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Inhibition of Human Immunodeficiency Virus Type 1 Replication in Human Cells by Debio-025, a Novel Cyclophilin Binding Agent▽†

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Debio-025 is a synthetic cyclosporine with no immunosuppressive capacity but a high inhibitory potency against cyclophilin A (CypA)-associated *cis-trans* prolyl isomerase (PPIase) activity. A lack of immunosuppressive effects compared to that of cyclosporine was demonstrated both in vitro and in vivo. For three cyclosporines, the inhibitory potential against PPIase activity was quantitatively correlated with that against human immunodeficiency virus type 1 (HIV-1) replication. Debio-025 selectively inhibited the replication of HIV-1 in a CD4$^+$ cell line and in peripheral blood mononuclear cells: potent activity was demonstrated against clinical isolates of various HIV-1 subtypes, including isolates with multidrug resistance to reverse transcriptase and protease inhibitors. Simian immunodeficiency virus and HIV-2 strains were generally resistant to inhibition by Debio-025; however, some notable exceptions of sensitive HIV-2 clinical isolates were detected. In two-drug combination studies, additive inhibitory effects were found between Debio-025 and 19 clinically used drugs of different classes. Clinical HIV-1 isolates that are naturally resistant to Debio-025 and that do not depend on CypA for infection were identified. Comparison of the amino acid sequences of the CypA binding domain of the capsid (CA) protein from Debio-025-sensitive and -resistant HIV-1 isolates indicated that resistance was mostly associated with an H87Q/P exchange. Mechanistically, cyclosporines competitively inhibit the binding of CypA to the HIV-1 CA protein, which is an essential interaction required for early steps in HIV-1 replication. By real-time PCR we demonstrated that early reverse transcription is reduced in the presence of Debio-025 and that late reverse transcription is almost completely blocked. Thus, Debio-025 seems to interfere with the function of CypA during the progression/completion of HIV-1 reverse transcription.

The introduction of highly active antiretroviral therapy has led to significant changes in disease progression and mortality of human immunodeficiency virus (HIV) type 1 (HIV-1) infection and its sequela, AIDS. HIV-1 infection has been turned into a chronic condition that can be treated and that may be manageable over many years (90). However, a significant proportion of patients still fail to have a complete response to treatment over a prolonged time and are at risk of virological rebound, which may lead to the emergence of drug-resistant virus variants (20). The 21 anti-HIV-1 drugs used at present target only three steps in the viral replication cycle, namely, virus fusion, reverse transcription, and the proteolytic processing of viral proteins (22). An inhibitor directed against a fourth step, envelope glycoprotein binding to the CCR5 coreceptor (24, 99), and an inhibitor of a fifth step, integration of viral DNA in the cellular genome (34), were recently approved by FDA. Further inhibitors of the last two steps are in clinical evaluation (23, 86). Thus, the four virus-coded proteins, the envelope glycoprotein, reverse transcriptase (RT), protease, and integrase, are the molecular targets of anti-HIV-1 chemotherapy in the clinic at present. This limits the number of possible combinations that may be used, because cross-resistance of virus strains against drugs targeted to the same viral molecule is common (61). Therefore, novel anti-HIV drugs directed against other steps in the viral replication cycle are highly needed.

Cyclophilin A (CypA) was established more than a decade ago to be a valid target in anti-HIV-1 chemotherapy (69, 70). The cellular protein CypA fulfills an essential function early in the HIV-1 replication cycle. It was found to bind specifically to the HIV-1 Gag polyprotein (48). A defined amino acid sequence around G89 and P90 of capsid protein p24 (CA) was identified as the binding site for CypA (17, 31). The affinity of

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CypA for CA promotes the incorporation of CypA into the virion particles during assembly (10, 30, 87). Experimental evidence indicates that the CypA-CA interaction is essential for HIV-1 replication; inhibition of this interaction impairs HIV-1 replication in human cells (10, 37, 81, 82). The step in the viral replication cycle where CypA is involved was demonstrated to be an event after penetration of the virus particle and before integration of the double-stranded viral DNA into the cellular genome (13, 51, 82).

CypA is a member of the immunophilin class of proteins. These ubiquitous cellular proteins possess *cis-trans* prolyl isomerase (PPIase) activities (27) and are assumed to be involved in protein folding and to function as chaperones in intracellular transport (78). Cyclophilins are also known to be the intracellular receptor molecules for cyclosporines (35), a class of cyclic undecapeptides produced by *Trichoderma polysporum* (25, 71). Binding of cyclosporines to cyclophilins leads to the blockade of the isomerase activity. The most prominent representative of this class of compounds is cyclosporine (Cs), a potent inhibitor of T-cell activation widely used in the clinic as an immunosuppressant in organ transplantation (11). When Cs is bound to CypA it forms a ternary complex with calcineurin. This binding inhibits the phosphatase activity of calcineurin, which is crucial for signal transduction in the activation cascade of T cells. Inhibition of calcineurin function is thus the molecular basis of the immunosuppressive action of Cs (8). The structure of Cs bound to its ligands has been elucidated: two separate domains in the undecapeptide Cs that are involved in binding to CypA and calcineurin, respectively, can be distinguished (40, 43, 66, 94, 98). The immunosuppressive capacity of Cs can therefore be separated from its affinity to CypA by chemical modification.

The anti-HIV-1 activity of Cs was first reported in 1988 (91). Evaluation of this drug and many derivatives for inhibition of HIV-1 replication revealed that nonimmunosuppressive Cs analogs had anti-HIV-1 activities equal to or even superior to those of immunosuppressive analogs (7, 10). A clear correlation was evident, however, between their antiviral activities and the ability of cyclosporines to bind to their cellular receptor protein, CypA. Modification of amino acids in the calcineurin binding domain of Cs, in particular, substitution of the undecapeptide at position 4, was shown to abolish the formation of the ternary complex with calcineurin and, thus, led to the loss of its immunosuppressive capacity (62, 96, 105). Binding to CypA and anti-HIV-1 activity were not impaired by such modifications. (Methyl Ile4)cyclosporine (NIM811), a nonimmunosuppressive cyclosporine with a high affinity for binding to CypA and also potent and selective anti-HIV-1 activity, was characterized in more detail (68). Mode-of-action studies revealed that NIM811 inhibited the CypA-CA interaction in a dose-dependent manner (10, 87) and that an early step in the replication cycle of HIV-1 was impaired in the presence of the compound (10, 51, 82). NIM811 was produced by fermentation of the fungus *Tolypocladium niveum*, followed by extraction and purification (89).

Chemical derivatization procedures for the production of a large variety of Cs derivatives are well established. A large amount of knowledge exists about the structure-activity relationships regarding immunosuppressive capacity, CypA binding, and anti-HIV-1 activity (7, 10, 41, 96, 105). We set out to synthesize a series of novel cyclosporines using Cs as a starting material. The goal was to produce by chemical derivatization nonimmunosuppressive cyclosporines with increased affinities for cyclophilin, which was expected to yield derivatives with improved anti-HIV-1 activities. The most promising compound in this series of derivatives turned out to be (D-MeAla3–EtVal4)cyclosporine (Debio-025; where Me and Et are methyl and ethyl, respectively) (97). The in vitro pharmacological profile of this novel CypA-blocking compound and, in particular, its anti-HIV-1 potential are described in this report.

TABLE 1. CypA PPIase inhibition and activity against HIV-1 replication

Compound	*Ki* (nM) for CypA PPIase inhibition	IC_{50} (μM) for HIV-1		
		IIIB in MT-4 cells[a]	IIIB in PBMCs[b]	SF2 in PBMCs[b]
Cs	9.79 ± 1.37	>0.83	0.45	0.54
NIM811	2.11 ± 0.32	0.31 ± 0.11	0.47	0.5
Debio-025	0.34 ± 0.12	0.099 ± 0.063	0.064	0.063
AZT	ND[c]	0.05 ± 0.01	0.06	0.014

[a] The CPE was determined by MTT staining; values are means ± standard deviation from four determinations.
[b] As determined by a p24 ELISA; values are from one determination.
[c] ND, not determined.

MATERIALS AND METHODS

Compounds and materials. Cs and NIM811 are natural metabolites of *Tolypocladium niveum* (also designated *Beauveria nivea*) and were obtained from Novartis AG, Basel, Switzerland. The synthesis of Debio-025 by the use of Cs as the starting material was based on the strategy for the synthesis of the (D-MeAla3–EtXaa4)cyclosporines described previously (41, 97).

3′-Azido-3′-deoxythymidine (AZT) was purchased from Sigma (St. Louis, MO) or Sigma-Aldrich (Bornem, Belgium). The other approved antiretroviral drugs used in the combination assays were obtained from the NIAID AIDS Research and Reference Reagent Program.

To prepare concentrated stock solutions, the cyclosporines were solubilized in ethanol or dimethyl sulfoxide (for the mixed lymphocyte reaction [MLR] assay) and were stored at −20°C; all other compounds were dissolved in dimethyl sulfoxide or sterile distilled H_2O as appropriate and were stored at −20°C.

Human recombinant CypA was purchased from Sigma AG, Buchs, Switzerland. The antigen keyhole limpet hemocyanin (KLH) was obtained from Pierce Science France SAS, Brebieres, France. Sprague-Dawley rats were purchased from a commercial breeder (Charles River Laboratories France, l'Arbresle, France).

Viruses. The following strains were obtained from the NIAID AIDS Research and Reference Reagent Program: HIV-1 strains IIIB/LAI, NL4-3, SF162, JR-CSF, and Ba-L; HIV-2 strains CDC310319 and CDC310342; and simian immunodeficiency virus (SIV) strain mac 251. HIV-1 strains IIIB/LAI and SF2, which were used for the assays whose results are presented in Table 1, were provided by Guido van der Groen (Institute of Tropical Medicine, Antwerp, Belgium).

The low-passage, lymphotropic clinical HIV-1 isolates WEJO, ROJO, TEKI, and SLKA were obtained from pediatric patients attending the AIDS Clinic at the University of Alabama at Birmingham and were isolated by the Southern Research Institute. The following additional HIV-1 isolates used in this study were obtained from the NIAID AIDS Research and Reference Reagent Program and were originally isolated from seropositive individuals or symptomatic patients from the indicated geographic locations: 92BR014, 93BR020, and 92BR025 from Brazil; 92RW016 from Rwanda; 92UG037 and 92UG046 from Uganda; CMU02 from Thailand; Jv1083 from Nigeria; and BCF01 from Cameroon. The SIV_{PBj14} isolate was recovered from a pig-tailed macaque at the Yerkes Primate Center and was provided by Mark Lewis; this isolate is referred to as SIV_{PBj}.

The multidrug-resistant isolates were obtained as follows: isolate G910-6 was from the NIAID AIDS Research and Reference Reagent Program; isolates 52-52 and 1064-52 were from William A. Schleif of Merck Research Laboratories, West Point, PA; and isolate MDR769, MDR1385, and MDR3761 were from

Thomas C. Merigan, Stanford University. HIV-1 G910-6 contains the mutations M41L, V60I, D67N, K70R, K83R, H208Y, T215Y, K219Q, L239V, R356K, and G359S in RT; this mutation pattern confers intermediate resistance to abacavir (ABC), zalcitabine (ddC), didanosine (ddI), and tenofovir (TDF) and high-level resistance to AZT and stavudine (d4T). HIV-1 52-52 contains the mutations L10R, M46I, L63P, A71V, V82T, and I84V in protease; this mutation pattern confers intermediate resistance to lopinavir (LPV) and high-level resistance to amprenavir (APV), atazanavir (ATV), indinavir (IDV), nelfinavir (NFV), rotinavir (RTV), and saquinavir (SQV). HIV-1 1064-52 contains the mutations L10I, I54V, L63P, A71T, V82F, and L90M in protease; this mutation pattern confers intermediate resistance to APV, ATV, and LPV and high-level resistance to IDV, NFV, RTV, and SQV. HIV-1 MDR769, MDR1385, and MDR3761 have been partially described previously (61). Highly drug-resistant HIV-1 clinical isolates are cross-resistant to many of the antiretroviral compounds in current clinical development. Briefly, MDR769 has the RT mutations M41L, A62V, K65R, D67N, V75I, F116Y, Q151M, Y181I, L210W, and T215Y and the protease mutations L10I, M36M/V, M46I, I54V, L63P, A71V, V82A, I84V, and L90M; this mutation pattern confers low-level resistance to efavirenz (EFV); intermediate resistance to lamivudine (3TC) and emtricitabine (FTC); and high-level resistance to ABC, AZT, d4T, ddC, ddI, TDF, delavirdine (DLV), nevirapine (NVP), APV, ATV, IDV, LPV, NFV, RTV, and SQV. MDR1385 has the protease mutations L10I, M36I, M46I, I54I/V, L63P, A71V, V82T, L90M, and I93L; this mutation pattern confers high-level resistance to APV, ATV, IDV, LPV, NFV, RTV, and SQV. In addition, although the mutation pattern in RT is not known, MDR1385 has been found to be resistant to AZT, ddI, 3TC, and d4T (R. G. Ptak, unpublished data). Finally, MDR3761 has the protease mutations L10I, K20I, M46I, L63P, A71I, G73T, I84V, L90M, and I93L; this mutation pattern confers intermediate resistance to LPV and high-level resistance to APV, ATV, IDV, NFV, RTV, and SQV. Similar to MDR1385, MDR3761 does not have a documented mutation pattern in the sequence for RT; however, resistance to AZT, ddI, 3TC, and d4T has been observed (R. G. Ptak, unpublished). Unless otherwise noted, resistance is predicted on the basis of the resistance analysis tools from the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/).

Aliquots of all viruses with predetermined titers were removed from the freezer (−80°C) thawed and rapidly to room temperature in a biological safety cabinet immediately before use.

Cells. The human T-cell leukemia virus-transformed T4 cell line MT-4 (50) and the human T4 lymphoblastoid cell line CEM-SS (53) were used for the cytopathic effect (CPE) inhibition assays. Jurkat cells (95) were used in the immunosuppression test. $CD4^+$ HeLa cells (TZM cells), which were used in the real-time PCR, and CEM-SS cells were obtained from the NIAID AIDS Research and Reference Reagent Program.

