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The bicyclams, a new class of potent human immunodeficiency virus inhibitors, block viral entry after binding

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Abstract

The bicyclams represent a new class of highly potent and selective HIV inhibitors. Time-of-addition experiments have previously shown that these compounds interfere with an early event in the viral replicative cycle. Additional experiments have now been carried out in order to investigate in more detail the mechanism of action of these promising compounds. As described in this paper, PCR experiments revealed that no viral DNA was formed following viral infection, thus confining the target(s) of action of the bicyclams to an early stage of HIV infection. An assay, using pseudotype virions containing the envelope of HIV-1 and the genome of a plaque-forming virus (Cocal Virus), pointed to viral entry as the main target of the bicyclams. HIV-1 strains resistant to two prototype bicyclams, JM2763 and SID791 (JM3100), were raised. Results obtained with SID791 with respect to syncytium formation induced by SID791-sensitive and -resistant HIV-1 strains and the cross-resistance observed for dextran sulfate, suggest inhibition of binding and/or fusion as a plausible target of SID791. Additional experiments enabled us to exclude SID791 and JM2763 as binding inhibitors and to conclude that bicyclams block the entry of cell-bound virus. Furthermore, a monoclonal antibody recognising the V3 loop of wild-type gp120 did not bind to this region in the two bicyclam-resistant strains. Our results point to gp120 as a possible target for the HIV-inhibitory effects of the bicyclams.

Keywords: Human immunodeficiency virus; Entry; Bicyclams

1. Introduction

Since the recognition of human immunodeficiency virus (HIV) as the causative agent of

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AIDS, much effort has been directed to the search for effective anti-HIV agents. Various compounds, acting at different sites of the replicative cycle of HIV, have been reported, some of which have entered clinical trials. However, the only inhibitors that are formally licensed for clinical use are reverse transcriptase inhibitors. The major drawbacks encountered with these and other compounds are their toxicity and/or the rapid emergence of virus-drug resistance. Therefore, new inhibitors targeted at other sites are desirable, and these compounds could be used either alone or in combination with existing drugs.

The bicyclams have been discovered as a new class of HIV inhibitors. These compounds act as highly potent and selective inhibitors of HIV-1 and HIV-2 in cell culture. The most active congener, SID791, is active against both HIV-1 and HIV-2 at 0.005 μM, while not being toxic at concentrations up to 500 μM, thus achieving a selectivity index of 100 000 or higher (De Clercq et al., 1994). 'Time-of-addition' experiments, whereby the compounds were added at different time intervals after virus infection, indicated that the bicyclams interfere with an early event in the viral replicative cycle. Experiments in which the sensitivity of the viral particle RNA to ribonuclease A was monitored, further pointed to fusion/uncoating as the most likely target for the anti-HIV action of the bicyclams (De Clercq et al., 1992, 1994). SID791 also proved active in a syncytium formation assay, suggesting a role in fusion inhibition. This paper describes further experiments aimed at elucidating the mechanism of action of these highly potent and selective HIV inhibitors.

2. Materials and methods

2.1. Compounds

The bicyclam derivatives JM2763 (1,1′-propylene-bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride tetrahydrate) and SID791 (JM3100; 1,1′-[1,4-phenylenebis-(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride dihydrate) were synthesized at Johnson Matthey (Bridger et al., 1995). The 8-chloro-TIBO derivative R86183 (TIBO derivative (+)-S-4,5,6,7-tetrahydro-8-chloro-5-methyl-6-(3-methyl-2-butenyl) -imidazo [4,5,1-*jk*][1,4]-benzodiazepin-2 (1*H*)-thione) was provided by the Janssen Research Foundation (Beerse, Belgium). Dextran sulfate (DS) of various molecular weight was provided by Pfeifer and Langen (Dormagen, Germany), dextran sulfate of MW 5000 and MW 500 000 and aurintricarboxylic acid (ATA) were obtained from Sigma (St. Louis, MO).

