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A Second Target for the Peptoid Tat/Transactivation Response Element Inhibitor CGP64222: Inhibition of Human Immunodeficiency Virus Replication by Blocking CXC-Chemokine Receptor 4-Mediated Virus Entry

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ABSTRACT

The peptoid CGP64222 has been previously demonstrated to inhibit the human immunodeficiency virus (HIV) Tat/transactivation response element complex formation. It has previously been shown that CGP64222 selectively inhibits HIV-1 long terminal repeat-driven gene expression and HIV-1$_{LAV}$ replication in lymphocytes. Here, we show that CGP64222 inhibits the replication of a wide range of laboratory strains of HIV-1 and HIV-2 in MT-4 cells. However, CGP64222 proved inactive in MT-4 cells against HIV-1 strains that are resistant to the bicyclams. The bicyclams are known to specifically interact with CXC-chemokine receptor 4, the main coreceptor used by T-tropic HIV strains to enter the cells. Mechanism of action studies revealed that CGP64222 can inhibit the HIV replicative cycle, also through a selective interaction with the CXC-chemokine receptor 4 coreceptor.

The replication of human immunodeficiency virus (HIV) requires the function of Tat, one of the regulatory gene products encoded by HIV (Arya et al., 1985; Sodroski et al., 1985). Tat transactivates expression of all viral genes that depend on the long terminal repeat (LTR) by increasing the rate of initiation and the processivity of transcription (Laspia et al., 1989; reviewed in Cullen, 1998). These activation events are mediated through the ability of Tat to interact with a *cis*-acting RNA sequence, the transactivation response element (TAR), located downstream of the transcriptional initiation site. Binding to TAR is mediated by a short, linear peptide domain of Tat that is predominantly composed of basic amino acids. This interaction may be considered a suitable target for the chemotherapy of HIV infection, because an inhibitor of the Tat/TAR interaction may have the potential to keep the virus in its dormant state. We have previously reported that a basic peptoid oligomer of nine residues, CGP64222 (Fig. 1), can effectively compete with Tat for binding to TAR. NMR spectroscopy indicated that CG64222 interacts with the TAR RNA in the region encompassing the UCU bulge and the 2 bp on either side of the bulge. CGP64222 induces a conformational change in TAR, and this change is mediated by direct contacts between an *N*-Arg side chain from the compound and G26 and U23 from TAR (Hamy et al., 1997). CGP64222 was also shown to block HIV-1$_{LAV}$ replication in PBLs (Hamy et al., 1997).

Numerous publications over the past 2 years have demonstrated the importance of chemokine receptors for HIV entry into the cells. Chemokines are chemotactic cytokines, which are classified as CC or CXC, depending on the positioning of conserved cysteine residues. The CXC-chemokine receptor 4 (CXCR4) mediates entry of T cell line-tropic (T-tropic) viruses, and this function can be inhibited by stromal cell-

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ABBREVIATIONS: HIV, human immunodeficiency virus; CMV, cytomegalovirus; LTR, long terminal repeat; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MFI, mean fluorescence intensity; M-tropic, macrophage-tropic; PBL, peripheral blood lymphocyte; PCR, polymerase chain reaction; PMA, phorbol-12-myristate-13-acetate; RANTES, regulated on activation normal T cell expressed and secreted; AZT, 3′-azido-2′,3′-dideoxythymidine; Ag, antigen; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription; SDF-1α, stromal cell-derived factor-1α; TAR, transactivation responsive element; T-tropic, T cell line-tropic; CXCR4, CXC-chemokine receptor 4; MIP-1α, macrophage inflammatory protein 1α.

derived factor-1α (SDF-1α), the natural ligand of CXCR4 (Oberlin et al., 1996). The CC-chemokine receptor CCR5 mediates entry of macrophage-tropic (M-tropic) viruses (Alkhatib et al., 1996). Bicyclams have been established as highly selective anti-HIV agents (De Clercq et al., 1992) that specifically block the CXCR4 coreceptor (Schols et al., 1997a,b; Donzella et al., 1998). Other antagonists of CXCR4 are ALX40−4C, a polycationic, nonapeptide solely existing of arginine residues (Doranz et al., 1997), and T22, which is also a positively charged peptide containing arginine and lysine residues (Murakami et al., 1997).

Here, we demonstrate that the polycationic peptoid CGP64222, designed as a Tat inhibitor, can also block early events in HIV-1 replication. In particular, this compound was shown to inhibit viral entry into the cells through blockade of the CXCR4 coreceptor.

Materials and Methods

Viruses, Cells and Drugs. The origins of the virus stock HIV-1 III$_B$ and RF (Popovic et al., 1984) and HIV-1 NDK (Spire et al., 1989) were described previously. The strains HIV-1 MN, the 3'-azido-2',3'-dideoxythymidine (AZT)-resistant HIV-1 strain RTMC (Larder and Kemp, 1989; a recombinant, AZT-resistant HIV-1 strain containing RT mutations D67N, K70R, T215F, and K219Q), HIV-2 ROD, and the HIV-1 M-tropic strains BaL and ADA were all obtained through the Medical Research Council AIDS reagent project (Herts, UK). The HIV-1 molecular clone NL4.3 was obtained from the National Institute of Allergy and Infectious Disease AIDS reagent program (Bethesda, MD). The HIV-1-resistant strains NL4.3$_{AMD3100}$^{R} (Esté et al., 1998), NL4.3$_{AMD2763}$ (De Vreese et al., 1996), NL4.3$_{DS5000}$^{R}, and NL4.3$_{AR177}$^{R} (Esté et al., 1998) were obtained as described previously. HIV-1 HE is a clinical isolate from a Belgian patient with AIDS. All virus stocks were prepared from the supernatants of infected MT-4 cells except for the strains tested in peripheral blood lymphocytes (PBLs), which were cultured on donor lymphocytes. HL$_{tat}$ cells (Felber et al., 1990) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS; MT-4 cells, Jurkat$_{tat}$ cells, Jurkat cells, and SUP-T1 cells (American Type Culture Collection, Rockville, MD) were grown and maintained in RPMI 1640 medium supplemented with 10% FCS. PBLs from healthy donors were isolated by density gradient centrifugation (Ficoll hypaque) and stimulated with phytohemagglutinin (1 μg/ml; Sigma Chemical Co., Bornem, Belgium) during 3 days at 37°C. The activated cells (phytohemagglutinin-stimulated blasts) were washed three times with PBS and inoculated with virus as described previously (Schols et al., 1997b).

Dextran sulfate was obtained from Sigma. AZT was synthesized by Christophe Pannecouque. AMD3100 and AMD2763 was kindly provided by Dr. G. Henson (AnorMED, Langley, British Columbia, Canada). Saquinavir was provided by N. Roberts (Roche Products, Welvyn Garden City, UK). Ro5-3335 was synthesized by Wayne A.

Spitze and Frantz Victor at Lilly Research Laboratories (Indianapolis, IN). The anti-CXCR4 monoclonal antibodies (mAb; clones 12G5 and 171) and the anti-CCR5 mAb (clone 2D7) were purchased from R and D Systems (Minneapolis, MN). The anti-CXCR4 mAb 2B11 was a kind gift of Dr. R. Förster and Dr. M. Lipp (Max Delbrück Center, Berlin-Buch, Germany).