Peripheral blood mononuclear cells (PBMCs) for the antiviral assays whose results are shown in Table 1 and for the MLR with human cells were purified from the buffy coats of HIV-negative donors kindly provided by the Belgian Red Cross (Bloedtransfusiecentrum, Leuven, Belgium). For all other drug susceptibility assays, fresh human blood was obtained commercially from Interstate Blood Bank, Inc. (Memphis, TN); PBMCs were isolated from fresh human blood from screened donors found to be seronegative for HIV and hepatitis B virus. Murine PBMCs for the MLR were isolated from the spleens of BALB/c or CBA/Ca mice.

PPIase inhibition assay. The inhibition of the PPIase activity of CypA was used to compare the inhibitory potential of cyclosporines as an indication of their binding affinity to CypA. The PPIase activity was measured in a chymotrypsin-coupled spectrophotometric assay based on the method described previously (32). The assay takes advantage of the high *trans* selectivity of chymotrypsin for peptides of the *N*-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide type. Thus, the hydrolysis of the *cis* form of this peptide is limited by the rate of *cis-trans* isomerization catalyzed by CypA. The assay was performed in a split-beam spectrophotometer set at 390 nm and at a temperature of 5°C. CypA was equilibrated for 1 h at 5°C with the cyclosporines by using a drug concentration range from 2 to 50 nM. The reaction was started by addition of the peptide, and the change in absorbance was monitored spectrophotometrically at 10 data points per second. The blank rates of hydrolysis (in the absence of CypA) were subtracted from the rates in the presence of CypA. The initial rates of the enzymatic reaction were analyzed by first-order regression analysis of the time course of the change in absorbance.

CPE inhibition assay with cell lines. MT-4 cells or CEM-SS cells, which are known to be highly permissive for HIV-1, were infected in their exponential growth phase essentially as described by Pauwels et al. (63). Inhibition of the CPE induced by HIV-1 strain IIIB/LAI after 5 or 6 days of infection was determined by measuring the viabilities of both HIV-1-infected and mock-infected cells.

The compound solutions were serially diluted and added to microtiter plates, and then the cell suspension was added, followed by addition of virus diluted to give from 85 to 95% cell killing at 5 or 6 days postinfection (multiplicity of infection, approximately 0.01). Samples were evaluated by obtaining triplicate measurements for 12 concentrations of drug at half-log dilutions. After 5 or 6 days of incubation, the viability of the cells was determined by staining with the soluble tetrazolium-based dye methyl tetrazolium sulfate (CellTiter reagent; Promega) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Bornem, Belgium). These dyes are metabolized by the mitochondrial enzymes of metabolically active cells to yield a soluble formazan product, allowing the rapid quantitative colorimetric analysis of cell viability and compound cytotoxicity. By using in-house computer programs, 50% inhibitory concentrations (IC_{50}s) and 50% cytotoxic concentrations (TC_{50}s) were calculated. AZT was evaluated in parallel as a positive control in the anti-HIV assays.

Antiviral activity assays with human PBMCs. Human PBMCs were purified over lymphocyte separation medium (density, 1.078 ± 0.002 g/ml; Cellgro; Mediatech, Inc.). The PBMCs were resuspended at 1×10^7 cells/ml in RPMI 1640 supplemented with 15% fetal bovine serum, 2 mM L-glutamine, and 4 μg/ml phytohemagglutinin P (PHA-P) and were incubated for 48 to 72 h at 37°C. After stimulation, the PBMCs were centrifuged and resuspended in RPMI 1640 with 15% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 20 U/ml recombinant human interleukin-2 (IL-2). The PBMCs were maintained in this medium at a concentration of 1×10^6 to 2×10^6 cells/ml with twice-weekly medium changes until they were used in the assay protocol. Monocyte-derived macrophages were depleted from the culture as the result of adherence to the tissue culture flask.

For the PBMC assay, PHA-P-stimulated cells from at least two healthy donors were pooled, diluted in fresh medium, and plated in the wells of a 96-well round-bottom microplate. Test drug dilutions were used in a standard format. After a 2-h preincubation (cells plus drug), a predetermined dilution of the virus stock was added to each test well (final multiplicity of infection, ≅0.1). Wells with cells and virus alone were used as a virus control. Separate plates were prepared identically without virus for drug cytotoxicity studies by using the methyl tetrazolium sulfate assay system (described above). The PBMC cultures were maintained for 7 days following infection, at which time cell-free supernatant samples were collected and assayed for RT activity in a microtiter plate-based RT reaction (16). The results presented in Table 1 were obtained with a modified PBMC preparation protocol, in which prior to stimulation, $CD8^+$ cells were removed from the cell population by adsorption onto anti-CD8-coated magnetic beads, as described by the manufacturer (Dynal Bioscience, Skoyen, Norway). In these assays HIV-1 replication was quantified by a p24 enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol (Perkin-Elmer, Belgium).

The IC_{50}s and TC_{50}s were calculated by using in-house computer programs. AZT was evaluated in parallel as a positive control.

Drug combination assays. Combination antiviral assays were performed with CEM-SS cells and the HIV-$1_{IIIB/LAI}$ strain of virus in a CPE inhibition assay, as described above. For each combination assay, five concentrations of drug A (i.e., the FDA-approved drugs 3TC, ABC, AZT, d4T, ddC, ddI, ETC, TFV, DLV, EFV, NVP, APV, ATV, IDV, LPV, NFV, RTV, SQV, and enfuvirtide) were tested in all possible combinations with eight concentrations of drug B (Debio-025). Three replicates were used to determine the antiviral efficacy of the combination, and two replicates were used to determine the cytotoxicity of the combination for uninfected CEM-SS cells. Each combination assay was performed at least three times.

The drug combination assay data were then analyzed by the method of Prichard and Shipman (65) by using the MacSynergy II program for data analysis and statistical evaluation. Briefly, the MacSynergy II program calculates the theoretical additive interactions of the drugs on the basis of the Bliss independence mathematical definition of expected effects for drug-drug interactions. The Bliss independence model is based on statistical probability and assumes that the drugs act independently to affect virus replication; this independent-effects model is also referred to as a dual-site model and was used for all combination analyses reported here. Theoretical additive interactions are calculated from the dose-response curves for each drug used individually. This calculated additive surface, which represents predicted or additive interactions, is then subtracted from the experimentally determined dose-response surface to reveal regions of nonadditive activity. The resulting surface would appear as a horizontal plane at 0% inhibition above the calculated inhibition if the interactions were merely additive. Any peaks above this plane of additivity would be indicative of synergy. Similarly, any depressions below the plane of additivity would indicate antago-

nism. The 95% confidence intervals around the experimental dose-response surface were used to evaluate the data statistically, and the volume of the peaks/depressions was calculated on the basis of the 95% confidence interval and was used to quantify the volume of synergy/antagonism produced. The volume of the peaks observed in the synergy plots (in units of concentration times concentration times percent, e.g., μM^2 %, nM^2 %, and nM μM %) was calculated by the program. This peak volume is the three-dimensional counterpart of the area under a three-dimensional dose-response surface and is a quantitative measure of synergy or antagonism.

Synergy was defined as drug combinations yielding synergy volumes greater than 50. Slightly synergistic activity and highly synergistic activity have been operationally defined as yielding synergy volumes of 50 to 100 and >100, respectively. Additive drug interactions have synergy volumes in the range of −50 to 50, while synergy volumes between −50 and −100 were considered slightly antagonistic and those <−100 were highly antagonistic.

In vitro assays for immunosuppression. IL-2 promoter activation upon T-cell stimulation was determined in a Jurkat T-cell line containing the β-galactosidase gene as a reporter under IL-2 promoter control (8).

For the MLR (49), responder cells were prepared from the spleens of BALB/c mice and PBMCs were purified by Ficoll high-density-gradient centrifugation. A suspension with a concentration of 10×10^6 viable cells per ml was prepared in complete RPMI 1640 medium. Stimulator cells were prepared from the spleens of CBA/Ca mice. The cell suspensions were put on ice and were irradiated (2,000 rads). The cell concentration was adjusted to 10×10^6 cells per ml.

In the MLR assay, six concentrations of Debio-025 ranging from 0.01 to 10 μM and of Cs, as the reference control, ranging from 0.005 to 5 μM were tested. Responder cells, stimulator cells, and test compound solution were mixed 1:1:1 in the wells of a flat-bottom microwell plate. After 102 h of culturing, [^{3}H]thymidine was added and proliferation was determined by measuring the incorporation of [^{3}H]thymidine after 18 h of additional incubation.

For the MLR assay with human cells, freshly isolated $CD4^+$ PBMCs from two healthy donors were mixed after one of the cell populations had been inactivated by irradiation (stimulator cells). After 5 days of cocultivation in the presence of test compound (0.83 μM), the proliferative response of the noninactivated cell population (responder cells) was determined by measurement of [^{3}H]thymidine incorporation. This assay was conducted reciprocally with the two populations, each being inactivated and stimulated in turn.

In vivo assay for immunosuppression. The potential immunosuppressive activity of Debio-025 was evaluated in the rat KLH model (39). Cs was used as a positive (immunosuppressive) control. Microemulsions of both compounds were prepared by using a cremophor-ethanol formulation. Young male Sprague-Dawley rats were assigned to one control and four treatment groups (six animals per group). Debio-025 was administered once daily by the oral route (via gavage) at 10, 25, or 50 mg/kg of body weight/day; and the reference drug (Cs) was administered at 10 mg/kg/day for 28 consecutive days. The control group received placebo microemulsion. On day 22, the KLH antigen (1 ml of a solution at 2.5 mg/ml) was administered by the subcutaneous route to all animals. At the end of treatment (day 29), venous blood was taken from the orbital sinus of all animals under anesthesia and the animals were euthanized. Serum was prepared and was kept at −20°C until analysis. Anti-KLH immunoglobulin M (IgM) levels were quantified by a specific ELISA method. For peripheral blood lymphocyte subset analysis, aliquots of whole blood were incubated with the following antibody combinations: anti-rat CD3-fluorescein isothiocyanate–anti-rat CD4-phycoerythrin for detection of T-helper cells and anti-rat CD3-fluorescein isothiocyanate–anti-rat CD8-phycoerythrin for T-cytotoxic/suppressor cells and total T lymphocytes. Analysis was performed on a Coulter EPICS XL-MCL flow cytometer, and the data were analyzed with EXPO32 software (Beckman Coulter, France).

Animal experiments were conducted in compliance with Animal Health regulations, in particular, Council Directive No. 86/609/EEC of 24 November 1986.

Sequencing. Virion-associated RNA was extracted (QIAmp viral RNA mini kit; Qiagen) from supernatant virus stocks, and the viral RNA was used as the template RNA for RT-PCR amplification (Superscript III One-Step RT-PCR system; Invitrogen). Forward primer SK145 (5′-AGT GGG GGG ACA TCA AGC AGC CAT GCA AAT-3′) and reverse primer SK39 (5′-TTT GGT CCT TGT CTT ATG TCC AGA ATG C-3′) (56) were used to amplify the CA region of the HIV-1 and HIV-2 isolates. The DNA sequence of both strands of the PCR-amplified region was subsequently determined by double-stranded DNA sequencing with primers SK145 and SK39 at the University of Alabama at Birmingham Center For AIDS Research Sequencing Core facility. SeqMan (DNAStar; Madison, WI) was used to analyze the resulting DNA sequence, which was used to determine the predicted amino acid sequence of the CA region of the viruses.

Quantitative real-time PCR. Strain NL4-3 virus stocks derived from the transfection of 293T cells were treated with 50 U/ml Turbo DNase (Ambion) for 60 min at 37°C. $CD4^+$ HeLa cells (TZM cells; 2×10^5 cells) were infected with 2×10^4 RT units of NL4-3, and the genomic DNA was isolated at various time points. Early reverse transcripts were quantified with primers ert2f (5′-GTGCC CGTCTGTTGTGTGAC-3′) and ert2r (5′-GGCGCCACTGCTAG AGATTT-3′) and the probe ERT2 (5′-[6-carboxyfluorescein]-CTAGAGATCCCTCAGA CCCTTTTAGTCAGTGTGG-[6-carboxytetramethylrhodamine]-3′) (9), and late reverse transcripts were quantified with primers MH531 (5′-TGTGTGCCC GTCTGTTGTGT-3′) and MH532 (5′-GAGTCCTGCGTCGAGAGAGC-3′) and the probe LRT-P (5′-[6-carboxyfluorescein]-CAGTGGCGCCCGAACAG GGA-[6-carboxytetramethylrhodamine]-3′) (18). The reaction mixtures contained the TaqMan universal master mixture (PE Biosystems), 300 nM primers, 100 nM probe, and 500 ng of genomic DNA. The PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C; the PCRs were performed on an ABI Prism 7700 apparatus (Applied Biosystems).

RESULTS

Correlation of anti-HIV-1 activity of cyclosporines with CypA PPIase inhibition. We evaluated a series of novel cyclosporines that had been generated by chemical modification of Cs for inhibition of the PPIase activity of CypA (97). The goal was to identify compounds with an increased potential to inhibit CypA PPIase activity, which was assumed to be based on the higher affinity of the compounds to CypA and, consequently, would lead to an improved capacity to block the interaction of CypA with the HIV-1 CA protein. Such derivatives were expected to exhibit potent anti-HIV-1 activities. The focus of chemical derivatization was on modification of the cyclophilin binding domain as well as on the generation of a nonimmunosuppressive molecule. Several of the most potent PPIase inhibitors were evaluated for their inhibition of HIV-1 strain IIIB/LAI replication in MT-4 cells and for their inhibition of strains IIIB/LAI and SF2 replication in PBMCs (data not shown).