2.2. Cells and viruses

The origin of the MT-4 cells, MOLT-4 and HUT-78 cells is described by Harada et al. (1985), Kikukawa et al. (1986) and Levy et al. (1984), respectively. All cells were propagated in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.1% sodium bicarbonate, 2 mM L-glutamine and 20 μg/ml gentamicin. HIV-1 NL4-3 is a molecular clone obtained from the National Institutes of Health (Bethesda, MD). HIV-1 IIIB was described by Popovic et al. (1984).

2.3. Analysis by PCR of the effect of SID791 on synthesis of viral DNA

MT-4 cells were infected with HIV-1 IIIB (moi = 1). Prior to the 1 h incubation at 37°C, allowing adsorption of the virus to the cell, SID791 was added at 0.3 μg/ml and 0.1 μg/ml, respectively. After 8, 30 and 54 h, 2×10^7 cells were harvested from mock- and virus-infected cells, and low-molecular weight DNA was isolated as described previously (Steinkasserer et al., 1995). DNA (300 ng) was subjected to 35 rounds of amplification in a total reaction mixture volume of 50 μl. Inhibition of formation of linear DNA was checked using the LTR-primer set (sense) 5′-CCTGTGAGC CTGCATGGAATGGATG-3′ (antisense) 5′-TGCTAGAGATTTTCCACACTGACTA AAAGG-3′ (Steinkasserer et al., 1995). The LTR amplification products were separated on agarose

gels and visualized by ethidium bromide staining. Mitochondrial DNA amplification was used as an internal PCR-standard.

2.4. Pseudotype assay

The construction of HIV-1 pseudotype virus containing a Cocal virus genome and a HIV envelope, and the assay, here used to measure the potential inhibitory effect of bicyclams on viral entry, are described by Gregory et al. (1993). Briefly, pseudotype virus was produced in a two-step procedure by superinfection of a HIV-1 infected cell line (usually 174XCEM) with Cocal Virus after 7–14 days. The culture was harvested 24–48 h later, yielding a mixture of Cocal Virus (COV) and phenotypically mixed particles, COV (HIV), in which COV contributes the genome and associated proteins, while HIV contributes the envelope. HeLa-CD4 cells were infected with this pseudotype virus in the presence of different concentrations of compound. The COV in the mixture was neutralised by anti-COV antiserum before infection. As a consequence, the plaques eventually produced would solely originate from COV(HIV). By calculating a relative pseudotype virus titre (the ratio of pseudotype virus titre in the presence of compound to pseudotype virus titre without compound) at the different concentrations tested, one can assess the EC_{50} for a compound in this assay as it equals the concentration of inhibitor at a relative pseudotype virus titre of 0.5.

2.5. Binding assays

The glycoprotein gp120 immunofluorescence assay is described by Schols et al. (1990). Briefly, HUT-78 cells persistently infected with HIV-1 IIIB were incubated with the compounds at the indicated concentrations for 20 min at room temperature. After incubation with anti-gp120 mAb (NEA 9284, DuPont De Nemours, Brussels, Belgium) for 50 min at 37°C and subsequent staining with FITC-conjugated $F(ab)_2$ fragments of rabbit anti-mouse immunoglobulin antibody (RaM-IgG-$F(ab)_2$-FITC) (Prosan, Ghent, Belgium), the cells were fixed and analysed by flow cytometry. The immunofluorescence virus binding assay was carried out as described by Schols et al. (1989a). Briefly, MT-4 cells were infected with a concentrated HIV-1 IIIB stock in the presence of the compound at the indicated concentrations and incubated for 30 min at 37°C. After incubation with a high-titred polyclonal antibody to HIV-1 for 50 min at 37°C and subsequent staining with FITC-conjugated secondary Ab, the cells were fixed and analysed by flow cytometry.

In an experiment to prove that bicyclams block the entry of already bound virus, MT-4 cells were incubated with HIV-1 IIIB (moi = 1) at 4°C to allow binding but not membrane fusion (McClure et al., 1992). After 1 h, cells were washed twice with cold PBS to remove unbound virus, resuspended in medium with compound ($100 \times EC_{50}$) and transferred to 37°C to allow for entry. Production of p24 was measured after 29 h, using the HIV p24 core profile ELISA-kit (DuPont Company, Wilmington, DE).

