Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5286–5290, June 1992
Medical Sciences

Potent and selective inhibition of human immunodeficiency virus (HIV)-1 and HIV-2 replication by a class of bicyclams interacting with a viral uncoating event

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Communicated by M. F. Perutz, February 24, 1992 (received for review November 22, 1991)

ABSTRACT **A series of bicyclams have been shown to be potent and selective inhibitors of human immunodeficiency virus (HIV). The compounds are inhibitory to the replication of various HIV-1 and HIV-2 strains in various human T-cell systems, including peripheral blood lymphocytes, at 0.14–1.4 μM, without being toxic to the host cells at 2.2 mM. The bicyclam JM2763 is active against 3′-azido-3′-deoxythymidine (zidovudine; AZT)-resistant HIV-1 strains and acts additively with AZT. Mechanism of action studies revealed that the bicyclams (i.e., JM2763) interact with an early event of the retrovirus replicative cycle, which could be tentatively identified as a viral uncoating event.**

The compounds that have been most extensively studied in the chemotherapy of human immunodeficiency virus (HIV) infections are the 2′,3′-dideoxynucleoside (ddN) analogues 3′-azido-3′-deoxythymidine (zidovudine; AZT), dideoxycytidine (ddC), and dideoxyinosine (ddI) (1). These compounds interfere with the virus-associated reverse transcriptase (RT). To this end, they need to be phosphorylated intracellularly by host enzymes to their 5′-triphosphate (ddNTP), which then interact as DNA chain terminators in the RT reaction (2). Other, nonnucleoside inhibitors of the HIV-1 RT have been recently identified (3–5) that directly interact with the enzyme at a specific target site, provisionally designated as the TIBO {tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione; see ref. 6} site.

In addition to the RT, several other stages in the HIV replicative cycle, starting from the virion binding to the cells to the ultimate release (budding) of the virus particles from the cells, have been envisaged as targets for therapeutic intervention (7–10). One such target is the HIV protease, and several HIV-1 protease inhibitors have been designed that appear to inhibit the enzyme, and consequently virus replication, with high specificity (11–16). The viral protease is essential for proper assembly of mature, fully infectious HIV particles. The uncoating process has also been proposed as a possible target for anti-HIV agents (17). This suggestion was based on the possible similarity in tertiary structure between the HIV capsid protein(s) and the capsid protein of picornaviruses, which have indeed proved sensitive to uncoating inhibitors (18).

Therapeutic strategies targeted at the HIV uncoating process would be worth pursuing, as they should block the release of the functional RNA inside the cells and thus interrupt the HIV replicative cycle before it proceeds to the RT step. To date, no compounds have been shown to act at the uncoating of HIV or retroviruses in general. In a review article (10), it was mentioned that the aromatic polycyclic diones hypericin and pseudohypericin may inhibit the process of uncoating. However, in the original publications (19, 20), these compounds were reported to directly inactivate retrovirions as well as to inhibit their assembly. Here we report on a newly discovered class of potent and selective HIV inhibitors, which seem to be targeted at a virus uncoating-associated process.

MATERIALS AND METHODS

Compounds. Cyclam (JM1498) was supplied by Aldrich. The isolation of the free base of JM1657 as an impurity in the large scale preparation of cyclam has been described (21). JM2762 and JM2763 were synthesized by published procedures (22). JM2849 and JM2936 were prepared at Johnson Matthey. The cyclam JM1498 was tested as the free base. Other compounds were tested as the octahydrochlorides.

Viruses, Cells, Antiviral Activity Assays, and Cytotoxicity Assays. Anti-HIV activity and cytotoxicity measurements in MT-4 cells (origin of the MT-4 cells is described in ref. 23) were based on viability of cells that had been infected or not infected with HIV and then exposed to various concentrations of the test compounds. After the MT-4 cells were allowed to proliferate for 5 days, the number of viable cells was quantified by a tetrazolium-based colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method in 96-well microtrays (24). Cytotoxicity measurements were also based on inhibition of the growth of human embryonic lung (HEL) cells and microscopically detectable alteration of the morphology of various confluent cell cultures—i.e., human embryonic skin–muscle fibroblasts and HeLa, Vero, and Madin–Darby canine kidney (MDCK) cells.