Analysis of HIV-1 Transactivation. Tat-dependent and independent transactivation of HIV-1 was monitored as described previously (Daelemans et al., 1997). For the Tat-dependent transactivation, HL$_{tat}$ cells were transfected with pHIV*LacZ* (Maio and Brown, 1988) or pCMVβ (Berger et al., 1988) plasmid DNA. pHIV*LacZ* was obtained from the National Institute of Allergy and Infectious Disease AIDS reagent program and contains the *LacZ* gene driven by the HIV-1 LTR promoter, and pCMVβ (Clontech, Palo Alto, CA) expresses the *LacZ* gene under control of the cytomegalovirus (CMV) immediate-early promoter. The compound Ro5-3335, previously reported as a specific HIV-1 LTR transactivation inhibitor (Hsu et al., 1991; Witvrouw et al., 1992), was used as reference compound. Additionally, Jurkat$_{tat}$ cells were transfected with pHIV*Luc* plasmid DNA, which contains the luciferase reporter gene under control of the HIV-1 LTR. pHIV*Luc* was obtained by ligating the *Kpn*I/*Hin*dIII HIV-1 LTR fragment from pHIV*LacZ* into the pGL3basic vector (Promega, Madison, WI). Ten million Jurkat cells were suspended in 200 μl of medium, and 15 μg of plasmid DNA was electroporated (260 V, 1050 μF, and infinite resistance) into the cells. Two hundred thousand electroporated cells were incubated in the presence of different concentrations of test compound in 96-well plates. For the Tat-independent transactivation, Jurkat cells were transfected with pHIV*Luc* and stimulated with phorbol-12-myristate-13-acetate (PMA; 5 μM). In all transactivation assays, inhibition of transactivation was measured by quantification of reporter gene activity 24 h after transfection. β-Galactosidase reporter gene activity was quantified with a colorimetric assay as described previously (Daelemans et al., 1997). Luciferase activity was measured by adding 100 μl of luciferase reagent (LucLite; Packard) to the same volume of cells according to the user's manual. The IC$_{50}$ value was calculated as being the inhibitor concentration that reduces reporter gene expression by 50%. Toxicity of the test compounds to the cells was performed either through quantification of total protein content according to the Bradford method (Bio-Rad, Hercules, CA) as described previously (Daelemans et al., 1997) or a tetrazolium-based viability assay (Celltiter 96 assay; Promega).

In Vitro Integration Assay. The inhibition of the HIV integrase 3'-processing and DNA strand transfer was monitored as described previously (Cherepanov et al., 1997).

Reverse Transcriptase Assay. Inhibition of HIV RT activity was monitored in vitro in standard reverse transcriptase assays of cell culture supernatant samples by incubating them with a standard RT reaction mixture as described previously (Willey et al., 1988). Briefly, the compound was added at increasing concentrations to the RT reaction mixture before the addition of culture medium containing virus. RT-dependent extension to oligo(dT) on synthetic poly(A)$^+$ RNA was determined by the in vitro incorporation of [^{32}P]dTTP into the synthesized product.

Quantification of Exposed CXCR4 and CCR5. SUP-T1 cells, U87.CD4.CXCR4, or U87.CD4.CCR5 cells were incubated with either CGP64222, the bicyclam AMD3100, or PBS for 30 min at 4°C before the cells were rinsed with PBS to remove unbound compound. The anti-CXCR4 and anti-CCR5 mAbs were then added, and the cells were washed and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Caltag Labs, San Francisco, CA). Cells were analyzed by a FACScan flow cytometer (Becton-Dickinson). The percentage of inhibition of mAb binding in the presence of different concentrations of the compound was calculated using the mean fluorescence intensity (MFI) values, as previously described (Schols et al., 1997b).

The biotinylated human macrophage inflammatory protein-1α (MIP-1α) fluorokine kits were purchased from R and D Systems. The
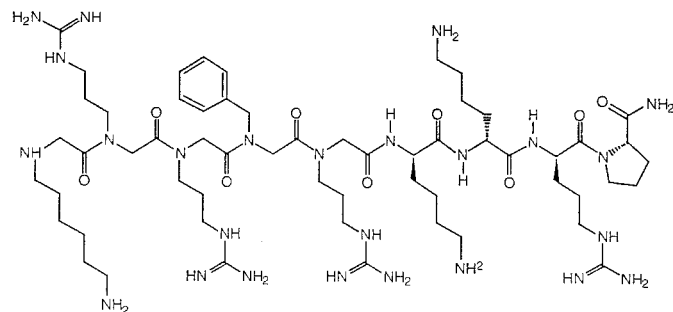

Fig. 1. Chemical structure of CGP64222.

binding of the biotinylated MIP-1α was performed according to the protocol of the manufacturer. Briefly, U87.CD4.CCR5 cells were incubated with CGP64222 for 30 min at 4°C, and then the staining protocol with biotinylated MIP-1α was started.

Intracellular Calcium Flux Assay. Exponentially growing SUP-T1 cells or U87.CD4.CCR5 cells were loaded for 45 min at room temperature with the fluorescent calcium indicator Fluo-4-AM (Molecular Probes, Leiden, the Netherlands) at 4 μM in cell culture medium. Thereafter, the cells were washed twice with calcium flux buffer (Hanks' balanced salt solution with 20 mM HEPES and 0.2% BSA, pH 7.4) and seeded onto a 96-well black-wall microplate (Costar, Cambridge, MA) at 3×10^5 cells/well. After a 20-min pre-incubation of the cells with the test compounds (CGP64222 or AMD3100) at the appropriate concentrations, the intracellular calcium flux in response to 5 ng/ml human recombinant SDF-1α or 2.5 ng/ml RANTES (regulated on activation normal T cell expressed and secreted; PeproTech, Rocky Hill, NJ) was monitored at 37°C as a function of time using a Fluorescent Imaging Plate Reader (FLIPR, Molecular Devices).

Antiviral Replication Assays. The antiviral activities of the compounds were determined in MT-4 cells by measuring inhibition of virus-induced cytopathogenicity. Briefly, MT-4 cells were infected with virus at 100× the 50% cell culture infective dose/ml in the presence of various concentrations of the test compound. The number of viable cells was determined after 5 days of incubation at 37°C, according to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method described previously (Pauwels et al., 1988).

The inhibitory effect of the compounds on the replication of HIV-1 strain III$_B$ in PBLs was monitored 7 days after infection by quantification of HIV-1 p24 core antigen (Ag) using an enzyme-linked immunosorbent assay (ELISA; DuPont, Wilmington, DE). These experiments were performed according to the ACTGDoD protocol.

U87.CD4 Transfectants. Astroglioma U87.CD4 cells, stably transfected with CXCR4 or CCR5 (kindly provided by Nathaniel R. Landau, Aaron Diamond AIDS Research Center, New York, NY), were cultured in Dulbecco's modified Eagle's medium containing 10% FCS. The parental U87.CD4 cells do not express either CXCR4 or CCR5. CXCR4- or CCR5-transfected cells were incubated with HIV-1 NL4.3 or HIV-1 BaL equivalent to 10^3 pg of p24, and viral replication was measured 6 days later with the p24 Ag ELISA.

Time-of-Addition Experiment in MT-4 Cells. MT-4 cells were infected with HIV-1 III$_B$ at a multiplicity of infection of more than 1, and the test compounds were added at different times (0, 1, 2, 3, 24 h) after infection, as described previously (De Clercq et al., 1992). Viral p24 antigen production was determined 29 h post infection by p24 Ag ELISA.