Debio-025 (Fig. 1) proved to be the most promising compound in this series of derivatives. Table 1 compares the ability of Debio-025 to inhibit PPIase and HIV-1 replication with the abilities of the nonimmunosuppressive cyclosporine NIM811, Cs, and the reference compound (AZT). The K_i value for PPIase inhibition of Debio-025 (0.34 ± 0.12 nM) was about 29-fold lower than that of Cs (9.79 ± 1.37 nM). For comparison, NIM811 (K_i, 2.11 ± 0.32 nM) was five times more potent than Cs in this regard. The relative potencies of the test compounds for the inhibition of HIV-1 replication correlated with the activity against PPIase: Debio-025 (IC_{50}s, 0.063 to 0.099 μM) proved to be a 5- to 10-fold more potent inhibitor than Cs, and NIM811 (IC_{50}s, 0.31 to 0.5 μM) was an approximately 2- to 3-fold more potent inhibitor than Cs (IC_{50}s, 0.45 to >0.83 μM). At the highest concentration used in these assays (0.83 μM), no cytotoxic effect was detectable for any of the cyclosporines. The reference compound, AZT (IC_{50}s, 0.05 to 0.014 μM), inhibited HIV-1 replication at 10 to 40 times lower concentrations than Cs.

We conclude from these results that the increased inhibitory potential for CypA PPIase activity of Debio-25 compared to the inhibitory potentials of Cs and NIM811, which indicates a higher affinity of binding of this compound to CypA, indeed translates, as expected, to the more potent inhibition of HIV-1 replication.

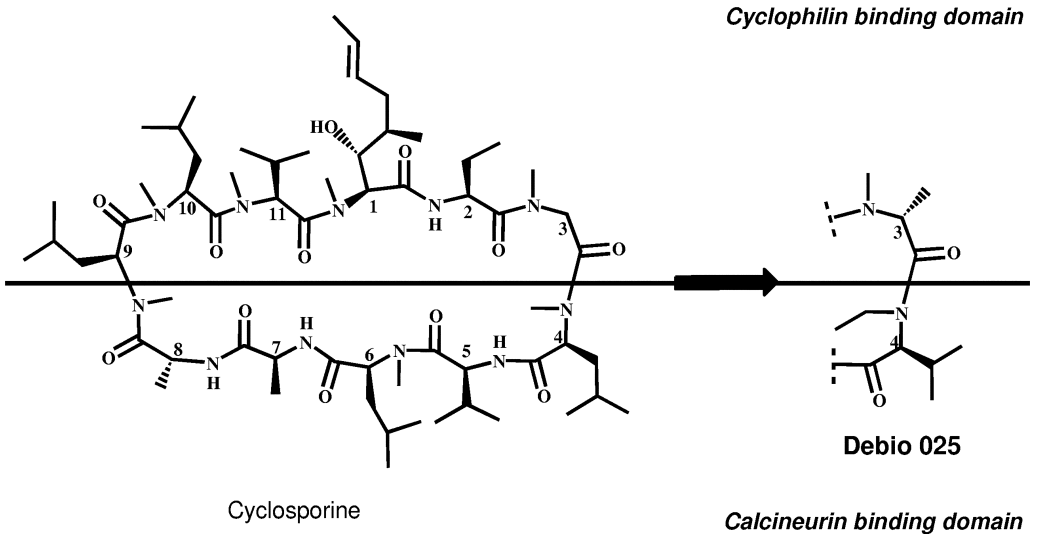

FIG. 1. Structural formulas of Cs and Debio-025. The top parts of the structures represent the cyclophilin binding domain, and the lower parts represents the calcineurin binding domains.

Lack of immunosuppressive activity of Debio-025 in vitro and in vivo. From structure-activity relationships published previously (7, 10, 62, 66, 105), we expected that the substitution of MeLeu at position 4 of Cs by EtVal in Debio-025 would abolish the immunosuppressive capacity of the cyclosporine. To prove the absence of immunosuppressive activity for Debio-025 in vitro, the compound was evaluated in the IL-2 reporter gene assay with Jurkat cells (8) and in the MLR (49) with murine and human primary cells. Cs is known to specifically block the signaling pathway in T lymphocytes, leading to IL-2 promoter activation upon T-cell stimulation (45), which is the molecular basis of its immunosuppressive activity. In a Jurkat cell line containing the β-galactosidase gene under IL-2 promoter control, Cs inhibited 50% of the PHA-phorbol myristate acetate-induced β-galactosidase activity at a concentration of 0.003 μM. For Debio-025, a more than 7,000-fold higher concentration (21.5 μM) was needed to bring about the same effect, and for NIM811 a more than 2,000-fold higher concentration (6.75 μM) was needed to bring about the same effect. Since these concentrations are known to be in the cytotoxic range for T-cell lines (68), these results essentially prove that Debio-025 and NIM811 have strongly reduced capacities to inhibit the signaling pathway in T lymphocytes leading to IL-2 promoter activation.

In the murine MLR, the IC_{50} of Cs for [^{3}H]thymidine incorporation after allogeneic stimulation was 0.056 μM, whereas Debio-025 exhibited an IC_{50} of 0.82 μM; thus, it is 15-fold less active than Cs in this assay. In a two-way MLR with human $CD4^+$ PBMCs, Cs at 0.83 μM reduced the proliferative response by a factor of 10, while Debio-025 as well as NIM811 at the same concentration showed no significant immunosuppressive activity (D. Jochmans, unpublished data).

To evaluate the potential immunosuppressive activity of Debio-025 in vivo, the rat KLH model (39) was used. Three concentrations of Debio-025 and Cs as the reference compound were administered orally to rats for 4 weeks. One week before the end of treatment, KLH was injected subcutaneously for antigenic stimulation. The immune responses were quantified by measuring IgM levels and by peripheral blood lymphocyte subset analysis. The data in Table 2 demonstrate that treatment with Debio-025 at 10, 25, or 50 mg/kg/day for 4 weeks did not have an effect on the IgM response in animals immunized with KLH, nor did it influence the relative amounts of lymphocyte subset populations. In animals given Cs at 10 mg/kg/day, significantly lower anti-KLH IgM levels as well as significantly reduced T lymphocytes and cytotoxic T-cell populations were observed. The amounts of T-helper cells were also reduced, but the difference did not reach significance.

Thus, Debio-025 was demonstrated to have significantly decreased or no immunosuppressive activity compared to that of Cs in vitro and in vivo.

Effects of Debio-025 on replication of various HIV-1 isolates in PBMCs. The antiviral efficacy of Debio-025 was evaluated in a standardized PBMC-based anti-HIV-1 assay with a panel of HIV-1 clinical isolates and laboratory strains from different geographic locations that included HIV-1 group M subtypes A, B, C, D, EA (E/A recombinant), F, and G, as well as HIV-1 group O. The panel included CCR5-tropic (R5), CXCR4-tropic (X4), and dual-tropic (R5X4) viruses; pediatric clinical

TABLE 2. Immunosuppressive capacity in vivo

Treatment	Dose (mg/kg)	IgM level (absorbance)[a]	Immunological response (% gated cells) after KLH immunization in rats[a]		
			T lymphocytes	T-helper cells	Cytotoxic T cells
Vehicle	0	0.87 ± 0.41	48.2 ± 5.7	30.1 ± 2.7	17.2 ± 4.1
Cs	10	0.18 ± 0.09[b]	33.6 ± 6.6[b]	23.1 ± 5.5	9.2 ± 1.3[b]
Debio-025	10	0.78 ± 0.41	52.4 ± 3.3	29.8 ± 12.1	17.6 ± 3.1
Debio-025	25	0.78 ± 0.029	53.4 ± 5.0	34.5 ± 2.9	18.0 ± 3.9
Debio-025	50	0.67 ± 0.41	50.0 ± 4.1	32.8 ± 5.3	16.1 ± 2.7

[a] The results are mean values ± standard deviation.
[b] $P < 0.01$ versus the results for the vehicle control.

TABLE 3. Efficacy of Debio-025 against various HIV-1 subtype isolates in PBMCs[a]

Virus	Subtype	Coreceptor usage[b]	IC_{50} (μM)[c]
92RW016	A	R5	0.008 ± 0.003
92UG037	A	R5	0.016 ± 0.012
NL4-3	B	X4	0.010 ± 0.007
JR-CSF	B	R5	0.015 ± 0.009
SF162	B	R5	0.015 ± 0.005
Ba-L	B	R5	3.53 ± 1.74
92BR014	B	R5X4	5.65 ± 2.43
SLKA	B[d]	R5	0.025[e]
TEKI	B[d]	R5	4.41 ± 3.12
ROJO	B[d]	X4	0.024 ± 0.022
WEJO	B[d]	X4	0.015 ± 0.006
92BR025	C	R5	0.015 ± 0.009
92UG046	D	X4	0.010 ± 0.005
CMU02	EA	X4	5.66 ± 0.42
93BR020	F	R5X4	5.53 ± 2.69
Jv1083	G	R5	6.25 ± 0.34
BCF01	O	R5	6.25 ± 0.98

[a] Efficacy was measured as RT activity.
[b] R5, CCR5; X4, CXCR4; R5X4, CCR5/CXCR4 dual tropic.
[c] Values are means ± standard deviations from 2 to 12 independent assays, depending on the virus isolate.
[d] Presumed subtype B.
[e] The value for HIV-1 SLKA is from a single assay.

isolates were also included. As summarized in Table 3, Debio-025 inhibited the replication of viruses from group M subtypes A, B, C, and D. The virus isolates from group M subtypes EA, F, and G and group O were not significantly inhibited by Debio-025; however, only one representative isolate of each subtype was tested. There was no detectable correlation between anti-HIV-1 activity and coreceptor usage, between anti-HIV-1 activity and the geographical location of virus isolation, or between anti-HIV-1 activity and whether the isolates were from pediatric populations versus adults. The IC_{50} values were mostly in the range of 0.01 to 0.02 μM. Cytotoxic effects were observed for Debio-025 in these assays with PBMCs only at 33.2 μM. Interestingly, in addition to the single isolates from group M subtypes EA, F, and G and group O, three subtype B HIV-1 strains were inhibited only by significantly higher concentrations by Debio-025, namely, 3 to 6 μM; thus, these viruses exhibited naturally occurring resistance to Debio-025. Recently, a much larger number of HIV-1 clinical isolates was evaluated for inhibition of replication by Debio-025: of 238 strains tested in infected $CD4^+$ HeLa cells, 36 (15%) were resistant to the compound to some degree (P. Gallay, unpublished data). The percentage of naturally resistant strains was similar in most group M subtypes (subtypes A, B, D, E, and F); however, only 2 of 35 isolates of subtype C and all 4 subtype G strains tested were resistant to Debio-025. Also, two of the eight group O isolates tested were naturally resistant; the one group N isolate in our collection was sensitive to Debio-025.

It should be emphasized that the naturally occurring resistance to Debio-025 that was observed is not an all-or-none phenomenon: the data presented in Fig. 2 compare the dose-response curves for Debio-025 against sensitive and resistant viruses and demonstrate a variety of inhibition curves for the seven Debio-025-resistant HIV-1 isolates listed in Table 3 and for the one resistant isolate listed in Table 4 (data described below), indicating that moderate to high degrees of natural resistance to Debio-025 occur with some HIV-1 isolates.

Effects of Debio-025 on replication of HIV-2 and SIV isolates in PBMCs. It has been reported previously that cyclosporines do not inhibit the replication of HIV-2 and SIV strains (14, 68) because these viruses do not depend on CypA for their replication in human cells. A lack of affinity of the HIV-2 CA protein for CypA and a lack of incorporation of CypA into HIV-2 virions were also demonstrated (10, 30, 87). The antiviral efficacy of Debio-025 against two SIV and two HIV-2 clinical isolates was evaluated in PBMCs. Both SIV strains, mac 251 and PBj, proved to be resistant to inhibition by Debio-025, as expected (IC_{50}s, 8.24 ± 1.37 and 7.97 ± 4.18 μM, respectively). Surprisingly, one of the two HIV-2 clinical isolates (isolate CDC310342) was found to be sensitive to the inhibition of replication by Debio-025, with an IC_{50} value (0.058 ± 0.046 μM) similar to the values for HIV-1 strains. The other HIV-2 isolate (isolate CDC310319) was resistant to Debio-025 (IC_{50}, 10.52 ± 0.35 μM). A larger number of HIV-2 clinical isolates and laboratory strains (in total, 33) infecting $CD4^+$ HeLa cells was recently evaluated for the inhibition of replication by Debio-025: three additional isolates were found to be sensitive to inhibition by Debio-025 (Gallay, unpublished). Thus, 4 of 33 HIV-2 isolates tested were effectively inhibited by Debio-025.

Amino acid sequences of CypA binding regions of CA protein from Debio-025-sensitive and -resistant HIV-1 and HIV-2 strains. To explain the different inhibitory activities of Debio-025 against the various strains of HIV-1 and HIV-2, the amino acid sequences of the CypA binding domains in the CA protein were determined for most of the HIV-1 isolates from Table 3 and for the two HIV-2 isolates described above. The sequence alignments for amino acids 85 to 99 of the CA proteins for the HIV-1 isolates are shown in Table 5. The amino acids directly involved in cyclophilin binding, namely, G89 and P90, were conserved in all strains. The most obvious amino acid change associated with resistance to Debio-025 was H87Q/P. Other amino acid changes, such as changes at positions 86, 91, and 96, were also observed in some strains showing high IC_{50} values and may also contribute to resistance. Remarkably, subtype G isolate Jv1083 contained a Q at positions 86 and 87 and an I at position 96. Similar results were reported previously (19) for a panel of HIV-1 isolates that were evaluated in infected $CD4^+$ HeLa cells.

