 5.96 (tdd, 1 H, J = 6.9, 10.2, 17.2 Hz), 5.82 (s, 1 H), 5.77 (tdd, 1 H, J = 6.8, 10.2, 16.9 Hz), 5.31 (m, 1 H), 4.98 (m, 1 H), 4.09 (m, 2 H), 3.94 (m, 2 H), 3.87 (s, 3 H), 3.79 (d, 1 H, J = 3.1 Hz), 3.21 (dd, 1 H, J = 9.2, 15.2 Hz), 2.99 (m, 2 H), 2.82 (dd, 1 H, J = 6.6, 13.3 Hz), 2.57 (m, 2 H), 2.09 (m, 2 H), 1.86 (m, 1 H), 1.62−1.70 (m, 2 H), 1.52−1.58 (m, 2 H), 1.43−1.52 (m, 3 H), 0.92 (d, 3 H, J = 6.6 Hz), 0.86 (d, 3 H, J = 6.6 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.8, 163.5, 149.6, 144.3, 138.7, 134.6, 130.3, 129.9, 127.2, 125.8, 122.6, 119.7, 119.5, 115.4, 114.8, 74.8, 73.5, 59.1, 56.0, 54.1, 53.0, 34.6, 34.0, 28.7, 27.7, 25.8, 20.5, 20.3. MS-ESI (*m/z*): 597.2 [M + Na]$^+$.

(2″*R*,13*S*)-*N*-[2″-Hydroxy-2″-(4-hydroxy-15-oxo-6,7,8,9,-10,11,12,13,14,15-decahydro-5-oxa-14-aza-benzocyclotridecen-13-yl)ethyl]-*N*-isobutyl-4-methoxybenzenesulfonamide (30). Acyclic diene **29** (42 mg) was cyclized under the conditions of olefin metathesis as described for **19** to give the corresponding cyclic olefin (37.7 mg, 75%, 1.9:1 mixture) as as a foam. IR (neat): 3347, 2958, 1633, 1596, 1577, 1535, 1497, 1463, 1332, 1290, 1260, 1152, 1091, 1025, 998, 807, 755 cm^{-1}; major isomer. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.84 (d, 1 H, J = 6.9 Hz), 7.67 (d, 2 H, J = 8.9 Hz), 7.51 (dd, 1 H, J = 2.0, 7.6 Hz), 7.02 (m, 2 H), 6.91 (d, 2 H, J = 8.9 Hz), 6.52 (s, 1 H), 5.60 (dd, 1 H, J = 6.12, 15.7 Hz), 5.55 (dd, 1 H, J = 5.5, 15.6 Hz), 4.26 (m, 2 H), 4.16 (m, 1 H), 3.95 (m, 1 H), 3.84 (s, 3 H), 3.15 (dd, 1 H, J = 3.2, 15.2 Hz), 3.00 (dd, 1 H, J = 8.4, 15.2 Hz), 2.90 (m, 2 H), 2.44 (m, 1 H), 2.35 (m, 1 H), 1.87 (m, 2 H), 1.81 (m, 1 H), 1.75 (m, 1 H), 1.67 (m, 2 H), 1.53 (m, 1 H), 0.83 (dd, 6 H, J = 6.7, 8.7 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.8, 163.3, 149.5, 144.6, 134.0, 130.5, 129.9, 128.4, 127.0, 124.6, 123.3, 120.5, 114.7, 74.1, 73.8, 58.7, 56.0, 54.1, 53.6, 32.4, 31.5, 27.6, 27.4, 24.4, 20.4, 20.4.

