ELSEVIER

Antiviral Research 29 (1996) 297–307

Antiviral Research

Antiviral activity of the bicyclam derivative JM3100 against drug-resistant strains of human immunodeficiency virus type 1

José A. Esté[a,*], Karen De Vreese[a], Myriam Witvrouw[a], Jean-Claude Schmit[a], Anne-Mieke Vandamme[a], Jozef Anné[a], Jan Desmyter[a], Geoffrey W. Henson[b], Gary Bridger[b], Erik De Clercq[a]

[a]*Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium*
[b]*Johnson-Matthey Pharmaceutical Research, 1401 King Road, West Chester, PA 19380, USA*

Received 7 November 1995; accepted 8 December 1995

Abstract

Bicyclams have recently been identified as potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) replication. The prototype of this series, JM3100 exhibits anti-HIV potency at concentrations ranging from 0.001 to 0.01 μg/ml. JM3100 proved to be active when tested against HIV strains resistant to the reverse transcriptase (RT) inhibitors 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine (DDI), 3TC, αAPA and TIBO, at roughly the same concentrations as for the wild-type strain. The virus was passaged in vitro in the presence of increasing concentrations of either TIBO or αAPA alone or in combination with JM3100. The combination between TIBO, or αAPA, and JM3100 delayed the development of TIBO- and αAPA-resistant strains, without emergence of resistance to JM3100. In separate experiments, it took more than 60 passages (300 days) in MT-4 cells and 20 passages (140 days) in peripheral blood lymphocyte (PBL) cells for the virus to become resistant to JM3100. The JM3100-resistant virus showed cross-resistance to sulfated polysaccharides such as dextran sulfate (DS), pentosan sulfate (PS), heparin and cyclodextrin sulfate (CDS), suggesting that these compounds may share a common mechanism of action. Furthermore, the inhibitory effect of JM3100 on virus-induced syncytium formation was enhanced in the presence of heparin. The results presented here provide further support for the bicyclams as attractive candidate drugs for the chemotherapy of HIV infections.

Keywords: Anti-HIV agents; Bicyclams; Resistance; JM3100

1. Introduction

The significant toxicity associated with the administration of approved antiviral drugs against human immunodeficiency virus (HIV) and the emergence of drug-resistant strains of HIV,

* Corresponding author. Tel.: + 32 16 332168; fax: + 32 16 337340.

SSDI 0166-3542(95)00936-G

severely diminish their therapeutic usefulness. The critical need to circumvent virus drug resistance has led to the development of a number of agents with similar or greater potency than the ones currently in use. These new drugs, when used in combination therapies, may help to prevent the emergence of drug-resistant HIV mutants and may also allow the use of individual drugs below their toxic concentrations (Tisdale et al., 1993).

Bicyclams derivatives which contain two monocyclam (1,4,8,11-tetracyclotetradecane) units, linked by either an aliphatic or aromatic linker, are potent inhibitors of HIV-1 and HIV-2 in vitro (De Clercq, 1992; De Clercq et al., 1994). The prototype of this series, compound JM3100, exhibits anti-HIV-1 potency at concentrations ranging from 0.001 to 0.01 μg/ml, while not being toxic at concentrations up to 250 μg/ml, thus achieving a selectivity index of 100 000 or higher (De Clercq et al., 1994). Bicyclams are targeted at an early step (fusion/uncoating) of virus replication (De Clercq, 1992). De Vreese et al. (1996a, 1996b) provided further evidence for a direct interaction of the bicyclams with the viral glycoprotein gp120.

When JM3100 was assayed in combination with either AZT or DDI for activity against HIV-1, additive effects were noted (De Clercq et al., 1994). Drug combinations that target different sites (i.e. reverse transcription and virus entry) in the HIV replicative cycle may not only exhibit additive or synergistic effects without accrued toxicity (Schols et al., 1991), but most important, these combinations may prevent or delay selection of resistant strains.

Rapid emergence of HIV resistance to non-nucleoside reverse transcriptase (RT) inhibitors (NNRTI) is the major drawback of these series of compounds. Virus strains resistant to compounds such as αAPA R89439 (Pauwels et al., 1993) or TIBO R82913 (Pauwels et al., 1990) are rapidly selected in culture and in vivo (Vandamme et al., 1994). Should JM3100 be active against those HIV strains that are resistant to other anti-HIV agents (i.e. RT inhibitors) and furthermore, prevent or delay the emergence of resistant strains to these drugs, this should substantially increase the potential usefulness of JM3100 in the treatment of HIV infections. The aim of this paper is to evaluate these possibilities.