Analysis of Inhibition of HIV-1 Viral DNA and Single-Spliced Viral RNA Production by Semiquantitative Polymerase Chain Reaction (PCR) in MT-4 cells. Two hundred thousand MT-4 cells were infected with HIV-1 III$_B$ (multiplicity of infection, 0.5) and incubated with different concentrations of the test compound. Twenty-four hours after infection, cells were counted and washed once with RPMI 1640. DNA was extracted from 1×10^5 cells (QIAamp Blood Kit; Qiagen, Studio City, CA), and PCR was performed in a total volume of 50 μl, mainly as described previously (Balzarini et al., 1992). The other half of the cells (1×10^5) was used for RNA extraction via TRIZOL and cDNA synthesis using the RNA-PCR kit from Perkin–Elmer Cetus (Norwalk, CT). The PCR was performed on 10 μl of cDNA in a total volume of 50 μl with 10 mM Tris · HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 200 μM concentration of dNTPs, 1 μM concentration of primers, and 1.25 U of Ampli-Taq DNA polymerase (Perkin–Elmer Cetus). The primers used were KPNA, 5′-AGAGTGGTGGTTGCTTCCTTCCACACAG-3′ sense and upstream of the major 5′ splice donor (Neumann et al., 1994), and AV14, 5′-CTCTCTCGACGCAGGACTCGGCTTGCTGAA-3′ anti-sense and downstream of the *env* splice acceptor, to amplify two fragments of 466 and 482 bp in the *env* region of HIV-1, one fragment of 665 bp in the *vpr* region, a 1053-bp fragment in the *vif* region, and a 1530-bp fragment in the *tat* region of HIV-1. As internal control, a 285-bp fragment of human β-actin RNA was amplified using the β-Actin Primer Pair (Promega). Five microliters of each PCR product were electrophoretically separated in a 6% acrylamide gel, and the DNA was stained with ethidium bromide. The HIV-specific bands of the inhibition experiments were compared with those obtained from a 2-fold dilution series of HIV-1 III$_B$-infected MT-4 cells (obtained from the same experiment) diluted with mock-infected MT-4 cells, and the β-actin bands of the inhibition experiment were compared with those obtained from a 2-fold dilution series of HIV-1 III$_B$-infected MT-4 cells (from the same inhibition experiment) diluted with medium.

Results

Inhibition of HIV-1 Transactivation. The transactivation assay allowed us to quantify Tat-dependent HIV-1 LTR transactivation in HL$_{tat}$ and Jurkat$_{tat}$ cells after transient transfection with pHIV*LacZ* or pHIV*Luc*, respectively. Tat-independent transactivation of the HIV-1 LTR by PMA was also measured in pHIV*Luc*-transfected Jurkat cells. After the Tat-dependent or independent transactivation of the HIV-1 LTR, the expression of the reporter genes was increased up to 12-fold with respect to the nontransfected cells. Thus, these assays provide a sensitive way to study HIV-1 LTR activity. The peptoid CGP64222, previously reported as an inhibitor of the Tat/TAR complex formation (Hamy et al., 1997), inhibited the Tat-dependent expression of the reporter gene with an IC$_{50}$ value of 20 μg/ml in HL$_{tat}$ cells (not shown) and an IC$_{50}$ value of 9.5 μg/ml in Jurkat$_{tat}$ cells (Fig. 2A). CGP64222 also inhibited the Tat-independent PMA-induced transactivation of the HIV-1 LTR in Jurkat cells with an IC$_{50}$ value of 13 μg/ml (Fig. 2B). Because a previous finding demonstrated an interaction of CGP64222 with TAR (Hamy et al., 1997), the Tat-independent anti-transactivation activity noted here is probably mediated through interference with cellular activators binding to TAR. The compound was not able to inhibit reporter gene expression in HeLa or Jurkat cells transiently transfected with a construct containing the reporter gene under the control of a Tat-independent CMV promoter (Fig. 2), thus proving the specificity of CGP64222 interaction with the HIV LTR promoter. Up to the highest concentration tested (50 μg/ml), CGP64222 was not cytotoxic as measured in parallel with a tetrazolium-based viability assay.

Effect on Integration and RT. Using the 3′-processing and DNA strand-transfer assays, CGP64222 was found not to interfere with the in vitro integration up to concentrations of 100 μg/ml (W. Pluymers, unpublished data). Possible inhibition of the HIV-1 reverse transcriptase by CGP64222 was examined using particle-associated enzyme. Virus isolated from HUT/4–3 cultures, constitutively producing pNL4.3, was lysed and incubated in the presence of CGP64222 at concentrations up to 70 μg/ml. In quadruplicate tests, no significant dose-dependent inhibition of the reverse transcriptase reaction was found (not shown).

Antiviral Activity Spectrum. We assessed the ability of CGP64222-treated cells to support the replication of different HIV-1 and HIV-2 strains. CGP64222 was examined for its inhibitory effect on the cytopathogenicity induced by the HIV-1 strains III$_B$, NL4.3, RF, NDK, MN, RTMC, and HE (a clinical isolate) and the HIV-2 strain ROD. CGP64222 was found to inhibit HIV-1 III$_B$ replication in MT-4 cells with an
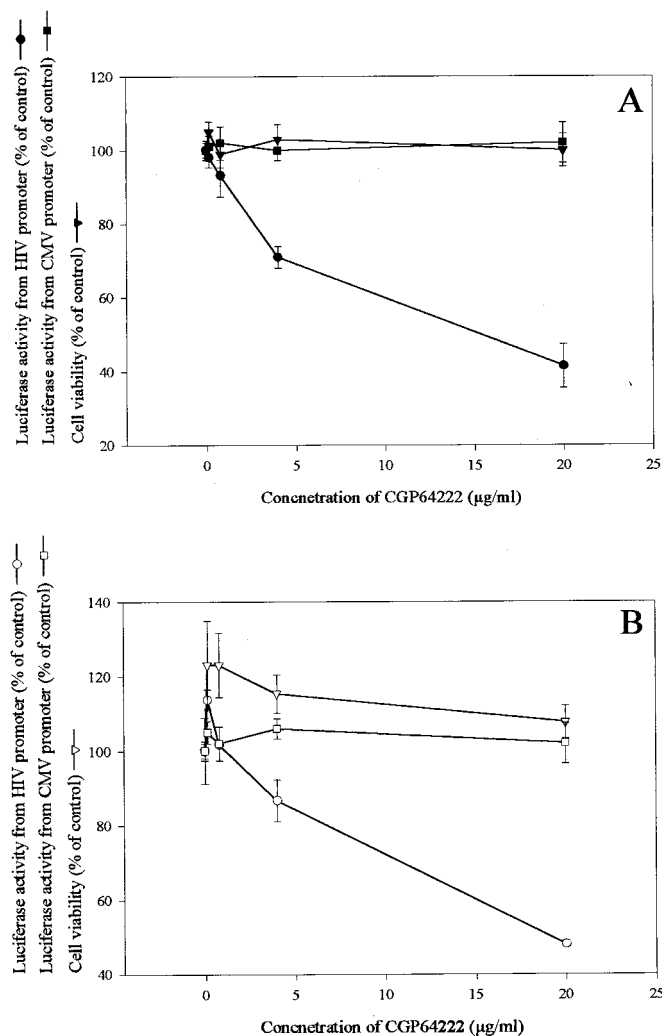
IC_{50} value of 8.4 μg/ml, whereas it was not cytotoxic at a concentration up to 125 μg/ml, resulting in a selectivity index of more than 15 (Table 1). The anti-HIV activity of CGP64222 in MT-4 cells was confirmed for a number of other HIV-1 strains, including the clinical isolate HE, the AZT-resistant strain RTMC, and the HIV-2 strain ROD (IC_{50} = 1.8–11.9 μg/ml; Table 1).