While the cytopathic effect of HIV-1 and HIV-2 in MT-4 cells was measured by the MTT procedure (24), for simian immunodeficiency virus (SIV) in MT-4 cells it was based on trypan blue exclusion (25). Viral antigen expression in

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Abbreviations: HIV, human immunodeficiency virus; AZT, 3′-azido-3′-deoxythymidine; RT, reverse transcriptase; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione; SIV, simian immunodeficiency virus; PBL, peripheral blood lymphocyte; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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HUT-78 or CEM cells was revealed by an indirect immunofluorescence procedure with polyclonal antibodies; the percentage of antigen-positive cells was determined by laser flow cytofluorometry (25). For the viral plaque formation in MT-4 cells and viral focus immunoassay in $CD4^+$ HeLa cells, see refs. 26 and 27, respectively. For the p24 antigen assays with peripheral blood lymphocytes (PBLs), see ref. 4; for the p24 antigen assays with MT-4 cells, see ref. 28. In both cases, p24 antigen was quantified by a sandwich ELISA. Murine sarcoma virus infection was monitored by scoring cell transformation (29). In all assays, virus multiplicity of infection (moi) was ≈0.01.

The origin of the different virus strains was as follows: III_B (LAI), ref. 30; RF, ref. 31; HE, isolated in our laboratory from an AIDS patient; ROD, ref. 32; EHO, ref. 33; MAC-251, ref. 34. The A012B, A012D, A018A, and A018C strains (28) were obtained through the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases (NIAID) (Bethesda, MD) (contributors were D. D. Richman for the viruses and the Division of AIDS/NIAID for the RT). The original stocks of HIV-1(III_B), HIV-2(ROD), HIV-2(EHO), and SIV(MAC-251) were provided by R. C. Gallo, L. Montagnier, L. Montagnier, and C. Bruck, respectively.

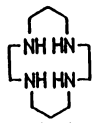
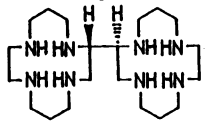
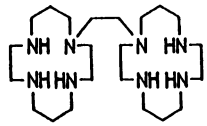
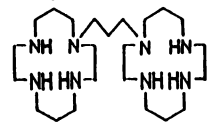
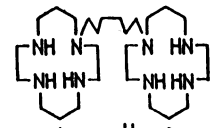
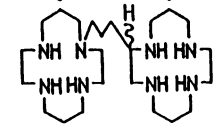
Time of Addition Experiment. MT-4 cells were infected with HIV-1(III_B) at a moi of >1, and the test compounds were added at different times (0, 1, 2, 3, . . . 22, 23, or 24 hr) after infection. Viral p24 antigen production was determined 29 hr postinfection by a sandwich ELISA. The compounds were added at a standardized concentration—that is, 100 times their 50% inhibitory concentration (IC_{50}) required to reduce by 50% the cytopathicity of HIV-1(III_B) (moi, 0.01) in MT-4 cells.

Drug Combination. The combined effect of JM2763 and AZT was examined in MT-4 cells infected with HIV-1(III_B) under the experimental conditions used for determination of the IC_{50} values (see above). Checkerboard 1:5 drug dilutions were prepared in three individual 96-well trays. After addition of virus and MT-4 cells, the trays were incubated for 5 days at 37°C. Cell viability was determined by the MTT procedure (24) and is expressed in OD units. To assess whether the drug combination resulted in a synergistic, additive, or antagonistic effect, a three-dimensional method of analysis [MacSynergy (35)] was used (R.P., unpublished data). Theoretical additive interactions were calculated from the dose–response curves of the individual drugs. The resulting dose–response surface was then compared with the actual dose–response surface. For an additive interaction the actual dose–response surface would coincide with the theoretical one, but any peaks above or below this surface would be indicative of synergy or antagonism, respectively. Significance was assessed by 95% confidence intervals around the experimentally obtained dose–response surface.