2. Materials and methods

2.1. Compounds

The bicyclam derivatives JM3100 and JM2763 were synthesized at Johnson/Matthey, West Chester, PA, as described elsewhere (Bridger et al., 1995). The αAPA derivative R89439 (loviride), 3TC (R97362), 8-chloro-TIBO R86183 (Pauwels et al., 1990), γ-cyclodextrin sulfate (γCDS) and β-cyclodextrin sulfate (βCDS) were provided by the Janssen Research Foundation. 3′-azido-3′-deoxythymidine (AZT) was obtained from Wellcome, UK. Polyvinylalcohol sulfate (PVAS) was provided by Dr. S. Görög (Chemical Works of Gedeon Richter Ltd., Hungary). Dextran sulfate (DS), heparin, aurintricarboxylic acid (ATA), 2′,3′-dideoxyinosine (DDI) and pentosan sulfate (PS) were obtained from Sigma.

2.2. 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 the viability of cells that had been infected or not infected with HIV and then exposed to various concentrations of the test compound. After the MT-4 cells were allowed to proliferate for 5 days, the number of viable cells was quantified by a tetrazolium-based colorimetric method (MTT method), as described by Pauwels et al. (1988). The HIV-1 NL43 strain is a molecular clone obtained from the National Institute of Health (Bethesda, MD). The HIV-1 mutant strains ADP141 (D67N, K70R, T215F, K219Q) and ADP144 (L74V) were developed by Drs. B. Larder and S. Kemp and provided through the Medical Research Council AIDS Reagent Project. The strains 13MB1 (L100I), 39MN1 (Y181C) and 3TC-HIV-1 (M184V) were isolated in our laboratory after serial passage of the IIIB strain in MT-4 cells (13MB1, 39MN1) and CEM cells (3TC-HIV-1) in the presence of

TIBO R82913, αAPA R89439 and 3TC, respectively.

2.3. Drug combinations

Experiments were carried out on MT-4 cells infected with HIV-1 (IIIB) under the conditions described above. Checkerboard 1:5 drug dilutions were prepared in three individual 96-well trays. Cell viability was determined by the MTT procedure. The drug combination effect was analyzed by the isobologram method as previously described (Baba et al., 1987). The 50% effective concentration (EC_{50}) was used for calculating the fractional inhibitory concentration (FIC). When the minimum FIC index, which corresponds to the FIC of compounds combined, is equal to 1, the combination is assumed to act in an additive fashion; when it is between 1.0 and 0.5, the combination acts subsynergistically, and when it is < 0.5 it acts synergistically (Schols et al., 1991).

2.4. Syncytium formation assay

MT-4 (clone 8) cells (1.8×10^6 cells/ml) were cultivated with persistently HIV-1-infected HUT-78 cells (2×10^5 cells/ml) in microtiter tray wells containing various concentrations of the test compound alone or in combination with JM3100. After a 24-h cocultivation period, the number of giant cells (syncytia) was recorded microscopically as described previously (Witvrouw et al., 1994a).

2.5. Resistance development

MT-4 cells were infected with HIV-1 (NL43) (Adachi et al., 1986) in medium containing the test compounds at 2–4 times their EC_{50} values. Cultures were incubated at 37°C until an extensive cytopathic effect was present (3–5 days). The culture supernatants were used for further passage in MT-4 cells in the presence of increasing (2- to 5-fold) concentrations of the test compounds. In combination studies, the concentration of JM3100 was maintained at a concentration between 0.01 and 0.02 μg/ml throughout.

Peripheral blood lymphocyte (PBL) cells were infected with HIV-1 (NL43) or with two clinical HIV-1 isolates in the presence of the test compound. HIV replication was measured (every 7–8 days) by a p24 antigen detection method (du-Pont). The p24 antigen-positive supernatant was further passaged in fresh PBL, thereby increasing by 2- to 5-fold the concentration of the test compound.

2.6. Sequence analysis of RT

RNA extraction of frozen culture supernatant was performed with RNAzol B (Biotecx Laboratories, USA). Isolated RNA was used for cDNA synthesis with the GeneAmp RNA polymerase chain reaction (PCR) kit (Perkin-Elmer, Brussels, Belgium). A 777-nucleotide base pair fragment (codons 1–259) of the RT gene was amplified in a nested PCR using the primers RT01/RT02 (Gu et al., 1992), followed by primers M13-USP-A35-biotinyl-M13-RSP-NE1 (modified from Larder et al., 1991). After DNA purification, the sequencing reactions were carried out with T7 DNA polymerase (AutoRead T7 Sequencing Kit, Pharmacia, Sweden) and sequencing products were analysed on an ALF sequencer (ALF manager, Pharmacia, Sweden). The sequences were exported to the software program GENEWORKS 2.4 (Intelligenetics Inc., UK) and aligned with the published RT sequence of the virus strain NL43.