Table 1 also shows the effect of CGP64222 on the replication of NL4.3-derived strains selected for their resistance to binding/fusion inhibitors. CGP64222 inhibited the replication of viruses resistant to DS5000 and AR177, two binding

Fig. 2. Effect of CGP64222 on the expression of luciferase in the transactivation assay in Jurkat cells. Cytotoxicity was determined in parallel with a tetrazolium-based viability assay (▼, ▽). Reporter gene activity was measured with a luciferase-dependent luminescence assay. Zero percent is the luminescence measured in untransfected cells, and 100% is the luminescence measured in transfected cells without the addition of drug. After Tat-dependent transactivation of the HIV-1 LTR, the expression of luciferase activity was increased up the 12-fold, whereas for the Tat-independent transactivation, the expression of luciferase was increased up to 6-fold. Experimental data are the average of two or three independent experiments, each performed in triplicate; error bars show the S.D. A, Jurkat$_{tat}$ cells were transfected with pHIV*Luc* or with pCMV*Luc*, and the inhibition of the Tat-dependent (●) or the HIV-independent (■) luciferase expression by CGP64222 was measured. B, Jurkat cells were transfected with pHIV*Luc* and stimulated with PMA. The inhibition of the Tat-independent LTR activation (○) or the HIV-independent transcription (□) was measured via the luciferase reporter gene.

inhibitors, with an IC_{50} value 5.9 and 14.5 μg/ml, respectively. However, CGP64222, at a concentration of 50 μg/ml, did not suppress the replication of viruses made resistant to the bicyclam derivatives AMD2763 and AMD3100 (Table 1).

CGP64222 Dose-Dependently Inhibits Binding of CXCR4-Specific mAbs. Because HIV-1 strains resistant to the bicyclams (which are CXCR4-specific antagonists) also showed resistance to CGP64222, the interaction of CGP64222 with CXCR4 was further investigated. The mAbs 12G5, 171, and 2B11 react specifically with the human CXCR4 protein and recognize this receptor on many T cell lines, such as the SUP-T1 T cell line (Endres et al., 1996). The 12G5 mAb (Labrosse et al., 1998) and 171 mAb mainly bind to the second loop of CXCR4, whereas 2B11 mAb (Förster et al., 1998) interacts with the first 63 amino-terminal amino acid residues of CXCR4. CGP64222 showed a clear dose-dependent inhibition of the interaction of the antibodies 12G5 and 171 with the CXCR4 receptor on SUP-T1 cells, although relatively high concentrations of inhibitor were required. At 50 μg/ml (36 μM), CGP64222 inhibited by 30 and 51% the binding of the 12G5 mAb (not shown) to CXCR4 and of the mAb 171 to CXCR4, respectively (Fig. 3C), whereas no effect was seen on the binding of the mAb 2B11 to the receptor at this concentration (Fig. 3F). The interference of CGP64222 with mAb binding to CXCR4 is a result of direct binding of CGP64222 to CXCR4, because the experiments were performed in a way that unbound CGP64222 was washed away before binding of the mAb to CXCR4 was measured. In comparison, AMD3100 at 8 ng/ml inhibited the binding of the mAb 12G5 to CXCR4 by 80%, which is in agreement with previous data (Schols et al., 1997b). In addition, AMD3100 (D. Schols, unpublished data) or SDF-1α (Förster et al., 1998) had no effect on the binding of the 2B11 mAb to CXCR4.

To investigate the specificity of the interaction of CGP64222 with CXCR4, competition of the binding of anti-CCR5 mAb 2D7 to U87.CD4 cells expressing CCR5 and of the binding of the anti-CXCR4 mAb 171 to U87.CD4 cells expressing CXCR4 was investigated (Fig. 4). At 50 μg/ml, CGP64222 did not inhibit the binding of 2D7 mAb to the CCR5 coreceptor (Fig. 4, F and G), whereas we could detect 38% inhibition of 171 mAb binding to U87.CD4 cells express-

TABLE 1
Inhibitory effect of CGP64222 on the replication of several HIV strains in MT-4 cells
Data are averages of two or three experiments, each performed in triplicate.

HIV Strain	Inhibition of Virus Replication IC_{50}[a]	Toxicity CC_{50}[b]	Selectivity Index
	$\mu g/ml$		
III$_B$	8.4	>125	>15
RF	1.8	>125	>69
NDK	2.4	>125	>52
MN	2.5	>125	>50
RTMC	11.9	>125	>10
HE	8.4	>125	>15
ROD	5.0	>125	>25
NL4.3	5.1	>125	>10
NL4.3$_{DS5000}$[R]	5.9	>125	>8
NL4.3$_{AR177}$[R]	14.5	>125	>4
NL4.3$_{AMD2763}$[R]	>50	>125	
NL4.3$_{AMD3100}$[R]	>50	>125	

[a] 50% inhibitory concentration. Anti-HIV activity was monitored by a tetrazolium-based viability assay in HIV-infected cells.
[b] 50% cytotoxic concentration. Cytotoxicity was measured in parallel with the anti-HIV activity via a tetrazolium-based viability assay in mock-infected cells.

ing the CXCR4 coreceptor (Fig. 4, B and C). The known CXCR4 antagonist AMD3100 completely blocked the binding of the 171 mAb to CXCR4 on U87.CD4.CXCR4 cells, whereas it had no effect on the interaction of the 2D7 mAb with CCR5 (Fig. 4, D and H).

In addition, CGP64222 did not inhibit the binding of biotinylated MIP-1α to U87.CD4.CCR5 cells, whereas as control, the anti-human MIP-1α blocking antibody included in the fluorokine kit almost completely blocked the binding of the biotinylated MIP-1α (Fig. 5)

These results suggest that the CGP64222 clearly interacts with the second extracellular loop (ECL2) of the CXCR4 receptor but not with the amino-terminal end of CXCR4. Also, CGP64222 is not able to block 2D7 mAb binding to CCR5 or biotinylated MIP-1α to CCR5.

Inhibition of SDF-1α-Triggered Calcium Flux. On binding of SDF-1α to its receptor CXCR4 or on binding of RANTES to the CCR5 receptor, a transient intracellular calcium flux can be measured. Inhibition of chemokine binding can be quantified by a reduction in calcium flux. Figure 6 shows a dose-dependent inhibition of SDF-1α-mediated intracellular calcium flux by CGP64222, whereas no inhibition of RANTES-induced calcium flux was observed with CGP64222 (Fig. 6B) at a concentration (50 μg/ml) that blocked the SDF-1α-mediated signaling through CXCR4. These data indicate that like AMD3100, CGP64222 acts as a CXCR4 antagonist.

Anti-HIV activity of CGP64222 in U87.CD4 transfectants. To confirm the interaction of CGP64222 with the CXCR4 receptor, the anti-HIV activity of the peptoid was tested in the astroglioma U87 cell line stably expressing CD4

and CXCR4 or CD4 and CCR5 (Deng et al., 1997). The T-tropic NL4.3 wild-type virus was used for infection of U87.CD4.CXCR4 cells (and was not able to infect the U87.CD4.CCR5 cells), and the M-tropic HIV-1 BaL strain was used for infection of U87.CD4.CCR5 cells (and does not replicate in the U87.CD4.CXCR4 cells; Schols et al., 1998). As controls, SDF-1α and RANTES were used. SDF-1α and RANTES, which are natural ligands for CXCR4 and CCR5, respectively, are inhibitory to the replication of the T-tropic and M-tropic virus, respectively. Table 2 shows the anti-HIV effect of CGP64222 in U87.CD4-transfected cells. The peptoid was found to inhibit NL4.3 replication in U87.CD4.CXCR4 cells at an IC$_{50}$ value of 1.1 μg/ml, whereas it did not inhibit the replication of HIV-1 BaL in U87.CD4.CCR5 cells. The Tat antagonist Ro5-3335 (Hsu et al., 1991; Witvrouw et al., 1992) was able to inhibit both NL4.3 and BaL replication in the U87.CD4 transfected cells with an IC$_{50}$ value varying between 0.5 and 1.8 μg/ml (Table 2).

