0026-895X/00/030641-00$3.00/0
MOLECULAR PHARMACOLOGY
Copyright © 2000 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 58:641–648, 2000

Vol. 58, No. 3
13023/845273
Printed in U.S.A.

MOL PHARM

MOLECULAR PHARMACOLOGY

Viral Entry as the Primary Target for the Anti-HIV Activity of Chicoric Acid and Its Tetra-Acetyl Esters

WIM PLUYMERS, NOURI NEAMATI, CHRISTOPHE PANNECOUQUE, VALERY FIKKERT, CHRISTOPHE MARCHAND, TERRENCE R. BURKE, JR., YVES POMMIER, DOMINIQUE SCHOLS, ERIK DE CLERCQ, ZEGER DEBYSER, and MYRIAM WITVROUW

Rega Institute for Medical Research, K. U. Leuven, Leuven, Belgium (W.P., C.P., V.F., D.S., E.D.C., Z.D., M.W.); Laboratory of Molecular Pharmacology, Division of Basic Sciences, National Cancer Institute, Bethesda, Maryland (N.N., C.M., T.R.B., Y.P.).

Received October 13, 1999; accepted June 1, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The antiviral activity of L-chicoric acid against HIV-1 has been attributed previously to the inhibition of HIV-1 integration. This conclusion was based on the inhibition of integrase activity in enzymatic assays and the isolation of a resistant HIV strain with a mutation (G140S) in the integrase gene. Here we show that the primary antiviral target of L-CA and its analogs in cell culture is viral entry. L- and D-chicoric acid (L-CA and D-CA) and their respective tetra-acetyl esters inhibit the replication of HIV-1 (III_B and NL4.3) and HIV-2 (ROD) in MT-4 cells at a 50% effective concentration (EC_{50}) ranging from 1.7 to 70.6 μM. In a time-of-addition experiment, L-CA, D-CA, L-CATA, and D-CATA were found to interfere with an early event in the viral replication cycle. Moreover, L-CA, D-CA, and their analogs did not inhibit the replication of virus strains that were resistant toward polyanionic and polycationic compounds at subtoxic concentrations. Furthermore, HIV-1 strains resistant to L-CA and D-CA were selected in the presence of L-CA and D-CA, respectively. Mutations were found in the V2, V3, and V4 loop region of the envelope glycoprotein gp120 of the L-CA and D-CA-resistant NL4.3 strains that were not present in the wild-type NL4.3 strain. Recombination of the *gp120* gene of the L-CA and D-CA resistant strain in a NL4.3 wild-type molecular clone fully rescued the phenotypic resistance toward L-CA and D-CA. No significant mutations were detected in the integrase gene of the drug-resistant virus strains. Although inhibition of HIV integrase activity by L-CA and its derivatives was confirmed in an oligonucleotide-driven assay, integrase carrying the G140S mutation was inhibited to the same extent as the wild-type integrase.

Considerable progress has been made in the treatment of patients infected with HIV, the causative agent of AIDS. Combination regimens that include potent reverse transcriptase and protease inhibitors are in clinical practice (Hammer et al., 1997). Nevertheless, therapy failure caused by the emergence of strains that are resistant to currently used drugs is common (Schinazi et al., 1996). Therefore, it is essential to find drugs targeted at alternative steps of the viral replication cycle.

The integrase of HIV is an attractive target for selective antiviral therapy because there is no known functional homolog in human cells (Thomas et al., 1996). The integration of retrotranscribed viral DNA into a host chromosome is an essential step in the replication cycle of retroviruses. The only viral enzyme required for efficient integration of retroviral cDNA is integrase (IN), a protein of 32 kDa encoded by the 3′-end region of the *pol* gene (Brown, 1997).

A vast series of compounds have been reported to inhibit the integrase activity in oligonucleotide assays; until recently, however, for none of these compounds has it been shown unambiguously that inhibition of the viral replication is caused by interference with the integration step (Neamati et al., 1997; Pommier et al., 1997). One promising compound is AR177 (Zintevir), a guanosine-quartet forming 17-mer composed of deoxyguanosine and thymidine (Rando et al., 1995). Zintevir is a potent inhibitor of HIV-1 integrase activity; nevertheless, we demonstrated that the compound owes its antiviral activity to an interaction with the viral envelope glycoprotein gp120 and not with integrase (Cherepanov et al., 1997; Esté et al., 1998). Recently, the antiviral effect of diketo acid inhibitors was described as being caused by the inhibition of the strand-transfer reaction of HIV-1 integrase (Hazuda et al., 2000).

Chicoric acid (CA; dicaffeoyltartaric acid) is a natural com-

Wim Pluymers is funded by a grant from the Flemish Institute supporting Scientific-Technological Research in Industry (IWT). Investigations were supported in part by the Biomedical Research Program of the European Commission (EC Biomed2 PL96–2161) and by grants of the Fonds voor Wetenschappelijk Onderzoek (FWO)-Vlaanderen (no.G.0104.98) and the Geconcerteerde Onderzoeksacties (GOA no.00/12) from the Flemish Community ("Vlaamse Gemeenschap").

ABBREVIATIONS: IN, integrase; CA, chicoric acid; DCQA, dicaffeoylquinic acid; SIV, simian immunodeficiency virus; DS, dextran sulfate; AZT, 3′-azido-3′-deoxythymidine; PHA, phytohemagglutinin; CPE, cytophatic effect; PCR, polymerase chain reaction; CATA, tetra-acetate form of chicoric acid.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

pound obtained from plants (Williams et al., 1996; Bonn, 1998). The chemical structure shows two carboxylic acid functionalities in the tartaric acid part of the molecule that are negatively charged at physiological pH and four weaker phenolic acid functions on the phenyl groups of the caffeic acid moieties (Fig. 1).

Dicaffeoylquinic acid (DCQA) derivatives and L-CA were shown to inhibit HIV-1 replication in cell culture at micromolar concentrations. This activity was attributed to the inhibition of integrase as observed in biochemical assays (Robinson et al., 1996b; McDougall et al., 1998), although it was suggested previously that 4,5-DCQA can inhibit HIV-replication in cell based systems, presumably by inhibition of the binding of gp120 to its cellular CD4 receptor (Mahmood et al., 1993). Molecular modeling suggested an interaction of DCQA with the core domain of HIV-1 integrase (Robinson et al., 1996a). Analogs of CA with improved activity against HIV integrase were synthesized (King et al., 1999). Inhibition of HIV integrase by DCQAs was shown to be irreversible (Zhu et al., 1999). An HIV-1 strain resistant to L-CA was selected and the observed resistance was attributed to a glycine-to-serine substitution at position 140 of the integrase (King and Robinson, 1998). CA was also found to improve the in vitro anti-HIV-1 effect of zidovudine and the protease inhibitor AG1350 (Robinson, 1998). We have recently confirmed the antiviral activity of both the D- and L-enantiomers of CA and their tetra-acetyl derivatives and their inhibition of integrase activity in an enzymatic assay (Lin et al., 1999).

Here we investigated in detail the mechanism underlying the antiviral effect of CA derivatives in cell culture. We have selected HIV-1 strains partially resistant to the drugs. Sequencing of these strains revealed mutations in the gene encoding gp120 but not in the integrase gene. Recombination of the *gp120* gene of the L-CA and D-CA resistant strain in a NL4.3 wild-type strain fully rescued the phenotypic resistance toward L-CA and D-CA. These results demonstrate that the phenotypic resistance to L-CA and D-CA can be completely explained by mutations in *gp120*. We conclude that the primary antiviral target of CA and its tetra-acetate esters is the envelope glycoprotein gp120 of HIV.

