ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1997, p. 2616–2620 Vol. 41, No. 12
0066-4804/97/$04.00+0

Sensitivity of Human Immunodeficiency Virus to Bicyclam Derivatives Is Influenced by the Three-Dimensional Structure of gp120

KAREN DE VREESE,† ILSE VAN NERUM,‡ KURT VERMEIRE, JOZEF ANNÉ, AND ERIK DE CLERCQ*

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Received 13 May 1997/Returned for modification 22 July 1997/Accepted 22 September 1997

The bicyclams are a new class of anti-human immunodeficiency virus (anti-HIV) compounds targeted at viral entry. From marker rescue experiments, it appears that the envelope gp120 glycoprotein plays an important role in the anti-HIV activity of the bicyclams. Bicyclam-resistant strains contain a number of amino acid changes scattered over the V2 to V5 region of gp120. Experiments aimed at estimating the relative importance of particular amino acid changes with regard to the overall resistance pattern are described. The sequences of some partially bicyclam-resistant virus strains, obtained during the resistance development process, were analyzed, and the corresponding 50% effective concentrations were determined. Selected mutations observed in bicyclam-resistant strains were introduced in the wild-type background by site-directed mutagenesis. In addition, some amino acids were back-mutated to their wild-type counterparts in an otherwise JM3100-resistant strain. The sensitivities of these mutant viruses to bicyclams were determined. Construction of chimeric viruses, carrying the V3 loop of JM3100-resistant virus in a wild-type HIV type 1 HXB2 background, enabled us to investigate the importance of the mutations in the V3 loop of JM3100-resistant virus. From the results described in the report, it can be concluded that single amino acid substitutions do not influence the observed resistance to JM3100. Also, the mutations in the V3 loop are not sufficient to engender even a partially resistant phenotype. We postulate that the overall conformation of gp120 determines the degree of sensitivity or resistance of HIV strains to bicyclams.

The bicyclams are a group of potent human immunodeficiency virus (HIV) inhibitors (3, 4) interfering with viral entry (7). To investigate the molecular target of these compounds, resistance to the prototype bicyclams JM2763 and JM3100 was developed by using the molecular clone HIV type 1 (HIV-1) strain NL4-3 (7). Via marker rescue experiments, particular regions of the genome of these bicyclam-resistant strains were exchanged for the corresponding regions in the wild-type strain. The results obtained enabled us to delineate the gp120 region(s) that determines the observed resistance to bicyclams. Sequence analysis revealed the presence of several mutations, scattered over the whole gp120 glycoprotein, but primarily clustered in the V3 loop (6). This paper describes experiments aimed at correlating particular amino acid changes in the strain NL4-3 gp120 with the resistant phenotype. In addition, the results obtained with the bicyclam-resistant strains originating from the clinical HIV-1 HE strain were analyzed and compared to the results obtained with HIV-1 NL4-3.

MATERIALS AND METHODS

Compounds. The bicyclam derivatives JM2763 (1,1′-propylene-bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride tetrahydrate) and JM3100 [1,1′-(1,4-phenylenebis-methylene)-bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride dihydrate] were synthesized at Johnson Matthey, West Chester, Pa. (1). 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).

Cells and viruses. The origin of the MT-4 cells is described by Harada et al. (10). The cells were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 0.1% sodium bicarbonate, 2 mM L-glutamine, and 20 μg of gentamicin per ml. HIV-1 NL4-3 is a molecular clone obtained from the National Institutes of Health (Bethesda, Md.). HIV-1 HE is a clinical strain isolated in our laboratory.

Resistance development. HIV-1 HE was passaged in the presence of increasing concentrations of JM2763, JM3100, or R86183. Starting concentrations for JM2763 were 2.5 (five times the 50% effective concentration [EC_{50}]), 10, and 100 μg/ml. JM3100 was supplemented at 0.02 (five times the EC_{50}), 0.1, 0.5, and 5 μg/ml. R86183 was added at 0.04 (five times the EC_{50}), 0.4, and 4 μg/ml. The concentrations of the compounds were increased when fulminant virus breakthrough was observed. The EC_{50} was determined by the cytopathic effect (CPE)–3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (CPE-MTT method) (13). The genomic DNA of HE-infected MT-4 cells was isolated via the QIAamp Blood Kit (Qiagen, Chatsworth, Calif.). The gp120 glycoproteins of wild-type and bicyclam-resistant HE strains were amplified with ULTma DNA polymerase (Perkin-Elmer Cetus, Foster City, Calif.) by using the forward primer V3F (base pairs 7035 to 7056) and the reverse primer T5R (base pairs 7701 to 7727). The PCR fragment was then cloned in the pCRScript SK(+) vector (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. Subsequently, the sequence of the V3 to V5 region of gp120 was determined.