Stage of Interaction with HIV Replicative Cycle in MT-4 Cells. To pinpoint at which stage the peptoid actually interacts with the HIV replicative cycle in MT-4 cells, a time-of-addition experiment was carried out (Fig. 7). The cells were infected at high virus multiplicity (multiplicity of
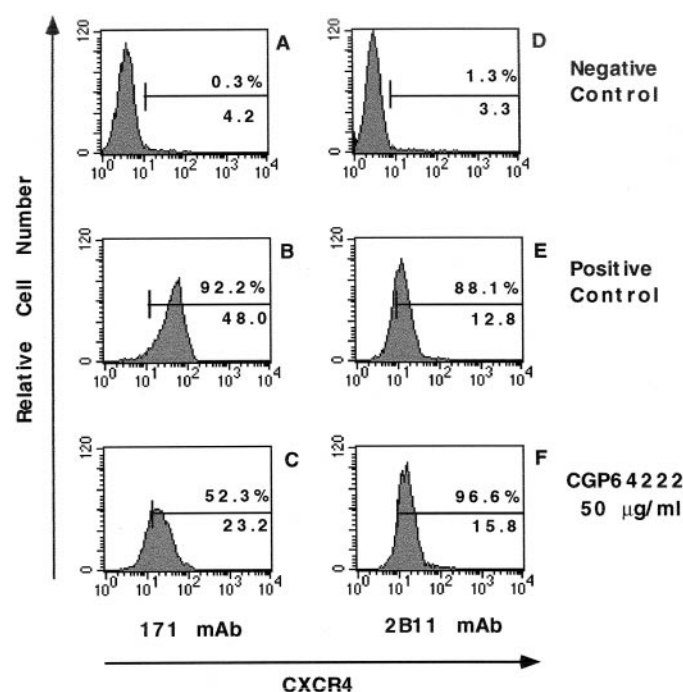
Fig. 3. Inhibition of the binding of the anti-CXCR4 mAb (171) to SUPT1 cells (left) and no inhibition of the binding of the anti-CXCR4 mAb (2B11) to SUPT1 cells (right) in the presence of CGP64222 (50 μg/ml; C and F). A and D, an isotype control mAb was used as negative control. B and E, the specific anti-CXCR4 mAb was used as positive control. The percentage of positive cells and the MFI values are indicated in each histogram. The percentage of inhibition can be calculated by comparing the MFI in presence of CGP64222 with the MFI of the positive control.
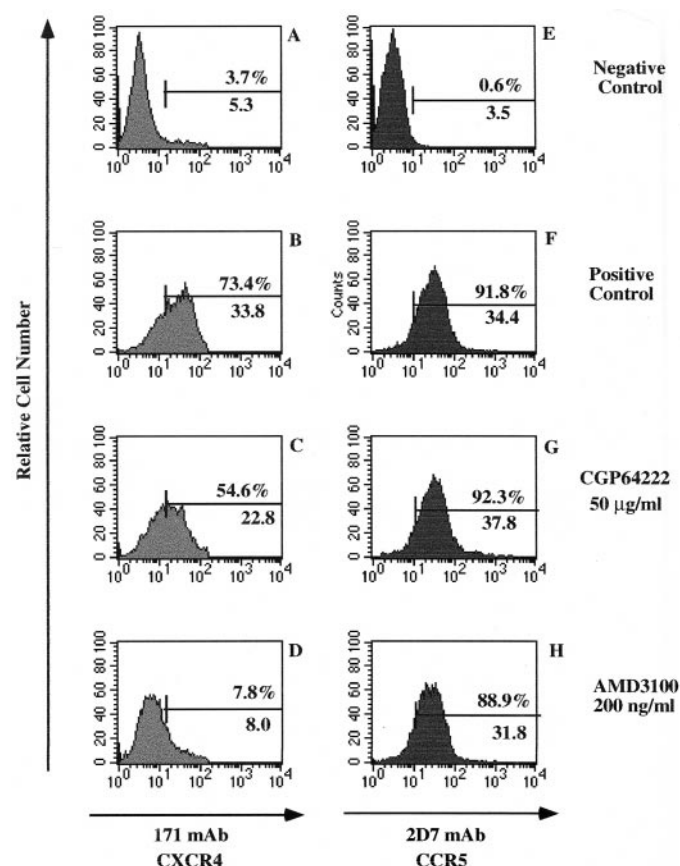
Fig. 4. Inhibition of the binding of the anti-CXCR4 mAb (171) to U87.CD4.CXCR4 cells (left) and no inhibition of the binding of the anti-CCR5 mAb (2D7) to U87.CD4.CCR5 cells (right) in the presence of CGP64222 (50 μg/ml; C and G) and AMD3100 (200 ng/ml; D and H). A and E, an isotype control mAb was used as negative control. B and F, the specific anti-CXCR4 mAb or anti-CCR5 mAb was used as positive control. The percentage of positive cells and the MFI values are indicated in each histogram. The percentage of inhibition can be calculated by comparing the MFI in presence of CGP64222 with the MFI of the positive control.

infection, 1), and the compounds were added every hour after infection during 24 hours. Depending on the stage at which a specific compound would act, and influenced by the intracellular metabolism and concentration, the addition of a given compound could be postponed for t hours without loss of activity. Dextran sulfate (100 μg/ml), which acts at the virus adsorption step (Baba et al., 1988; Mitsuya et al., 1988), must be added together with the virus ($t = 0$) to be active. For AZT (0.5 μg/ml), which, after its intracellular phosphorylation, acts at the RT step (Huang et al., 1990), the addition to the cells could be delayed for up to 5 h ($t = 5$) after infection. The protease inhibitor saquinavir (2 μg/ml), which interacts with a late event in the virus cycle (assembly of mature virions; Dreyer et al., 1989), is still effective if added as late as 21 h after infection ($t = 21$). Like dextran sulfate, the peptoid CGP64222 (150 μg/ml) has to be added together with the virus ($t = 0$) to be active against viral replication in MT-4 cells (Fig. 7). This indicates that CGP64222 must interact with an early stage of the viral replicative cycle in MT-4 cells.

We then further analyzed the inhibition by CGP64222 of viral DNA and single-spliced viral RNA production in MT-4 cells. If inhibition by CGP64222 is mediated exclusively by interference with the Tat/TAR interaction, we should see no inhibition of new viral DNA formation by reverse transcription (RT) after virus entry, whereas there should be inhibition of viral RNA production. Tat inhibition would result in diminished levels of HIV-1 specific single-spliced RNA levels as the result of interference with transactivation. To test this hypothesis, we analyzed viral DNA formation and viral single-spliced RNA production in MT-4 cells at 24 h after infection using a semiquantative PCR assay. Inhibition of HIV-1 III$_B$ replication by increasing concentrations of CGP64222 was correlated with a decrease in new viral DNA formation (Fig. 8). There was a dose-dependent decrease in proviral