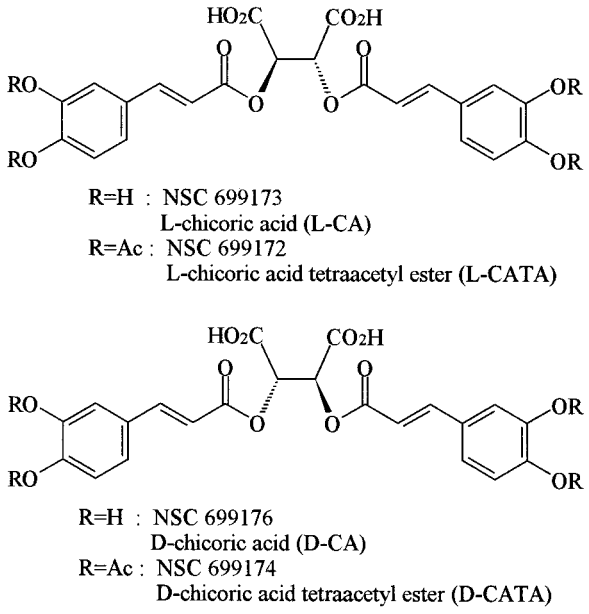

Fig. 1. Chemical structures of L-CA, D-CA, L-CATA, and D-CATA.

Materials and Methods

Compounds. L-CA, D-CA, and their tetra-acetate esters (L-CATA and D-CATA) were synthesized according to the method described by Zhao and Burke (1998). Dextran sulfate (DS) (MW 5000) was purchased from Sigma (St. Louis, MO, USA). The bicyclam derivatives AMD3100 and AMD2763 were provided by AnorMED (Langley, BC, Canada) and were synthesized as described previously (Bridger et al., 1995). 3′-Azido-3′-deoxythymidine (AZT) was synthesized according to the method described by Horwitz et al. (1964). The oligonucleotide AR177 was provided by Aronex Pharmaceutical (Woodlands, TX). Ritonavir was kindly provided by Abbott Laboratories (Abbott Park, IL).

Viruses, Cells, Antiviral Activity Assays, and Cytotoxicity Assays. Anti-HIV activity and cytotoxicity measurements in MT-4 cells (Harada et al., 1985) were based on viability of cells that had been infected or not infected with HIV in the presence of various concentrations of the test compounds. After the MT-4 cells had been allowed to proliferate for 5 days, the number of viable cells was quantified by a tetrazolium-based colorimetric method (MT-4/MTT assay or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium method) as described by Pauwels et al. (1988).

The origin of the virus stocks has been described previously: the HIV-1 strains used were HIV-1(III_B) (Popovic et al., 1984) and HIV-1(NL 4.3) (Adachi et al., 1986), a molecular clone obtained from the National Institute of Health (Bethesda, MD). HIV-2 (ROD) (Barré-Sinoussi et al., 1983) stocks were obtained from the culture supernatant of HIV-2-infected cell lines. Simian immunodeficiency virus [SIV (MAC251)] was originally isolated by Daniel et al. (1987) and was obtained from C. Bruck (Smith Kline-RIT, Rixensart, Belgium); SIV (MAC251) stocks were prepared from supernatant of SIV-infected MT-4 cells. The strains NL4.3/DS5000^{res} (Esté et al., 1997), NL4.3/AMD3100^{res}, NL4.3/AMD2763^{res} (De Vreese et al., 1996), and NL4.3/AR177^{res} (Esté et al., 1998) were selected in our laboratory after serial passage of the HIV-1 strain NL4.3 in MT-4 cells in the presence of increasing concentrations of the respective drugs.

Peripheral blood mononuclear cells from healthy donors were isolated by density centrifugation (Lymphoprep; Nycomed Pharma AS Diagnostics, Oslo, Norway) and stimulated with phytohemagglutinin (PHA) (Sigma Chemical Co., Bornem, Belgium) for 3 days. The activated cells (PHA-stimulated blasts) were washed three times with PBS and viral infections were done as described by the AIDS clinical trial group protocols (Japour et al., 1993). HIV-infected or mock-infected PHA-stimulated blasts were cultured in the presence of 25 U/ml of interleukin-2 and varying concentrations of the drugs. Supernatant was collected at day 7 and HIV-1 core antigen (p24 Ag) in the supernatant was analyzed by the p24 Ag enzyme-linked immunosorbent assay (NEN, Brussels, Belgium).

Selection of L-CA- and D-CA Resistant HIV-1 NL4.3. L-CA- and D-CA-resistant HIV-1 strains were obtained after sequential passaging of HIV-1 NL4.3 virus in the presence of increasing concentrations of L-CA or D-CA, respectively, in MT-4 cells. At the start of the selection, NL4.3 virus was inoculated on MT-4 cells in the presence of 5 μM L-CA or D-CA. When the cytopathic effect (CPE) was observed, cell culture supernatant was used as inoculum to infect new MT-4 cells at the same concentration of compound. The second time CPE was observed, the concentration of L-CA or D-CA was increased two times. After serial passaging, we were able to culture resistant virus in the presence of 63.2 μM L-CA and 84.3 μM D-CA, respectively.

Time of Addition Experiment. (Pauwels et al., 1990) MT-4 cells were infected with HIV-1 (III_B) at a multiplicity of infection of 0.5, and the test compounds were added at different times after infection. Viral p24 Ag production was determined at 31 h after infection by the p24 Ag enzyme-linked immunosorbent assay (NEN).

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

Virus Adsorption Assay. In this assay, the inhibitory effects of L- and D-CA, DS, and the bicyclam AMD3100 on virus adsorption to MT-4 cells were measured. We evaluated the binding capacity of NL4.3 wild-type strain in parallel with the L-CA- and D-CA-resistant NL4.3 strains. Therefore, MT-4 cells (5×10^5 cells per tube) were incubated with the respective HIV strains (corresponding to 100 ng of p24) in the presence or absence of serial dilutions of the test compounds. After a 2-h incubation at 37°C, the cells were washed extensively with PBS to remove the unadsorbed virus particles. Then the cells were lysed with PBS containing 0.5% of tergitol Nonidet P-40 (Sigma, St. Louis, MO). The amount of p24 Ag was determined by the p24 Ag Elisa.