3. Results

3.1. Activity of JM3100 against HIV-1 strains that are resistant to RI inhibitors

The comparative effects of six compounds against wild-type and HIV-1 strains resistant to AZT, DDI, 3TC, αAPA R89439 and TIBO R86183 are presented in Table 1. All the strains tested proved sensitive to the bicyclam JM3100 (EC_{50} values ranging from 0.004 to 0.035 μg/ml). JM3100 was not toxic to the host cells at a concentration of 250 μg/ml (Table 1). Strain ADP141 showed 100-fold resistance to AZT, strain 3TC-HIV-1 > 3000-fold resistance to 3TC, 13MB1 > 500-fold resistance to TIBO R86183 and 39MN1 > 3000-fold resistance to αAPA

Table 1
Activity of JM3100 and RT inhibitors against different HIV-1 strains

HIV-1 strain [c]	Compounds (EC_{50} = μg/ml) [a]					
	JM3100	AZT	DDI	3TC	TIBO	αAPA
ADP141	0.035	0.02	1.6	–	0.001	0.02
ADP144	0.006	0.0001	0.6	0.04	0.0004	0.002
3TC-HIV-1	0.026	0.0002	3.6	>250	13.2	0.0006
13MB1	0.013	0.0001	0.4	0.04	0.4	0.007
39MN1	0.004	0.0002	0.4	0.04	0.06	2.8
IIIB	0.005	0.0002	0.7	0.08	0.0007	0.0009
CC_{50} (μg/ml) [b]	>250	3.2	>125	>250	20	>125

[a] EC_{50} = 50% effective concentration, based on the inhibition of HIV-induced cytopathicity in MT-4 cells, as determined by the MTT method.
[b] CC_{50} = 50% cytotoxic concentration, based on the viability of MT-4 cells by the MTT method.
[c] Strains were as follows: The HIV-1 mutant strains ADP141 (D67N, K70R, T215F, K219Q), ADP144 (L74V) provided through the Medical Research Council AIDS Reagent Project. The strains 13MB1 (L100I), 39MN1 (Y181C) and 3TC-HIV-1(M184V) were isolated in our laboratory after serial passage of the IIIB strain in MT-4 cells (13MB1, 39MN1) and CEM cells (3TC-HIV-1) in the presence of TIBO R82913, α-APA R89439 and 3TC, respectively.

R89439. Strain 3TC-HIV-1 showed a 5-fold resistance to DDI. The M184V mutation, which confers resistance to 3TC, has been previously reported to confer cross-resistance to DDI up to 5-fold the EC_{50} necessary for the parental virus (Gu et al., 1992). PCR analysis showed that the ADP144 strain contains a mutation in the RT coding region at codon 74 (L–V); however, in our assay, this strain failed to demonstrate any resistance to DDI, which contrasts with what has been previously reported (St.Clair et al., 1991).

3.2. Drug combinations

JM3100 was assayed in combination with either αAPA R89439 or TIBO R86183 for activity against HIV-1 (IIIB) in MT-4 cells (Fig. 1) and the inhibitory action of the combined drugs was evaluated by the isobologram method. The minimum FIC value fell below the 0.5 level (0.3 for both the combination of TIBO with JM3100 and of αAPA with JM3100), indicating a moderate synergistic action for these combinations. No reduction in the viability of MT-4 cells was observed with any of the combinations used.

3.3. Resistance development to JM3100

HIV-1 strains resistant to the bicyclam JM3100 were raised in MT-4 cells and in PBL. In MT-4 cells the HIV-1 strain NL43 remained sensitive to the drug for at least 120 days (30 passages) and became only resistant (300-fold) after 240 days (60 passages). The NL43 strain became 170-fold resistant to the prototype bicyclam JM2763 after 100 days (25 passages) and 380-fold resistant to TIBO R86183 after 40 days (10 passages) (Fig. 2) (De Vreese et al., 1996b).

In PBL, 100-fold resistance was obtained after 140 days (20 passages) of HIV-1 NL43 in the presence of JM3100. The virus remained sensitive to the drug for at least 56–63 days (8–10 passages). Resistance of the clinical HIV-1 isolates CI1 (260-fold) and CI2 (360-fold) was obtained after 74 days (10 passages) (Table 2).

3.4. Resistance development in combinations with JM3100

Passage of HIV-1 NL43 in the presence of the non-nucleoside RT inhibitors αAPA R89439 and TIBO R86183 resulted in rapid development of drug resistant virus. However, when selection of HIV-1 variants resistant to αAPA and TIBO was

carried out in the presence of JM3100 a delay in the emergence of resistance was observed (Fig. 3), without loss of sensitivity to JM3100 (Table 3). We also noted that full cytopathic effect (CPE) in the drug combination cultures took about 3–4 days longer to reach that for the wild-type virus. These experiments were carried out at a constant JM3100 concentration of 0.02 μg/ml, only 4-fold higher than the EC_{50} required in the anti-HIV assay.