2.6. Resistance development

HIV-1 NL4-3 was passed in MT-4 cells (2×10^5 cells/ml) in the presence of increasing concentrations of JM2763, SID791 or R86183. Initial concentrations were approximately $5 \times EC_{50}$. Concentrations of the compounds were increased 2-fold when fulminant virus breakthrough was observed. In the first three to four passages, 1 ml cells plus virus was passed in order to keep as much virus as possible in the culture; after three to four passages only cell-free supernatant (1 ml) was passed onto fresh cells plus compound. EC_{50} measurements were done via the MTT-method as described previously (Pauwels et al., 1988). EC_{50} was defined as the concentration of compound required to protect 50% of the virus-infected cells against viral cytopathicity (cytopathic effect; CPE).

2.7. Inhibition of syncytium formation

HUT-78 cells persistently infected with wild-type or bicyclam-resistant HIV-1 NL4-3 virus (resistant virus was capable of growing in 500 μg/ml JM2763 or SID791) were co-cultured with

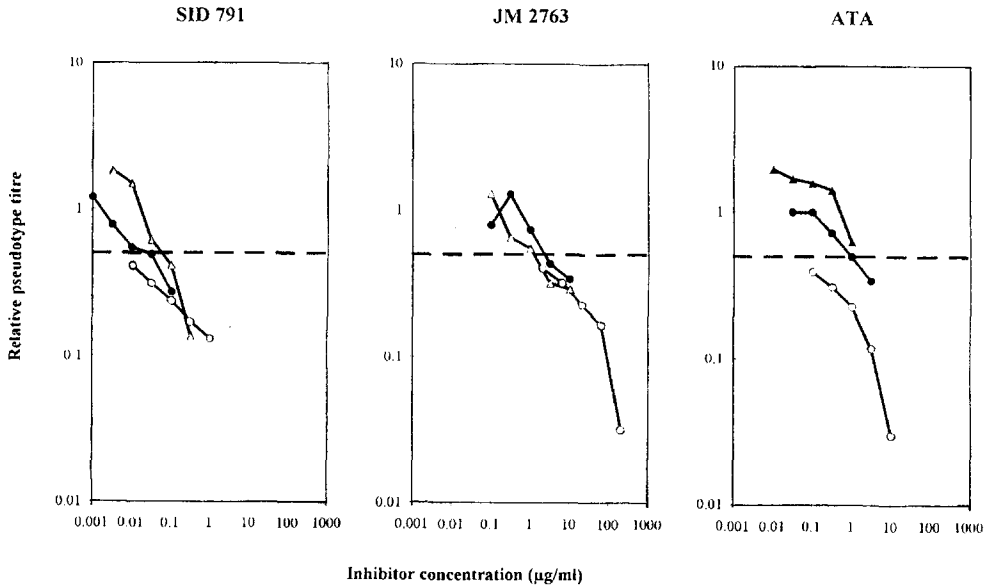

Fig. 1. Inhibition of viral entry by the bicyclam derivatives JM2763 and SID791 and by aurintricarboxylic acid (ATA). The relative pseudotype titre is the ratio of pseudotype virus titre in the presence of compound over pseudotype virus titre without drug. The dashed line represents a relative pseudotype titre of 0.5, therefore the corresponding concentration equals the EC_{50} of the compound in this assay. Results obtained in the same experiment are presented by identical symbols.

MOLT-4 cells at 1×10^6 cells/ml each in the presence of JM2763, SID791 or dextran sulfate at different concentrations. Syncytium formation was scored microscopically after 20-24 h and further analysed by laser flow cytofluorography as described previously (Schols et al., 1989b). EC_{50} was defined as the concentration of compound required to inhibit syncytium formation by 50%.