Genotypic Analysis of gp120 Encoding Sequences. MT-4 cells were infected with the HIV-1(NL4.3) strains in vitro selected in the presence of L-CA and D-CA. DNA extraction of proviral DNA was performed using the QIAamp blood Kit (Qiagen, Westburg, Leusden, The Netherlands). A 2105-nucleotide base pair fragment (codons 1 to 445) of *gp120* was amplified in a nested polymerase chain reaction (PCR) using Expand High Fidelity PCR system (Boehringer Mannheim, Roche, Germany), which is composed of an enzyme mix containing thermo-stable *Taq* DNA and Pwo DNA polymerase with 3′-5′ exonuclease proofreading capacity. The outer PCR reaction was performed on a Perkin Elmer Gene Amp PCR system 9600 and the inner PCR reaction on a Biometra Trioblock (Westburg) using the primers AV310 (5′-AGC AGG ACA TAA T/CAA GGT AGG-3′ corresponding to position 5447–5467 of NL4.3) and AV311 (5′ GGA GAA GTG AAT TAT ATA AG/AT ATA AAG TAG-3′ corresponding to position 7630–7659 of NL4.3), followed by the primers AV312 (5′-AGA A/GGA C/TAG ATG GAA CAA GCC CCA G-3′ corresponding to position 5549–5573 of NL4.3) and AV313 (5′-GAC CTG GAG GAG GAA/G ATA TGA G/AGG A-3′ corresponding to position 7605–7629 of NL4.3). The outer cycling conditions were as follows: a first denaturation step of 3 min at 95°C followed by 40 cycles consisting of 45 sec at 95°C, 30 sec at 50°C, and 2 min at 72°C. A final extension was performed at 72°C for 10 min. For the inner cycling, the following conditions were used: after 3 min at 95°C, 30 cycles of 45 sec 95°C, 30 sec 58°C, 2 min 72°C and 10 min 72°C extension.

PCR products were purified and concentrated using the PCR purification kit (Qiagen, Westburg, Leusden, The Netherlands). To carry out the sequencing reaction, the ABI PRISM Dye terminator cycle sequencing core kit (Perkin Elmer, Brussels, Belgium) was used. The primers used to sequence the entire *gp120* gene were: AV304 (5′-ACA TGT GGA AAA ATG ACA TGG T-3′ corresponding to position 6504–6525 of NL4.3), AV305 (5′-GAG TGG GGT TAA TTT TAC ACA TGG-3′ corresponding to position 6552–6575 of NL4.3), AV306 (5′-TGT CAG CAC AGT ACA ATG TAC ACA-3′ corresponding to position 6946–6969 of NL4.3), AV307 (5′-TCT TCT TCT GCT AGA CTG CCA T-3′ corresponding to position 6987–7008 of NL4.3), AV308 (5′-TCC TCA GGA GGG GAC CCA GAA ATT-3′ corresponding to position 7313–7336 of NL4.3), AV309 (5′-CAG TAG AAA AAT TCC CCT CCA CA-3′ corresponding to position 7333–7355 of NL4.3), and AV313 (5′-GAC CTG GAG GAG GAA/G ATA TGA G/AGG A-3′ corresponding to position 7605–7629 of NL4.3). The samples were loaded on the ABI PRISM 310 Genetic Analyser (Perkin Elmer, Brussels, Belgium). The sequences were analyzed using the software program Geneworks 2.5.1 (Intelligenetics Inc., Oxford, UK).

***gp120*-Recombination.** MT-4 cells were subcultured at a density of 500,000 cells/ml on the day before transfection. Cells were pelleted and resuspended in phosphate-buffered saline at a concentration of 3.1×10^6 cells/ml. For each transfection, 2.5×10^6 cells (0.8 ml) were used. Transfections were performed by electroporation using an EASYJECT (Eurogentec, Seraing, Belgium). Cells were cotransfected with 10 μg of a linearized *gp120*-deleted NL4.3 clone (V. Fikkert, C. Pannecouque, P. Cherepanov, C. Van Laethem, E. De Clercq, A.M. Vandamme, and M. Witvrouw, in preparation.) and 2 μg of purified and concentrated AV312-AV313 inner PCR product (PCR Purification Kit; Qiagen, Westburg, The Netherlands). The electroporation conditions were 300 μF and 300 V. After 30-min incubation at room temperature, the transfected cell suspension in 5 ml of culture medium was incubated at 37°C in a humidified atmosphere with 5% CO_2. When full CPE was observed in the culture (about 8 days after transfection), cells were pelleted, and the supernatant containing the recombinant virus was stored in 1 ml aliquots at −80°C for subsequent infectivity and drug susceptibility determinations in the MT-4/MTT assay.

TABLE 1
Antiviral activity and cytotoxicity of L-CA and D-CA and their tetraacetate esters

Compound	EC_{50} (μM)[a]				CC_{50} (μM)[b]
	HIV-1		HIV-2	SIV	
	III_B	NL4.3	ROD	MAC251	
L-CA	5.2 ± 0.1	12.7 ± 8.5	70.6 ± 36.9	>115	115.0 ± 21.0
L-CATA	4.0 ± 0.3	8.0 ± 2.8	25.5 ± 13.8	>74	74.0 ± 1.7
D-CA	1.7 ± 0.1	10.9 ± 6.5	8.5 ± 3.1	>111	111.0 ± 25.0
D-CATA	3.8 ± 1.2	7.8 ± 2.6	5.0 ± 1.9	>93	93.0 ± 8.1
DS	0.08 ± 0.06	0.08 ± 0.08	0.008 ± 0.006	0.7 ± 0.4	>25

[a] 50% effective concentration, or concentration required to inhibit the viral cytopathic effect by 50% in MT-4 cell cultures.
[b] 50% cytotoxic concentration, or concentration required to inhibit the viability 50% of MT-4 cell by 50%.
All data represent the mean ± S.D. for at least two separate experiments.

TABLE 2
Antiviral activity of L-CA and D-CA and their tetraacetate esters against HIV strains resistant to entry inhibitors

Compound	EC_{50} (μM)[a] (Fold Resistance)[b]				
	NL4.3 WT	NL4.3 DS^{res}	NL4.3 $AR177^{res}$	NL4.3 $AMD2763^{res}$	NL4.3 $AMD3100^{res}$
L-CA	12.7 ± 8.5	>115 (>9)	>115 (>9)	>115 (>9)	>115 (>9)
L-CATA	8.0 ± 2.8	>74 (>9)	>74 (>9)	>74 (>9)	>74 (>9)
D-CA	10.9 ± 6.5	>111 (>10)	>111 (>10)	>111 (>10)	>111 (>10)
D-CATA	7.8 ± 2.6	>93 (>12)	>93 (>12)	>93 (>12)	>93 (>12)
DS	0.08 ± 0.08	>25 (>313)	3.9 (48)	6.1 (77)	>25 (>313)

WT, wild-type.
[a] 50% effective concentration, or concentration required to inhibit the viral cytopathic effect by 50% in MT-4 cell cultures.
[b] Fold resistance is the ratio of EC_{50} determined for the resistant strain divided by the EC_{50} obtained for the HIV-1 NL4.3WT strain.
All data represent mean ± S.D. for at least two separate experiments.

aspet

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

HIV-1 Integrase Assays. The recombinant enzyme preparation of wild-type His-tagged integrase the substrate and target DNA were as described previously (Cherepanov et al., 1997; Debyser et al., 2000). The following high-performance liquid chromatography-purified deoxynucleotides were purchased from Amersham-Pharmacia Biotech: INT1, 5′-TGTGGAAAATCTCTAGCAGT; INT2, 5′-ACTGCTAGAGATTTTCCACA; T35, 5′-ACTATACCAGACAATAATTGTCTGGCCTGTACCGT; and SK70, 5′-ACGGTACAGGCCAGACAATTATTGTCTGGTATAGT. The oligonucleotides INT1 and INT2 correspond to the U5 end of the HIV-1 long-terminal repeats. The 3′-processing, overall integration, and strand-transfer assays were slightly modified from published procedures. The final reaction mixture for the 3′-processing assays contained 20 mM HEPES, pH 7.5, 5 mM dithiothreitol, 10 mM $MgCl_2$, 75 mM NaCl, 5% (v/v) polyethylene glycol 8000, 15% (v/v) dimethyl sulfoxide, 30 nM concentrations of the oligonucleotide substrate, and 230 nM His-tag IN in a volume of 10 μl. Reactions were started by the addition of the enzyme. Inhibitors were incubated shortly with the reaction components before the addition of IN. Reactions were allowed to proceed at 37°C for 7 min and stopped by the addition of formamide dye. In the overall integration assay, the reaction is allowed to proceed for 60 min before the addition of formamide dye.