Analysis of intermediate passages. MT-4 cells (30×10^4/ml) were infected with a stock of different partially resistant HIV-1 NL4-3 for which the EC_{50} was known. Genomic DNA was isolated via the QIAamp Blood kit (Qiagen). The gp120 regions of these proviruses were then amplified with the primers V3F and T5R and cloned in the pCRScript SK(+) vector (Stratagene). The sequence was analyzed by using the Sequenase, version 2.0, kit (United States Biochemical Corp., Buckinghamshire, England). Marker rescue experiments were performed as described previously (6). Briefly, 20 μg of cloned DNA excised from the cloning vector at polylinker restriction sites was precipitated with 8 μg of restriction enzyme-digested NL4-3 wild-type DNA and was transfected into MT-4 cells by electroporation (settings, 250 V and 960 μF). The EC_{50}s for the recombinant viruses were determined by the CPE-MTT method. The gp120 glycoproteins of these viruses were amplified and analyzed via fluorescent cycle sequencing with the ABI Prism Dye Terminator cycle sequencing core kit (Perkin-Elmer Cetus).

Site-directed mutagenesis. The gp120 regions of wild-type virus and JM3100-resistant HIV-1 NL4-3, previously cloned in pCRScript (6), were excised with the restriction enzymes *Sac*I and *Kpn*I and were ligated into the pALTER-1 vector. Oligonucleotide-directed mutagenesis was performed with the Altered Sites II in vitro mutagenesis system (Promega, Leiden, The Netherlands). The different mutations introduced and the oligonucleotides used are summarized in Table 1. The mutants obtained were confirmed by sequence analysis. Subsequently, infectious virus that contained the appropriate changes was isolated by the marker rescue technique. The EC_{50} was determined by the CPE-MTT method. The gp120 glycoproteins of these viruses were amplified and analyzed by fluorescent cycle sequencing.

Construction of chimeric viruses by exchange of the V3 region. The V3 regions of wild-type virus and JM3100-resistant HIV-1 NL4-3 were amplified with spe-

* Corresponding author. Mailing address: Rega Institute for Medical Research, K. U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium. Phone: 32-16-33.73.67. Fax: 32-16-33.73.40.

† Present address: Innogenetics NV, B-9052 Ghent, Belgium.

‡ Present address: Fruitteeltcentrum, B-3001 Heverlee, Belgium.

TABLE 1. Mutations introduced and oligonucleotides used in the site-directed mutagenesis procedure

Background	Original amino acid sequence (region)	Mutation[a]	Primer sequence (5′ to 3′)[b]	Position
Wild type	−NNNTR− (V3)	−NYNSTR−	CGGATACTTTTTCT***A***GT***ACTGTTGTA***GTTGGGTCTTGTAC[c]	7101–7137
Wild type	R_{272} (V3)	T	GGATACTTTTT***G***TTGTATTGTTG	7114–7136
JM3100 resistant	T_{272} (V3)	R	GGATACTTTTT***C***TTGTACTGTTGTAG	7114–7136
Wild type	A_{297} (V3)	T	GTTACAATGTG***T***TTGTCTCATATTTCC	7184–7210
JM3100 resistant	T_{297} (V3)	A	GTTACAATGTG***C***TTGTCTCATATGTCC	7184–7210
Wild type	−FNSTWFNSTWSTEG− (V4)	FNSTWDSTEG	GACCCTTCAGTACTCCAAGTACTATTAAACAG***C***TGTGTTGAATTAC[d]	7368–7428
Wild type	P_{385} (V4)	L	GTTTTATTCTGCAT***A***GGAGTGTGATTG	7452–7478
JM3100 resistant	L_{385} (V4)	P	TTGTTTTATTCTGCAT***G***GGAGTGTGATTGTGTCACTTCC	7442–7480
Wild type	A_{297}/P_{385} (V3/V4)	T/L[e]	GTTACAATGTG***T***TTGTCTCATATTTCC	7184–7210