Comparison of the corresponding amino acid sequence in CA protein of HIV-2 clinical isolates CDC310319 and CDC310342 yielded the surprising result that sensitive HIV-2 strain CDC310342 contains exactly the same sequence in this region as the HIV-1 clade A strains (Table 6). Resistant HIV-2 strain CDC310319, however, has the characteristic H87P exchange and additional changes at positions 86, 91, 93, and 96; its sequence thus resembles the HIV-2 subtype A consensus sequence. Further characterization of CypA binding capacities and determination of the amino acid sequences in the cyclo-

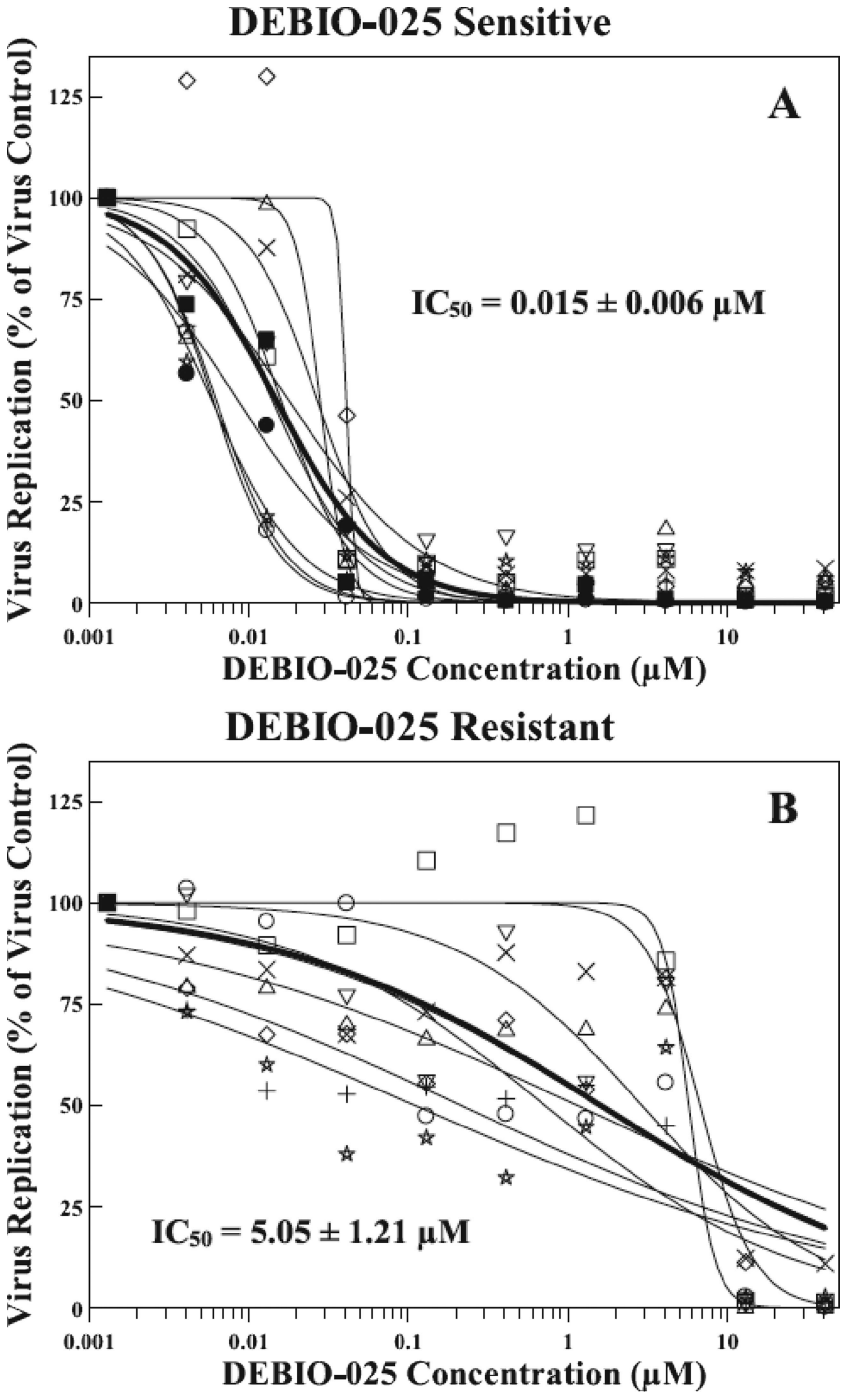

FIG. 2. Comparison of the inhibitory effects of Debio-025 against sensitive and resistant HIV-1 isolates. (A) Inhibitory effects against 10 Debio-025-sensitive isolates: 92RW016 (☆), 92UG037 (●), NL4-3 (○), JR-CSF (+), SF162 (□), SLKA (△), ROJO (◇), WEJO (▽), 92BR025 (×), and 92UG046 (■). (B) Inhibitory effects against eight Debio-025-resistant isolates: Ba-L (+), 92BR014 (□), TEKI (◇), CMU02 (☆), 93BR020 (▽), Jv1083 (△), BCF01 (×), and 52-52 (○). The activity of Debio-025 against HIV-1 isolates in human PBMCs was determined; curve fitting was performed with the XLfit4 (version 4.2.2) software add-in (IDBS, Guildford, Surrey, United Kingdom) for Microsoft Excel; regular lines indicate the results from one representative PBMC assay for each virus isolate, and each data point represents the mean of three replicates per concentration of Debio-025 tested in the assay; boldface lines indicate the global curve fits for the 10 sensitive combined and the 8 resistant isolates combined; IC_{50} values represent the means ± standard deviations of the mean IC_{50} values determined for the 10 sensitive and 8 resistant virus isolates whose data are included here (see Table 4 for data virus isolate 52-52 and Table 3 for data for all other virus isolates).

philin binding domains of the CA proteins of the four sensitive isolates and a number of resistant HIV-2 isolates is ongoing.

Antiviral spectrum of Debio-025. To determine the spectrum of antiviral activity of Debio-025, we evaluated this compound in cellular assays against a panel of viruses from different classes (data are available in Table S1 in the supplemental material). Cs and positive reference drugs for the respective viruses were included as controls. Debio-025 and Cs had no

TABLE 4. Efficacy of Debio-025 against multidrug-resistant strains of HIV-1 in PBMCs[a]

Virus	Drug resistance phenotype	IC_{50} (μM)[b]
$HIV\text{-}1_{1064\text{-}52}$	APV, ATV, LPV, IDV, NFV, RTV, SQV	0.007 ± 0.001
$HIV\text{-}1_{MDR3761}$	LPV, APV, ATV, IDV, NFV, RTV, SQV, AZT, ddI, 3TC, d4T	0.16 ± 0.14
$HIV\text{-}1_{G910\text{-}6}$	ABC, ddC, ddI, TDF, AZT, d4T	0.029 ± 0.029
$HIV\text{-}1_{MDR769}$	APV, ATV, IDV, LPV, NFV, RTV, SQV, 3TC, FTC, ABC, AZT, d4T, ddC, ddI, TDF, EFV, DLV, NVP	0.012 ± 0.006
$HIV\text{-}1_{MDR1385}$	APV, ATV, IDV, LPV, NFV, RTV, SQV, AZT, ddI, 3TC, d4T	0.058 ± 0.023
$HIV\text{-}1_{52\text{-}52}$	LPV, APV, ATV, IDV, NFV, RTV, SQV	3.12 ± 2.57

[a] Efficacy was measured as RT activity.
[b] Values are mean ± standard deviation from two independent assays.

inhibitory effects on the replication of coxsackie B3 virus, influenza virus type A and type B, human parainfluenza virus type 3, herpes simplex virus type 1 and type 2, vesicular stomatitis virus, respiratory syncytial virus, or vaccinia virus. Also, no specific inhibitory effect of Debio-025 or Cs on the growth of human papillomavirus-positive cells could be detected. Debio-025 was observed to have weak activity against varicella-zoster virus, human cytomegalovirus, sudden acute respiratory syndrome-associated human coronavirus, feline coronavirus, and hepatitis B virus. The cytotoxic effects of Debio-025 in these cell lines were observed over a concentration range of 8 to 25 μM.

Since Cs had been reported to inhibit the replication of hepatitis C virus (HCV) in vitro (52, 92), we were particularly interested in studying whether Debio-025 exhibits anti-HCV activity. The antiviral activity was assessed by monitoring the effect of Debio-025 on HCV (genotype 1b) subgenomic replicon replication either by determination of luciferase activity in Huh 5-2 cells or by quantitative RT-PCR with viral RNA isolated from Huh 5-2 cells (60). The IC_{50}s for the inhibition of viral replication in Huh 5-2 cells were 0.22 ± 0.025 μM for Debio-025 and 2.3 ± 0.33 μM for Cs when luciferase activity was monitored. The concentrations that reduced the growth of exponentially growing Huh 5-2 cells by 50% were 22 μM for Debio-025 and 10 μM for Cs. The potent anti-HCV activity of Debio-025 was corroborated by quantitative RT-PCR with Huh 5-2 cells and two other subgenomic replicon-containing cell lines (60). Debio-025 and Cs had no inhibitory effect on the replication of other members of the family *Flaviviridae*, namely, bovine viral diarrhea virus and yellow fever virus (isolate 17D).

Effects of Debio-025 on replication of multidrug-resistant HIV-1 isolates in PBMCs. Since Debio-025 exhibits a mode of action for the inhibition of HIV-1 replication different from the modes of action of all approved anti-HIV-1 drugs, it was expected to be active against virus strains that were resistant to single or multiple inhibitors of other steps of virus replication. Table 4 summarizes the antiviral activities of Debio-025 against six different HIV-1 strains resistant to multiple drugs targeted at RT or protease. Five of these multidrug-resistant strains were inhibited by Debio-025 with low IC_{50} values (0.07 to 0.16 μM). One isolate, however, strain 52-52, proved to be naturally resistant (IC_{50}, 3.12 μM). As described above, resistance to Debio-025 is mostly associated with defined mutations in the CypA binding region of CA. Indeed, isolate 52-52 was found to carry the H87Q and M96I mutations in CA (Table 5). Therefore, the mutations in protease leading to the protease inhibitor resistance associated with this virus are not likely to be the cause of Debio-025 resistance. This interpretation is supported by the data for strains 1064-52, MDR3761, MDR769, and MDR1385, which are also protease inhibitor resistant but which were inhibited efficiently by Debio-025.

Combination of Debio-025 with approved anti-HIV-1 drugs. It can be anticipated that in the clinic a new anti-HIV-1 drug will always be used in combination with approved HIV-1 inhibitors. Therefore, it was important to demonstrate that Debio-025 is not antagonistic in inhibiting HIV-1 replication when it is used with approved drugs. The anti-HIV-1 efficacy

TABLE 5. Amino acid sequences of the CypA binding region in CA of HIV-1 strains

Virus	Subtype	CA sequence at amino acid:															Debio-025 IC_{50} (μM)
		85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	
92RW016	A	P	V	H	A	G	P	I	P	P	G	Q	M	R	E	P	0.008
92UG037	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.016
NL4-3	B	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	0.010
JR-CSF	B	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	0.015
Ba-L	B	—	—	Q	—	—	—	V	A	—	—	—	I	—	D	—	3.53
92BR014	B	—	—	Q	—	—	—	V	A	—	—	—	—	—	—	—	5.65
92BR025	C	—	—	—	—	—	—	V	A	—	—	—	—	—	—	—	0.015
92UG046	D	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	0.010
CMU02	EA	—	A	—	—	—	—	N	—	—	—	—	—	—	—	—	5.66
93BR020	F	—	T	Q	—	—	—	—	—	—	—	—	I	—	—	—	5.53
Jv1083	G	—	Q	Q	—	—	—	—	—	—	—	—	I	—	—	—	6.25
BCF01	O	T	P	P	V	—	—	L	—	—	—	—	I	—	—	—	6.25
52-52	B[a]	—	—	Q	—	—	—	—	A	—	—	—	I	—	—	—	3.12

[a] Presumed subtype B.

TABLE 6. Amino acid sequences of the Cyp binding region in CA of HIV-2 strains

Virus	Subtype	CA sequence at amino acid[a]:															Debio-025 IC_{50} (μM)
		85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	
Consensus sequence	HIV-2 A	P	I	P	*	G	P	L	P	A	G	Q	L	R	E/D	P	ND[b]
CDC310319	HIV-2	—	L/W	—	*	—	—	M	—	—	—	—	—	—	—	—	10.52
CDC310342	HIV-2	—	V	H	A	—	—	I	—	P	—	—	M	—	—	—	0.058
92RW016	HIV-1 A	—	V	H	A	—	—	I	—	P	—	—	M	—	—	—	0.008

[a] *, no amino acid. L/W and E/D, mixed populations.
[b] ND, not determined.

and cytotoxicity of Debio-025 were evaluated in two-drug combination studies with the nucleoside RT inhibitors 3TC, ABC, AZT, d4T, ddC, ddI, ETC, and TFV; the nonnucleoside RT inhibitors DLV, EFV, and NVP; the protease inhibitors APV, ATV, IDV, LPV, NFV, RTV, and SQV; and the fusion inhibitor enfuvirtide.

Each two-drug combination was tested three to six times in CEM-SS T-lymphocytic cells acutely infected with laboratory-adapted strain $HIV\text{-}1_{IIIB}$. Viral replication and its inhibition were quantified by measuring virus-induced CPEs at the experimental end point. The cytotoxicity of each two-drug combination was evaluated in parallel by the antiviral assays. The eight concentrations of Debio-025 (0.2 to 500 nM) used in these evaluations were selected in order to test a range of concentrations broad enough to provide a complete dose-response curve. Similarly, the concentrations of the approved drugs used for the combination were selected to provide a dose-response curve as complete as possible under the limitations of five total concentrations. A positive antagonism control, namely, d4T in combination with ribavirin, was tested in parallel with each of the assays performed. The results from these two-drug combination experiments were analyzed at the 95% confidence level by using the MacSynergy II three-dimensional model of Prichard and Shipman (65) for statistical evaluation of the assays with the combinations. The synergy and antagonism volumes calculated at the 95% confidence level are summarized in Table 7. On the basis of the synergy criteria defined in Materials and Methods, the test compound Debio-025 interacted with all 19 FDA-approved drugs, and the combinations achieved antiviral efficacy in an essentially additive fashion over the concentration ranges examined. A slightly synergistic interaction was observed between Debio-025 and 3TC in two of the three experiments performed. The average interaction of Debio-025 with AZT appeared to indicate a slight antagonism; however, this antagonism was observed in only one of the six experiments performed and is reflected in the high standard deviation for this value. Therefore, the overall interpretation is that Debio-025 interacts with AZT in an additive manner. The interactions of Debio-025 with the other 17 drugs were found to be strictly additive. In contrast, the positive antagonism control of d4T in combination with ribavirin exhibited a highly antagonistic interaction in all experiments (mean antagonism volume, 330 μM^2 %) (data not shown). The overall interpretation of these data suggests that antagonism of the antiviral effects of the 19 approved antiretroviral drugs evaluated in this study should not be an issue associated with the use of Debio-025 in the clinical setting.