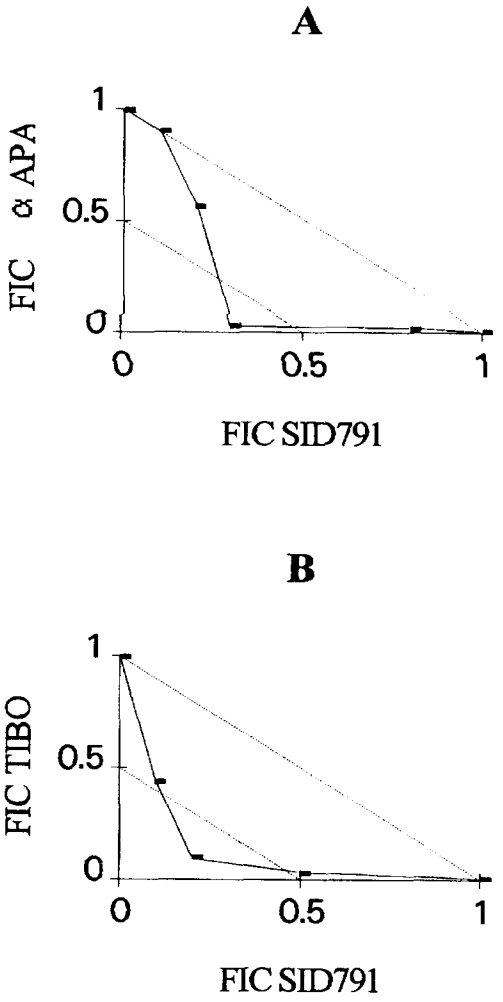

Fig. 1. Isobologram representation of the combined inhibitory effects of JM3100 and αAPA R89439 (panel A) and TIBO R86183 (panel B) on the cytopathicity of HIV-1 for MT-4 cells. Broken lines represent the unity lines for FIC equal to 1 and 0.5, respectively.

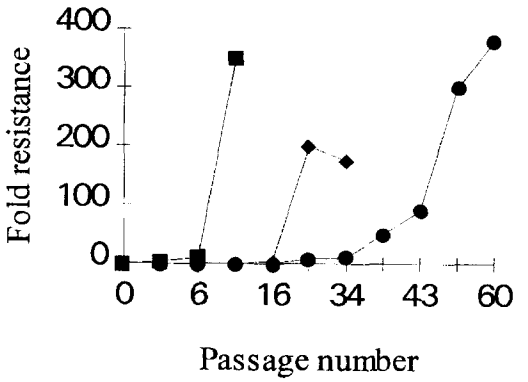

Fig. 2. Resistance development of HIV-1 NL43 to the bicyclams JM2763(◆) and JM3100(●), and to TIBO R86183 (■). At different passages EC_{50} values were determined and compared to the wild-type EC_{50}.

To confirm the delay in the emergence of resistant virus, we performed DNA sequence analysis within the RT region obtained by PCR amplification of cDNA from virus recovered after nine passages in the presence of either αAPA, or αAPA with JM3100 and after 10 passages in the presence of either TIBO alone, or TIBO with JM3100. These passages resulted in the loss of sensitivity to the NNRTIs of the virus passaged in the presence of the NNRTI alone. However, the combination of αAPA or TIBO with JM3100 delayed the appearance of the mutations associated with resistance to NNRTI (Table 4).

3.5. Cross-resistance of JM3100 resistant virus to polyanions

JM3100-resistant virus (HIV-1 NL43) raised in MT-4 cells, was fully resistant to heparin, dextran sulfate (DS, MW 1500), pentosan sulfate(PS), β-cyclodextrin sulfate (βCDS) and γ-cyclodextrin sulfate (γCDS), and partially cross-resistant to aurintricarboxylic acid (ATA) (36-fold) and PVAS (14-fold) (Table 5).

3.6. Giant cell formation

Anti-HIV-1 (IIIB) activity of checkerboard dilutions of heparin and JM3100 was tested in the HIV cytopathicity assay, and analyzed by the

Table 2
Sensitivity of clinical HIV-1 isolates to JM3100 after serial passage in PBL in the presence of the drug

HIV-1 strains [a]	EC_{50} (μg/ml)	Fold resistance [b]	Number of passages in PBL (days)
wtNL43	0.004	–	
JM3100^r NL43	0.41	103	20 (140)
wtCI1	0.01	–	
JM3100^r CI1	2.6	260	10 (74)
wtCI2	0.01	–	
JM3100^r CI2	3.6	360	10 (74)

[a] Resistant strains (r) were obtained after passage of wild-type (wt) HIV strains (NL43) or isolates 1 (CI1) and 2 (CI2) in PBL, in the presence of JM3100. Virus growth (breakthrough) was monitored by p24 antigen expression in the supernatant of cultured PBL.
[b] Fold resistance: ratio EC_{50} for the resistant strain to EC_{50} for the wild-type strain.