2.8. Recognition of wild-type and bicyclam-resistant virus by V3-specific antibodies

MT-4 cells were infected with wild-type, JM2763-resistant and SID791-resistant HIV-1 NL4-3 virus, respectively, and incubated with either of the monoclonal antibodies, mAb 9284 or mAb 9305 (DuPont de Nemours, Brussels, Belgium), each recognising a different region of the V3 loop of gp120. mAb 9284 binds to the epitope NTRKSIRIQRG of the V3-loop, while mAb 9305 recognises the adjacent epitope GPGRAFVTIG. After subsequent staining with FITC-labelled anti-mouse immunoglobulin antibody (Prosan, Ghent, Belgium), the cells were analysed by laser flow cytofluorography.

3. Results

3.1. Inhibition of synthesis of viral DNA

SID791 inhibited the formation of linear viral DNA in a very effective manner, as checked by PCR using the LTR-primer set: no DNA could be detected at 0.3 μg/ml of compound used; only a trace amount of DNA was detected in the sample treated with SID791 at 0.1 μg/ml.

3.2. Inhibition of viral entry

The infection of HeLa-CD4 cells by pseudovirions, composed of a HIV envelope and a Cocal virus genome, was inhibited by the bicyclams at an EC_{50} that is 4- to 10-fold higher than the EC_{50} found in a cytopathic assay (Fig. 1). The EC_{50} of JM2763 was around 2 μg/ml in this assay, while CPE was inhibited by 50% at 0.5 μg/ml. Similarly, for SID791 an EC_{50} of about 0.05 μg/ml was

Table 1
Inhibition of binding tested for bicyclams, DS and ATA as detected by FACS analysis

Compound	Concentration (μg/ml)	% inhibition of binding	
		HIV-I IIIB + CD4$^+$ cells[a]	Anti-gp120 mAb+ persistently infected HUT-78[b]
ATA[c]	25	84	86
DS[d]	25	53	85
JM2763	500	6	0
	100	0	5
SID791	200	7	11
	100	0	6

[a]MT-4 cells were infected with a concentrated HIV-1 IIIB stock in the presence of the compound at the indicated concentrations. After incubation with a high-titred polyclonal antibody to HIV-1 and subsequent staining with FITC-conjugated secondary Ab, the cells were fixed and analysed by flow cytometry.
[b]HUT-78 cells persistently infected with HIV-1 IIIB were incubated with the compounds at the indicated concentrations, then treated with anti-gp120 (mAb 9284) and subsequently stained with FITC-conjugated secondary Ab, fixed and analysed by flow cytometry.
[c]Aurintricarboxylic acid.
[d]Dextran sulfate MW 5000.

obtained, while the EC_{50} in the cytopathic assay was about 0.005–0.01 μg/ml. Since ATA is a known inhibitor of entry (Cushman et al., 1992), this compound was included as a control. The EC_{50} obtained in the pseudotype assay was about 1 μg/ml, 50% inhibition of CPE being seen at 0.5 μg/ml (Fig. 1).

3.3. Binding assays

JM2763 and SID791 had no effect on binding of anti-CD4 mAb (OKT4A) to the CD4 cell receptor (data not shown). In a series of assays it was shown that DS and ATA effectively blocked the binding of anti-gp120 monoclonal antibody to persistently infected cells and prevented binding of the virus to the cell (Table 1). On the other hand, neither JM2763 nor SID791 showed any activity in each of these assays (Table 1). Also, when administered during binding at 4°C, no inhibitory effect of the bicyclams was noted, but they blocked the entry of virus that was already bound to the cells (Table 2).

3.4. Resistance development to the bicyclam derivatives JM2763 and SID791 and 8-chloro-TIBO (R86183)

HIV-1 strains resistant to two prototype bicyclams, JM2763 and SID791, were raised. Resistance development to the 8-chloro-TIBO R86183 was included as a control. HIV-1 NL4-3 was passed in MT-4 cells in the presence of increasing concentrations of either JM2763, SID791 or R86183. After ten passages the virus showed 380-fold resistance to R86183 (Fig. 2).

On the other hand, HIV-1 NL4-3 became resistant (172-fold) to JM2763 after 25 passages and it took more than 60 passages for the virus to become resistant to SID791: 300-fold resistance after the 63rd passage (Fig. 2). This observation parallels the results achieved with HIV-1 IIIB in CEM cells, where no resistance development to SID791 was observed following 30 passages of HIV-1 IIIB in CEM cells in the presence of various drug concentrations (De Clercq et al., 1994).