Importantly, there was no evidence of synergistic cytotoxicity over the drug concentrations examined for Debio-025 (highest concentration tested, 500 nM) (data not shown). This was not unexpected, since none of the drugs were cytotoxic over the concentration ranges evaluated. Much higher concentrations of all drugs would be required to examine potential synergistic cytotoxicity interactions. However, it is important to document that no notable synergistic toxicities were observed at the concentrations at which the FDA-approved drugs displayed potent antiviral properties.

Mode-of-action studies. It is well documented that cyclosporines abrogate CypA-CA interactions in human cells (10, 48, 87). This interaction of CypA with a defined region around G89 and P90 of the CA protein is important for efficient HIV-1 replication in human cells. Experimental evidence indicated that CypA exerts its function early in the viral replication cycle, i.e., after penetration of the capsid particle into the cytoplasm

TABLE 7. Efficacy of Debio-025 in combination with 19 FDA-approved anti-HIV-1 drugs in CEM-SS cells[a]

Compound group and compound	Synergy vol (nM^2 % or nM μM %)	Antagonism vol (nM^2 % or nM μM %)
Nucleoside RT inhibitors		
3TC	56.9 ± 27.9	7.0 ± 6.6
ABC	23.6 ± 17.5	22.3 ± 19.6
AZT	35.6 ± 39.4	43.4 ± 79.1
d4T	18.8 ± 28.5	10.1 ± 17.2
ddC	12.2 ± 10.9	15.8 ± 13.7
ddI	26.8 ± 37.3	20.3 ± 21.9
FTC	20.0 ± 25.5	9.7 ± 8.5
TFV	28.1 ± 26.0	12.7 ± 11.1
Nonnucleoside RT inhibitors		
DLV	43.6 ± 37.4	16.4 ± 12.7
EFV	32.5 ± 34.1	17.7 ± 11.4
NVP	95.9 ± 63.3	18.0 ± 17.9
Protease inhibitors		
APV	38.8 ± 34.4	0.1 ± 0.2
ATV	14.8 ± 22.9	44.1 ± 39.7
IDV	47.8 ± 29.7	24.9 ± 29.0
LPV	1.3 ± 1.5	30.1 ± 47.7
NFV	17.5 ± 17.3	38.3 ± 48.4
RTV	4.6 ± 7.7	20.4 ± 14.4
SQV	29.3 ± 34.9	26.7 ± 21.4
Fusion inhibitor enfuvirtide	27.8 ± 21.5	0 ± 0

[a] HIV-1 strain IIIB-induced CPE; synergy and antagonism volumes were calculated at the 95% confidence level; values are means ± standard deviations from three to six independent determinations.

and before integration of the double-stranded DNA provirus into the cellular genome (13, 51, 82). For Debio-025, we demonstrated (19) that this compound dose dependently inhibited the incorporation of CypA into the virus particles, which indicates that Debio-025 interferes with the CypA-CA interaction in the virus-producing cell as well as in the target cell. Comparison of the amino acid sequences of the CypA binding site in the CA protein (Table 5) for the inhibition-sensitive and -resistant HIV-1 strains revealed that a mutation changing H87 to Q or P is relevant for the resistant phenotype. The results of specific site-directed mutagenesis studies with the NL4-3 clone were published previously (19) and led to similar conclusions. Furthermore, the generated NL4-3 variants containing the H87Q amino acid exchange did incorporate CypA in the viral particles in the absence of Debio-025 and did not incorporate it in the presence of the drug. The same result was obtained for Debio-025-resistant strains 93BR020, Jv1083, and BCF01 (Gallay, unpublished). This indicated that the naturally Debio-025-resistant virus strains are also able to incorporate CypA into their virions, presumably by interaction with the CA protein, but that their replication in human cells is not significantly impaired when this interaction is blocked by Debio-025. Thus, the replication of the Debio-025-resistant HIV-1 strains in human cells seem to be CypA independent.

To define the point of attack in the viral replication cycle for Debio-025, we monitored the reverse transcription and nuclear import of the viral genome of the HIV-1 NL4-3 wild-type strain in infected $CD4^+$ HeLa cells in the absence and the presence of Debio-025. Genomic DNA was extracted at 2, 16, and 28 h postinfection. Early reverse transcripts (a hallmark of entry), late reverse transcripts (a hallmark of complete reverse transcription), and two long terminal repeat circles (a hallmark of nuclear import) were amplified by PCR with specific pairs of primers. The PCR products were then analyzed by Southern blot with a linearized ^{32}P-radiolabeled NL4-3-specific probe. We found that Debio-025 only slightly decreased the accumulation of early reverse transcripts within the cytosols of infected cells but strongly reduced the formation of late reverse transcript products. The complete absence of two long terminal repeat circles in Debio-025-treated infected cells indicated that it has an inhibitory effect on the completion of viral double-stranded DNA, which obviously led to a lack of transport to the nucleus (Gallay, unpublished).

To further prove that the block in the viral replication cycle exerted by Debio-025 is progression/completion of reverse transcription, a real-time PCR experiment was performed. Figure 3 demonstrates that, in the presence of Debio-025, early reverse transcription took place, although to a largely reduced extent, and that late reverse transcription was almost completely blocked. In fact, at 2 h postinfection early RT products are similar in quantity in Debio-025-treated cells and untreated control cells, which also shows that Debio-025 does not affect HIV-1 entry. At 6 h postinfection, the amount of early cDNA in the control is at its highest level, while in the presence of Debio-025 the amount of early cDNA is less than that at 2 h, indicating degradation of the newly made cDNA. These results are in agreement with the assumption that CypA exerts an important function during the progress of reverse transcription. Disruption of the CypA-CA interaction by Debio-025 presumably leads to blockade of an event, e.g., uncoating, which is necessary for progression/completion of reverse transcription and, consequently, to the lack of transport of the preintegration complex to the nucleus.

DISCUSSION

Here we report on the potent anti-HIV-1 activity of Debio-025, a novel nonimmunosuppressive cyclosporine that was prepared by chemical modification of Cs (41, 97). It has previously been reported that nonimmunosuppressive cyclosporines have anti-HIV-1 activities equal to or even superior to those of immunosuppressive ones (7, 10, 68, 89). Structure-activity relationships revealed a strong correlation between the antiviral activities of cyclosporines and the cyclophilin binding capacities of the compounds (10). Luban et al. (48) have demonstrated that the HIV-1 Gag protein binds to CypA. Upon binding, HIV-1 CA amino acid residues 86 to 93, which form an exposed loop, fill the active site of the CypA peptidyl PPIase (17, 30, 31). The peptidyl-prolyl bond linking CA residues G89 and P90 may undergo catalytic isomerization; however, the significance of the CypA catalytic activity that assists in the folding or unfolding of the CA protein is not proven; CypA may also function as a chaperone during intracellular transport of the viral particle after infection. So far, all CypA variants with reduced catalytic activities have a concomitant reduction in substrate affinity (26); thus, the isomerase activity cannot be separated from the binding function. The binding of CypA to the HIV-1 CA protein as well as PPIase activity are known to be inhibited by Cs (12, 48) and the nonimmunosuppressive cyclosporine NIM811 (10, 87).

We used the PPIase assay to evaluate a series of chemically derived new cyclosporines with the goal of identifying novel potent HIV-1 inhibitors. A compound with an increased inhibitory potential against the CypA isomerase activity was expected to possess an enhanced affinity to CypA and thus should compete more efficiently with the CA protein for binding to CypA. As a consequence, CypA function in the replication cycle of HIV-1 should be impaired, resulting in the less efficient replication of the virus. NIM811 had been reported to have a higher affinity of binding to CypA than Cs and to be a more potent inhibitor of HIV-1 than Cs (10, 68). Debio-025 proved to be the most potent inhibitor of PPIase when its inhibitory potency was compared to the inhibitory potencies of Cs and NIM811, which indeed correlated well with the relative inhibitory capacities against HIV-1 replication for the three cyclosporines. Debio-025 contains three distinct modifications compared to the structure of Cs. One modification is in the cyclophilin binding domain; namely, the sarcosine at position 3 was exchanged for D-methyl alanine, which led to the increased inhibition of PPIase, presumably caused by the higher binding affinity of Debio-025 for CypA. The other two modifications are located in the calcineurin binding domain; namely, the methyl leucine at position 4 was replaced by ethyl valine. On the basis of previous results (7, 10, 62, 98, 105), these modifications were expected to reduce the affinity of the Debio-025–CypA complex for calcineurin. Indeed, Debio-25 proved to have significantly reduced immunosuppressive activity, as was demonstrated (i) in vitro in the IL-2 reporter gene assay with Jurkat cells, (ii) in the MLR with murine and human primary cells, and (iii) in vivo in the rat KLH model. It has previously

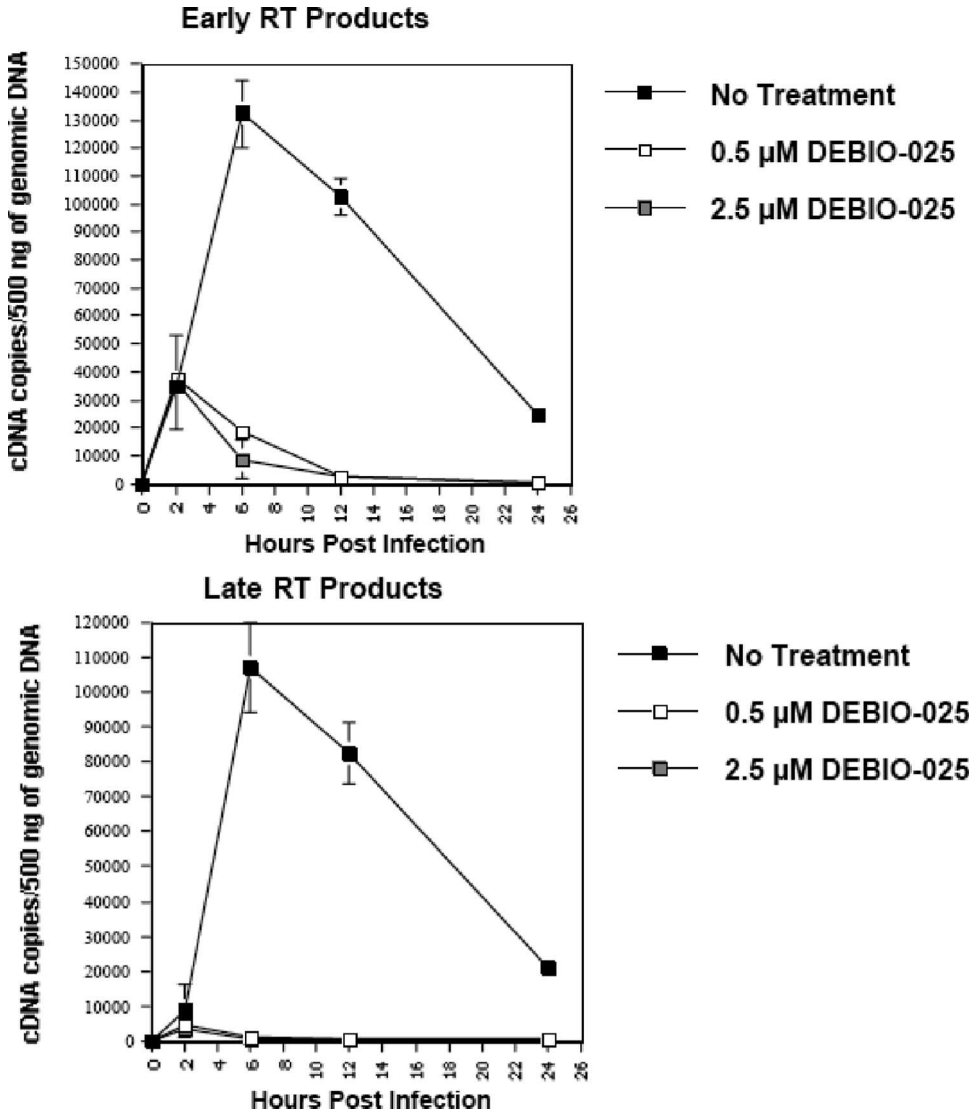

FIG. 3. Debio-025 blocks HIV-1 infection during early reverse transcription. $CD4^+$ HeLa cells (TZM) were infected with DNase-treated NL4-3 HIV-1 together with Debio-025 or without Debio-025 (0.5 or 2.5 μM). Target cell DNA was isolated at the indicated times and was used to detect early (top) and late (bottom) reverse transcripts. Data are the means ± standard deviations from duplicate experiments.

been argued that immunosuppression per se may have a beneficial effect in HIV disease (4). However, clinical results demonstrated that immunosuppressive treatment early in HIV infection is by no means beneficial but, on the contrary, enhances the progression to AIDS (64, 75). Therefore, we believe that a cyclosporine derivative with only antiviral potential and no immunosuppressive activity should be preferred for the treatment of HIV disease.

The anti-HIV-1 activity of Debio-025 against a series of laboratory strains and clinical isolates from different geographic locations was demonstrated in primary cells. Debio-025 inhibited the replication of viruses that use CXCR4 or CCR5 as a coreceptor. HIV-1 strains from most group M subtypes were sensitive to inhibition by Debio-025; however, certain isolates were found to be naturally resistant to this compound.

The comparison of the amino acid sequences of the CypA binding domains of the CA protein from Debio-025-sensitive and -resistant isolates of HIV-1 indicated that the most relevant amino acid change associated with resistance is H87Q/P. Other amino acid changes, like V86A/T/Q/P, I91V/N/L, and/or M96I, may also contribute to resistance. In a previous publication (19), we described that the introduction of mutations in these four positions of CA into laboratory strain NL4-3, which normally relies on CypA for replication and which is thus sensitive to Debio-025, conferred resistance to the viral constructs. The replication of these viruses proved to be independent of the CypA function. The existence of HIV-1 strains naturally resistant to Debio-025 suggests that some clinical isolates may have developed strategies to replicate in human cells independently from CypA.