MCSYNERGY program (Prichard et al., 1990). No significant synergistic effect between these two drugs was noted (data not shown). However, when analyzed in the giant cell assay, checkerboard dilutions of heparin and JM3100 revealed a potentiating effect of heparin on the inhibitory action of JM3100 against syncytium formation. Heparin alone did not inhibit syncytium formation at a concentration of 200 μg/ml; however, in combination with JM3100 the concentration of the latter compound required to inhibit syncytium formation by 50% was reduced 100-fold in a concentration-dependent manner (Fig. 4). AZT did not have any effect on the action of JM3100 against syncytium formation (data not shown).

4. Discussion

Much progress has been made in recent years in the field of anti-HIV therapy. A number of valuable agents (i.e. AZT, DDI, DDC, D4T) are currently available for the treatment of HIV infection. Unfortunately, these agents are only partially effective when used in monotherapy. This has been attributed, at least in part, to the development of virus drug resistance by HIV. New and more effective candidate drugs are required that alone, or more likely, in combination with drugs currently used, will help to overcome resistance development.

The bicyclam JM3100 is a potent inhibitor of HIV-1 and HIV-2 in vitro, with a selectivity index greater than 100 000. The mode of action of bicyclams has not been totally elucidated yet. However, it has been shown that bicyclams interfere with an early event in the viral replicative cycle that occurs after virus binding to the CD4 receptor but precedes the RT process. Fusion/uncoating seems to be the most likely target (De Clercq et al., 1992, 1994).

Our results ascertained that JM3100 is able to inhibit the replication of HIV-1 strains that are resistant to HIV RT inhibitors currently in therapeutic use (AZT and DDI), as well as drugs which have already entered clinical trials (3TC, αAPA (loviride) and 8-chloro-TIBO) (Goldthorpe et al., 1995). Therefore, JM3100 may be a good candidate drug in patients who require alternative therapy due to the emergence of drug-resistant virus.

De Clercq et al. (1994) have shown that combinations of JM3100 with AZT or of JM3100 with DDI primarily act in an additive manner. We have observed modest synergism when JM3100 is used in combination with αAPA R89439 or TIBO R86183, two NNRTIs to which resistance develops after a few passages in cell culture. Such rapid resistance development severely limits the therapeutic value of these inhibitors except possibly when they are used in combination with other HIV-1 inhibitors. We studied the effects of combinations of αAPA with JM3100 and of TIBO with JM3100 on the emergence of resistant virus during in vitro passage. JM3100 was able to delay the development of αAPA-resistant and TIBO-resistant virus (Fig. 3) without the emergence of resistance to JM3100. The slower growth of the virus in the drug combination of NNRTI and SID791

could mean that these combinations may cause a reduced overall yield from a single-cycle replication or that the time to complete a single cycle of replication is longer than with the wild-type virus. We envision that combinations with higher concentrations of JM3100 (which are easily obtainable in rabbit serum after subcutaneous administration (Witvrouw et al., 1996)) and NNRTIs not only could slow down viral growth but totally suppress ('knock-out') (Balzarini et al., 1993) virus replication and prevent emergence of NNRTI-resistant strains.

Using the HIV-1 strain NL43, we have found that resistant variants arose only after prolonged passaging in the presence of gradually increasing concentrations of the bicyclam JM3100. Similar results were obtained when clinical isolates were passaged in the presence of increasing concentrations of the compound. After 74 days (10 passages) resistant virus was recovered. DNA sequence analysis of the NL43-derived strains that are resistant to JM3100 has revealed that loss of sensitivity to the drug is based exclusively on mutations in the HIV gp120 protein and that up to 11 mutations may be required to achieve marked resistance of HIV-NL43 virus to the bicyclams (De Vreese et al., 1996a). The HIV gp120 region is highly variable among HIV strains (Ghiara et al., 1994); therefore, it is possible that HIV quasispecies containing some, but not all, of these mutations may be present in the culture and may be more readily selected by the treatment with the drug. Also the possibility remains that mutations other than those described by De Vreese et al. (1996a), are responsible for the loss of sensitivity to JM3100.