HIV-1 NL4-3 grown in the presence of, and having developed resistance to JM2763, remained sensitive to SID791 (Fig. 3). In contrast, HIV-1 NL4-3 grown in the presence of SID791 acquired full cross-resistance (>740-fold) to JM2763 at the same passage at which the virus became resistant to JM2763 when passed in the presence of JM2763 (Fig. 4).

The HIV-1 NL4-3 strains resistant to JM2763, SID791 and R86183 were also tested for their

Table 2
Inhibition of entry of already bound virus

Primary incubation at 4°C in the presence of compound[a]	29 h incubation at 37°C in the presence of compound[a]	Amount of p24 (pg/ml) produced
—[b]	—[b]	1263
—	ATA	78
—	DS	284
—	JM2763	0
—	SID791	13
—	AZT	33
ATA	—	0
DS	—	0
JM2763	—	1045
SID791	—	922
AZT	—	1196

[a]Compound concentration used was 100 × EC_{50} obtained in a cytopathic assay.
[b]No compound.

sensitivity towards the compounds AZT and DDI. The bicyclam- and TIBO-resistant strains proved to be fully sensitive to these dideoxynucleoside analogues (data not shown).

3.5. Inhibition of syncytium formation induced by wild-type and resistant HIV-1 NL4-3 strains

Syncytium formation in co-cultures of MOLT-4 cells with HUT-78 cells persistently infected with wild-type HIV-1 NL4-3, was inhibited by JM2763 only at an EC_{50} of 64.4 μg/ml (Table 3A). However, SID791 inhibited syncytium formation at an EC_{50} of 0.26 μg/ml (Table 3A), which is about 100-fold higher than the EC50 required for this compound to inhibit viral cytopathicity (Table 3B). HIV-1 NL4-3 that had been grown solely in the presence of JM2763 developed resistance to JM2763 and up to 10-fold cross-resistance to SID791, as monitored by both virus-induced cytopathicity (Table 3B) and syncytium formation (Table 3A). HIV-1 NL4-3 that had been grown solely in the presence of SID791 developed resistance to both SID791 and JM2763 (Table 3A and Table 3B). Unexpectedly, the SID791-resistant virus also showed full resistance to dextran sulfate, as based upon both syncytium formation (Table 3A) and viral cytopathicity (Table 3B).

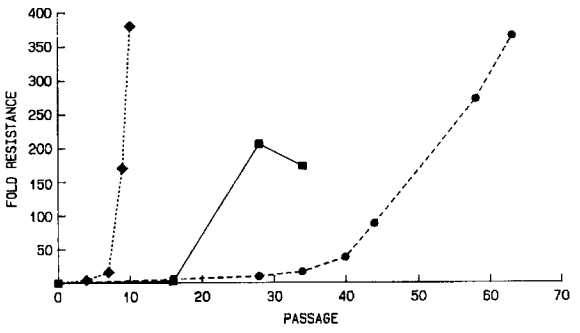

Fig. 2. Rate of resistance development of HIV-1 NL4-3 to the bicyclams JM2763 and SID791, and to TIBO R86183. At different passages EC_{50} values were determined and compared with wild-type EC_{50}. The ratio of EC_{50} (passage n) to EC_{50} (passage 0) is displayed in function of passage n for JM2763 (■) SID791 (●) and R86183 (◆).

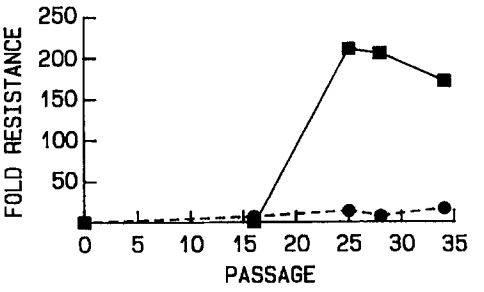

Fig. 3. Rate of development of resistance of HIV-1 NL4-3 to JM2763 (■) and of cross-resistance to SID791 (●).

