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The Molecular Target of Bicyclams, Potent Inhibitors of Human Immunodeficiency Virus Replication

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Bicyclams are a novel class of antiviral compounds which act as potent and selective inhibitors of the replication of human immunodeficiency virus type 1 (HIV-1) and HIV-2. They block an early step in the viral life cycle following adsorption to the CD4 receptor and preceding reverse transcription. To identify the molecular target of these compounds, we genetically analyzed variants of the HIV-1 molecular clone NL4-3, which developed resistance against two structurally related bicyclams, JM2763 and the more potent SID791. The resistant strains were obtained after long-term passaging in MT-4 cells in the presence of progressively increasing compound concentrations. Recombinants between selected genes of the resistant strains and the parental NL4-3 provirus were generated by adapting the marker rescue technique to MT-4 cells. The bicyclam-resistant phenotype was rescued by transferring the envelope gp120 gene of bicyclam-resistant virus into the NL4-3 parental genetic background. In the gp120 genes of the resistant strains, we identified several mutations leading to amino acid substitutions in the V3 loop. Furthermore, two substitutions of highly conserved amino acids in close proximity to the disulfide bridges of the V3 and V4 loops were found in both SID791- and JM2763-resistant strains. Additional mutations in regions encoding V3, C4, V5, and C5 were present in SID791-resistant viruses. Recombination experiments with overlapping parts of the envelope gene indicated that most, if not all, of the mutations were necessary to develop the fully SID791 resistant phenotype. The mutations in the C-terminal part of gp120 downstream of the V3 loop sequence conferred partial resistance to JM2763 but did not significantly decrease susceptibility to SID791. The genetic data and the biological properties of the resistant viruses point to inhibition of entry and fusion as the mode of action of the HIV-inhibitory bicyclams. A possible mechanism of binding of bicyclams to gp120 leading to inhibition of unfolding of gp120 and its shedding from the gp41 fusion domain is discussed.

Antiviral chemotherapy of human immunodeficiency virus (HIV) disease focuses primarily on inhibitors of the viral reverse transcriptase and protease. Clinical studies with these compounds showed that they are not able to permanently suppress the chronic virus replication and progressive $CD4^+$ T-cell depletion. Eventually, drug-resistant variants arise and rapidly fill up the pool of replicating virus, although immediately after initiation of therapy, a transient reduction of virus load by up to 99% can be obtained (21, 38). Concomitant with this temporal reduction in virus load, a transient increase in $CD4^+$ T-cell counts in blood is observed. To replenish the constantly shrinking pool of $CD4^+$ T cells, it will be necessary to keep the virus replication at low levels for a prolonged period of time (13). Most probably, this can be achieved only by treatment with a combination of antiviral drugs (20). These should be directed against different viral targets in order to avoid a rapid selection of resistant strains.

Recently, a new class of selective and potent inhibitors of HIV type 1 (HIV-1) and HIV-2 replication, the bicyclams, has been described (14, 15). These compounds inhibit an early step in the viral replication cycle preceding reverse transcription of the viral genome. Studies on infection of cells with virus particles harboring metabolically labeled, radioactive viral genomes showed that in the presence of bicyclams, the genomic RNA was protected from degradation by RNase A when tested at a time point in the infection cycle at which the genomes of viruses in untreated cultures were accessible to RNase. Therefore, inhibition of fusion and/or uncoating was proposed as a potential mode of action of these compounds (14). In fact, SID791, the most potent representative of the series of bicyclams, also inhibits envelope-mediated fusion to $CD4^+$ T-cell lines, although at concentrations considerably higher than those necessary to block de novo infections (15). This finding indicated that the viral envelope may serve as a target molecule. This mechanism would offer new points of attack against the massive turnover of viruses in vivo.

A critical parameter in determining the clinical efficacies of antiviral drugs is the rate of resistance development. This can be estimated from experiments in vitro in which the virus is passaged in the presence of increasing concentrations of the compound being tested, whereby a starting concentration is chosen that allows virus replication to occur at a low level (3, 22, 23, 25, 29). We have isolated resistant variants of the HIV-1 proviral clone NL4-3 after long-term passaging in the presence of the bicyclam JM2763 or SID791. The biological phenotypes of these bicyclams have been described by De Vreese et al. (16).