The strand transfer was assayed in the following way: 30 nM DNA substrate was preincubated with 230 nM IN at 37°C for 5 min to allow the cleavage reaction to occur. The composition of the reaction mixture was identical with that in the processing assay. After 5 min, 1 μl of excess target DNA (final concentration, 250 nM) with or without inhibitor was added, and the samples were incubated at 37°C for 1 h. This excess target DNA competitively blocks further binding of IN to the viral substrate. Reactions were stopped by the addition of formamide dye and products were separated in a 15% denaturing polyacrylamide/urea gel. Autoradiography was performed by exposing the wet gel to X-ray film (CURIX RP1, Agfa, Germany). Quantification of the results was performed using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Creation of the G140S IN Mutant Using Site-Directed Mutagenesis. The G140S mutant was generated by using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Two oligonucleotides, G140S+ (26-mer, 5′-TGTAGGGAATGCTAAATTCCTGCTTG-3′), and G140S− (28-mer, 5′-GATCAAGCAGGAATTTAGCATTCCCTAC-3′) embedding the 140 mutation were designed and purchased from Bioserve (Laurel, MD). PCR performed with these two primers and pET-15b-IN1–288/F185K/C280S plasmid (Jenkins et al., 1996) as starting template, produced the pET-15b-IN1–288/F185K/C280S/G140S plasmid containing the desired mutation. After transformation of *Escherichia coli* strain XL1-Blue by this plasmid, five colonies were sequenced and all of them contained the G140S mutation.

Expression and Purification of F185K/C280S/G140S Integrase from *E. coli*. pET-15b-IN1–288/F185K/C280S/G140S plasmid was expressed in *E. coli* strain BL21 as described previously (Jenkins et al., 1996) with the following modifications. Cells were grown in 1000 ml of LB medium (Digene, Beltsville, MD) containing 50 μg/ml of ampicillin until absorbance reached 0.8 at 600 nm. Protein expression was induced for 3 h by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside, and cells were harvested and resuspended in lysis buffer containing 25 mM HEPES, pH 7.5, 1 M NaCl, 5 mM imidazole, 2 mM β-mercaptoethanol, and 0.3 mg/ml lysozyme. After 30 min on ice and sonication, lysed cells were centrifuged for 20 min at 30,000*g* and the supernatant was applied onto a nickel-Sepharose column. Integrase retained on the column was

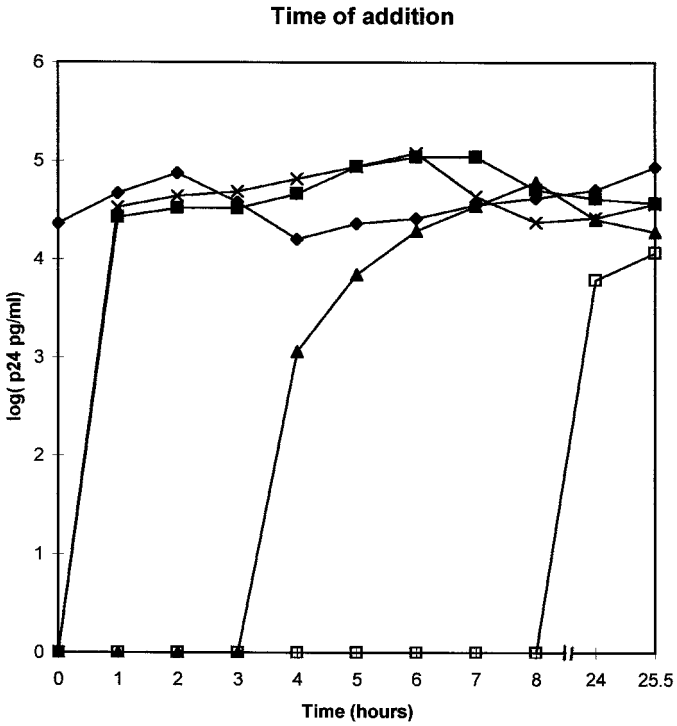

Fig. 2. Time-of-addition experiment. MT-4 cells were infected with HIV-1(III_B) at a multiplicity of infection of 0.5, and the test compounds were added at different times after infection. Viral p24 Ag production was determined at 31 h after infection and is expressed as the $\log_{10}$ of the p24 Ag content in pg/ml. ◆, control; ■, DS (20 μM); ▲, AZT (1.9 μM); ×, Ritonavir (2.8 μM); □, L-CA (50 μM).

TABLE 3

Inhibitory effects of antiviral compounds against HIV-1 (NL4.3) strains selected for resistance against L-CA or D-CA

	EC_{50} (μM)[a] (Fold Increase)[b]		
	NL4.3 WT	NL4.3 L-CA^{res} [c]	NL4.3 D-CA^{res} [d]
L-CA	12.7 ± 8.5	66.8 (5)	>115 (>9)
L-CATA	8.0 ± 2.8	62.0 (8)	37.0 (5)
D-CA	10.9 ± 6.5	>111 (>10)	>111 (>10)
D-CATA	7.8 ± 2.6	75.7 (10)	92.0 (12)
DS	0.08 ± 0.08	2.1 (26)	1.24 (16)

WT, wild type.

[a] 50% effective concentration, or concentration required to inhibit the viral cytopathic effect by 50% in MT-4 cell cultures.

[b] Fold resistance is the ratio of EC_{50} determined for the resistant strain divided by the EC_{50} obtained for the HIV-1 NL4.3WT strain.

[c] Virus strain selected after 23 passages in MT-4 cells in the presence of increasing concentrations of L-CA (up to 63.2 μM).

[d] Virus strain selected after 26 passages in MT-4 cells in the presence of increasing concentrations of D-CA (up to 84.3 μM).

All data represent mean ± S.D. for at least two separate experiments.

TABLE 4

Mutations in the *gp120* of wild-type NL4.3, L-CA^{res} NL4.3, and D-CA^{res} NL4.3

Amino Acid Position (Region)	NL4.3 Wild-Type Strain		NL4.3 L-CA^{res} Strain		NL4.3 D-CA^{res} Strain	
	Codon	Amino Acid	Codon	Amino Acid	Codon	Amino Acid
160 (V2)	AGC	S	AAC	N	AAC/AGC	N/S
295 (V3)	AAT	N	GAT	D	AAT/GAT	N/D
372 (V4)	ACT	T	AAT	N	ACT	T
378 (V4)	ACT	T	AAT	N	ACT	T
379 (V4)	GAA	E	GAA/CAA	E/Q	GAA/AAA	E/K

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

washed with a buffer containing 25 mM HEPES, pH 7.5, 0.5 M NaCl, 2 mM β-mercaptoethanol, and an increasing imidazole concentration from 20 to 250 mM. The protein was then eluted with the same buffer containing 750 mM imidazole and dialysed overnight against 25 mM HEPES, pH 7.5, 1 M NaCl, 2 mM, 2 mM EDTA, 10 mM dithiothreitol, 2 mM β-mercaptoethanol, 100 mM imidazole, and 10% glycerol.