Hydrogenation of above olefin mixture for 1 h following the procedure for **26** resulted in saturated macrocycle **30** as a colorless solid (15.7 mg) in 71% yield; mp 74−75.5; $[\alpha]_D^{23}$ +29.9 (*c* 0.73, $CHCl_3$). IR (neat): 3348, 2951, 2931, 1635, 1596, 1577, 1534, 1497, 1464, 1332, 1288, 1260, 1151, 1091, 1025, 996, 807, 755 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.66 (d, 2 H, J = 8.9 Hz), 7.27 (m, 2 H), 7.04 (m, 2 H), 6.92 (d, 2 H, J = 8.9 Hz), 5.95 (s, 1 H), 4.12 (m, 2 H), 3.91 (m, 1 H), 3.85 (bs, 4 H), 3.08 (d, 2 H, J = 4.2, Hz), 2.86 (d, 2 H, J = 7.5 Hz), 1.93 (m, 1 H), 1.86 (m, 1 H), 1.79 (pentet, 1 H, J = 6.8 Hz), 1.69 (m, 4 H), 1.58 (m, 2 H), 1.48 (m, 3 H), 1.42 (m, 2 H), 0.86 (d, 3 H, J = 6.6 Hz), 0.82 (d, 3 H, J = 6.6 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.1, 163.4, 149.4, 144.4, 130.2, 129.9, 128.5, 125.1, 122.2, 119.3, 114.7, 74.1, 73.4, 59.0, 56.0, 54.2, 52.7, 29.5, 27.8, 27.5, 26.3, 24.1, 21.5, 21.4, 20.4, 20.3. HRMS-ESI (*m/z*) [M + K]$^+$calcd for $C_{29}H_{42}N_2O_7NaS$, 585.2610; found, 585.2591.

(1″*S*,1‴*R*)-N-(1″-[{2‴-Benzo[1⁗,3⁗]dioxole-5⁗-sulfonyl)isobutyl-amino]-1‴-hydroxyl-ethyl}hept-6″-enyl)-2-but-3′-enyloxy-3-hydroxy-benzamide (32). Acid **10** and amine **31** were coupled according to the method used for **19** to give **32** (38 mg, 57%) as a yellow oil: $[\alpha]_D^{23}$ −12.2 (*c* 0.18, $CHCl_3$). IR (neat): 3355, 1638, 1576, 1536, 1331 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.68 (d, 1 H, J = 8.6 Hz), 7.48 (dd, 1 H, J = 2.2, 7.3 Hz), 7.34 (dd, 1 H, J = 1.8, 6.4 Hz), 7.20 (d, 1 H, J = 1.8 Hz), 7.10 (m, 2 H), 6.88 (d, 1 H, J = 8.2 Hz), 6.09 (s, 2 H), 5.96 (tdd, 1 H, J = 6.9, 10.3, 17.1 Hz), 5.77 (m, 2 H), 5.33 (dd, 1 H, J = 1.4, 17.2 Hz), 5.29 (d, 1 H, J = 10.3 Hz), 4.98 (m, 1 H), 4.92 (d, 1 H, J = 10.2 Hz), 4.09 (m, 2 H), 3.97 (m, 2 H), 3.79 (d, 1 H, J = 3.29 Hz), 3.79 (d, 1 H, J = 3.3 Hz), 3.21 (dd, 1 H, J = 9.0, 15.1 Hz), 3.04 (d, 1 H, J = 2.6 Hz), 3.00 (m, 1 H), 2.85 (dd, 1 H, J = 6.7, 13.4 Hz), 2.57 (m, 2 H), 2.04 (dd, 2 H, J = 6.8, 11.9 Hz), 1.87 (ddd, 1 H, J = 6.7, 6.7, 14.8 Hz), 1.64 (m, 2 H), 1.45 (m, 2 H), 1.39 (m, 1 H), 0.92 (d, 3 H, J = 6.6 Hz), 0.87 (d, 3 H, J = 6.6 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.5, 151.5, 149.2, 148.3, 143.9, 138.6, 134.1, 131.6, 126.8, 125.4, 123.1, 122.2, 119.3, 119.1, 114.6, 108.4, 107.5, 102.4, 74.4, 73.1, 58.7, 53.6, 52.9, 34.1, 33.6, 29.7, 28.8, 27.2, 25.7, 20.1, 19.8. MS-ESI (*m/z*): 625.2 [M + Na]$^+$. HRMS-ESI (*m/z*) [M + Na]$^+$ calcd for $C_{31}H_{42}N_2O_8NaS$, 625.2562; found, 625.2560.