[a] D, deletion of five amino acids (FNSTW).
[b] The mutated nucleotides are indicated in boldface and italics.
[c] An additional *Sca*I restriction site was created to facilitate detection of mutant plasmid after mutagenesis.
[d] An additional *Pvu*II restriction site was created to facilitate detection of mutant plasmid after mutagenesis.
[e] This combination of mutations was obtained by introducing the mutation $A_{297}T$ into a mutant plasmid already carrying the $P_{385}L$ mutation.

cially designed primers containing a restriction site (5). Subsequently, the V3 region was cloned into a wild-type HXB2 background with the pJJ25-pJJ5 plasmid vector system (5). The resulting chimeric viruses were transfected into MT-4 cells, and the sensitivity of the resulting virus was determined by the CPE-MTT method.

RESULTS

Analysis of intermediate passages. Specifications about the strains chosen, the passage at which the particular strain was isolated, and the mutations present in the clones are summarized in Table 2. However, the cloning procedure implied that one copy was selected from a heterogeneous mixture of PCR fragments. When analyzing a strain that has been cultivated for 5 to 10 passages in the presence of a high concentration of bicyclam, the mix probably contains only viruses carrying the mutations necessary for this resistance. On the contrary, when amplifying DNA from partially resistant virus from an intermediate passage, a nonrepresentative sequence could be selected. Proof for this possibility was given by two JM3100-resistant clones from passage 28 that were analyzed: their sequences differed, and one clone carried more mutations than the other clone (Table 2). Therefore, it is possible that the sequence of the clone is not related to the EC_{50} obtained for the partially resistant virus. On the other hand, recombination of the cloned viruses via marker rescue yields a homogeneous population of viruses for which the EC_{50} can be determined, and this EC_{50} can then be directly related to the sequences obtained.

Thus, marker rescue was carried out with these clones. Since we were not certain what level of resistance could be expected, the recombinant viruses had to be grown without the selection pressure of the compound. This implies the risk of selecting for wild-type virus instead of the wanted mutant (presumably originating from trace amounts of uncleaved DNA in the preparation). This was the case for JM3100-resistant virus from passages 28 and 42 in the experiments (data not shown). It can be concluded that the results (Table 3) obtained by using this approach must be interpreted with caution. The cloned JM2763-resistant virus from passage 25 was no longer resistant to JM2763 after marker rescue. It missed some mutations that were probably necessary ($A_{297}T$ and $P_{385}L$). On the other hand, the marker rescue experiments confirmed that JM2763-resistant virus from passage 16 was not resistant to JM2763, even with two mutations present. Therefore, these amino acid changes are probably not important with regard to resistance. Nevertheless, they could be important when combined with mutations in other regions.

Site-directed mutagenesis. Some amino acids in V3 and one in V4 from wild-type virus were changed to their counterparts in mutant viruses. In addition, these amino acids were back-mutated to those of the wild type in an otherwise JM3100-resistant virus. Furthermore, the effects of the insertion of one amino acid in the V3 loop and the deletion of a pentapeptide in the V4 loop of gp120 were investigated. Finally, a possibly important combination of both A_{297} and P_{385} was evaluated. These two amino acids are situated in the proximity of disulfide bridges. According to Ryser et al. (14), the reduction of disulfide bridges by a membrane-bound protein disulfide isomerase might be the first and necessary step in a cascade of conformational changes in gp120 initiated after binding to $CD4^+$

TABLE 2. Analysis of sequences of partially bicyclam-resistant NL4-3 viruses

Strain	Passage no.[a]	Fold resistance to the following:		Mutations detected[b]												
		JM2763	JM3100	$N_{269}Y$	Insertion S	$R_{272}T$	$S_{274}R$	$Q_{278}H$	$I_{288}V$	$N_{293}H$	$A_{297}T$	DV4	$P_{385}L$	$Q_{410}E$	$S_{433}P$	$V_{457}I$
JM2763 resistant	16	2	3	0	0	0	+	+	0	0	0	0	0	0	0	0
	25	200	11	0	0	0	+	+	+	0	0	+	0	0	0	0
	28	200	6	0	0	0	+	+	+	0	+	+	+	0	0	0
JM3100 resistant	28	450	7	0	0	0	+	+	+	0	+	0	+	+	0	0
				0	0	0	+	+	+	0	+	+	+	+	+	0
	42	>740	60	0	+	+	+	+	+	+	+	+	+	+	+	+
	60	>740	200	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] One passage corresponds to 3 to 4 days.
[b] 0, not present; +, present; D, deletion of five amino acids (FNSTW) in the V4 loop.