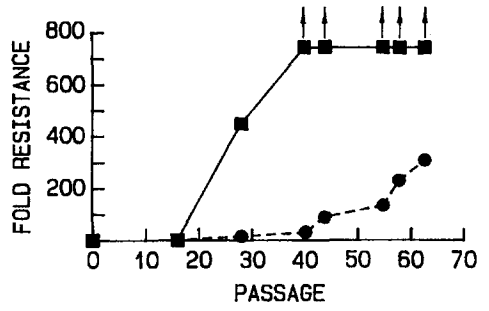

Fig. 4. Rate of development of resistance of HIV-1 NL4-3 to SID791 (●) and of cross-resistance to JM2763 (■). The symbol (↑) indicates that the EC_{50} exceeded the highest compound concentration used in the assay.

This is, to our knowledge, the first time that development of HIV resistance to dextran sulfate has been reported. When testing the effects of dextran sulfate samples of various molecular weight on the wild-type and resistant viruses, a molecular weight-dependent resistance pattern was observed; resistance was higher for dextran sulfate with lower molecular weight (Table 4).

3.6. Recognition of wild-type and resistant virus by V3-specific antibodies

The binding ability of the monoclonal antibody mAb 9305 to gp120 of wild-type was not altered as compared with that of the resistant virus. However, when repeating this experiment with a monoclonal antibody specific for another domain of the V3-loop of gp120 (mAb 9284), the resistant viruses were not recognised by this antibody (Table 5).

4. Discussion

The bicyclam derivatives represent a new and promising class of HIV inhibitors. The most potent congener, SID791, is active against both HIV-1 and HIV-2 at nanomolar concentrations, while not being cytotoxic at concentrations up to 500 μM (De Clercq et al., 1994). Time-of-addition experiments pointed to an inhibitory action of the compounds early in the viral life cycle, situated before reverse transcription (De Clercq et al., 1992, 1994). This observation has now been confirmed since viral DNA could not be detected in PCR experiments after infection of cells with HIV in the presence of SID791. An experiment, whereby a concentration-dependent inhibitory effect of bicyclams on viral RNA degradation was noticed, pointed to uncoating as a tentative target (De Clercq et al., 1992, 1994). However, this step in the viral life-cycle cannot be assayed directly and is ill-defined. In addition, the uncoating process may be innately linked to virus-cell fusion. Therefore, we investigated in more detail the effects of the bicyclams on binding and fusion, for which assays are readily available. The fusion process can be considered as an essential step in the viral entry event.

A pseudotype assay provides a convenient tool to monitor viral entry, by disconnecting it from subsequent steps in viral replication. The inhibitory effect of a compound on processes involved in HIV entry can be measured by counting the emerging plaques after infection of HeLa-CD4 cells with pseudotype virus in the presence of inhibitor. Both JM2763 and SID791 showed an EC_{50} very similar to the EC_{50} values obtained in a cytopathic assay, indicating that inhibition of entry correlates with inhibition of viral replication.

As viral entry comprises both binding of the virus to the cell and fusion of the viral membrane with the cell membrane, each step was investigated in more detail. Inhibition of binding can be examined by several assays, either using gp120 expressed by infected cells or gp120 as part of the whole virus. Interference of inhibitor with binding to CD4 can also be measured. All these assays proved to be negative for the bicyclams, excluding binding of HIV to the cell as a possible target.

Another assay to distinguish binding from fusion was based on the fact that at 4°C only binding takes place and fusion does not occur (McClure et al., 1992). Adding a binding inhibitor during the incubation at 4°C will inhibit the binding of the virus to the cell. Adding that same binding inhibitor only after the incubation at 4°C will have no further effect on binding, as the virus is already bound to the cell. However, a fusion inhibitor will inhibit the subsequent fusion at 37°C and, as a consequence, reduce the p24 levels

Table 3
Inhibitory effect of bicyclams on (A) syncytium formation tested in co-culture with MOLT-4 and (B) cytopathicity of wild-type and bicyclam-resistant HIV-1 NL4-3