RESULTS AND DISCUSSION

A variety of macrocyclic polyamines, with the cyclam 1,4,8,11-tetraazacyclotetradecane (JM1498) used as the prototype, were synthesized and analyzed for anti-HIV activity. The monocyclam had slight activity against HIV-1 and HIV-2. Compounds in which two cyclam units were linked in various ways (bicyclams), in particular JM1657 and JM2763, were found to be active against HIV-1 and HIV-2 at a concentration as low as 0.14 μM (Table 1), with a selectivity index of >5000 (Table 2). The anti-HIV activity of JM1657 and JM2763 was observed with several strains of both HIV-1

Table 1. Anti-HIV activity of mono- and bicyclams in MT-4 cells

Compound	Structure	CC_{50}, μM	IC_{50}, μM		Selectivity index	
			HIV-1(III_B)	HIV-2(ROD)	HIV-1(III_B)	HIV-2(ROD)
JM1498 FW = 200.33		1248	399	150	3	8
JM1657 8HCl FW = 690.33		319	0.144	1.01	2215	316
JM2762 8HCl·2H_2O FW = 754.4		349	18.6	124.6	19	3
JM2763 8HCl·4H_2O FW = 804.47		>622	0.248	1.00	>2500	>625
JM2849 8HCl·2H_2O FW = 810.52		290	0.616	0.616	470	470
JM2936 8HBr·2H_2O FW = 1124.08		>445	1.96	3.56	>225	>125

Data represent mean values for two to six experiments. IC_{50}, 50% inhibitory concentration for cytopathicity of HIV-1(III_B) or HIV-2(ROD) in MT-4 cells. Viral MOI = 0.01 [this corresponds to 100 $CCID_{50}$ (100 times the 50% cell culture infective dose)]. CC_{50}, 50% cytotoxic concentration for mock-infected MT-4 cells. The selectivity index (SI) corresponds to the ratio CC_{50}/IC_{50}.

Table 2. Antiviral activity spectrum of the bicyclams JM1657 and JM2763

				JM1657				JM2763			
Virus	Strain	Cell	Assay	IC_{50}, μM	IC_{90}, μM	CC_{50}, μM	SI	IC_{50}, μM	IC_{90}, μM	CC_{50}, μM	SI
HIV-1	III_B	MT-4	CPE/MTT	0.43	1.16	362	842	0.37	0.87	>1865	>5,040
HIV-1	RF	MT-4	CPE/MTT	0.29	0.87	362	1248	0.37	0.87	>1865	>5,040
HIV-1	HE	MT-4	CPE/MTT	0.29	0.87	362	1248	0.50	1.19	>1865	>3,730
HIV-2	ROD	MT-4	CPE/MTT	0.29	0.87	362	1248	0.75	1.99	>1865	>2,487
HIV-2	EHO	MT-4	CPE/MTT	0.29	0.87	362	1248	1.37	3.60	>1865	>1,361
HIV-1	III_B	HUT-78	Antigen expression	0.43	1.45	>362	>842	0.99	3.98	>1865	>1,865
HIV-1	III_B	CEM	Antigen expression	0.72	2.32	>724	>1006	1.24	5.10	>1865	>1,504
HIV-2	ROD	CEM	Antigen expression	0.14	0.58	>724	>5171	0.37	1.49	>1243	>3,360
HIV-1	III_B	MT-4	Plaque formation	0.43	—	—	—	1.24	—	—	—
HIV-1	III_B	$CD4^+$ HeLa	Focus immunoassay	0.43	—	—	—	3.73	—	—	—
HIV-1	III_B	PBL	p24 antigen	1.45	3.77	≥724	≥500	1.86	3.73	>1865	>1,003
HIV-1	A012B	MT-4	p24 antigen	—	—	—	—	0.12	—	>1865	>15,542
HIV-1	A012D*	MT-4	p24 antigen	—	—	—	—	0.25	—	>1865	>7,460
HIV-1	A018A	MT-4	p24 antigen	—	—	—	—	2.49–373	—	>1865	—
HIV-1	A018C*	MT-4	p24 antigen	—	—	—	—	0.37	—	>1865	>5,041
SIV	MAC_{251}	MT-4	CPE/TB	—	—	—	—	249	—	>622	>2.5
MSV	Moloney	C3H/3T3	Transformation	>29	—	>29	—	622	—	>622	—

IC_{50}, CC_{50}, and SI are explained in Table 1. All data represent median values for several separate determinations. —, Not determined. MSV, murine sarcoma virus; CPE, cytopathic effect; TB, trypan blue.
*Resistant to AZT (IC_{50} of AZT, 0.75 μM for A012D and 1.12 μM for A018C, as compared to 0.007 μM for A012B).