TABLE 3. Analysis of mutations detected in partially resistant NL4-3 strains after marker rescue

Strain	Passage no.[a]	Fold resistance to the following:		Mutations detected[b]												
		JM2763	JM3100	$N_{269}Y$	Insertion S	$R_{272}T$	$S_{274}R$	$Q_{278}H$	$I_{288}V$	$N_{293}H$	$A_{297}T$	DV4	$P_{385}L$	$Q_{410}E$	$S_{433}P$	$V_{457}I$
JM2763 resistance	16	4	2	0	0	0	+	+	0	0	0	0	0	0	0	0
	25	4	8	0	0	0	+	+	+	0	0	+	0	0	0	0
	28	200	8	0	0	0	+	+	+	0	+	+	+	0	0	0
JM3100 resistant	60	>740	73	+	+	+	+	+	+	+	+	+	+	+	0	0

[a] One passage corresponds to 3 to 4 days.
[b] 0, not present; +, present; D, deletion of five amino acids (FNSTW) in the V4 loop.

cells. Mutation of these amino acids may result in a conformational change at the base of the loops, hindering the bracket formation of bicyclams with sulfide anions.

Table 4 presents the EC_{50}s obtained for the different mutants recovered via marker rescue. Because the degree of resistance could not be foreseen, no compound was added during growth of the recombinant virus. As discussed earlier, in some instances this approach resulted in the recovery of wild-type virus instead of the mutants for which we were aiming. Furthermore, recombination was sometimes not complete, resulting in the loss of the mutations of the V5 region. Apparently, substitution of only one amino acid did not influence the observed resistance to JM3100. Also, the combination of $A_{297}T$ and $P_{385}L$ did not lead to alteration of the sensitivity. This argues against the hypothesis that these amino acids would be of paramount importance for the inhibitory activity of JM3100 (see above). Unexpectedly, the reversion of T_{272} to R in the JM3100-resistant strain lowered the cross-resistance to JM2763. This observation remains to be explained, especially because the $R_{272}T$ change does not occur in JM2763-resistant virus. Equally puzzling is the increased susceptibility of the wild-type virus to JM2763 following the mutation of one amino acid or the deletion in V4. The slightly decreased EC_{50}s obtained for the JM3100-resistant virus carrying single amino acid changes could be due to the lack of the mutations in V5 (incomplete recombination). However, twofold differences in EC_{50}s are, in general, considered not significant.

Construction of chimeric viruses by exchange of the V3 region. The V3 loop of HIV gp120 has been reported to be very important in the fusion process. Assuming that the bicyclams are fusion inhibitors, the accumulation of mutations in the V3 region of bicyclam-resistant strains makes sense. The question is whether these mutations alone are sufficient to confer at least partial resistance to the bicyclams.

To this end, chimeric constructs were made by using an HIV-1 HXB2 background (pJJ25-pJJ5 plasmid vector system) in which the V3 region of wild-type or JM3100-resistant HIV-1 was inserted. MT-4 cells were transfected with the resulting chimeric viruses, and the sensitivity of the emerging virus was determined. No significant difference between the EC_{50} of HIV-1 HXB2 carrying wild-type V3 and that of HIV-1 HXB2 carrying JM3100-resistant V3 could be detected. The EC_{50}s of JM2763 and JM3100 for wild-type HIV-1 NL4-3 cloned in a wild-type HXB-2 background were 0.39 and 0.0045 μg/ml, respectively. Those for JM3100-resistant HIV-1 NL4-3 cloned in a wild-type HXB-2 background were 0.64 and 0.0068 μg/ml, respectively (1.6- and 1.5-fold resistance, respectively, compared to that for wild-type virus). Consequently, the mutations of V3 are not sufficient either to confer resistance to JM3100 or to allow for cross-resistance to JM2763.