IN Assays Using the F185K/C280S Integrase and its G140S Mutant. These methods were performed essentially as described previously (Mazumder et al., 1999).

Results

Antiviral Activity Spectrum. The two enantiomers of CA (L-CA and D-CA) and their respective tetra-acetate esters (L-CATA and D-CATA) (Fig. 1) proved active against different strains of HIV-1 (III_B, NL4.3) and against HIV-2 (ROD) with EC_{50} values ranging from 1.7 to 70.6 μM, whereas SIV (MAC251) was not sensitive to their inhibitory effects at subtoxic concentrations in MT-4 cells (Table 1). L-CA and D-CA were toxic for the cells at 115 and 111 μM, respectively, resulting in a selectivity index of 22 for L-CA and 65 for D-CA against the replication of HIV-1(III_B) in MT-4 cells. L- and D-CATA showed a selectivity index of 18 and 24, respectively. The CA derivatives showed reduced activity against HIV-1 strains that were resistant toward DS 5000, the G-quartet AR177, and the bicyclams AMD2763 or AMD3100 (Table 2). These compounds are known to interfere with viral entry and the resistance of viral strains against the inhibitory effects of these compounds is caused by mutations in gp120 (De Vreese et al., 1996; Esté et al., 1997; Esté et al., 1998). The bicyclams are specific CXCR4 antagonists and do not interact directly with *gp120* (Schols et al., 1997). The cross-resistance observed with the CA derivatives against viruses known to be resistant to gp120-targeted compounds suggests an interaction of the CA analogs targeted at the envelope glycoprotein gp120.

When the CA derivatives were evaluated for their antiviral activity in peripheral blood mononuclear cells the activity was markedly reduced (data not shown). A likely explanation is the strong esterase activity in these cells that may lead to hydrolysis of the CA and its analogs to tartaric acid and caffeic acid, two molecules that are deprived of any anti-HIV activity (McDougall et al., 1998). The esterase is predominantly intracellular, but we cannot exclude that dying cells release the esterase in the medium.

Time (Site) of Intervention. A time-of-addition experiment was performed to pinpoint the possible step(s) in the replicative cycle of HIV-1 that is (are) inhibited by D- and L-CA. HIV-1 replication, as measured by p24 Ag production, could only be inhibited by DS if added at the time of infection. Addition of the nucleoside reverse transcriptase inhibitor AZT could be delayed up to 4 h after infection without loss of inhibitory activity. Addition of L- and D-CA at different concentrations could not be delayed, like DS, more than 1 h after infection as is shown in Fig. 2. These results indicate that CA and its analogs interact with an early stage (binding/fusion) of the viral replicative cycle.

Selection of HIV-1 (NL4.3) Mutant Strains. HIV-1 strains resistant to L- and D-CA were generated in MT-4 cells by passaging the virus in the presence of increasing concentrations of L- and D-CA, respectively. An HIV-1(NL4.3) selected strain, NL4.3 L-CA^{res} was able to grow in the presence of 63.2 μM L-CA after 23 passages. NL4.3 L-CA^{res} proved to be 5- to 10-fold resistant to L-CA (and its derivatives) and 26-fold resistant to DS (Table 3). NL4.3 D-CA^{res} was obtained after 26 passages in the presence of increasing concentrations of D-CA (final concentration was 84.3 μM). NL4.3 D-CA^{res} was 5-to 12-fold resistant to D-CA (and its derivatives) and 16-fold resistant to DS (Table 3).

Proviral DNA of this Resistant Virus (NL4.3 D-CA^{res}) was isolated and sequencing revealed several mutations in the *gp120* gene, which are summarized in Table 4. The integrase gene was sequenced as well; no mutations were found except for one substitution (C56Y), described earlier as a polymorphism in the HIV-1 integrase gene (Pommier et al., 1997).

Virus Adsorption Assay. To confirm that the anti-HIV activity of L-CA and D-CA is attributed to the inhibition of virus binding or fusion to the cells, a virus adsorption assay was performed. A known adsorption inhibitor DS and a fusion inhibitor AMD3100 were included as control specimens. Cells were infected with wild-type, L-CA- or D-CA-resistant NL4.3 strain. DS, L-CA, and D-CA inhibited the binding of

TABLE 5
Inhibition of HIV binding to MT-4 cells by CA derivatives DS and AMD3100

	IC_{50} (μM)[a]		
	NL4.3 WT	NL4.3 L-CA^{res} [b]	NL4.3 D-CA^{res} [c]
L-CA	14.3 ± 15.6	>210	>210
D-CA	2.1 ± 1.0	>210	>210
DS	0.01 ± 0.001	>2	>2
AMD3100	>1	>1	>1

WT, wild type.
[a] 50% effective concentration, or concentration required to inhibit virus binding to MT-4 cells by 50%.
[b] Virus strain selected after 23 passages in MT-4 cells in the presence of increasing concentrations of L-CA (up to 63.2 μM).
[c] Virus strain selected after 26 passages in MT-4 cells in the presence of increasing concentrations of D-CA (up to 84.3 μM).
All data represent mean values ±S.D. for at least two separate experiments.

TABLE 6
Sensitivity of HIV-1 strains selected in vitro for resistance against L-CA and D-CA and the corresponding *gp120*-recombined resistant strains in comparison to the parental NL4.3 wild-type strain.

Virus Strains[b]	IC_{50}[a]			
	DS	AMD3100	L-CA	D-CA
NL4.3	0.08 ± 0.08	0.01 ± 0.006	12.7 ± 8.5	10.9 ± 6.5
NL4.3 RV	0.11 ± 0.06	0.01 ± 0.008	14.2 ± 3.6	20.9 ± 5.4
L-CA^{res}	2.3 ± 0.4 (29)	0.02 ± 0.004 (2)	66.8 ± 5.4 (5)	>111 (>10)
L-CA^{res} RV	2.6 ± 0.9 (24)	0.02 ± 0.002 (2)	63.3 ± 42.8 (4)	>111 (>5)
D-CA^{res}	1.7 ± 0.9 (21)	0.02 ± 0.001 (2)	>115 (>9)	>111 (>10)
D-CA^{res} RV	1.9 ± 0.6 (17)	0.02 ± 0.004 (2)	>115 (>8)	>111 (>5)

[a] 50% inhibitory concentration IC_{50} (μM) in MT-4 cells.
[b] IC_{50} values are given for the wild-type or resistant selected strains and the wild-type or resistant strains with recombined *gp120* gene. In parentheses are the fold increases in IC_{50} value of the resistant strains compared with the wild-type strains.

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

aspet

wild-type virus to the cells with an IC_{50} of 0.01 μM, 14.3 μM, and 2.1 μM, respectively. As expected, the CXCR4 antagonist AMD3100 (Schols et al., 1997) did not inhibit virus adsorption (Table 5). The inhibitors DS, L-CA, and D-CA lost their inhibitory effect on the cell binding if the L-CA- and D-CA-resistant NL4.3 strains were used. These results indicate that the mechanism of anti-HIV action of L-CA and D-CA is based on inhibition of adsorption. Compounds had no effect on the binding of mAb to CD4 or CXCR4 (data not shown).