Virus	EC_{50} (μg/ml)[a]		
	Dextran sulfate	JM2763	SID791
(A) HUT-78/NL4-3 WT[b]	7.55	64.4	0.26
HUT-78/NL4-3 JM2763[r,c]	66.0 (8)	>500 (>7)	1.3 (5)
HUT-78/NL4-3 SID791[r,d]	>500 (>66)	>500 (>7)	500 (1900)
(B) NL4-3 WT[e]	0.075	0.161	0.002
NL4-3 JM2763[r,f]	0.014	127.5 (792)	0.043 (22)
NL4-3 SID791[r,g]	>500.0 (>6667)	>500.0 (>3105)	0.546 (243)

[a]EC_{50} was defined as the concentration of compound required to inhibit (A) syncytium formation or (B) viral cytopathicity by 50%.
[b]HUT-78 cells persistently infected with wild-type HIV-1 NL4-3.
[c]HUT-78 cells persistently infected with HIV-1 NL4-3 cultivated in the presence of 500 μg/ml of JM2763.
[d]HUT-78 cells persistently infected with HIV-1 NL4-3 cultivated in the presence of 500 μg/ml of SID791.
[e]Wild-type HIV-1 NL4-3.
[f]HIV-1 NL4-3 cultivated in the presence of 500 μg/ml of JM2763.
[g]HIV-1 NL4-3 cultivated in the presence of 500 μg/ml of SID791.
[r]Resistant strain.
Numbers in parenthes represent the fold increase in EC_{50} as compared with wild-type EC_{50}.

after 29 h, while a fusion inhibitor administered during the incubation at 4°C will have no inhibitory effect. Table 2 clearly shows that SID791 behaved as could be expected from a fusion inhibitor. JM2763 acted in a similar way. When ATA or DS was added after binding, it was noted that the p24 count of the samples was considerably lower than the positive control sample without added compound either during or after binding. Thus, ATA and DS also may exert an inhibitory activity on fusion, although their first and main target is binding. Addition of these compounds after binding may still have an effect on a subsequent step in the viral life-cycle, thus supporting the hypothesis of DS being involved both in binding and fusion, as suggested by Callahan et al. (1991).

Inhibition of cell-cell fusion is observed both with SID791 and JM2763 at an EC_{50} that is about 100-fold the EC_{50} obtained in a cytopathic assay (see Table 3A and Table 3B for EC_{50} values). HIV-1 NL4-3 strains resistant to both bicyclam derivatives were developed and tested in a syncytium formation assay as well as a cytopathic assay. These assays confirmed the likely role of SID791 as an inhibitor of fusion. Further evidence for a target of SID791 located at the viral envelope glycoproteins stems from the findings obtained with dextran sulfate: SID791-resistant virus proved cross-resistant to dextran sulfate in both the cytopathicity and syncytium assays. Callahan et al. (1991) postulated that dextran sulfate binds to gp120 sites involved in virus binding to the cell surface as well as fusion (syncytium formation). Selection pressure of SID791 may cause alterations in viral glycoprotein regions that are also involved in an interaction with dextran sulfate. The observed cross-resistance with regard to dextran sulfate depends on its molecular weight: cross-resistance seems inversely proportional to molecular weight (Table 4). This phenomenon might be explained by the charge of the dextran sulfate molecules; a higher molecular weight corresponds to a more negatively charged molecule, that will be more strongly attached to the positively charged V3 loop of gp120. A mutation in that region might have more impact on interaction with less charged molecules, as the interaction is already weaker. Alternatively, the mutations could induce a conformational change of gp120 in which the interaction of the virus with the cell is not further affected by dextran sulfate.