and HIV-2 in different cell types, including PBLs (Table 2). Irrespective of the method used to assess anti-HIV activity—i.e., inhibition of viral cytopathicity, antigen expression, p24 production, plaque, or focus formation—the bicyclams invariably inhibited HIV-1 and HIV-2 within the concentration range of 0.14–1.4 μM. Also, AZT-resistant HIV-1 strains were as sensitive as AZT-sensitive HIV-1 strains to JM2763 (Table 2). The sensitivity of the clinical HIV-1 isolate A018A to JM2763 varied considerably from one assay to another [IC_{50}, 2.5 to >373 μM (Table 2)], whereas against the HIV-1(III_B) strain JM2763 was consistently active at an IC_{50} of 0.25–0.37 μM. The reason for the aberrant behavior of the A018A isolate remains to be clarified. SIV [SIV_{mac251} strain (Table 2)] and other SIV strains [i.e., SIV_{agm3} and SIV_{mndGB1} (data not shown)] and murine sarcoma virus [Moloney strain (Table 2)] were not sensitive to JM1657 and JM2763. Various other viruses (influenza A, influenza B, parainfluenza 3, measles, respiratory syncytial, reo-1, polio 1, Coxsackie B-4, Sindbis, Semliki forest, vesicular stomatitis, herpes simplex 1, herpes simplex 2, varicella-zoster, and cytomegalovirus) were also insensitive to JM1657 and JM2763 at concentrations up to 579 and 497 μM, respectively. JM2763 was nontoxic to the host cells at the highest concentration tested: 1.86 mM for MT-4, HUT-78, CEM, or PBL cells; 0.62 mM for C3H/3T3 cells; 0.50 mM for embryonic skin–muscle, HeLa, Vero, or MDCK cells; or 0.25 mM for HEL cells.

When JM2763 was assayed against HIV-1(III_B) in combination with AZT, the compounds appeared to act in an additive fashion at the concentrations used (Fig. 1) (confidence limits, 95%). The method used for analysis of drug combinations appeared suitable to clearly distinguish additive combinations (JM2763 plus AZT) from synergistic combinations (i.e., TIBO + AZT, ddI + ribavirin) and antagonistic combinations (i.e., AZT plus ribavirin) (R.P., unpublished data).

In attempts to resolve the mechanism of action of the bicyclams, we found that, unlike dextran sulfate, pentosan polysulfate, and the polyoxometalate $K_{13}[Ce(SiW_{11}O_{39})_2]$ (JM1590), which inhibit HIV-1(III_B) binding to MT-4 cells (for method, see ref. 36) by ≈90% at a concentration of 25 μg/ml, JM1657 and JM2763 at 25 μg/ml (36 and 31 μM, respectively) do not affect virus binding to the cells (data not shown).

Also, JM1657 and JM2763 did not cause any inhibition of giant cell (syncytium) formation if added to cocultures of persistently HIV-1(III_B)-infected HUT-78 cells and MOLT-4 cells at concentrations ranging from 0.16 to 500 μg/ml (0.231–724 μM for JM1657; 0.199–621 μM for JM2763). When tested under the same conditions, dextran sulfate, pentosan polysulfate, and JM1590 inhibited syncytium formation by 50% at a concentration of 10–15 μg/ml (see also refs. 37 and 38). Thus, at the concentrations that are effective in inhibiting viral cytopathicity, JM1657 and JM2763 fail to inhibit the formation of giant cells in a direct syncytium formation assay. In this respect, JM1657 and JM2763 clearly differ from those compounds [i.e., mannose-specific plant lectins (39), succinylated concanavalin A (40), and succinylated human serum albumins (41)] that inhibit the formation of giant cells (in a direct syncytium formation assay) at concentrations similar to those required to inhibit viral cytopathicity.

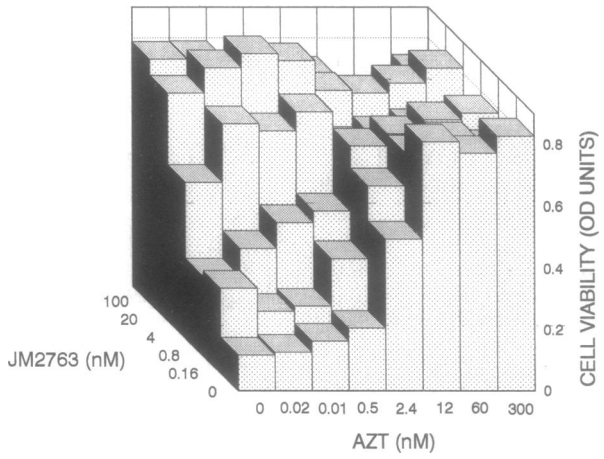


FIG. 1. Drug combination. Combined effects of JM2763 and AZT were evaluated in MT-4 cells infected with HIV-1(III_B). Cell viability was determined by the MTT method (24) and is expressed in OD units. Data represent median values for three observations.