***gp120*-Recombination.** To verify that the mutations in *gp120* were sufficient to explain the resistant phenotype, recombinant viruses were constructed that contain the *gp120* gene of the strains selected in vitro for resistance against L-CA and D-CA. These recombinant strains are referred to as L-CA^{res}RV and D-CA^{res}RV. The resistant profiles of the recombinant viruses were similar to the in vitro selected strains: both L- and D-CA^{res}RV were less susceptible to inhibition by DS, L-CA, and D-CA compared with the NL4.3RV strain in which the wild-type *gp120* gene is recombined (Table 6). No cross-resistance was observed toward the fusion inhibitor AMD3100.

Integrase Assays. Integrase catalyzes two subsequent reactions: 3′-processing and DNA strand transfer (Fig. 3A). It can also catalyze a reversal of the integration reaction, denoted the disintegration reaction (Fig. 3B). We have previously established enzymatic assays that can evaluate potential inhibition of both the 3-processing or strand-transfer reaction independently from each other (Cherepanov et al., 1997). All molecules tested inhibited the 3′-processing reaction of HIV-1 IN at concentrations that were in accordance with the reported values (Table 7). The compounds were inactive in the DNA strand-transfer assay (Table 7). The IC_{50} values obtained for the overall integration reaction (3′-processing + DNA strand transfer) were similar to the values obtained for the 3′-processing reaction.

Sensitivity of the G140S IN Mutant toward the Compounds. An HIV strain resistant to L-CA was reported to contain the G140S substitution in the integrase gene (King and Robinson, 1998). We now have constructed and purified recombinant HIV-1 IN carrying this mutation. The enzymatic activity of the mutant enzyme was somewhat lower than that of the wild-type enzyme, especially with respect to the DNA strand transfer activity (data not shown). The wild-type integrase used in this assay carried F185K/C280S mutations to increase the solubility (Jenkins et al., 1996). The G140S mutation was introduced in this soluble mutant. All CA derivatives tested inhibited the 3′-processing activity and the disintegration activity of HIV-IN and the G140S mutant to the same extent (Table 8). The compounds also inhibited IN-DNA cross-linking within a similar range for both enzymes (data not shown). Taken together, these data show that the CAs without any discrimination can inhibit wild-type IN and the G140S mutant. This indicates that the G140S mutation in the IN gene described earlier cannot be hold responsible for the resistant phenotype of the selected virus.

Discussion

We have shown that the anti-HIV activity of CA and its derivatives (L-CA, D-CA, L-CATA, and D-CATA) in cell culture is caused by interference of the compounds with viral entry, much alike polyanionic molecules such as dextran sulfate (DS) and the G-quartet AR177 (Zintevir). The structure of CA contains several negatively charged residues, which is a point of similarity with other polyanionic compounds. However, CAs have only a limited number of negative charges in contrast with the other described polyanionic anti-HIV compounds.

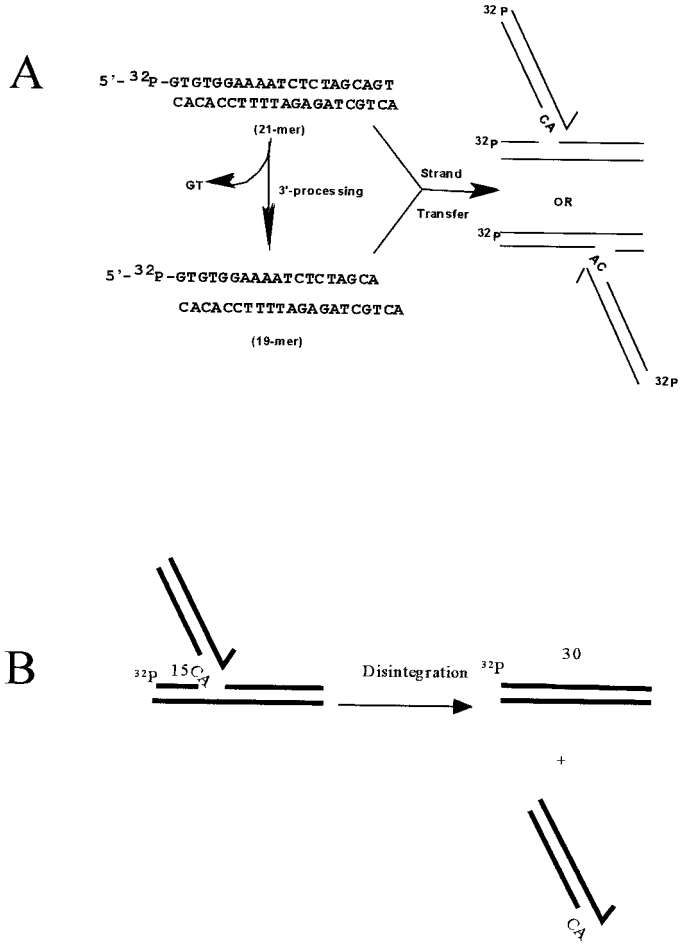

Fig. 3. HIV-1 integrase catalytic assays. A, a 21-mer blunt-end oligonucleotide corresponding to the U5 end of the HIV-1 proviral DNA, 5′ end-labeled with ^{32}P, reacts with purified HIV-1 integrase. The initial step involves nucleolytic cleavage of two bases from the 3′ end, resulting in a 19-mer oligonucleotide. The second step, 3′-end joining or strand transfer, involves joining of this recessed 3′ end to the 5′ end of an integrase-induced break in another identical oligonucleotide, which serves as the target DNA. B, HIV-1 integrase disintegration assay. The substrate oligonucleotide mimics a strand transfer step product (i.e., a Y oligonucleotide containing a 15-mer oligonucleotide 5′-end-labeled with ^{32}P). HIV-1 integrase-mediated disintegration generates a 30-mer oligonucleotide.

TABLE 7
Inhibition of the 3′-processing, strand transfer, and overall integration reaction

Compound	IC_{50} (μM)		
	3′-Processing reaction	Strand transfer reaction	Overall reaction
L-CA	1.0 ± 0.3	>105	0.2 ± 0.2
L-CATA	4.0 ± 2.1	>78	0.6 ± 0.06
D-CA	0.5 ± 0.1	>105	0.3 ± 0.02
D-CATA	4.5 ± 2.3	>78	2.4 ± 0.08
DS	0.07 ± 0.02	0.4 ± 0.1	ND

ND, not determined.
All data represent mean values ± S.D. for at least two independent experiments.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

We have shown that CA and CATA inhibit the virus replication of different HIV-1 (III_B, NL4.3) strains and HIV-2 (ROD) strain with EC_{50} values ranging from 1.7 to 70.6 μM. The cytotoxicity (CC_{50}) for MT-4 cells is in the range of 74 to 115 μM. The loss of antiviral activity of CA and CATA against HIV-1 NL4.3 strains that are resistant to inhibitors of viral entry (DS, AR177, AMD2763, AMD3100) and that are known to contain mutations in the viral envelope *gp120* suggested to us that the antiviral activity of CA may involve an interaction with gp120.

In a time-of-addition experiment, the addition of inhibitors can be postponed after infection as long as the replication step with which the inhibitor interferes has not taken place. Like DS, CA had to be present at the time of virus adsorption to inhibit HIV replication, confirming the interaction with an early process in the viral replication cycle. For a "true" integrase inhibitor, one should be able to postpone addition of the compound for more than 6 h after infection.