To identify the genes responsible for resistance, marker rescue experiments with the parental proviral genome were performed. The fully resistant phenotype of $SID791^r$ viruses is characterized by a 200- to 400-fold increase in the EC_{50}s (defined as the compound concentrations required to protect 50% of the virus-infected cells against viral cytopathicity) for both

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TABLE 1. Primers used to amplify genes of resistant NL4-3 virus strains

Rescued genes	Sequence (nt)[a]	
	5′ primer (recombination site)	3′ primer (recombination site)
psi, *gag*	558–588 (*Bss*HII)	2398–2370 (*Apa*I)
vpr, *vpu*, *gp120*	5520–5541 (*Sal*I)	7772–7748 (*Bsa*BI)
vpr, *vpu*, *gp120-V1V2*	5520–5541 (*Sal*I)	6977–6954 (*Stu*I)
gp120-V2V5	6588–6613 (*Stu*I)	7728–7703 (*Bsa*BI)
gp120-V2-gp41	6588–6613 (*Stu*I)	8812–8788 (*Bam*HI)
gp120-V4-gp41	7374–7399 (*Bsa*BI)	8812–8788 (*Bam*HI) or 9092–9045 (*Xho*I)

[a] Numbering of nucleotides (nt) is according to the NL4-3 sequence stored in the GenBank database, accession number M19921.

SID791 and JM2763 relative to the NL4-3 wild type (wt). The JM2763^r strain shows a similar increased resistance to JM2763 but retains sensitivity to SID791. Of both bicyclam-resistant strains, full resistance was rescued by transferring the gp120 *env* gene into the parental NL4-3 wt genetic background. The *gag* gene products are also involved in the early steps of infection, but the *gag* genes of the resistant strains did not contribute to resistance, as the corresponding recombinants were as sensitive to bicyclams as NL4-3 wt.

Sequencing of the gp120 genes of both JM2763^r and SID791^r viruses identified several mutations in the coding region of the V3 loop and, in addition, single mutations in V2, V4, V5, C4, and C5. Strikingly, two amino acids, which are in close proximity to the disulfide bridges of V3 and V4 and are highly conserved among HIV-1 isolates, appear to be mutated in both bicyclam-resistant strains. A model for the inhibitory action of bicyclams is discussed.

MATERIALS AND METHODS

Compounds. The bicyclams JM2763 (1,1′-propylene-bis-1,4,8,11-tetraazacyclotatradecane octa-hydrochloride tetrahydrate) and SID791 [or JM3100; 1,1′-(1,4-phenylenebis-(methylene))-bis-1,4,8,11-tetraazacyclotetradecaneoctahydrochloridedihydrate] were synthesized by Johnson Matthey as described previously (6), and their antiviral activities were reported previously (14, 15).

Cell culture and generation of resistant variants. The HIV-1 molecular clone NL4-3 was obtained from the National Institute of Allergy and Infectious Diseases AIDS reagent program. To prepare a virus stock, CEM T cells were transfected with NL4-3 proviral DNA. To select bicyclam-resistant virus variants, MT-4 cells were infected at a multiplicity of infection of 0.01, corresponding to 100 times the 50% cell culture infective dose, and at a starting concentration of 0.08 μg of SID791 or 0.4 μg of JM2763 per ml. Every 3 to 4 days, supernatants were transferred to fresh cultures, and the concentration of bicyclam was raised two- to threefold when massive syncytia were detected microscopically. SID791^r strains developed after 63 passages in MT-4 cells at a final compound concentration of 500 μg/ml, and JM2763^r strains when isolated after 25 passages also at a compound concentration of 500 μg/ml.

Antiviral activity assays. The antiviral activities of bicyclams were monitored by the tetrazolium-based 3-4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide (MTT) procedure in microtiter plates as previously described (31). All EC_{50} determinations were performed in MT-4 cells at a multiplicity of infection of 0.01, which in the absence of inhibitor led to complete cytopathic effect (CPE) after 5 days in culture. To quantify the drug sensitivities of the resistant virus strains, one passage in MT-4 cells in the absence of compound was performed to obtain a compound-free virus stock.

Marker rescue experiments. Different parts of the proviral genome of bicyclam-resistant NL4-3 strains were isolated by PCR amplification of total cellular DNA, purified from infected MT-4 cells at day 4 after infection. Preparative PCR was performed with 15 μg of total cellular DNA, purified by the Trizol reagent protocol (Gibco BRL, Gaithersburg, Md.), and 5 μg of each primer in a total volume of 500 μl according to a standard protocol (Perkin-Elmer, Norwalk, Conn.). The PCR primers were selected to generate DNA fragments with overhangs of 100 to 400 bp at single restriction sites in the NL4-3 genome. The sequences of the PCR primers are summarized in Table 1, and the corresponding genome fragments are depicted in Fig. 1. NL4-3 wt proviral DNA was cleaved with single-cutter restriction enzymes to excise the genome regions for replacement by mutant DNA through homologous recombination. Restriction enzyme-digested NL4-3 wt DNA, precipitated by ethanol, together with PCR DNA, purified by the Wizard PCR Preps system (Promega, Madison, Wis.), was used to transfect MT-4 T cells by electroporation. All manipulations with cells were performed at room temperature. Preincubations of cells with DNA on ice were found to drastically reduce the transfection rate. Briefly, 10 to 15 μg of NL4-3 wt DNA and 5 to 8 μg of purified PCR DNA (about fivefold molar excess) in a total volume of 10 to 20 μl were mixed in a 1-ml electroporation cuvette with 10^7 cells suspended in 200 μl of prewarmed (37°C) culture medium (RPMI 1640 supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine) and electropulsed (settings, 0.25 kV and 960 μF). Immediately thereafter, the cells were resuspended in 15 ml of prewarmed fresh medium supplemented with 5 ml of old culture medium. At 48 h after transfection, cultures were split in two portions, and 20 μg of SID791 or