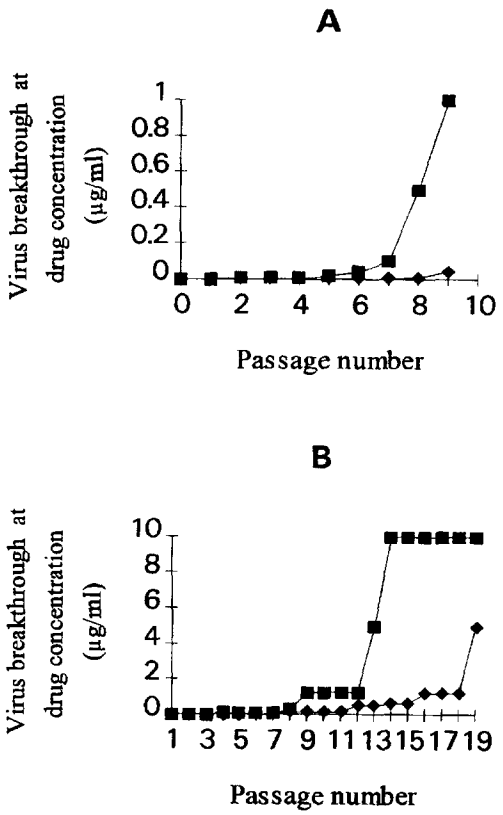

Fig. 3. Development of resistance to αAPA R89439 (panel A) or TIBO R86183 (panel B) in the absence (■) or presence (◆) of 0.02 μg/ml JM3100. MT-4 cells were infected with HIV-1 NL43 and incubated in the presence of the compounds. Virus recovered from culture supernatants was serially passaged in the presence increasing concentrations of the compound until full CPE was detected.

The rate by which drug-resistant variants may arise in HIV-1 populations may depend on a number of factors such as the mutation frequency, viral load, and the number of mutations needed to confer the resistant phenotype (Domingo and Holland, 1994). Quasispecies of HIV with mutations associated with AZT and 3TC resistance from patients undergoing no drug therapy have been identified (Najera et al., 1995); these natural resistant quasispecies contain single amino acid substitutions that confer the resistant phenotype. In the case of JM3100, many mutations are required to obtain highly resistant virus. It is improbable then that such high-level resistant virus is a natural dominant quasispecies if so many mutations are required.

It was shown that HIV-1 NL43-derived strains that are resistant to JM3100 are cross-resistant to different polyanions (Table 5). The JM3100-resistant strain is fully cross-resistant to the bicyclam JM2763, to the polyanions DS, heparin, PS, βCDS and γCDS, and partially cross-resistant to ATA and PVAS. This cross-resistance phenomenon further supports the notion that gp120 acts as the target molecule of JM3100 since it has been shown that polysulfates inhibit gp120-depen-

Table 3
Sensitivity of HIV-1 NL43 to different inhibitors after serial passage of the virus in the presence of TIBO R86183 or αAPA R89439 alone or in the presence of JM3100.

HIV-1 strain	Compound	EC_{50} (μg/ml)	Fold resistance
wtNL43	αAPA	0.0005	–
	TIBO	0.0003	–
	JM3100	0.003	–
	AZT	0.0002	–
αAPA^{r}NL43 (after 9 passages)	αAPA	2.4	>4000
	JM3100	0.004	–
	AZT	0.0003	–
αAPA^r NL43+SID791 (after 9 passages)	αAPA	0.0048	9
	JM3100	0.008	3
	AZT	0.0004	2
TIBO^r NL43 (after 10 passages)	TIBO	0.07	230
	JM3100	0.003	–
	AZT	0.0001	–
TIBO^{r}NL43+SID791 (after 10 passages)	TIBO	0.003	10
	JM3100	0.002	–
	AZT	0.0001	–

MT-4 cells were infected with HIV-1 NL43 and incubated in the presence of TIBO or αAPA alone or in combination with 0.02 μg/ml JM3100. Virus recovered from the culture supernatants was serially passaged in the presence of increasing concentrations of the compound until full CPE was detected. After 10 passages (αAPA) or 13 passages (TIBO), virus was titered and EC_{50} were determined by the MTT method in MT-4 cells.

Table 4
DNA sequence analysis of the RT coding region of drug-resistant virus derived by passage of HIV-1 NL43 in the presence of either αAPA or TIBO alone or in combination with JM3100

Virus	Inhibitor(s)	Amino acid at position:			Expected phenotype
		100	103	181	
Consensus	—	L	K	Y	Wild type
Passage 9	αAPA	L	K	C	Resistant
Passage 9	αAPA+JM3100	L	K	Y	Wild type
Passage 10	TIBO	L/I mix	K	Y/C mix	Resistant
Passage 10	TIBO+JM3100	L	K	Y/C mix	Partially resistant
Passage 19	TIBO	L	K/Nmix	C	Resistant
Passage 19	TIBO+JM3100	L	N	C	Resistant

dent binding of virions to the CD4 receptor (Witvrouw et al., 1994b). DS, ATA, heparin and CDS may share a common mechanism of action in inhibiting virus binding; however, a second mechanism is suggested by the fact that heparin and CDS, unlike DS and ATA, do not inhibit syncytium formation at concentrations up to 200 μg/ml. Furthermore, JM3100 does not inhibit virus binding to CD4 at 100 μg/ml, and inhibits syncytium formation with an EC_{50} 100-fold higher than the EC_{50} for CPE inhibition. However, this EC_{50} was reduced up to 100-fold when JM3100 was used in combination with heparin, pointing to complementary activities between both compounds. Javaherian et al. (1995) have proposed a putative binding site for heparin at the amino acid sequence RKSIR present in the V3 loop of HIV-1 isolates IIIB, MN and SF2. This amino acid