DNA production that was not due to toxicity, because β-actin RNA formation was not impaired. In a parallel experiment, the RT inhibitor AZT showed a dose-dependent inhibition of viral DNA formation, whereas the protease inhibitor saquinavir had no effect on viral DNA formation (not shown). In fact, AZT inhibited both viral DNA and single-spliced viral
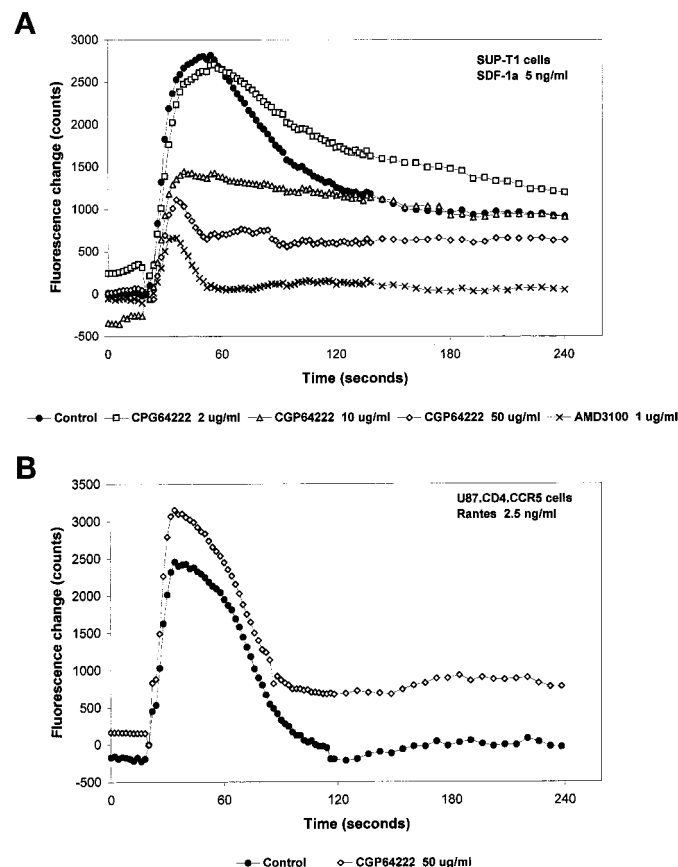
Fig. 6. A, concentration-dependent reduction of SDF-1α-induced intracellular calcium flux by CGP64222 in SUP-T1 cells. SUP-T1 cells were preincubated with CGP64222 at 0, 2, 10, and 50 μg/ml or with AMD3100 at 1 μg/ml. The cells were then stimulated with 5 ng/ml SDF-1α. The transient increase in fluorescence is a measure for the transient increase in intracellular calcium concentration on binding of the chemokine (SDF-1α) to its receptor (CXCR4). The control represents the response of the cells elicited by 5 ng/ml SDF-1α in the absence of any antagonist. Each curve shows the average of four wells. B, U87.CD4.CCR5 cells were preincubated with CGP64222 at 50 μg/ml. The cells were stimulated with 2.5 ng/ml RANTES. The transient increase in fluorescence is a measure for the increase in intracellular calcium concentration on binding of RANTES to its CCR5 receptor. The control represents the response of the cells elicited by chemokine in the absence of any antagonist.

Fig. 5. Lack of inhibition of the binding of biotinylated MIP-1α to U87.CD4.CCR5 cells in the presence of CGP64222 (50 μg/ml; D). A, only the avidin-FITC was added as negative control. B, the biotinylated MIP-1α and avidin-FITC were added as positive control. D, the biotinylated MIP-1α and avidin-FITC were added in the presence of CGP64222 (50 μg/ml), C, the biotinylated MIP-1α and avidin-FITC were added in the presence of the blocking antibody. The percentage of positive cells and MFI values are indicated in each histogram. The percentage of inhibition can be calculated by comparing the MFI in presence of CGP64222 with the MFI of the positive control.

TABLE 2

Anti-HIV activity of CGP64222, SDF-1α, RANTES, and Ro5-3335 in U87.CD4 cells transfected with CXCR4 or CCR5

Virus yield was monitored in the cell-free supernatant from U87.CD4 transfected cells 6 days after infection using a viral p24 antigen ELISA.

Compound	Inhibition of Virus Replication		Toxicity CC$_{50}$
Coreceptor HIV strain	CXCR4 NL4.3	CCR5 BaL	
	$\mu g/ml$		
CGP64222	1.1	>50	>50
SDF-1α	0.27	>1	>1
RANTES	>1	0.61	>1
Ro5-3335	0.5	1.8	5

Fig. 7. Time-of-addition experiment. MT-4 cells were infected with HIV-1 III_B at multiplicity of infection of more than 1, and the test compounds were added at different times post infection. Viral p24 Ag production was determined 29 h after infection.

RNA production (Fig. 8), with the latter being a direct result of the inhibition of proviral DNA synthesis. CGP64222 also inhibited viral RNA synthesis, but this seems to be completely secondary to inhibition of proviral DNA production (as observed for AZT). We could not detect any additional inhibitory effect with CGP64222 on viral RNA production as a result of inhibition of transactivation.

Inhibition of M-Tropic HIV Strains in PBLs. The effect of CGP64222 on virus production in acutely infected PBLs with the M-tropic HIV-1 strains BaL and ADA was examined. HIV-1 BaL is assumed to exclusively use CCR5 as coreceptor, and ADA uses mainly CCR5 (eventually, CCR2b and CCR3; Alkhatib et al., 1996). CGP64222 inhibited the replication of the HIV-1 strains BaL and ADA with an IC_{50} value of 1.2 and 0.7 μg/ml, respectively, and in the absence of cytotoxicity ($CC_{50} > 50$ μg/ml; Table 3).

Discussion

The search for drugs that can block the transactivation of HIV started several years ago (reviewed in Daelemans et al., 1999). In a recent report, a peptoidic compound, CGP64222, that was able to effectively compete with Tat for binding to TAR RNA was described (Hamy et al., 1997). We also showed here that CGP64222 is inhibiting Tat-dependent as well as Tat-independent transactivation of the HIV-1 LTR promoter, whereas it has no effect on the CMV control promoter. This confirms our previous data (Hamy et al., 1997) that the peptoid is specifically interacting with the HIV-1 LTR promoter. In vitro experiments previously showed that this interaction is with the TAR RNA. Our results suggest that CGP64222 not only inhibits Tat/TAR binding but also inhibits promoter activity in the absence of Tat, most probably through interference with cellular activators.

In this study, we also found that CGP64222 indeed is a potent inhibitor of the replication of various HIV-1 laboratory strains and clinical isolates and of HIV-2 (Table 1). The

Fig. 8. Inhibition of viral DNA and RNA formation as measured by PCR after infection with HIV-1 III_B. Acutely infected MT-4 cells were cultured in the absence (0) or in the presence of different concentrations of compound before infection. −, Mock-infected MT-4 cells. β-actin RNA production was measured as control for toxicity of the test compounds. For RNA, only the major 482-bp fragment in the *env* region of HIV-1 is shown. HIV-1 III_B RNA and DNA PCR products were loaded together i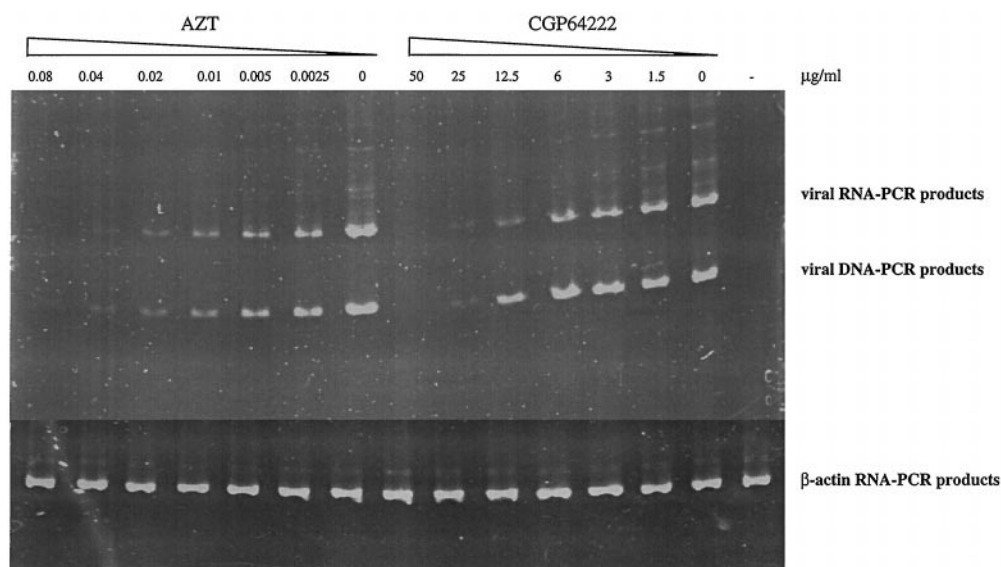n a single slot of the polyacrylamide gel (top). β-Actin RNA-PCR products were loaded on a separate gel (bottom). There is a dose-dependent inhibition of viral DNA synthesis and, hence, viral RNA production in the presence of the RT inhibitor AZT. CGP64222 is inhibiting the viral DNA synthesis, and no additional effect on viral RNA production could be detected.