An analysis of 2,599 HIV-1 capsid sequences from the Los Alamos database indicated that 19.74% of strains contain the H87Q substitution; also, substitutions in CA amino acids 86, 91, and 96 exist naturally in a significant proportion of virus

isolates (19). Experimentally, we found that 36 of 238 strains, i.e., 15%, were resistant to Debio-025 inhibition in $CD4^+$ HeLa cells (Gallay, unpublished). Natural resistance to Debio-025 occurred in most subtypes of HIV-1 group M at a rate of 10 to 18%, with the following exceptions: 4 of 4 isolates of subtype G were resistant, and only 2 of 36 (5.5%) resistant virus strains of subtype C were detected. Group O virus isolates proved to be sensitive or resistant to Debio-025. Interestingly, the consensus sequence for subtype G contains H87Q, which may be the reason for the resistance to Debio-025 observed for this virus subtype. The correlation of resistance with the group O sequence of the CypA binding region is not so obvious: the group O consensus sequence contains P residues at positions 86 and 87; the group O strain tested here, strain BCF01, carries the V86P mutation as well as the H87P mutation and is resistant to Debio-025. Wiegers and Kräusslich (100) reported that group O strains with a P at position 86 and an A or P at position 87 can be either sensitive or resistant to Cs.

Previously, an IC_{50} value of 0.08 μM for strain 92BR014 had been determined at The Scripps Research Institute (19). In the present study, strain 92BR014 proved to be resistant to Debio-025 (IC_{50}, 5.65 μM) when it was tested at the Southern Research Institute. Sequencing of both virus stocks revealed that the isolate used at the Southern Research Institute carried the H87Q mutation, which was not the case for the sensitive virus used at The Scripps Research Institute.

Monocytotropic HIV-1 strain Ba-L was found to be resistant to Debio-025 inhibition at the Southern Research Institute (IC_{50}, 3.53 μM) as well as at The Scripps Research Institute (IC_{50}, 1.9 μM) (19). Sequencing revealed that the virus used at The Scripps Research Institute had only the H87Q mutation in the CA protein, while the one used at the Southern Research Institute carried, in addition, the mutations I91V and M96I, similar to previously published Ba-L sequences (GenBank accession numbers AB221005, AB253432, and AY713409). Kootstra et al. (44) showed that replacement of the CypA binding region in CA of strain HXB2 by that of Ba-L resulted in a retroviral vector that was less sensitive to Cs and that also appeared to be resistant to simian cell restriction factor. This Ba-L-derived CA sequence contains, in addition to H87Q, the mutations A88P and I91V. Recently, a more detailed analysis (67) revealed that either a Q at position 87 or a P at position 88 resulted in resistance to Cs, which coincided with unrestricted infection in simian cells. The I91V substitution alone was not sufficient for overriding the postentry restriction in simian cells, and the virus proved sensitive to Cs treatment.

A correlation of substitutions within the CypA binding region of CA with Cs resistance in human cells was also reported by Ikeda et al. (42). Those authors identified a triple capsid mutation (V86P, H87Q, and I91V) that rendered HIV-1 resistant to Cs. These virus variants, too, showed an increased capacity to replicate in simian cells.

These results altogether nicely corroborate our finding that a mutation at position 87 is the most relevant change that leads to resistance to cyclosporines. The correlation between resistance to restriction in simian cells and the CypA independence of replication in human cells was previously reported in more detail by us (19) and others (36, 42, 44, 57, 67, 88).

We did not detect the previously described mutation A92E or G94D (1, 12) in the sequences of the CypA binding loop from naturally resistant virus isolates. Mutants carrying these mutations had been selected from strain NL4-3 when it infected $CD4^+$ HeLa cells and was passaged in the presence of NIM811. These mutants showed a NIM811-dependent or -resistant phenotype, depending on the cell line used (103). However, these mutants proved to be fully sensitive to Cs, NIM811, and Debio-025 when they were grown in primary cells (72; B. Rosenwirth, unpublished data; Gallay, unpublished).

Recently, it was reported that determinants outside the CypA binding loop of CA, namely, a T54A amino acid change in α helix 3, can also modulate the dependence of HIV-1 infection on CypA in human cells (102). Similar to the phenotype of mutants A92E and G94D, infection by the T54A mutant was inhibited or stimulated by Cs in a target cell-specific manner. Mutations in α helix 3 have been reported to reduce the susceptibility of HIV-1 to restriction in simian cells (58).

Taken together, these results support the assumption that CypA binding to the HIV-1 CA protein protects the incoming viral capsid from a restriction factor(s) in human cells and, on the other hand, promotes the restriction of HIV-1 replication in simian cells. The protein TRIM5α has been identified as the relevant restriction factor for HIV-1 replication in simian cells (74, 84); this restriction requires CypA. However, the stimulatory effect of CypA on HIV-1 infectivity was reported to be independent of human TRIM5α (73, 79, 85), which suggests that CypA protects HIV-1 from a yet unknown antiviral activity in human cells.

HIV-2 and SIV were reported to be independent from CypA in their replication (14, 68), and their CA proteins showed no affinity to CypA (17, 87). Thus, they were expected to be resistant to the antiviral activity of cyclosporines. We confirmed the lack of sensitivity of two SIV strains to Debio-025; however, surprisingly, one of two HIV-2 clinical isolates was found to be sensitive to inhibition by Debio-025. Evaluation of the activity of Debio-025 31 against further HIV-2 isolates led to the identification of three additional sensitive strains. Comparison of the CA sequence of one sensitive HIV-2 isolate to that of one resistant HIV-2 isolate revealed that the resistant strain contained the consensus HIV-2 subtype A sequence at positions 85 to 99, whereas the corresponding sequence of the sensitive HIV-2 strain was identical to the consensus sequence of HIV-1 subtype A. Further characterization of the Debio-025-sensitive HIV-2 strains will reveal whether their CA protein binds to CypA and whether they represent chimeras or intermediates between HIV-1 and HIV-2.

Debio-025 was evaluated broadly for its ability to inhibit the replication of a large panel of viruses from different classes. The compound proved to be inactive against most viruses tested or showed only moderate inhibition of doubtful significance of varicella-zoster virus, cytomegalovirus, human and feline coronaviruses, and hepatitis B virus. We could not detect any activity of Cs, NIM811, or Debio-025 against vaccinia virus, which is at variance to the findings of a study published previously (21). Debio-025 was found to have a very pronounced inhibitory effect, however, on the replication of HCV. Cs and NIM811 had previously been reported to inhibit the replication of HCV (52, 92). We compared the potential of Debio-025 to inhibit HCV in vitro with that of Cs (60): Debio-025 was demonstrated to be 5 to 10 times more potent than Cs in a

series of assays. Debio-025 had no inhibitory effect against other members of the family *Flaviviridae*.

Thus, Debio-025 is a potent and selective inhibitor of HIV-1 and HCV replication. The common target for Debio-025 in the replication cycles of HIV-1 and HCV appears to be a cyclophilin: CypA plays an essential role early in the replication of HIV-1 by binding to the CA protein, an interaction that is inhibited competitively by cyclosporines; CypB was recently reported (93) to act as a functional regulator of the HCV RNA polymerase; the anti-HCV activity of cyclosporines is assumed to be based on inhibition of the interaction of CypB with the HCV replication complex.

A major problem for the success of anti-HIV-1 highly active antiretroviral therapy is the selection of multidrug-resistant viruses (61). Since the target molecule for the anti-HIV-1 activity of Debio-025 is CypA, which is different from the targets of all other approved anti-HIV-1 drugs, it was expected that Debio-025 would inhibit the replication of multidrug-resistant virus isolates. Five of these multidrug-resistant strains were indeed potently inhibited by Debio-025; one protease inhibitor-resistant isolate, however, proved to be naturally resistant to inhibition by Debio-025. We assume that this resistance to Debio-025 is not caused by the mutations in the protease that lead to protease inhibitor resistance, since this isolate contains both the H87Q and the M96I mutations in the CypA binding region of CA. Gatanaga et al. (33) reported that in HIV-1 variants resistant to protease inhibitors, multiple amino acid substitutions frequently emerged in noncleavage sites of the Gag protein; in particular, the H87Q/P mutation in the CypA binding loop was observed. Those authors presented evidence that the H87Q/P substitutions in the CypA binding loop confer a replication advantage to the protease inhibitor-resistant viruses; the magnitude of that advantage, however, was dependent on the cell type used. Structural modeling analysis indicated that the H87Q/P mutation affects the conformation of the CypA binding motif, thereby rendering HIV-1 replication independent of CypA in human cells (33).

In the clinic, a new anti-HIV-1 drug will always be used in combination with approved HIV-1 inhibitors. Debio-025 was evaluated in combination with 19 approved drugs of different classes, namely, nucleoside RT inhibitors, nonnucleoside RT inhibitors, protease inhibitors, and one fusion inhibitor. Debio-025 interacted with all of 19 FDA-approved drugs, and the interactions achieved antiviral efficacy in essentially an additive fashion over the concentration ranges examined. There was no evidence of synergistic cytotoxicity over the concentrations of Debio-025 examined. These data suggest that antagonism of the antiviral effects of Debio-025 should not be an issue in the clinical setting when it is used with the approved antiretroviral drugs.

The modes of action of cyclosporines in inhibiting HIV-1 replication have been studied extensively (for recent reviews, see references 47 and 80). Cyclosporines bind to the active site of CypA and competitively inhibit the interaction of CypA with the CA protein of HIV-1. This interaction between CypA and the loop around G89 and P90 of CA is important for the efficient replication of HIV-1 in human cells. By means of this affinity to the CA protein, CypA is incorporated into the virions that are produced and is carried over to the target cell in new infections. It has been reported that virus particles produced in the presence of Cs or NIM811 are less infectious (13, 51, 82, 87), which pointed to the importance of producer cell CypA. However, more recent studies yielded compelling evidence that target cell CypA plays the most relevant role early in HIV-1 infection (37, 44, 81). It may be possible that the producer cell CypA and the target cell CypA act at different steps during early infection and perform different functions. It is generally agreed that CypA plays a role at a step after penetration of the virion into the cell and before integration of the newly made double-stranded proviral DNA into cellular DNA. Thus, CypA may promote uncoating of the incoming viral particle and/or reverse transcription and/or translocation of the preintegration complex to the nucleus (13, 15, 37, 51, 82). For Debio-025, we demonstrated (19) that, as expected, this cyclosporine dose dependently inhibited the incorporation of CypA into the virus particles, indicating interference with the CypA-CA interaction. The naturally Debio-025-resistant virus strains were still able to incorporate CypA; thus, the CypA-CA interaction took place. However, their replication in human cells was not impaired when this interaction was blocked by Debio-025.

To further define the mode of action of Debio-025, we demonstrated by real-time PCR that, in the presence of Debio-025, early reverse transcription is reduced and late reverse transcription is almost completely blocked. A significant inhibitory effect of Debio-025 on the early RT transcripts was detected only from 6 h postinfection on. The data indicate that the newly made cDNA is degraded in the presence of Debio-025.

Thus, CypA probably exerts a relevant function during the progression and/or completion of reverse transcription. Debio-025 disrupts the CypA-CA interaction, which presumably leads to blockade of this function. The early events in virus replication can be assumed to occur in a highly ordered fashion at the right time and at the right intracellular location. If there is a blockade in this sequence of events, the chance increases that the viral replication complex is destroyed by restriction factors or is transported to a compartment of the cell where infection is abortive. According to this hypothesis, the function of CypA may be that of a chaperone that stabilizes or transports the viral replication complex during reverse transcription to the right location before restriction factors can abort the infection. Eventually, CypA may also mediate the removal of the CA protein, which is known to be a precondition for transport of the preintegration complex to the nucleus (101).

Debio-025 was also evaluated in vivo in the SCID/hu Thy/Liv mouse model (6). The compound, given at 30 mg/kg/day orally, significantly inhibited HIV-1 RNA and CA protein production in SCID/hu Thy/Liv mice when strain NL4-3 or JD was used for infection. Remarkably, HIV-1 strain Ba-L was inhibited to only a minor (nonsignificant) degree with higher concentrations of Debio-025, which corroborates the relative in vitro resistance of strain Ba-L to inhibition by this compound. The pharmacokinetic behavior of Debio-025 in animals and humans proved to be favorable: good oral bioavailability in an appropriate formulation and a long half-life in blood were demonstrated (83). The toxicology results obtained with several species did not reveal any side effects representing serious obstacles to the further clinical development of Debio-025 (J.-M. Dumont, unpublished data).

In conclusion, we have presented here the pharmacological profile of the nonimmunosuppressive cyclosporine Debio-025 with regard to its anti-HIV-1 activity. In addition to its potent inhibitory potential against HIV-1 infection, this compound also exhibited remarkable activity against HCV replication in vitro. Initial clinical trials with patients with HIV-1 or HCV infection and in individuals with HIV-1 and HCV double infections were performed and yielded promising results (28, 29, 38, 83); further clinical evaluation is ongoing. The cyclophilin-blocking compound Debio-025 represents a promising candidate drug for the treatment of HIV-1 and HCV infections, in particular, in patients coinfected with these two viruses.