Development of resistance to bicyclams by the clinical isolate HIV-1 HE. Development of resistance to JM2763 by clinical isolate HIV-1 HE occurred very rapidly. Even when starting at 100 μg of JM2763 per ml (200 times the EC_{50}), a full CPE was observed after three passages. Complete resistance (EC_{50}, >200 μg/ml) was obtained after four passages. In contrast, the addition of JM3100 at a concentration of 0.5 μg/ml did not result in a detectable CPE. After five passages, no virus was recovered from the supernatant, even when continuing the culture in the absence of compound. However, when starting with JM3100 at five times the EC_{50}, resistant virus (EC_{50}, >200 μg/ml) could be recovered after eight passages. At six passages, full cross-resistance to JM2763 was observed. The development of resistance to R86183 was included as a control. In this case, virus growing in the presence of R86183 at 20 μg/ml (2,000 times the EC_{50}) was recovered after five passages (Fig. 1).

Preliminary data on the sequence analysis of gp120 of wild-type and bicyclam-resistant HIV-1 HE. The marker rescue technique was applied to the wild-type and bicyclam-resistant HE strains in order to find out which part of the genome is responsible for the observed resistance. Unfortunately, the method failed to provide recombinant viruses. A major problem that was encountered consisted of the selection of resistant strains when growing the recombinant viruses under the pressure of exposure to the compound, even when starting with wild-type HE.

Since the marker rescue experiments carried out with HIV-1 NL4-3 pointed to the V2 to V5 region of gp120 as being important for the observed resistance, the V3 to V5 region of the resistant HE strains was analyzed. Preliminary experiments revealed the mutations summarized in Table 5. None of the mutations matched the amino acid substitutions detected in the resistant NL4-3 strains. Moreover, the mutations leading to JM2763 resistance were not detected in the JM3100-resistant strain.

When comparing the sequences of the wild types of HIV-1

TABLE 4. Determination of EC_{50}s for mutant viruses

Background	Mutation	EC_{50} (μg/ml)	
		JM2763	JM3100
Wild type	None	0.45	0.006
	$A_{297}T$	0.12	0.004
	$P_{385}L$	0.16	0.006
	DV4[a]	0.10	0.004
	Combination $A_{297}T$ + $P_{385}L$	0.28	0.002
JM3100 resistance	None	>200	0.230
	$T_{297}A$	>200	0.120
	$T_{272}R$	32.9	0.140
	$L_{385}P$	>200	0.110

[a] D, deletion of five amino acids (FNSTW) in the V4 loop.

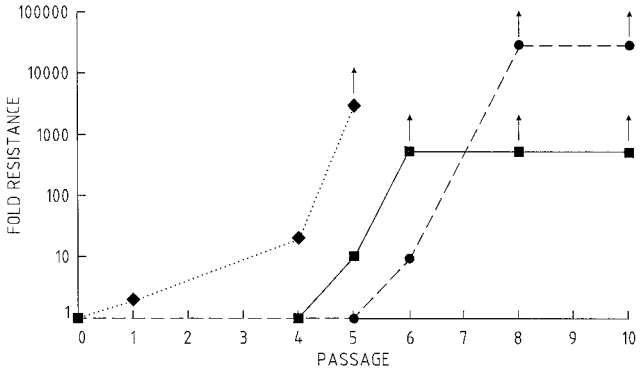

FIG. 1. Rate of development of resistance to the bicyclams JM2763 and JM3100 and to the TIBO compound R86183 by HIV-1 HE. At different passages EC_{50}s were determined and were compared with the wild-type EC_{50}. The ratio of EC_{50} (passage *n*) to EC_{50} (passage 0) is displayed as a function of passage *n*. The arrows indicate that the EC_{50} was greater than 250 μg/ml.

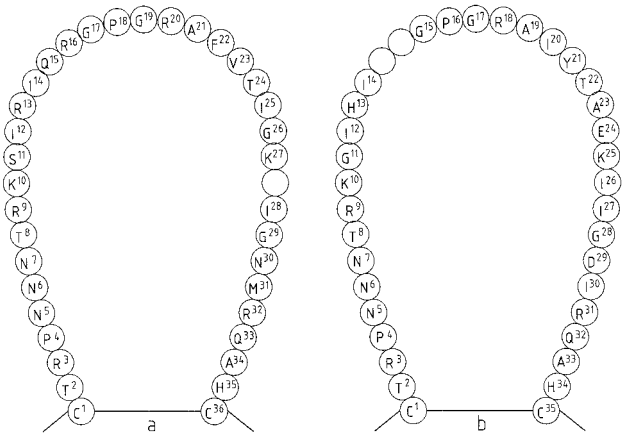

FIG. 2. V3 loops of wild-type HIV-1 NL4-3 (a) and HIV-1 HE (b).