In a direct virus-cell binding assay, binding of wild-type HIV but not of L-CA- or D-CA-resistant virus was clearly inhibited by DS, L-CA, and D-CA, whereas the bicyclam AMD3100, a CXCR4 antagonist, was inactive in the binding assay. These data provide direct proof that the CA derivatives block virus adsorption.

To identify the molecular target(s) of CA, HIV strains resistant to the compounds were developed in cell culture. We have previously shown that HIV-1 strains that are resistant to inhibitors targeted at the binding/fusion step of replication can emerge after sequential passaging of the virions in cell culture in the presence of increasing concentrations of the compounds. The resistance to L-CA developed after 23 passages of HIV-1 NL4.3 in MT-4 cells in the presence of increasing concentrations of the compound. The resistant strain was able to replicate in the presence of L-CA at concentrations up to 63.2 μM. DNA sequence analysis of the *gp120*-encoding region showed the emergence of mutations in the L-CA-resistant strain that were not present in the wild-type strain. The mutation found in the integrase gene (C56Y) has been described as a polymorphism (Pommier et al., 1997). To definitively rule out the role of the integrase gene in the resistant phenotype, the *gp120* gene of each resistant virus was recombined in a wild-type NL4.3 virus, deleted for *gp120*. The recombinant viruses were capable of infecting MT-4 cells. The resistance profile of the recombined virus toward the compounds L-CA, D-CA, DS, and AMD3100 is essentially the same as the resistant strain selected in vitro. It can thus be concluded that the resistance phenotype can be completely rescued by the recombination of the *gp120* gene.

The genotypic analysis of the gp120 coding region reveals a typical mutation in the V3 loop, N295D, that has already been described for DS and SOF-1α (Esté et al., 1997; Schols et al., 1998). In addition, four novel mutations (S160N, T372N, T378N, and E379Q) were selected in the presence of L-CA, whereas in the presence of D-CA, the mutations S160N and E379K arose in *gp120*. Further investigations regarding the mechanism of interference by different compounds with gp120 will be described elsewhere (V. Fikkert, C. Pannecouque, P. Cherepanov, C. Van Laethem, E. De Clercq, A.M. Vandamme, and M. Witvrouw, in preparation.).

Theoretically, it cannot be excluded that further passage of CA-resistant HIV strains in further-increasing concentrations of L-CA might eventually lead to the emergence of mutations in the integrase gene. However, it should be pointed out that selection of resistance occurred at concentrations of L-CA that were already toxic for the host cell. Furthermore, the instability of the ester function linking the tartaric and the caffeic acid moieties confounds achieving high drug levels in cell culture. Synthesis of congeners with esters being replaced by amide functions could lead to more stable and potentially more active compounds.

The CA derivatives inhibited the enzymatic activities of both wild-type integrase and G140S mutant enzyme to the same extent. The G140S mutation was found after passaging an HIV strain in the presence of L-CA (King and Robinson, 1998). Our results question the role of this mutation in the observed resistance to CA. In light of our results, it would be interesting to sequence the *gp120* glycoprotein of the reported (King and Robinson, 1998) virus strain. However, the G140S mutated integrase carries F185K/C280S mutations to increase solubility (Jenkins et al., 1996). It might be possible that these additional mutations affect the resistance of the G140S enzyme to CA.

The CA derivatives L-CA, L-CATA, and their enantiomers act primarily on the binding of viral gp120 with the cells. The precise mechanism of interaction warrants further investigation. In the case of integrase inhibitors, proof of inhibition of the integration step in cell culture is rather difficult to achieve (Hazuda et al., 2000), emphasizing the need for developing a good cellular integration system. The development of a human cell line stably expressing HIV-1 integrase at high expression levels will be helpful in the development of a cellular integration assay (Cherepanov et al., 2000). Assays with purified preintegration complexes that more closely resemble the actual integration step in the infected cell should also contribute to this end. In fact, L-CA was found to be inactive in an HIV-1 preintegration complex assay in a microtiter plate format (Hansen et al., 1999).

The observation of the inhibitory effect of CA on HIV entry does not necessarily compromise the future of L-CA and its analogs as anti-HIV-1 drugs. On the contrary, because of

TABLE 8
Comparison of the inhibitory profile of CA derivatives on wild-type and G140S mutant integrase

Compound	IC_{50} (μM)[a]					
	Wild-Type Integrase[b]			G140S Mutant Integrase		
	3′-Processing	Overall Reaction	Disintegration	3′-Processing	Overall Reaction	Disintegration
L-CA	1.4 ± 0.8	0.9 ± 0.2	2.0	0.9 ± 0.1	0.8 ± 0.1	1.8
L-CATA	7.0 ± 3.0	5.0 ± 3.0	3.3	2.5 ± 0.7	2.0 ± 0.1	2.0
D-CA	2.0 ± 1.0	1.8 ± 1.0	2.0	2.5 ± 0.7	1.8 ± 0.4	2.0
D-CATA	11.0 ± 7.0	6.0 ± 3.0	10.0	2.0 ± 0.1	2.5 ± 0.7	5.0

[a] Values with standard deviation are mean values for at least two independent experiments.
[b] Soluble mutant recombinant integrase (F185K/C280S) was used as wild-type whereas the mutant form carries an additional G140S mutation.

aspet

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

renewed interest in viral entry as a potential antiviral target and because binding and fusion of HIV to the cell are mediated in part by the gp120 molecule, these inhibitors of gp120 ought to be further developed. CA derivatives clearly belong to a new class of HIV entry inhibitors, as indicated by the emergence of novel resistance mutations in the viral *gp120* glycoprotein on repeated exposure to the compound.

Acknowledgments

We are grateful to Sandra Claes, Kristien Erven, Cindy Heens, and Barbara Van Remoortel for excellent technical assistance.

References

Adachi A, Gendelman H, Koenig S, Folks T, Willey R, Rabson A and Martin M (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol* **59:**284–291.

Barré-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vézinet-Brun F, Rouzioux C, Rozenbaum W and Montagnier L (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for AIDS. *Science (Wash DC)* **220:**868–871.

Bonn D (1998) Green coffee beans may yield new class of anti-HIV-1 agents. *Lancet* **352:**1039.

Bridger G, Skerlj R, Thornton D, Padmanabhan S, Martellucci SA, Henson GW, Abrams MJ, Yamamoto N, De Vreese K and Pauwels R (1995) Synthesis and structure-activity relationship of phenylenebis(methylene)-linked bis-tetraazamacrocycles that inhibit HIV replication. Effects of macrocyclic ring size and substituents on the aromatic linker. *J Med Chem* **38:**366–378.

Brown PO (1997) Integration of retroviral DNA. *Curr Topics Microbiol Immunol* **157:**19–47.

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 in the integrase of human immunodeficiency virus type 1. *Mol Pharmacol* **52:**771–780.

Cherepanov P, Pluymers W, Claeys A, Proost P, De Clercq E and Debyser Z (2000) High-level expression of active HIV-1 integrase from a synthetic gene in human cells. *FASEB J* **14:**1389–1399.

Daniel MD, Letvin NL, Sehgal PK, Hunsmann G, Schmidt DK, King NW and Desrosier RC (1987) Long-term persistent infection of macaque monkeys with simian immunodeficiency virus. *J Gen Virol* **68:**3183–3189.