(2′*R*,14*S*)-Benzo[1″,3″]dioxole-5″-sulfonic Acid [2′-Hydroxy-2′-(4-hydroxy-16-oxo-7,8,9,10,11,12,13,14,15,16-decahydro-6*H*-5-oxa-15-aza-benzocyclotetradecen-14-yl)ethyl]isobutyl-amide (33). Acyclic diene **32** (31 mg) was subjected to the conditions of olefin metathesis as described for **22** to provide the corresponding cyclic olefin (29 mg, 9:1 mixture) in 95% yield. IR (neat): 3350, 1634, 1576, 1530, 1330 cm^{-1}. ^{1}H NMR of major (trans) diastereomer (500 MHz, $CDCl_3$): δ 7.53 (m, 1 H), 7.31 (m, 1 H), 7.18 (m, 1 H), 7.11 (m, 2 H), 6.83 (d, 1 H, J = 8.2 Hz), 6.08 (s, 2 H), 5.8 (m, 1 H), 5.5 (m, 2 H), 4.22 (d, 1 H, J = 4.9 Hz), 3.98 (m, 3 H), 3.9 (m, 1 H), 3.21 (m, 1 H), 3.07 (dd, 1 H, J = 8.5, 15.2 Hz), 2.93 (m, 2 H), 2.60 (m, 1 H), 2.50 0.85 (d, 3 H, J = 6.8 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.8, 151.7, 149.4, 148.6, 144.8, 133.1, 132.6, 129.8, 128.5, 127.1, 125.6, 123.4, 123.3, 120.1, 108.7, 108.0, 102.7, 74.4, 58.4, 55.3, 53.3, 34.2, 29.5, 27.3, 26.9, 25.3, 22.7, 20.5, 20.3.

Hydrogenation of above olefin mixture according to the procedure for **26** afforded **33** (20 mg, 91%) as a white solid; mp 64−65.5 °C; $[\alpha]_D^{23}$ +15.5 (*c* 0.62, $CHCl_3$). IR (neat): 3348, 1637, 1532, 1329 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38 (dd, 1 H, J = 3.1, 6.5 Hz), 7.31 (m, 2 H), 7.17 (d, 1 H, J = 1.7 Hz), 7.07 (m, 2 H), 6.83 (d, 1 H, J = 8.2 Hz), 6.07 (s, 2 H), 5.91 (s, 1 H), 4.20−4.04 (m, 2 H), 3.90−3.80 (m, 2 H), 3.76 (d, 1 H, J = 3.1 Hz), 3.16−3.07 (m, 2 H), 2.92 (dd, 1 H, J = 5.3, 13.4 Hz), 2.85 (dd, 1 H, J = 7.0, 13.4 Hz), 1.91 (m, 1H), 1.80 (m, 1 H), 1.69 (m, 3 H), 1.51−1.41 (m, 8 H), 1.26 (m, 2 H), 0.88 (d, 3 H, J = 6.7 Hz), 2.3 (m, 2 H), 1.87 (m, 1 H), 1.73 (m, 1 H),1.5 (m, 5 H), 0.87 (d, 3 H, J = 6.7 Hz), 6.6), 0.83 (d, 3 H, J = 6.6 Hz). ^{13}C (125 MHz, $CDCl_3$): δ 166.8, 151.9, 149.7, 148.7, 144.5, 132.0, 128.0, 125.5, 123.5, 122.6, 119.5, 108.8, 107.9, 102.7, 75.5, 74.1, 59.0, 54.0, 53.3, 28.5, 28.3, 27.5, 26.6, 24.4, 24.2, 23.5, 23.2, 20.5, 20.3. HRMS-ESI (*m/z*) [M + Na]$^+$ calcd for $C_{29}H_{40}N_2O_8SNa$, 599.2403; found, 599.2378.