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REFERENCES

1. **Aberham, C., S. Weber, and W. Phares.** 1996. Spontaneous mutations in the human immunodeficiency virus type 1 *gag* gene that affect viral replication in the presence of cyclosporins. J. Virol. **70:**3536–3544.
2. **Andrei, G., R. Sienaert, C. McGuigan, E. De Clercq, J. Balzarini, and R. Snoeck.** 2005. Susceptibilities of several clinical varicella-zoster virus (VZV) isolates and drug-resistant VZV strains to bicyclic furano pyrimidine nucleosides. Antimicrob. Agents Chemother. **49:**1081–1086.
3. **Andrei, G., R. Snoeck, J. Piette, P. Delvenne, and E. De Clercq.** 1998. Antiproliferative effects of acyclic nucleoside phosphonates on human papillomavirus (HPV)-harboring cell lines compared with HPV-negative cell lines. Oncol. Res. **10:**523–531.
4. **Andrieu, J. M., P. Even, and A. Venet.** 1986. AIDS and related syndromes as a viral-induced autoimmune disease of the immune system: an anti-MHC II disorder. Therapeutic implications. AIDS Res. **2:**163–174.
5. **Balzarini, J., E. Keyaerts, L. Vijgen, H. Egberink, E. De Clercq, M. Van Ranst, S. S. Printsevskaya, E. N. Olsufyeva, S. E. Solovieva, and M. N. Preobrazhenskaya.** 2006. Inhibition of feline (FIPV) and human (SARS) coronavirus by semisynthetic derivatives of glycopeptide antibiotics. Antivir. Res. **72:**20–33.
6. **Bare, J., J.-M. Dumont, K. Besseghir, and C. Stoddart.** 2004. Potent *in vivo* anti-HIV-1 activity of the nonimmunosuppressive cyclosporine UNIL-025, abstr. 110. Keystone Symp. HIV Pathogenesis.
7. **Bartz, S. R., E. Hohenwalter, M. K. Hu, D. H. Rich, and M. Malkovsky.** 1995. Inhibition of human immunodeficiency virus replication by nonimmunosuppressive analogs of cyclosporin A. Proc. Natl. Acad. Sci. USA **92:**5381–5385.
8. **Baumann, G., G. Zenke, R. Wenger, P. Hiestand, V. Quesniaux, E. Andersen, and M. H. Schreier.** 1992. Molecular mechanisms of immunosuppression. J. Autoimmun. **5**(Suppl. A)**:**67–72.
9. **Besnier, C., Y. Takeuchi, and G. Towers.** 2002. Restriction of lentivirus in monkeys. Proc. Natl. Acad. Sci. USA **99:**11920–11925.
10. **Billich, A., F. Hammerschmid, P. Peichl, R. Wenger, G. Zenke, V. Quesniaux, and B. Rosenwirth.** 1995. Mode of action of SDZ NIM 811, a nonimmunosuppressive cyclosporin A analog with activity against human immunodeficiency virus (HIV) type 1: interference with HIV protein-cyclophilin A interactions. J. Virol. **69:**2451–2461.
11. **Borel, J. F., F. Di Padova, J. Mason, V. Quesniaux, B. Ryffel, and R. Wenger.** 1989. Pharmacology of cyclosporine (Sandimmune). Pharmacol. Rev. **41:**239–242.
12. **Braaten, D., C. Aberham, E. K. Franke, L. Yin, W. Phares, and J. Luban.** 1996. Cyclosporine A-resistant human immunodeficiency virus type 1 mutants demonstrate that *gag* encodes the functional target of cyclophilin A. J. Virol. **70:**5170–5176.
13. **Braaten, D., E. K. Franke, and J. Luban.** 1996. Cyclophilin A is required for an early step in the life cycle of human immunodeficiency virus type 1 before the initiation of reverse transcription. J. Virol. **70:**3551–3560.
14. **Braaten, D., E. K. Franke, and J. Luban.** 1996. Cyclophilin A is required for the replication of group M human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus SIV(CPZ)GAB but not group O HIV-1 or other primate immunodeficiency viruses. J. Virol. **70:**4220–4227.
15. **Braaten, D., and J. Luban.** 2001. Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. EMBO J. **20:**1300–1309.
16. **Buckheit, R. W., Jr., and R. Swanstrom.** 1991. Characterization of an HIV-1 isolate displaying an apparent absence of virion-associated reverse transcriptase activity. AIDS Res. Hum. Retrovir. **7:**295–302.
17. **Bukovsky, A. A., A. Weimann, M. A. Accola, and H. G. Gottlinger.** 1997. Transfer of the HIV-1 cyclophilin-binding site to simian immunodeficiency virus from Macaca mulatta can confer both cyclosporin sensitivity and cyclosporin dependence. Proc. Natl. Acad. Sci. USA **94:**10943–10948.
18. **Butler, S. L., M. S. Hansen, and F. D. Bushman.** 2001. A quantitative assay for HIV DNA integration *in vivo*. Nat. Med. **7:**631–634.
19. **Chatterji, U., M. D. Bobardt, R. Stanfield, R. G. Ptak, L. A. Pallansch, P. A. Ward, M. J. Jones, C. A. Stoddart, P. Scalfaro, J. M. Dumont, K. Besseghir, B. Rosenwirth, and P. A. Gallay.** 2005. Naturally occurring capsid substitutions render HIV-1 cyclophilin A independent in human cells and TRIM-cyclophilin-resistant in owl monkey cells. J. Biol. Chem. **280:**40293–40300.
20. **Cheung, P. K., B. Wynhoven, and P. R. Harrigan.** 2004. 2004: which HIV-1 drug resistance mutations are common in clinical practice? AIDS Rev. **6:**107–116.
21. **Damaso, C. R., and N. Moussatche.** 1998. Inhibition of vaccinia virus replication by cyclosporin A analogues correlates with their affinity for cellular cyclophilins. J. Gen. Virol. **79:**339–346.
22. **De Clercq, E.** 2005. Emerging anti-HIV drugs. Expert Opin. Emerg. Drugs **10:**241–273.
23. **DeJesus, E., D. Berger, M. Markowitz, C. Cohen, T. Hawkins, P. Ruane, R. Elion, C. Farthing, L. Zhong, A. K. Cheng, D. McColl, and B. P. Kearny for the 183-0101 Study Team.** 2006. Antiviral activity, pharmacokinetics, and dose response of the HIV-1 integrase inhibitor GS-9137 (JTK-303) in treatment-naive and treatment-experienced patients. J. Acquir. Immune Defic. Syndr. **43:**1–5.
24. **Dorr, P., M. Westby, S. Dobbs, P. Griffin, B. Irvine, M. Macartney, J. Mori, G. Rickett, C. Smith-Burchnell, C. Napier, R. Webster, D. Armour, D. Price, B. Stammen, A. Wood, and M. Perros.** 2005. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob. Agents Chemother. **49:**4721–4732.
25. **Dreyfuss, M., E. Harri, H. Hoffmann, H. Kobel, W. Pache, and H. Tscherter.** 1976. Cyclosporin A and C. New metabolites from Trichoderma polysporum. Eur. J. Appl. Microbiol. **3:**125–133.
26. **Eisenmesser, E. Z., O. Millet, W. Labeikovsky, D. M. Korzhnev, M. Wolf-Watz, D. A. Bosco, J. J. Skalicky, L. E. Kay, and D. Kern.** 2005. Intrinsic dynamics of an enzyme underlies catalysis. Nature **438:**117–121.
27. **Fischer, G., H. Bang, and C. Mech.** 1984. Determination of enzymatic catalysis for the *cis-trans*-isomerization of peptide binding in proline-containing peptides. Biomed. Biochim. Acta **43:**1101–1111.
28. **Flisiak R., A. Horban, P. Gallay, M. Bobardt, S. Selvarajah, A. Wiercinska-Drapalo, E. Siwak, I. Cielniak, J. Higersberger, J. Kierkus, C. Aeschlimann, P. Grosgurin, V. Nicolas-Metral, J.-M. Dumont, H. Porchet, R. Crabbe, and P. Scalfaro.** The cyclophilin inhibitor Debio-025 shows potent anti-hepatitis C effect in human immunodeficiency virus co-infected hepatitis C patients. Hepatology, in press.
29. **Flisiak, R., A. Horban, J. Kierkus, J. Stanczak, I. Cielniak, G. P. Stanczak, A. Wiercinska-Drapalo, E. Siwak, J. Higersberger, C. Aeschlimann, P. Grosgurin, V. Nicolas, J.-M. Dumont, H. Porchet, R. Crabbe, and P. Scalfaro.** 2006. The cyclophilin inhibitor Debio-025 has a potent dual anti-HIV and anti-HCV activity in treatment-naive HIV/HCV co-infected subjects. Hepatology **44**(Suppl. 1)**:**609A.
30. **Franke, E. K., H. E. Yuan, and J. Luban.** 1994. Specific incorporation of cyclophilin A into HIV-1 virions. Nature **372:**359–362.
31. **Gamble, T. R., F. F. Vajdos, S. Yoo, D. K. Worthylake, M. Houseweart, W. I. Sundquist, and C. P. Hill.** 1996. Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. Cell **87:**1285–1294.
32. **Garcia-Echverria, C., J. L. Kofron, P. Kuzmic, and D. H. Rich.** 1993. A continuous spectrophotometric direct assay for peptidyl prolyl *cis-trans* isomerases. Biochem. Biophys. Res. Commun. **191:**70–75.
33. **Gatanaga, H., D. Das, Y. Suzuki, D. D. Yeh, K. A. Hussain, A. K. Ghosh, and H. Mitsuya.** 2006. Altered HIV-1 Gag protein interactions with cyclophilin A (CypA) on the acquisition of H219Q and H219P substitutions in the CypA binding loop. J. Biol. Chem. **281:**1241–1250.
34. **Grinsztejn, B., B. Y. Nguyen, C. Katlama, J. M. Gatell, A. Lazzarin, D. Vittecoq, C. J. Gonzalez, J. Chen, C. M. Harvey, and R. D. Isaacs.** 2007. Safety and efficacy of the HIV-1 integrase inhibitor raltegravir (MK-0518) in treatment-experienced patients with multidrug-resistant virus: a phase II randomised controlled trial. Lancet **369:**1261–1269.