JM2763 was also examined for its ability to interact directly with the cellular CD4 receptor for HIV and the viral envelope glycoprotein gp120. Under conditions in which aurintricarboxylic acid (42) effectively interacted with the CD4 receptor (as monitored by interference with the binding of OKT4A/Leu3a monoclonal antibody), and under conditions in which dextran sulfate (43) effectively blocked the binding of anti-gp120 monoclonal antibody to persistently HIV-1-infected HUT-78 cells, JM2763 (at 0.124 mM) had no effect on either the CD4 cell receptor or viral gp120 glycoprotein.

Also, the RT could be excluded as a target for the bicyclams. JM2763 did not inhibit the activity of HIV-1 recombinant RT, HIV-2 recombinant RT, HIV-1(III_B) virion-derived RT, HIV-2(ROD) virion-derived RT, whether directed by poly(A)·oligo(dT) or poly(C)·oligo(dG) as template·primer, at concentrations up to 5 mM. Under the same test conditions, the polyoxometalate JM1590 effected a 50% reduction in HIV-1 RT activity [with poly(A)·oligo(dT) as template·primer] at 3.1 nM, and the TIBO derivative R82150 effected a 50% reduction in HIV-1 RT activity [with poly(C)·oligo(dG) as template·primer] at 0.56 μM.

JM2763 (1 mM) did not prove inhibitory to recombinant HIV-1 protease under conditions (BACHEM protocol) in which acetyl pepstatin (5 μM) showed effective inhibition of the enzyme. Neither JM1657 nor JM2763 nor AZT (0.15 mM) caused an appreciable (>15%) reduction of p24 antigen production by chronically HIV-1-infected HUT-78 cells, under conditions where the HIV protease inhibitor Ro31-8959 [compound XVII (14)] reduced p24 antigen production by ≈85%.

Preincubation of HIV-1(III_B) with JM1657 or JM2763 at concentrations up to 100 μg/ml (144 and 124 μM, respectively) did not cause a decrease in virus infectivity (≈10^6 cell culture ID_{50}/ml), which indicates that the bicyclams, unlike hypericin and pseudohypericin (20), do not directly inactivate the virus. In fact, when hypericin was evaluated for its inhibitory effects on the cytopathicity of HIV-1 or HIV-2 in MT-4 cells, no activity was observed at subtoxic concentrations (50% cytotoxic concentration, 5 μM).

To pinpoint at which stage the bicyclams actually interact with the HIV replicative cycle, a time-of-addition experiment was carried out (Fig. 2). The cells were infected at high virus multiplicity to ensure that the virus replicative steps would be synchronized in the whole cell population, and the compounds were added 1, 2, 3, . . . 22, 23, or 24 hr after infection. Depending on the stage at which they interact and the need for intracellular metabolism, addition of the compounds could be delayed for n hr without loss of activity. Dextran sulfate, which acts at the virus adsorption step (44, 45), must be added together with the virus (n = 0) to be active. For AZT and ddI, which, following their intracellular phosphorylation, act at the RT step (2), addition to the cells could be delayed until ≈5 hr (n = 5) after infection, and for the TIBO derivatives (R82150, R82913), which do not need intracellular transformations before they can interact with their target enzyme (HIV-1 RT) (4, 6), the addition could be delayed by another 2 hr (n = 7). The protease inhibitor Ro31-8959 [compound XVII (14)], which interacts with a late event in the virus cycle (assembly of mature virions) (11–16) was still effective if added as late as 21 hr after infection (n = 21). From the time-of-addition experiment, it appeared that the bicyclams JM1657 and JM2763 (n = 1 or 2) had to interact with a process following virus adsorption but preceding reverse transcription, which means virus–cell fusion and/or uncoating. As mentioned above, the bicyclams are not inhibitory in the direct syncytium formation assay at concentrations that are inhibitory to HIV replication. This makes them clearly different from previously reported virus–cell fusion inhibitors (39–41).