compound retained full activity against an AZT-resistant HIV-1 strain. An intriguing observation, however, was that CGP64222 was inactive against HIV strains resistant to bicyclams (Table 1), suggesting that in MT-4 cells CGP64222 and bicyclams might be acting on the same target. Bicyclams have recently been shown to be selective antagonists of the CXCR4 coreceptor (Schols et al., 1997a; Donzella et al., 1998). Numerous publications over the past 2 years have demonstrated the importance of chemokine receptors for HIV entry. The following observations further confirmed the hypothesis of a common target for CGP64222 and the bicyclams. On the one hand, CGP64222 interfered in a dose-dependent manner with the binding of specific anti-CXCR4 mAbs to the second loop of the CXCR4-coreceptor on SUPT-1 T cells (Fig. 3). However, it did not block the binding of a specific mAb binding to the amino-terminal end of CXCR4 and the binding of the CCR5 mAb 2D7 to the CCR5 coreceptor (Fig. 4). CGP64222 does not appear to interact directly with CCR5 because it does not inhibit the binding of biotinylated MIP-1α or 2D7 mAb to the CCR5 receptor (Fig. 5). These results suggest that CGP64222 specifically binds to the second extracellular loop of CXCR4 in vitro, as has also been demonstrated for AMD3100 (Labrosse et al., 1998). The inhibitory potency against the anti-CXCR4 mAb binding was more than 1000-fold lower than that of the bicyclam AMD3100. This differential effect could be due to the fact that the anti-CXCR4 mAbs are binding toward a slightly different epitope than CGP64222. However, this differential activity can also be explained by the fact that the peptoid has an IC$_{50}$ value between 1 and 10 μg/ml against T-tropic HIV strains in MT-4 cells, whereas the AMD3100 IC$_{50}$ value is 1000-fold more active (1–10 ng/ml; Schols et al., 1997a). Second, in calcium flux experiments, CGP64222 proved to inhibit functionally and specifically the binding of SDF-1α to the CXCR4 receptor (Fig. 6). Third, the fact that HIV replication in U87.CD4.CXCR4 cells, but not in U87.CD4.CCR5 cells, is inhibited by CGP64222 demonstrates that this compound specifically interferes with the CXCR4 receptor (Table 3). Furthermore, in the time-of-addition experiments in MT-4 cells, we could clearly show that CGP64222 interacts with an early event in the viral replication cycle (Fig. 7). Because the compound was equally active against the NL4.3$_{DS5000}$^{R} and the NL4.3$_{AR177}$^{R}, strains made resistant to the binding inhibitors DS5000 or AR177, compared with the NL4.3 wild-type strain, we can exclude that the compound behaves as a pure virus binding inhibitor. In addition, CGP64222 inhibited the viral DNA production in MT-4 cells, confirming that the peptoid interacts with HIV replication at a stage before or coinciding with RT (Fig. 8). No additional inhibitory effect on viral RNA production in MT-4 cells could be found because

the production of single-spliced viral RNA was inhibited in a similar way as for the RT inhibitor AZT. Therefore, all the data are consistent with an activity of the peptoid at the virus/cell fusion process mediated through the CXCR4 coreceptor binding in MT-4 cells.

MT-4 cells are human T lymphotropic virus-1-transformed human T cells that express the human T lymphotropic virus-1 Tax transactivator, which is also able to induce the HIV LTR promoter. Therefore, in these cells, HIV transactivation inhibitors are very difficult to monitor, but all other inhibitory effects on viral replication can be evaluated. Our data in MT-4 cells therefore suggest that CGP64222, previously shown to be able to block the Tat/TAR interaction, is also capable of interacting with the virus-cell binding process through inhibition of the CXCR4 coreceptor and that this target is the only operational target for the anti-HIV activity of CGP64222 in MT-4 cells. However, in PBLs, the peptoid interferes with the replication of both T-tropic viruses (HIV-1$_{LAV}$; Hamy et al., 1997), which use the CXCR4 coreceptor, and M-tropic viruses (BaL), which use the CCR5 coreceptor (Table 3). These latter results suggest that in PBLs, besides the CXCR4 coreceptor target, the peptoid is still capable of inhibiting viral replication through a postentry event, most likely the Tat/TAR interaction. This differential effect seen with CGP64222 in MT-4 cells and PBLs reflects the cell type specificity of Tat inhibitors, as previously demonstrated for other Tat antagonists (Witvrouw et al., 1992).

In conclusion, our findings indicate that CGP64222, an inhibitor of the Tat/TAR interaction, also shows inhibitory activity against HIV replication through interference with virus entry consequent to blocking CXCR4 coreceptor binding.

References

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM and Berger EA (1996) CC CKR5: A RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science (Wash DC)* **272:**1955–1958.
Arya SK, Guo C, Josephs SF and Wong-Staal F (1985) Trans-activator gene of human T-lymphotropic virus type III (HTLV-III). *Science (Wash DC)* **229:**69–73.
Baba M, Pauwels R, Balzarini J, Arnout J, Desmyter J and De Clercq E (1988) Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus *in vitro. Proc Natl Acad Sci USA* **85:**6132–6136.
Balzarini J, Pérez-Pérez M-J, San-Felix A, Schols D, Perno C-F, VanDamme A-M, Camarasa M-J and De Clercq E (1992) 2'5'-Bis-O-(*tert*-butyldimethylsilyl)-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide)pyrimidine (TSAO) nucleoside analogues: Highly selective inhibitors of human immunodeficiency virus type 1 that are targeted at the viral reverse transcriptase. *Proc Natl Acad Sci USA* **89:**4392–4396.
Berger J, Hauber J, Hauber R, Geiger R and Cullen B (1988) Secreted placental alkaline phosphatase: A powerful new quantitative indicator of gene expression in eukaryotic cells. *J Virol* **68:**1179–1185.
Cherepanov P, Esté JA, Rando RF, Ojwang JO, Reekmans G, Steinfeld R, David G, De Clercq E and Debyser Z (1997) Mode of interaction of G-quartets with the integrase of human immunodeficiency virus type 1. *Mol Pharmacol* **52:**771–780.
Cullen BR (1998) HIV-1 auxiliary proteins: Making connections in a dying cell. *Cell* **93:**685–692.
Daelemans D, Esté JA, Witvrouw M, Pannecouque C, Jonckheere H, Aquaro S, Perno C-F, De Clercq E and VanDamme A-M (1997) *S*-Adenosylhomocysteine hydrolase inhibitors interfere with the replication of human immunodeficiency virus type 1 through inhibition of the LTR transactivation. *Mol Pharmacol* **52:**1157–1163.
Daelemans D, VanDamme A-M and De Clercq E (1999) Human immunodeficiency virus (HIV) gene regulation inhibitors as target for antiviral therapy. *Antiviral Chem Chemother* **10:**1–14.
De Clercq E, Yamamoto N, Pauwels R, Baba M, Schols D, Nakashima H, Balzarini J, Debyser Z, Murrer BA, Schwartz D, Thornton D, Bridger G, Fricker S, Henson G, Abrams M and Picker D (1992) Potent and selective inhibition of human

TABLE 3
Inhibitory effect of CGP64222 on the replication of M-tropic HIV-1 strains in PBLs
Data are averages of two separate experiments.