Table 5
Anti-HIV activity of bicyclams and polyanions against HIV-1 wild type and SID791-resistant virus

Compound	EC_{50} (μg/ml) [a]			CC_{50} (μg/ml) [c]
	Wild type NL43	JM3100^r NL43	Syncytium [b] formation	
JM3100	0.009	0.9	3	>250
JM2763	0.4	96	>50	>250
DS5000	0.3	>125	2.2	>250
DS1500	0.4	>125	200	>250
Heparin	0.4	97	>125	>250
ATA	0.3	10.9	3.3	>250
PS	0.20	>125	14	>250
βCDS	0.3	105	>500	>250
γCDS	1.7	>125	>500	>250
PVAS	2.4	34	2	>250
AZT	0.0002	0.0016	>2	>2

[a] EC_{50} = 50% effective concentration based, on the inhibition of HIV-induced cytopathicity in MT-4 cells, as determined by the MTT method.
[b] Based on the inhibition of giant cell (syncytium) formation between HUT-78/IIIB cells and MT-4 clone 8 cells.
[c] CC_{50} = 50% cytotoxic concentration, based on the viability of MT-4 cells by the MTT method.

sequence is also present in the V3 loop of strain NL43 and two of the mutations associated with JM3100 resistance are located in this small peptide stretch. The cross-resistance of JM3100-resistant virus to heparin, the synergism between these two compounds in the syncytium formation assay, and the mutations at the flanking region of the V3 loop in the JM3100-resistant strains suggest that JM3100 and heparin interact with similar or overlapping amino acid motifs. This possibility is the subject of further research. Unravelling the mechanism of action of JM3100 will shed some light on the way by which sulfated polysaccharides interfere with the interaction between the viral gp120 and its cell receptor.

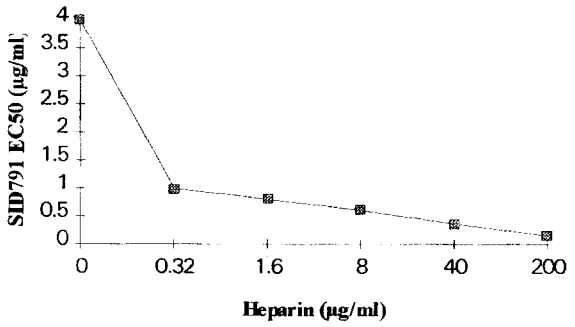

Fig. 4. Inhibitory effect of JM3100 on HIV-1-induced syncytium formation in the presence of heparin. The EC_{50} is based on inhibition of giant cell formation between persistently HIV-1 (strain IIIB) infected HUT-78 cells and uninfected MT-4 (clone 8) cells. Heparin alone did not inhibit syncytium formation at concentrations up to 200 μg/ml.

The results presented here further support the potential usefulness of JM3100 for the therapy of HIV-1, as an alternative treatment modality or in combination with other HIV inhibitors.

Acknowledgements

We thank Anja Van Cauwenberge, Barbara Van Remoortel and Patrick Seldeslachts for excellent technical assistance. This work was supported in part by the Biomedical and Health Research Programme of the European Commission, the Janssen Research Foundation, the Belgian Geconcerteerde Onderzoeksacties and the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek. J.A.E. is a fellow from the Venezuelan Consejo Nacional de Investigaciones Científicas y Tecnológicas (BID-CONICIT).

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.

Baba, M., Pauwels, R., Balzarini, J., Herdewijn, P., De Clercq, E. and Desmyter, J. (1987) Rivabirin antagonizes inhibitory effects of pyrimidine 2′,3′-dideoxynucleosides but enhances inhibitory effects of purine 2′,3′-dideoxynucleosides on replication of human immunodeficiency virus in vitro. Antimicrob. Agents Chemother. 31, 1613–1617.

Balzarini, J., Karlsson, A., Pérez-Pérez, M., Camarasa, M., Tarpley, W. and De Clercq, E. (1993) Knocking-out concentrations of HIV-1-specific inhibitors completely suppress HIV-1 infection and prevent the emergence of drug-resistant virus. Virology 196, 576–585.

Bridger, G., Skerlj, R., Thornton, D., et al. (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.

De Clercq, E. (1992) Human immunodeficiency virus inhibitors targeted at virus-cell fusion and/or viral uncoating. Int. J. Immunother. 8, 115–123.