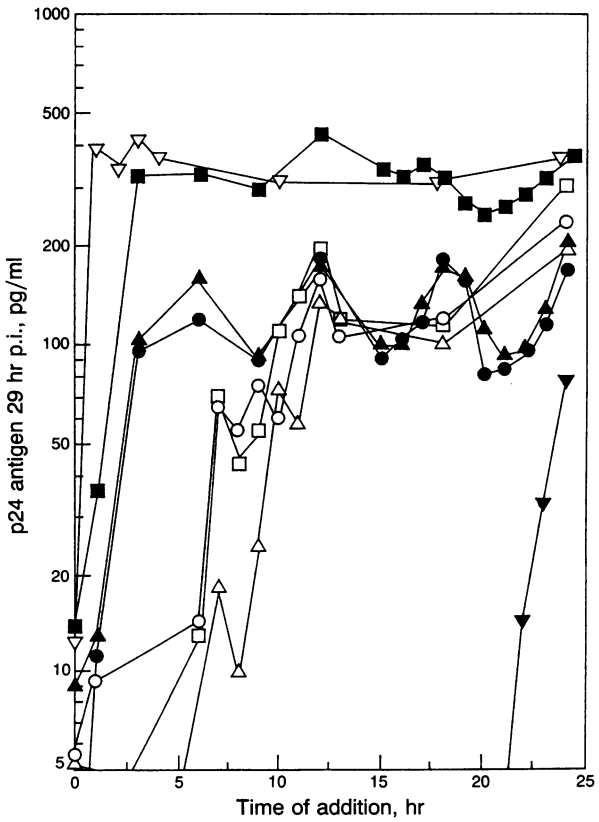


FIG. 2. Time of addition experiment. MT-4 cells were infected with HIV-1(III_B) at MOI >1, and the test compounds were added at different times post infection (p.i.). Viral p24 antigen production was determined 29 hr p.i. ▽, Dextran sulfate (M_r, 5000) (10 μM); ■, JM1590 (3.1 μM); ▲, JM1657 (28.9 μM); ●, JM2763 (24.9 μM); ○, AZT (0.19 μM); □, ddI (423 μM); △, R82913 (0.155 μM); ▼, Ro31-8959 (0.065 μM).

To obtain further evidence for the inhibitory effect of JM2763 on HIV uncoating (or fusion), experiments were designed whereby the viral RNA harvested from cells that had just been infected was monitored for its sensitivity to degradation by RNase. It was reasoned that if uncoating (fusion) would be hampered, the viral capsid (or envelope) proteins would remain associated with the viral RNA genome and thus the RNA should be protected against RNase attack. When MT-4 cells were exposed to radiolabeled HIV-1(III_B) particles (45) at a very high MOI (MOI = 10) and then treated with different concentrations of JM2763 or JM1657, viral RNA harvested from the cells 5 hr after infection showed a concentration-dependent resistance to degradation by RNase A; viral RNA harvested from HIV-infected cells treated with other anti-HIV agents (i.e., AZT, ddI, R82913, or Ro31-8959) did not show this increased resistance to degradation by RNase A (detailed data to be reported elsewhere).

Thus, it may be postulated that the bicyclams interact with the HIV uncoating (fusion) process. Their precise mode of action remains to be studied. If targeted at uncoating rather than fusion, the bicyclams represent the first compounds reported to interfere with the uncoating of retroviruses. They represent a newly discovered class of anti-HIV agents. Their high specificity, as has been demonstrated with several HIV-1 and HIV-2 strains in several cell systems, together with their unique mode of action, makes the bicyclams attractive candidate drugs for the chemotherapy and prophylaxis of HIV infections.

We thank A. Vandamme, M. Witvrouw, R. Snoeck, M. Hosoya, H. Azijn, A. Absillis, and A. Camps for their help with the experi-

ments; C. Callebaut for editorial assistance; and E. K. Barefield (Georgia Institute of Technology, Atlanta) for the gift of *d,l*-2,2'-bicyclam (JM1657). These investigations were supported in part by the AIDS Basic Research Programme of the European Community and by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek and the Belgian Geconcerteerde Onderzoeksacties.

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