HIV-1 Strain	Inhibition of Virus Replication IC$_{50}$[a]	Toxicity CC$_{50}$[a]
	$\mu g/ml$	
BaL	1.2	>50
ADA	0.7	>50

[a] Anti-HIV activity was monitored by quantification of p24 viral antigen production in the culture supernatant.
[b] Cytotoxicity was measured in parallel by trypan blue exclusion.

immunodeficiency virus HIV-1 and HIV-2 replication by a class of bicyclams interacting with a viral uncoating event. *Proc Natl Acad Sci USA* **89:**5286–5290.

De Vreese K, Reymen D, Griffin P, Steinkasserer A, Werner G, Bridger GJ, Esté JA, James W, Henson G, Desmyter J, Anné J and De Clercq E (1996) The bicyclams, a new class of potent human immunodeficiency virus inhibitors, block viral entry after binding. *Antiviral Res* **29:**209–219.

Deng H, Unutmaz D, KewalRamani VN and Littman DR (1997) Expression cloning of new receptors used by simian and human immunodeficiency viruses. *Nature (Lond)* **388:**296–300.

Donzella GA, Schols D, Lin SW, Este JA, Nagashima KA, Maddon PJ, Allaway GP, Sakmar TP, Henson G, De Clercq E and Moore JP (1998) AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nat Med* **4:**72–77.

Doranz BJ, Grovit-Ferbas K, Sharron MP, Mao SH, Goetz MB, Daar ES, Doms RW and O'Brien WA (1997) A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as and HIV-1 coreceptor. *J Exp Med* **186:**1395–1400.

Dreyer GB, Metcalf BW, Tomaszek TA, Carr TJ, Chandler AC, Hyland L, Fakhoury SA, Magaard VW, Moore ML, JE Strickler, Debouck C and TD Meek (1989) Inhibition of human immunodeficiency virus 1 protease *in vitro*: Rational design of substrate analogue inhibitors. *Proc Natl Acad Sci USA* **86:**9752–9756.

Endres MJ, Clapham PR, Marsh M, Ahuja M, Turner JD, McKnight A, Thomas JF, Stoebenau-Haggarty B, Choe S, Vance PJ, Wells TNC, Power CA, Sutterwala SS, Doms RW, Landau NR and JA Hoxie (1996) CD4-independent infection by HIV-2 is mediated by fusin/CXCR4. *Cell* **87:**745–756.

Esté JA, Cabrera C, Schols D, Cherepanov P, Gutierrez A, Witvrouw M, Pannecouque C, Debyser Z, Rando RF, Clotet B, Desmyter J and De Clercq E (1998) Human immunodeficiency virus glycoprotein gp120 as the primary target for the antiviral action of AR177 (Zintevir). *Mol Pharmacol* **53:**340–345.

Felber BK, Drysdale CM and Pavlakis GN (1990) Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. *J Virol* **64:**3734–3741.

Förster R, Kremmer E, Schubel A, Breitfeld D, Kleinschmidt A, Nerl C, Bernhardt G and Lipp M (1998) Intracellular and surface expression of the HIV-1 coreceptor CXCR4/fusin on various leukocyte subsets: Rapid internalization and recycling upon activation. *J Immunol* **160:**1522–1531.

Hamy F, Felder ER, Heizmann G, Lazdins J, Aboul-Ela F, Varani G, Karn J and Klimkait T (1997) An inhibitor of the Tat/TAR RNA interaction that effectively suppresses HIV-1 replication. *Proc Natl Acad Sci USA* **94:**3548–3553.

Hsu M-C, Schutt D, Holly M, Slice LW, Sherman MI, Richman DD, Potash MJ and Volsky DJ (1991) Inhibition of HIV replication in acute and chronic infections in vitro by a tat antagonist. *Science (Wash DC)* **254:**1799–1802.

Huang P, Farquhar D and Plunkett W (1990) Selective action of 3′-azido-3′-deoxythimidine 5′-triphosphate on viral reverse transcriptases and human DNA polymerases. *J Biol Chem* **265:**11914–11918.

Labrosse B, Brelot A, Heveker H, Sol N, Schols D, De Clercq E and Alizon M (1998) Determinants for sensitivity of human immunodeficiency virus coreceptor CXCR4 to the bicyclam AMD3100. *J Virol* **72:**6381–6388.

Larder BA and Kemp SD (1989) Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science (Wash DC)* **246:**1155–1158.

Laspia MF, Rice AP and Mathews MB (1989) HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. *Cell* **59:**283–292.

Maio JJ and Brown FL (1988) Regulation of expression driven by human immuno-deficiency virus type 1 and human T-cell leukemia virus type E long terminal repeats in pluripotential human embryonic cells. *J Virol* **62:**1398–1407.

Mitsuya H, Looney DJ, Kuno S, Ueno R, Wong-Staal and Broder S (1988) Dextran sulfate suppression of viruses in the HIV family: Inhibition of virion binding to CD4$^+$ cells. *Science (Wash DC)* **240:**646–649.

Murakami T, Nakajima T, Koyanagi N, Tachibana K, Fujii N, Tamamura H, Yoshida N, Waki M, Matsumoto A, Yoshie O, Kishimoto T, Yamamoto N and Nagasawa T (1997) A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. *J Exp Med* **186:**1389–1393.

Neumann M, Harrison J, Saltarelli M, Hadziyannis E, Erfle V, Felber BK and Pavlakis GN (1994) Splicing variability in HIV type 1 revealed by quantitative RNA polymerase chain reaction. *AIDS Res Hum Retroviruses* **10:**1531–1542.

Oberlin E, Amara A, Bachelerie F, Bessia C, Virelizier JL, Arenzana-Seisdedos F, Schwartz O, Heard JM, Clark-Lewis I, Legler DF, Loetscher M, Baggiolini M and Moser B (1996) The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature (Lond)* **382:**833–835.

Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, Desmyter J and De Clercq E (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J Virol Methods* **20:**309–321.

Popovic M, Sarngadharan MG, Read E and Gallo RC (1984) Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science (Wash DC)* **224:**497–500.

Schols D, Esté JA, Cabrera C and De Clercq E (1998) T-cell-line-tropic human immunodeficiency virus type 1 that is made resistant to stromal cell-derived factor 1α contains mutations in the envelope gp120 but does not show a switch in coreceptor use. *J Virol* **72:**4032–4037.

Schols D, Esté JA, Henson G and De Clercq E (1997a) Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. *Antiviral Res* **35:**147–156.

Schols D, Struyf S, Van Damme J, Esté JA, Henson G and De Clercq E (1997b) Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. *J Exp Med* **186:**1383–1388.

Sodroski J, Rosen C, Wong SF, Salahuddin SZ, Popovic M, Arya SK, Gallo RC and Haseltine WA (1985) Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. *Science (Wash DC)* **227:**171–173.

Spire B, Sire J, Zachar V, Rey F, Barré-Sinoussi F, Galibert F, Hampe A and Chermann JC (1989) Nucleotide sequence of HIV-1-NDK: A highly cytopathic strain of human immunodeficiency virus. *Gene* **81:**275–284.

Willey RL, Smith DH, Lasky LA, Theodore TS, Earl PL, Moss B, Capon DJ and Martin MA (1988) In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. *J Virol* **62:**139–147.

Witvrouw M, Pauwels R, VanDamme A-M, Schols D, Reymen D, Yamamoto N, Desmyter J and De Clercq E (1992) Cell-type specific anti-human immunodeficiency virus type 1 activity of the transactivation inhibitor Ro5-3335. *Antimicrob Agents Chemother* **36:**2628–2633.

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