NL4-3 and HE, a marked difference was noted. The V4 hydrophobic profile of wild-type HE is quite similar to that of the bicyclam-resistant NL4-3 strains, both of which lack the second hydrophobic peak. This could already give an indication for the explanation of the much faster development of resistance seen with HE. It is likely that the lack of a second hydrophobic domain in V4 leads to a different conformation which allows for a much easier escape from the inhibitory action of the bicyclams. Also, the V3 loops of wild-type NL4-3 and wild-type HE (Fig. 2) are quite distinct, which by itself may lead to a different conformation.

DISCUSSION

Experiments were carried out to investigate the importance of particular amino acid changes in the envelope gp120 glycoprotein with regard to the development of resistance to bicyclam derivatives.

From site-directed mutagenesis of selected amino acids likely to be involved in the development of resistance, it was not possible to deduce which amino acids were important. Mutagenesis studies with gp120 have mostly concentrated on the effect of amino acid substitutions in the tip of the V3 loop (GPGRAF) on tropism and viral entry. This region is not altered in bicyclam-resistant strains. However, Okada and Gurney (12) included R_{272} and N_{293} in one of their studies. They changed R_{272} to S and N_{293} to D to investigate the effect of these amino acids on the anti-HIV activities of polyanionic compounds like heparin and dextran sulfate. Because S and T both are uncharged polar amino acids, the R-to-S switch is comparable to the R_{272}T mutation observed in the JM3100-resistant strain. The N-to-D mutation (D is an acidic amino acid) is in contrast to the N_{293}H mutation (H is a basic amino acid) occurring in the bicyclam-resistant strains. Okada and Gurney (12) concluded that single basic amino acid substitutions at positions 272 and 293 in the V3 domain were not sufficient to alter the inhibitory activities of sulfated polysaccharides such as dextran sulfate and heparin. These observations are in agreement with our findings concerning the effects of substitutions of single amino acids on the sensitivity of HIV to bicyclams.

TABLE 5. Mutations in gp120 of bicyclam-resistant HIV-1 HE

Amino acid position (region)[a]	Wild-type HE		JM3100-resistant HE		JM2763-resistant HE	
	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid
283 (V3)	AGA	R	**G**GA	G	AGA	R
311 (C3)	AAG	K	AAG	K	A**G**G	R
407 (C4)	GUC	V	GUC	V	**A**UC	I
408 (C4)	ACA	T	ACA	T	A**AU**	N

[a] Numbering refers to the corresponding position in HIV-1 NL4-3; numbering of amino acids and domain classification is according to the NL4-3 sequence published in the GenBank database (accession no. M19921).

Cloning of the V3 loop of JM3100-resistant virus into a wild-type background did not change the phenotypic sensitivity to bicyclams. This means that the mutations clustered in the V3 loop do not suffice but that, instead, the whole set of amino acid changes scattered over the whole gp120 molecule seems to be necessary to confer reduced sensitivity to bicyclam derivatives. This points to the importance of the three-dimensional structure of gp120 and also supports the reported interactions between several domains of gp120 (9, 11, 18). Apparently, the changes brought about by the detected amino acid mutations alter the conformation of gp120 in such a way that gp120 at least partially escapes the inhibitory actions of the bicyclams.

Alternatively, some mutations may compensate others that would otherwise lower the infectivity of the virus. This phenomenon is known for protease inhibitors (15), and reports from Willey et al. (17) have indicated that a glycosylation site-deleting substitution in the C2 domain, which reduces HIV infectivity, could be functionally compensated for by a reversion substitution in the C1 domain. Moore and colleagues (11) explored the topologies of the gp120 glycoproteins of the mutant and revertant and found that the C2 substitution disrupted the CD4 binding site, which was restored to its wild-type function by the C1 compensatory substitution. Furthermore, the C2 change caused an abnormal exposure of a large segment of the C1 domain, and the segment was restored to its wild-type conformation by the reversion substitution in C1 (11). Likewise, some of the mutations observed in the bicyclam-resistant strains may not be involved in an interaction with gp120 but might simply be required to compensate for other amino acid changes that by themselves would disrupt the conformation necessary for productive infection.