De Clercq, E., Yamamoto, N., Pauwels, R., et al. (1992) 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. Proc. Natl. Acad. Sci. U.S.A. 89, 5286–5290.

De Clercq, E., Yamamoto N., Pauwels, R., et al. (1994) Highly potent and selective inhibition of human immunodeficiency virus by the bicyclam derivative JM 3100. Antimicrob. Agents Chemother. 38, 668–674.

De Vreese, K., Kofler-Mongold, V., Leutgeb, C., et al. (1996a) The molecular target of bicyclams, potent inhibitors of human immunodeficiency virus replication. J. Virol., 70, 689–696.

De Vreese, K., Reymen, D., Griffin, P., et al. (1996b) The bicyclams, a new class of potent human immunodeficiency virus inhibitors, block viral entry after binding. Antiviral Res., in press.

Domingo, E. and Holland, J. (1994) Mutation rates and rapid evolution of RNA viruses. In: S.S. Morse (Ed), Evolutionary Biology of Viruses, pp. 161–184. Raven Press, New York.

Ghiara, J., Stura, E., Satanfield, R., Profy, A. and Wilson, I. (1994) Crystal structure of the principal neutralization site of HIV-1. Science 264, 82–85.

Goldthorpe, S., Kinchington, D. and Lovelidge, D. (1995) Current antiviral agents. Int. Antiviral News 3, 92–98.

Gu, Z., Gao, Q., Li, X., Parniak, M. and Wainberg, M. (1992) Novel mutation in the human immunodeficiency virus type 1 reverse transcriptase gene that encodes cross-resistance to 2′,3′-dideoxycytidine. J. Virol. 66, 7128–7135.

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 229, 563–566.

Javaherian, K., Khiroya, R., Quinlan, C., Boyd, J. and Hanham, K. (1995) V3-mediated heparin neutralization of HIV type 1. AIDS Res. Hum. Retroviruses 10, 1421–1423.

Larder, B., Kellam, P. and Kemp, S. (1991) Zidovudine resistance predicted by direct detection of mutations in DNA from human immunodeficiency virus infected lymphocytes. AIDS 5, 137–144.

Najera, I., Holguin, A., Quiñones- Mateu, M., et al. (1995) *pol* Gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy. J. Virol. 69, 23–31.

Pauwels, R., Balzarini, J., Baba, M., et al. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods 20, 309–321.

Pauwels, R., Andries, K., Desmyter, J., et al. (1990) Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. Nature 343, 470–474.

Pauwels, R., Andries, K., Debyser, Z., et al. (1993) Potent and highly selective human immunodeficiency virus type 1 (HIV-1) inhibition by a series of α-anilinophenylacetamide derivatives targeted at HIV-1 reverse transcriptase. Proc. Natl. Acad. Sci. U.S.A. 90, 1711–1715.

Prichard, M., Prichard, L., Baguley, W., Nassiri, M. and Shipman, C. (1990) Three-dimensional analysis of the synergistic cytotoxicity of ganciclovir and zidovudine. Antimicrob. Agents Chemother. 35, 1060–1065.

Schols, D., De Clercq, E., Witvrouw, M., et al. (1991) Sulphated cyclodextrins are potent anti-HIV agents acting synergistically with 2′,3′-dideoxynucleoside analogues. Antiviral Chem. Chemother. 2, 45–53.

St.Clair, M., Martin, J. and Tudor-Williams, G. (1991) Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science 253, 1557–1559.

Tisdale, M., Kemp, S., Parry, N. and Larder, B. (1993) Rapid in vitro selection of human immunodeficiency virus type I resistant to 3′-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. Proc. Natl. Acad. Sci. U.S.A. 90, 5653–5656.

Vandamme, A.-M., Debyser, Z., Pauwels, R., et al. (1994) Characterization of HIV-1 strains isolated from patients treated with TIBO R82913. AIDS Res. Hum. Retrovir. 10, 39–46.

Witvrouw, M., Esté, J.A., Quiñones-Mateu, M., et al. (1994a) Activity of a sulfated polysaccharide extracted from the red seaweed *Aghardiella tenera* against human immunodeficiency virus and other enveloped viruses. Antiviral Chem. Chemother. 5, 297–303.

Witvrouw, M., Desmyter, J. and De Clercq, E. (1994b) Antiviral portrait series: 4. Polysulfates as inhibitors of HIV and other enveloped viruses. Antiviral Chem. Chemother. 5, 345–359.

Witvrouw, M., Sifert, J.M., Henson, G., Martellucci, S.A., Desmyter, J. and De Clercq, E. (1996) Pharmacokinetics of the anti-HIV bicyclam SID791 (JM3100) in rabbits, as determined by both analytical and bioassay methods. Antiviral Chem. Chemother., 7, 27–30.