JM2763 is not very active in inhibiting syncytium formation. However, the fusion of virus

Table 4
Inhibitory effect of dextran sulfate (DS) samples of various molecular weight (MW) on the cytopathicity of wild-type and resistant HIV-1 NL4-3 strains

Compound	EC_{50} (μg/ml)		
	NL4-3 WT	NL4-3 JM2763^r	NL4-3 SID791^r
DS MW 1000	3.25	179.00 (55)	>250 (>77)
DS MW 1500	0.725	77.03 (106)	>250 (>345)
DS MW 3400	0.280	2.32 (8)	>250 (>893)
DS MW 5000	0.230	0.620	>250 (>1086)
DS MW 10 000	0.407	0.663	55.00 (135)
DS MW 40 000	0.631	0.797	17.74 (28)
DS MW 70 000	0.756	0.826	8.58 (11)
DS MW 500 000	0.825	0.965	4.00 (5)
JM2763	0.698	>250 (>358)	>250 (>358)
SID791	0.007	0.143 (20)	3.41(487)

^{r}Resistant strain.
Numbers in parentheses represent the fold resistance compared with wild-type EC_{50}.

particles with cells, thus viral entry, may not be identical to fusion between uninfected and infected cells and requirements might be different, leading to the eventual observation that a compound is active in an assay for viral entry but not in a syncytium formation experiment. Such a compound (MS 8209) was described by Pleskoff et al. (1995). This amphotericin B derivative had to be present during the phase of virus-cell contact in order to be active, but it did not prevent the binding of recombinant gp120 to $CD4^+$ cells. Furthermore, it blocked the entry of virus already bound to the cell, but it was found not active in a syncytium formation assay. Pleskoff et al. (1995) developed an indirect assay to address the post-binding events of HIV entry and observed a clear inhibition with MS 8209 in this assay. They concluded that the antiviral effect of MS 8209 is mediated, at least in part, by an interaction with the HIV-1 envelope proteins, possibly by interference with virus-cell fusion. Other compounds, e.g. heparin, seem to share a similar mode of action. Like JM2763, heparin blocks HIV entry but not syncytium formation (Baba et al., 1990). Heparin was recently shown not to inhibit the gp120-CD4 interaction (Harrop et al., 1994). Moreover, SID791-resistant virus proved to be highly cross-resistant to heparin (data not shown), as it is to JM2763.

Table 5
Analysis of binding ability of two V3-specific monoclonal antibodies to gp120 of wild-type and bicyclam-resistant HIV-1 NL4-3 viruses

Virus	% viral antigen expression as detected by[a]	
	mAb 9284	mAb 9305
NL4-3 WT	72.7	77.9
NL4-3 JM2763^r	3.3	83.7
NL4-3 SID791^r	2.1	84.5

[a]MT-4 cells were infected with wild-type (NL4-3 WT), JM2763-resistant (NL4-3 JM2763^r) or SID791-resistant (NL4-3 SID791^r) HIV-1 NL4-3 virus and incubated with either mAb 9284 or mAb 9305. FITC-labeled secondary antibodies were detected via flow cytometry analysis.

Further evidence that gp120 is involved in the inhibitory effect of the bicyclams stems from the observation that a monoclonal antibody with specificity for the NTRKSIRIQRG region in the V3 loop of gp120 was not able to bind to the bicyclam-resistant strains. Apparently, resistance causes a mutation(s) in this, or a nearby, region that alters the access to the epitope. This effect is very specific, as the binding ability of a monoclonal antibody recognizing an adjacent region was not altered. Indeed, sequencing analysis of the resistant strains could not detect any muta-

tions in the epitope of mAb 9305, while several mutations were observed in the epitope of mAb 9284 (De Vreese et al., 1996).

Finally, marker rescue experiments carried out by De Vreese et al. (1996) confirm the important role of gp120 with regard to bicyclam resistance. Recombination of gp120 of bicyclam-resistant strains into a wild-type background fully reconstituted the resistance, proving that mutations in this region could solely account for the observed resistance (De Vreese et al., 1996).

The data presented are strongly indicative of virus-cell fusion and/or cell-cell fusion as a target for the bicyclam derivatives. Moreover, the results reported here and the data presented by De Vreese et al. (1996) prove that mutations within the envelope glycoprotein gp120 are responsible for the observed resistance. These findings suggest that to achieve their anti-HIV activity, the bicyclam derivatives must interact with a specific region of the envelope glycoprotein gp120.

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