The data obtained in the resistance experiments with HE provide further evidence that the conformation of gp120 is likely to be very important in the interaction with bicyclams. The development of resistance to bicyclams by the HE strain occurred very rapidly. This could be due to the presence of a

less sensitive or drug-resistant strain in the HIV-1 HE pool. This strain would then be selected during passage in the presence of the compound. In this respect, the development of resistance to JM3100 in peripheral blood lymphocytes starting from a clinical isolate also required less time (70 days to obtain 300-fold less sensitive virus) than that required for NL4-3 (8). The mutations detected in the resistant HE strains were different from those occurring in the resistant NL4-3 viruses. The amino acid change in the JM3100-resistant HE strain appeared to be located in the rather conserved tip of the V3 loop, changing GPGRAF to GPG**G**AF and thereby deleting a positive charge. This change has been linked to a switch from a syncytium-inducing strain to a less syncytium inducing or a non-syncytium-inducing strain. However, the phenotypic behavior of this JM3100-resistant strain pointed to the contrary: the induced CPE was stronger than that observed for the wild-type HE.

The mutations found in the JM2763-resistant HE strains were also different from those found in the JM3100-resistant HE strain. Because the charge of the amino acids was not changed by these mutations, the possibility that they contribute to resistance is rather low. However, the location of the two last amino acids mutated in the C4 region is very close to the Q-to-E change detected in JM3100-resistant NL4-3. The importance of these mutations remains to be elucidated. The interesting observation that the HE wild type possesses the same hydrophobic profile in the V4 region of gp120 as the bicyclam-resistant NL4-3 viruses might be related to the much faster development of resistance for the HE strain than for the NL4-3 strain. The sequence of gp120 of HE is quite different from that of NL4-3, probably resulting in a somewhat different three-dimensional conformation of the glycoprotein.

It thus appears that for the follow-up of bicyclam resistance in patients, the whole gp120 conformation will have to be taken into consideration. A model based on the three-dimensional structure of gp120 should be established to predict which amino acid changes lead to resistance. Unfortunately, our knowledge of the three-dimensional structure of gp120 is very limited, and it will not be easy to unravel it. Recently, a model of the isolated V3 loop was proposed (2), but it remains to be seen if studies on isolated regions of gp120 will reveal much about the fundamental processes in which this glycoprotein is involved, because the undoubtedly numerous intramolecular interactions between the different domains may be assumed to play an important role in the function of gp120.

After submission of the manuscript of this report, results obtained by Schols et al. (16) demonstrated that the bicyclams directly interact with fusin (also known as CXCR-4), a coreceptor for T-tropic viruses. M-tropic strains, which mainly use CCR5 as a coreceptor, are not inhibited by the bicyclams (16). Mutant T-tropic viruses that are selected for in the presence of drug can escape the inhibitory action of the compound. Due to the changes in the amino acid sequence of gp120 and the concomitant conformational changes in the three-dimensional structure of gp120, the bicyclam-resistant viruses might be forced to use another epitope of CXCR-4 or they may need less CXCR-4 to enter the cell. Proof for these possibilities is given by the fact that the observed resistance for the NL4-3 strain is never complete. HIV-1 HE, on the contrary, rapidly develops complete resistance to the bicyclams, suggesting that this virus may use another coreceptor besides CXCR-4 to enter the target cells (15a).

ACKNOWLEDGMENTS

We thank Anja Van Cauwenberge, Barbara Van Remoortel, Cindy Heens, Kristien Erven, Sonia Van Dooren, and Christel Van Laethem for excellent technical assistance. The special primer set used to amplify the V3 region and the plasmids pJJ25 and pJJ5 were kindly provided by J.-J. de Jong.

This work was supported in part by the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek, the Belgian Geconcerteerde Onderzoeksacties, and the Biomedical Research Programme of the European Commission.