De Vreese K, Kofler-Mongold V, Leutgeb C, Weber V, Vermeire K, Schacht S, Anné J, De Clercq E, Datema R and Werner G (1996) The molecular target of bicyclams, potent inhibitors of human immunodeficiency virus replication. *J Virol* **70:**689–696.

Debyser Z, Cherepanov P, Pluymers W and De Clercq E (2000) Assays for the Evaluation of HIV-1 Integrase Inhibitors, in *Methods in Molecular Biology. Nuclease Methods and Protocols* (Schein CH ed), The Humana Press, Inc., Totowa, NJ, in press.

Esté JA, Cabrera C, Schols D, Cherepanov P, 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.

Esté JA, Schols D, De Vreese K, Van Laethem K, Vandamme A-M, Desmyter J and De Clercq E (1997) Development of resistance of human immunodeficiency virus type 1 to dextran sulfate associated with the emergence of specific mutations in the envelope gp120 glycoprotein. *Mol Pharmacol* **52:**98–104.

Hammer SM, Squires KE, Hughes MD, Grimes JM, Demeter LM, Currier JS, Eron JJ, Feinberg JE, Balfour HH, Deyton LR, Chodakewitz JA and Fischl MA (1997) A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 counts of 200 per cubic millimeter or less. *N Engl J Med* **337:**725–733.

Hansen MST, Smith GJ, Kafri T, Molteni V, Siegel JS and Bushman FD (1999) Integration complexes derived from HIV vectors for rapid assays *in vitro*. *Nat Biotechnol* **17:**578–582.

Harada S, Koyanagi Y and Yamamoto N (1985) Infection of HTLV-III/LAV in HTLV-I carrying cells MT-2 and MT-4: An application in a plaque assay. *Science (Wash DC)* **229:**563–566.

Hazuda DJ, Felock P, Witmer M, Wolfe A, Stillmock K, Grobler JA, Espeseth A, Gabryelski L, Schleif W, Blau C and Miller MD (2000) Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science (Wash DC)* **287:**646–650.

Horwitz JP, Chua J, Noel M (1964) Nucleoside V. The monomesylates of 1-(2′-deoxy-β-D-lyxofuranosyl) thymine. *J Org Chem* **29:**2076–2078.

Japour AJ, Mayers DL, Johnson VA, Kuritzkes DR, Beckett LA, Arduino J-M, Lane J, Black RL, Reichelderfer PS, D'Aquila RT and Crumpacker CS (1993) Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. *Antimicrob Agents Chemother* **37:**1095–1101.

Jenkins TM, Engelman A, Ghirlando R and Craigie R (1996) A soluble active mutant of HIV-1 integrase: Involvement of both the core and carboxyl-terminal domains in multimerization. *J Biol Chem* **271:**7712–7718

King PJ, Ma G, Miao W, Jia Q, McDougall BR, Reinecke MG, Cornell C, Kuan J, Kim TR and Robinson WE Jr (1999) Structure-activity relationships: Analogues of dicaffeoylquinic and dicaffeoyltartaric acids as potent inhibitors of human immunodeficiency virus type 1 integrase and replication. *J Med Chem* **42:**497–509.

King PJ and Robinson WE Jr (1998) Resistance to the anti-human immunodeficiency virus type 1 compound l-chicoric acid results from a single mutation at amino acid 140 of integrase. *J Virol* **72:**8420–8424.

Lin Z, Neamati N, Zhao H, Kiryu Y, Turpin JA, Aberham C, Strebel K, Kohn K, Witvrouw M, Pannecouque C, Debyser Z, De Clercq E, Rice WG, Pommier Y and Burke TR Jr (1999) Chicoric acid analogues as HIV-1 integrase inhibitors. *J Med Chem* **42:**1401–1414.

Mahmood N, Moore PS, De Tommasi N, De Simone F, Colman S, Hay AJ and Pizza C (1993) Inhibition of HIV infection by caffeoylquinic acid derivatives. *Antivir Chem Chemother* **4:**235–240.

Mazumder A, Neamati N, Sunder S, Owen J and Pommier Y (1999) Retroviral integrase: A novel target in antiviral development. Basic in vitro assays with the purified enzyme, in *Antiviral Methods and Protocols* (Kinchington D and Schinazi R eds) pp 327–335, The Humana Press, Inc, Totowa, NJ.

McDougall B, King PJ, Wu BW, Hostomsky Z, Reinecke MG, Robinson WE Jr (1998) Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase. *Antimicrob Agents Chemother* **42:**140–146.

Neamati N, Sunder S and Pommier Y (1997) Design and discovery of HIV-1 integrase inhibitors. *Drug Discovery Today* **2:**487–498.

Pauwels R, Andries K, Desmyter J, Schols D, Kukla MJ, Breslin HJ, Raeymaekers A, Van Gelder J, Woestenborghs R, Heykants J, Schellekens K, Janssen MAC, De Clercq E and Janssen PAJ (1990) Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. *Nature (Lond)* **343:**470–474.

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.

Pommier Y, Pilon AA, Bajaj K, Mazumder A and Neamati N (1997) HIV-1 integrase as a target for antiviral drugs. *Antimicrob Agents Chemother* **8:**463–483.

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

Rando RF, Ojwang J, Elbaggari A, Reyes JR, Tinder R, McGrath MS and Hogan ME (1995) Suppression of immunodeficiency virus type 1 activity *in vitro* by oligonucleotides which form intramolecular tetrads. *J Biol Chem* **270:**1754–1760.

Robinson WE Jr (1998) L-chicoric acid, an inhibitor of human immunodeficiency virus type 1 (HIV-1) integrase, improves on the in vitro anti-HIV-1 effect of zidovudine plus a protease inhibitor (AG1350). *Antivir Res* **39:**101–111.

Robinson WE Jr, Cordeiro M, Abdel-Malek S, Jia Q, Chow SA, Reinecke MG and Michell WM (1996a) Dicaffeoylquinic acid inhibitors of human immunodeficiency virus integrase: Inhibition of the core catalytic domain of human immunodeficiency virus integrase. *Mol Pharmacol* **50:**846–855.

Robinson WE Jr, Reinecke MG, Abdel-Malek S, Jia Q and Chow SA (1996b) Inhibitors of HIV-1 replication that inhibit HIV integrase. *Proc Natl Acad Sci USA* **93:**6326–6331.

Schinazi RF, Larder BA and Mellors JW (1996) Mutations in retroviral genes associated with drug resistance. *Int Antivir News* **4:**95–107.

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

Thomas M and Brady L (1997) HIV integrase: A target for AIDS therapeutics. *Trends Biotechnol* **15:**167–172.

Williams CA, Goldstone F and Greenham J (1996) Flavonoids, cinnamic acids and coumarins from the different tissues and medicinal preparations of *Taraxacum officinale*. *Phytochemistry* **42:**121–127.

Zhao H and Burke TR Jr (1998) Facile syntheses of (2R,3R)-(−)- and (2S,3S)-(+)-chicoric acids. *Synth Commun* **28:**737–740.

Zhu K, Cordeiro ML, Atienza J, Robinson WE and Chow SA (1999) Irreversible inhibition of human immunodeficiency virus type 1 integrase by dicaffeoylquinic acids. *J Virol* **73:**3309–3316.

Send reprint requests to: Dr. Myriam Witvrouw, Rega Institute for Medical Research, K. U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium. E-mail: myriam.witvrouw@uz.kuleuven.ac.be

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012