Antiviral Assay. The activity of designated compounds against HIV-1LAI was determined as previously described.[3a] Briefly, MT-2 cells (2 × 10^3/well) were exposed to 100 50% tissue culture infectious doses (TCID50s) of HIV-1LAI in the presence of various concentrations of an agent, examined in 96 well microculture plates, and incubated at 37 °C for 7 days (final volume: 200 μL/well). After the medium was removed from each well, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (7.5 mg/mL) in phosphatate-buffered saline was added to each well in the plate, followed by incubation at 37 °C for 2 h. After incubation,

to dissolve the formazan crystals, 100 μL of acidified 2-propanol containing 4% (v/v) Triton X-100 was added to each well and the optical density (570 nm) was measured in a microplate reader (Vmax; Molecular Devices, Sunnyvale, CA). All assays were conducted in duplicate.

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Supporting Information Available: HRMS and HPLC data for compounds **19–22**, **25–27**, **30**, **32**, and **33**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) To analyze the protein–ligand interactions of this class of inhibitors, crystallographic studies of the HIV-1 protease complex of inhibitor **26** are being attempted.

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Spontaneous recovery of hemoglobin and neutrophil levels in Japanese patients on a long-term Combivir® containing regimen

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Abstract

Objective: In order to evaluate long-term toxicity of Combivir, we retrospectively reviewed clinical records of HIV-1 infected cases under treatment with Combivir-containing regimen and we analyzed the clinical data compared to other NRTIs-containing regimens.
Study design: A total of 55 patients who were on Combivir and 39 on a control regimen were examined.
Results: After starting treatment with Combivir-containing regimens viral load and $CD4^+$ T-cell count improved as well as the control group. Rates of adverse events in Combivir group and ZDV (400 mg/day) + 3TC group were 50.9% (28/55) and 60% (12/20), respectively. Some of these Japanese patients who started Combivir regimen as a first-line HAART (primary Combivir group) showed some decrease in hemoglobin levels or neutrophil counts within 6 months. However, a significant recovery of these indices of hematological toxicities occurred in patients who continued the regimen for 18–24 months.
Conclusion: Our findings suggest that the safety of 600 mg of ZDV is similar to 400 mg/day of ZDV and the existence of mechanisms that compensate for anemia and for the neutropenia associated with long-term use of Combivir.

Keywords: Combivir; Zidovudine; Lamivudine; Hemoglobin; Neutrophil; Long-term treatment

1. Introduction

Prognosis of HIV infections dramatically improved after introduction of highly active anti-retro viral therapy (HAART). However, the occurrence of adverse events and drug resistance during long-term use of anti-retrovirals are now big issues (Yeni et al., 2002; Dieleman et al., 2002). Present HAART also has a problem to maintain a high adherence because of the pill burden and patients' quality of life is affected. Combivir® is a fixed dose combination tablet containing zidovudine (ZDV) and lamivudine (3TC) (Eron et al., 2000). Each tablet contains 300 mg of ZDV and 150 mg of 3TC and has been widely used as a nucleoside reverse transcriptase inhibitor (NRTI) component of HAART against HIV-1 infection.

HIV infection and AIDS are known to be associated with a significant hematological toxicity, including anemia, neutropenia, and thrombocytopenia (Moses et al., 1998). In addition, studies with zidovudine have shown that this drug may compound the hematological toxicity of HIV and lead to an independent development of anemia and neutropenia (Wilde and Langtry, 1993). Consistent with these observations, the incidence of anemia or neutropenia in mildly or asymptomatic adults treated with zidovudine was between 1.1% and 9.7%, whereas in adults with AIDS or the AIDS related complex it ranged from 15% to as high as 61% (Wilde

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