REFERENCES

1. **Bridger, G. J., R. T. Skerlj, D. Thornton, S. Padmanabhan, S. A. Martelucci, G. W. Henson, M. J. Abrams, N. Yamamoto, K. De Vreese, R. Pauwels, and E. De Clercq.** 1995. Synthesis and structure-activity relationships 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.
2. **Catasti, P., J. D. Fontenot, E. M. Bradbury, and G. Gupta.** 1995. Local and global structural properties of the HIV-MN V3 loop. J. Biol. Chem. **270:**2224–2232.
3. **De Clercq, E., N. Yamamoto, R. Pauwels, M. Baba, D. Schols, H. Nakashima, J. Balzarini, Z. Debyser, A. A. Murrer, D. Schwartz, D. Thornton, G. Bridger, S. Fricker, G. Henson, M. Abrams, and D. Picker.** 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. USA **89:**5286–5290.
4. **De Clercq, E., N. Yamamoto, R. Pauwels, J. Balzarini, M. Witvrouw, K. De Vreese, Z. Debyser, B. Rosenwirth, P. Peichl, R. Datema, D. Thornton, R. Skerlj, F. Gaul, S. Padmanabhan, G. Bridger, G. Henson, and M. Abrams.** 1994. Highly potent and selective inhibition of human immunodeficiency virus by the bicyclam derivative JM3100. Antimicrob. Agents Chemother. **38:**668–674.
5. **de Jong, J. J., J. Goudsmit, W. Keulen, B. Klaver, W. Krone, M. Tersmette, and A. de Ronde.** 1992. Human immunodeficiency virus type 1 clones chimeric for the envelope V3 domain differ in syncytium formation and replication capacity. J. Virol. **66:**757–765.
6. **De Vreese, K., V. Kofler-Mongold, C. Leutgeb, V. Weber, K. Vermeire, S. Schacht, J. Anné, E. De Clercq, R. Datema, and G. Werner.** 1996. The molecular target of bicyclams, potent inhibitors of human immunodeficiency virus replication. J. Virol. **70:**689–696.
7. **De Vreese, K., D. Reymen, P. Griffin, A. Steinkasserer, G. Werner, G. Bridger, J. Esté, W. James, G. W. Henson, J. Desmyter, J. Anné, and E. De Clercq.** 1996. The bicyclams, a new class of potent human immunodeficiency virus inhibitors, block viral entry after binding. Antivir. Res. **29:**209–219.
8. **Esté, J. A., K. De Vreese, M. Witvrouw, J.-C. Schmit, A.-M. Vandamme, J. Anné, J. Desmyter, G. W. Henson, G. Bridger, and E. De Clercq.** 1996. Antiviral activity of the bicyclam derivative JM3100 against drug-resistant strains of human immunodeficiency virus type 1. Antivir. Res. **29:**297–307.
9. **Freed, E. O., and M. A. Martin.** 1994. Evidence for a functional interaction between the V1/V2 and C4 domains of human immunodeficiency virus type 1 envelope glycoprotein gp120. J. Virol. **68:**2503–2512.
10. **Harada, S., Y. Koyanagi, and N. Yamamoto.** 1985. Infection of HTLV-III/LAV in HTLV-1-carrying cells MT-2 and MT-4 and application in a plaque assay. Science **229:**563–566.
11. **Moore, J. P., R. L. Willey, G. K. Lewis, J. Robinson, and J. Sodroski.** 1994. Immunological evidence for interactions between the first, second, and fifth conserved domains of the gp120 surface glycoprotein of human immunodeficiency virus type 1. J. Virol. **68:**6836–6847.
12. **Okada, T., and M. E. Gurney.** 1995. Single basic amino acid substitutions at positions 302 or 320 in the V3 domain of HIV-1 type 1 are not sufficient to alter the antiviral activity of dextran sulfate and heparin. AIDS Res. Hum. Retroviruses **11:**571–575.
13. **Pauwels, R., J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyter, and E. De Clercq.** 1988. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods **20:**309–321.
14. **Ryser, H., E. M. Levy, R. Mandel, and G. J. DiSciullo.** 1994. Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. Proc. Natl. Acad. Sci. USA **91:**4559–4563.
15. **Schinazi, R. F., B. A. Larder, and J. W. Mellors.** 1996. Mutations in retroviral genes associated with drug resistance. Int. Antivir. News **4:**95–107.

15a. **Schols, D.** Personal communication.

16. **Schols, D., J. A. Esté, G. Henson, and E. De Clercq.** 1997. Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. Antivir. Res. **35:**147–156.
17. **Willey, R. L., E. K. Ross, A. J. Buckler-White, T. S. Theodore, and M. A. Martin.** 1989. Functional interaction of constant and variable domains of human immunodeficiency virus type 1 gp120. J. Virol. **63:**3595–3600.
18. **Wyatt, R., M. Thali, S. Tilley, A. Pinter, M. Posner, D. Ho, J. Robinson, and J. Sodroski.** 1992. Relationship of the human immunodeficiency virus type 1 gp120 third variable loop to a component of the CD4 binding site in the fourth conserved region. J. Virol. **66:**6997–7004.