35. **Handschumacher, R. E., M. W. Harding, J. Rice, R. J. Drugge, and D. W. Speicher.** 1984. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science **226:**544–546.
36. **Hatziioannou, T., S. Cowan, U. K. Von Schwedler, W. I. Sundquist, and P. D. Bieniasz.** 2004. Species-specific tropism determinants in the human immunodeficiency virus type 1 capsid. J. Virol. **78:**6005–6012.
37. **Hatziioannou, T., D. Perez-Caballero, S. Cowan, and P. D. Bieniasz.** 2005. Cyclophilin interactions with incoming human immunodeficiency virus type 1 capsids with opposing effects on infectivity in human cells. J. Virol. **79:**176–183.
38. **Hermann, E., R. Flisiak, A. Horban, R. Crabbe, H. Porchet, V. Nicolas, P. Scalfaro, and S. Zeuzem.** 2007. Viral kinetics during 14-day treatment with Debio-025 demonstrate efficient blocking of viral replication in different HCV genotypes without signs of emerging viral resistance. J. Hepatol. **46**(Suppl. 1)**:**S40.
39. **Herzyk, D. J., and E. R. Gore.** 2004. Adequate immunotoxicity testing in drug development. Toxicol. Lett. **149:**115–122.
40. **Huai, Q., H. Y. Kim, Y. Liu, Y. Zhao, A. Mondragon, J. O. Liu, and H. Ke.** 2002. Crystal structure of calcineurin-cyclophilin-cyclosporin shows common but distinct recognition of immunophilin-drug complexes. Proc. Natl. Acad. Sci. USA **99:**12037–12042.
41. **Hubler, F., T. Ruckle, L. Patiny, T. Muamba, J. F. Guichou, M. Mutter, and R. Wenger.** 2000. Synthetic routes to NEtXaa4-cyclosporin A derivates as potential anti-HIV drugs. Tetrahedron Lett. **41:**7193–7196.
42. **Ikeda, Y., L. M. Ylinen, M. Kahar-Bador, and G. J. Towers.** 2004. Influence of *gag* on human immunodeficiency virus type 1 species-specific tropism. J. Virol. **78:**11816–11822.
43. **Jin, L., and S. C. Harrison.** 2002. Crystal structure of human calcineurin complexed with cyclosporin A and human cyclophilin. Proc. Natl. Acad. Sci. USA **99:**13522–13526.
44. **Kootstra, N. A., C. Munk, N. Tonnu, N. R. Landau, and I. M. Verma.** 2003. Abrogation of postentry restriction of HIV-1-based lentiviral vector transduction in simian cells. Proc. Natl. Acad. Sci. USA **100:**1298–1303.
45. **Kronke, M., W. J. Leonhard, J. M. Depper, S. K. Arya, F. Wong-Staal, R. C. Gallo, T. A. Waldmann, and W. C. Greene.** 1984. Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. Proc. Natl. Acad. Sci. USA **81:**5214–5218.
46. **Lebeau, I., G. Andrei, F. Dal Pozzo, J. R. Beadle, K. Y. Hostetler, E. De Clercq, J. van den Oord, and R. Snoeck.** 2006. Activities of alkoxyalkyl esters of cidofovir (CDV), cyclic CDV, and (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine against orthopoxviruses in cell monolayers and in organotypic cultures. Antimicrob. Agents Chemother. **50:**2525–2529.
47. **Luban, J.** 2007. Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection. J. Virol. **81:**1054–1061.
48. **Luban, J., K. L. Bossolt, E. K. Franke, G. V. Kalpana, and S. P. Goff.** 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell **73:**1067–1078.
49. **Meo, T.** 1979. The MLR test in the mouse, p. 227–239. *In* I. Lefkovitz and B. Pernis (ed.), Immunological methods. Academic Press, Inc., New York, NY.
50. **Miyoshi, I., S. Yoshimoto, I. Kubonishi, H. Taguchi, Y. Shiraishi, Y. Ohtsuki, and T. Akagi.** 1981. Transformation of normal human cord lymphocytes by co-cultivation with a lethally irradiated human T-cell line carrying type C virus particles. Gann **72:**997–998.
51. **Mlynar, E., D. Bevec, A. Billich, B. Rosenwirth, and A. Steinkasserer.** 1997. The non-immunosuppressive cyclosporin A analogue SDZ NIM 811 inhibits cyclophilin A incorporation into virions and virus replication in human immunodeficiency virus type 1-infected primary and growth-arrested T cells. J. Gen. Virol. **78:**825–835.
52. **Nakagawa, M., N. Sakamoto, N. Enomoto, Y. Tanabe, N. Kanazawa, T. Koyama, M. Kurosaki, S. Maekawa, T. Yamashiro, C. H. Chen, Y. Itsui, S. Kakinuma, and M. Watanabe.** 2004. Specific inhibition of hepatitis C virus replication by cyclosporin A. Biochem. Biophys. Res. Commun. **313:**42–47.
53. **Nara, P. L., W. C. Hatch, N. M. Dunlop, W. G. Robey, L. O. Arthur, M. A. Gonda, and P. J. Fischinger.** 1987. Simple, rapid, quantitative, syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. AIDS Res. Hum. Retrovir. **3:**283–302.
54. **Neyts, J., A. Meerbach, P. McKenna, and E. De Clercq.** 1996. Use of the yellow fever virus vaccine strain 17D for the study of strategies for the treatment of yellow fever virus infections. Antivir. Res. **30:**125–132.
55. **Neyts, J., D. Reymen, D. Letourneur, J. Jozefonvicz, D. Schols, J. Este, G. Andrei, P. McKenna, M. Witvrouw, S. Ikeda, J. Clement, and E. De Clercq.** 1995. Differential antiviral activity of derivatized dextrans. Biochem. Pharmacol. **50:**743–751.
56. **Ou, C. Y., S. Kwok, S. W. Mitchell, D. H. Mack, J. J. Sninsky, J. W. Krebs, P. Feorino, D. Warfield, and G. Schochetman.** 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science **239:**295–297.
57. **Owens, C. M., B. Song, M. J. Perron, P. C. Yang, M. Stremlau, and J. Sodroski.** 2004. Binding and susceptibility to postentry restriction factors in monkey cells are specified by distinct regions of the human immunodeficiency virus type 1 capsid. J. Virol. **78:**5423–5437.
58. **Owens, C. M., P. C. Yang, H. Gottlinger, and J. Sodroski.** 2003. Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells. J. Virol. **77:**726–731.
59. **Paeshuyse, J., J. M. Chezal, M. Froeyen, P. Leyssen, H. Dutartre, R. Vrancken, B. Canard, C. Letellier, T. Li, H. Mittendorfer, F. Koenen, P. Kerkhofs, E. De Clercq, P. Herdewijn, G. Puerstinger, A. Gueiffier, O. Chavignon, J. C. Teulade, and J. Neyts.** 2007. The imidazopyrrolopyridine analogue AG110 is a novel, highly selective inhibitor of pestiviruses that targets the viral RNA-dependent RNA polymerase at a hot spot for inhibition of viral replication. J. Virol. **81:**11046–11053.
60. **Paeshuyse, J., A. Kaul, E. De Clercq, B. Rosenwirth, J. M. Dumont, P. Scalfaro, R. Bartenschlager, and J. Neyts.** 2006. The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication *in vitro*. Hepatology **43:**761–770.
61. **Palmer, S., R. W. Shafer, and T. C. Merigan.** 1999. Highly drug-resistant HIV-1 clinical isolates are cross-resistant to many antiretroviral compounds in current clinical development. AIDS **13:**661–667.
62. **Papageorgiou, C., X. Borer, and R. R. French.** 1994. Calcineurin has a very tight-binding pocket for the side chain of residue 4 of cyclosporin. Bioorg. Med. Chem. Lett. **4:**267–272.
63. **Pauwels, R., J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyter, and E. De Clercq.** 1988. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods **20:**309–321.
64. **Phillips, A., M. A. Wainberg, R. Coates, M. Klein, A. Rachlis, S. Read, F. Shepherd, H. Vellend, S. Walmsley, P. Halloran, and M. Fanning.** 1989. Cyclosporine-induced deterioration in patients with AIDS. CMAJ **140:**1456–1460.
65. **Prichard, M. N., and C. Shipman, Jr.** 1990. A three-dimensional model to analyze drug-drug interactions. Antivir. Res. **14:**181–206.
66. **Quesniaux, V. F., M. H. Schreier, R. M. Wenger, P. C. Hiestand, M. W. Harding, and M. H. Van Regenmortel.** 1987. Cyclophilin binds to the region of cyclosporine involved in its immunosuppressive activity. Eur. J. Immunol. **17:**1359–1365.
67. **Rits, M. A., K. A. van Dort, C. Munk, A. B. Meijer, and N. A. Kootstra.** 2007. Efficient transduction of simian cells by HIV-1-based lentiviral vectors that contain mutations in the capsid protein. Mol. Ther. **15:**930–937.
68. **Rosenwirth, B., A. Billich, R. Datema, P. Donatsch, F. Hammerschmid, R. Harrison, P. Hiestand, H. Jaksche, P. Mayer, P. Peichl, V. Quesniaux, F. Schatz, H. J. Schuurmann, R. Traber, R. Wenger, B. Wolff, G. Zenke, and M. Zurini.** 1994. Inhibition of human immunodeficiency virus type 1 replication by SDZ NIM 811, a nonimmunosuppressive cyclosporine analog. Antimicrob. Agents Chemother. **38:**1763–1772.
69. **Rosenwirth, B., A. Billich, A. Steinkasserer, F. Hammerschmid, P. Peichl, H. Göttlinger, R. Traber, and R. Wenger.** 1995. Cyclophilin A as a novel target in anti-HIV-1 chemotherapy. Int. Antivir. News **3:**62–63.
70. **Rosenwirth, B., A. Billich, A. Steinkasserer, P. Peichl, P. Donatsch, A. Lawen, R. Wenger, and R. Traber.** 1995. SDZ NIM 811: anti-HIV nonimmunosuppressive natural cyclosporin. Drugs Future **20:**579–583.
71. **Ruegger, A., M. Kuhn, H. Lichti, H. R. Loosli, R. Huguenin, C. Quiquerez, and A. von Wartburg.** 1976. Cyclosporin A, ein immunosuppressiv wirksamer Peptidmetabolit aus Trichoderma polysporum. Helv. Chim. Acta **59:**1075–1092.
72. **Saini, M., and M. J. Potash.** 2006. Novel activities of cyclophilin A and cyclosporin A during HIV-1 infection of primary lymphocytes and macrophages. J. Immunol. **177:**443–449.
73. **Sayah, D. M., and J. Luban.** 2004. Selection for loss of Ref1 activity in human cells releases human immunodeficiency virus type 1 from cyclophilin A dependence during infection. J. Virol. **78:**12066–12070.
74. **Sayah, D. M., E. Sokolskaja, L. Berthoux, and J. Luban.** 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature **430:**569–573.
75. **Schwarz, A., G. Offermann, F. Keller, I. Bennhold, J. L'Age-Stehr, P. H. Krause, and M. J. Mihatsch.** 1993. The effect of cyclosporine on the progression of human immunodeficiency virus type 1 infection transmitted by transplantation—data on four cases and review of the literature. Transplantation **55:**95–103.
76. **Siddiqi, S. M., X. Chen, S. W. Schneller, S. Ikeda, R. Snoeck, G. Andrei, J. Balzarini, and E. De Clercq.** 1994. An epimer of 5′-noraristeromycin and its antiviral properties. J. Med. Chem. **37:**1382–1384.
77. **Snoeck, R., T. Sakuma, E. De Clercq, I. Rosenberg, and A. Holy.** 1988. (*S*)-1-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine, a potent and selective inhibitor of human cytomegalovirus replication. Antimicrob. Agents Chemother. **32:**1839–1844.
78. **Snyder, S. H., and D. M. Sabatini.** 1995. Immunophilins and the nervous system. Nat. Med. **1:**32–37.
79. **Sokolskaja, E., L. Berthoux, and J. Luban.** 2006. Cyclophilin A and TRIM5alpha independently regulate human immunodeficiency virus type 1 infectivity in human cells. J. Virol. **80:**2855–2862.
80. **Sokolskaja, E., and J. Luban.** 2006. Cyclophilin, TRIM5, and innate immunity to HIV-1. Curr. Opin. Microbiol. **9:**404–408.
81. **Sokolskaja, E., D. M. Sayah, and J. Luban.** 2004. Target cell cyclophilin A

modulates human immunodeficiency virus type 1 infectivity. J. Virol. **78:** 12800–12808.
82. **Steinkasserer, A., R. Harrison, A. Billich, F. Hammerschmid, G. Werner, B. Wolff, P. Peichl, G. Palfi, W. Schnitzel, E. Mlynar, and B. Rosenwirth.** 1995. Mode of action of SDZ NIM 811, a nonimmunosuppressive cyclosporin A analog with activity against human immunodeficiency virus type 1 (HIV-1): interference with early and late events in HIV-1 replication. J. Virol. **69:** 814–824.
83. **Steyn, D., D. Richman, C. Aeschlimann, J.-M. Dumont, P. Grosgurin, B. Rosenwirth, C. Loveday, H. Porchet, V. Nicolas, and P. Scalfaro.** 2006. A double-blind placebo-controlled study in HIV-1-infected subjects on the safety, pharmacokinetics and antiviral effect of cyclophilin A targeting Debio-025, abstr. 516. 13th Conf. Retrovir. Opportunistic Infect.
84. **Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier, and J. Sodroski.** 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature **427:**848–853.
85. **Stremlau, M., B. Song, H. Javanbakht, M. Perron, and J. Sodroski.** 2006. Cyclophilin A: an auxiliary but not necessary cofactor for TRIM5alpha restriction of HIV-1. Virology **351:**112–120.
86. **Strizki, J. M., C. Tremblay, S. Xu, L. Wojcik, N. Wagner, W. Gonsiorek, R. W. Hipkin, C. C. Chou, C. Pugliese-Sivo, Y. Xiao, J. R. Tagat, K. Cox, T. Priestley, S. Sorata, W. Huang, M. Hirsch, G. R. Reyes, and B. M. Baroudy.** 2005. Discovery and characterization of Vicriviroc (SCH 417690), a CCR5 antagonist with potent activity against human immunodeficiency virus type 1. Antimicrob. Agents Chemother. **49:**4911–4919.
87. **Thali, M., A. Bukovsky, E. Kondo, B. Rosenwirth, C. T. Walsh, J. Sodroski, and H. G. Gottlinger.** 1994. Functional association of cyclophilin A with HIV-1 virions. Nature **372:**363–365.
88. **Towers, G. J., T. Hatziioannou, S. Cowan, S. P. Goff, J. Luban, and P. D. Bieniasz.** 2003. Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. Nat. Med. **9:**1138–1143.
89. **Traber, R., H. Kobel, H. R. Loosli, H. Senn, B. Rosenwirth, and A. Lawen.** 1994. [Melle^4] cyclosporin, a novel natural cyclosporin with anti-HIV activity. Antivir. Chem. Chemother. **5:**331–339.
90. **Vella, S., and L. Palmisano.** 2000. Antiretroviral therapy: state of the HAART. Antivir. Res. **45:**1–7.
91. **Wainberg, M. A., A. Dascal, N. Blain, L. Fitz-Gibbon, F. Boulerice, K. Numazaki, and M. Tremblay.** 1988. The effect of cyclosporine A on infection of susceptible cells by human immunodeficiency virus type 1. Blood **72:**1904–1910.
92. **Watashi, K., M. Hijikata, M. Hosaka, M. Yamaji, and K. Shimotohno.** 2003. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology **38:**1282–1288.
93. **Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno.** 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol. Cell **19:**111–122.
94. **Weber, C., G. Wider, B. Von Freyberg, R. Traber, W. Braun, H. Widmer, and K. Wuthrich.** 1991. The NMR structure of cyclosporin A bound to cyclophilin in aqueous solution. Biochemistry **30:**6563–6574.
95. **Weiss, A., R. L. Wiskocil, and J. D. Stobo.** 1984. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pre-translational level. J. Immunol. **133:**123–128.
96. **Wenger, R. M.** 1986. Cyclosporine and analogues—isolation and synthesis—mechanism of action and structural requirements for pharmacological activity. Fortschr. Chem. Org. Naturst. **50:**123–136.
97. **Wenger, R. M., M. Mutter, and T. Ruckle.** January 2000. Novel cyclosporin with improved activity profile. Patent WO 00/01715. U.S. patent 6,927,208 B1, August 2005.
98. **Wenger, R. M., T. G. Payne, and M. H. Schreier.** 1985. Cyclosporine: chemistry, structure-activity relationships and mode of action. Prog. Clin. Biochem. Med. **3:**157–191.
99. **Westby, M., M. Lewis, J. Whitcomb, M. Youle, A. L. Pozniak, I. T. James, T. M. Jenkins, M. Perros, and E. van der Ryst.** 2006. Emergence of CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4-using virus reservoir. J. Virol. **80:**4909–4920.
100. **Wiegers, K., and H. G. Kräusslich.** 2002. Differential dependence of the infectivity of HIV-1 group O isolates on the cellular protein cyclophilin A. Virology **294:**289–295.
101. **Yamashita, M., and M. Emerman.** 2004. Capsid is a dominant determinant of retrovirus infectivity in nondividing cells. J. Virol. **78:**5670–5678.
102. **Yang, R., and C. Aiken.** 2007. A mutation in alpha helix 3 of CA renders human immunodeficiency virus type 1 cyclosporin A resistant and dependent: rescue by a second-site substitution in a distal region of CA. J. Virol. **81:**3749–3756.
103. **Yin, L., D. Braaten, and J. Luban.** 1998. Human immunodeficiency virus type 1 replication is modulated by host cyclophilin A expression levels. J. Virol. **72:**6430–6436.
104. **Ying, C., A. Holy, D. Hocková, Z. Havlas, E. De Clercq, and J. Neyts.** 2005. Novel acyclic nucleoside phosphonate analogues with potent anti-hepatitis B virus activities. Antimicrob. Agents Chemother. **49:**1177–1180.
105. **Zenke, G., G. Baumann, R. Wenger, P. Hiestand, V. Quesniaux, E. Andersen, and M. H. Schreier.** 1993. Molecular mechanisms of immunosuppression by cyclosporins. Ann. N. Y. Acad. Sci. **685:**330–335.
106. **Zoidis, G., N. Kolocouris, G. B. Foscolos, A. Kolocouris, G. Fytas, P. Karayannis, E. Padalko, J. Neyts, and E. De Clercq.** 2003. Are the 2-isomers of the drug rimantadine active anti-influenza A agents? Antivir. Chem. Chemother. **14:**153–164.