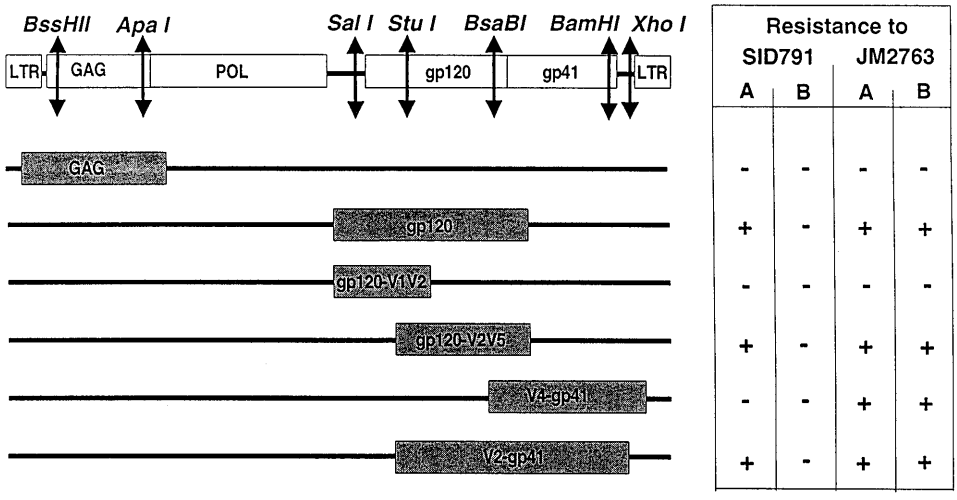

FIG. 1. Schematic representation of recombinant genomes generated by marker rescue and the resistance phenotypes of the corresponding recombinant viruses. The genome organization of HIV-1 NL4-3 with the single restriction sites used for marker rescue is shown on the left. The various genome segments which have been amplified from proviral DNA of bicyclam-resistant strains to replace the corresponding piece in the NL4-3 wt genome by homologous recombination are illustrated below. The resistance phenotypes of the corresponding recombinant viruses, generated either from the SID791^r—(columns A) or JM2763^r—(columns B) strain, is indicated at the right. It was determined by judging virus growth (+) microscopically as indicated by formation of syncytia and CPE in transfected cultures supplemented with 20 μg of either SID791 or JM2763 per ml. The resistance phenotype of recombinant virus in supernatants of transfected substance-free cultures was confirmed in de novo infections performed in the presence of similar bicyclam concentrations. LTR, long terminal repeat.

TABLE 2. Inhibition of NL4-3 strains and recombinants by bicyclams and dextran sulfate

NL4-3 strain[a] (passage no. in MT-4 cells) or recombinant virus[b] (recombination sites)	EC_{50} (μg/ml)[c] for inhibition by:		
	SID791	JM2763	Dextran sulfate
NL4-3			
wt (63)	0.007	0.56	0.54
SID791^r (63)	2.22	>250	>250
JM2763^r (34)	0.097	245.0	4.06
Recombinant virus			
SID791^r-gp120 (*Sal*I-*Bsa*BI)	1.09	>250	>250
JM2763^r-gp120 (*Sal*I-*Bsa*BI)	0.032	66.8	13.7
NL4-3 wt-gp120 (*Sal*I-*Bsa*BI)	0.005	0.35	1.24

[a] The bicyclam-resistant NL4-3 virus strains were of passage 63 (SID791^r virus) or 34 (JM2763^r virus) cultured in MT-4 cells in the presence of 500 μg of the corresponding bicyclam per ml. NL4-3 wt was passaged 63 times in MT-4 cells in the absence of compound.

[b] Recombinant viruses, obtained from supernatants of cells transfected as described for the marker rescue procedure, were used to prepare a virus stock in fresh MT-4 cells, cultured in compound-free medium. This virus stock was used for determination of the 50% cell culture infective doses and EC_{50}s in MT-4 cells.

[c] Determined by the MTT procedure as described in Materials and Methods.

JM2763 per ml was added to one of them. Cells were inspected daily for the appearance of syncytia and cell death. PCR fragments of the *env* gene were cloned in the pCR-Script SK(+) cloning vector (Stratagene, La Jolla, Calif.) for sequencing and further marker rescue experiments. DNA sequencing was performed by the enzymatic chain termination method, using Sequenase 2.0 (United States Biochemicals, Cleveland, Ohio). In marker rescue experiments with cloned DNA, excised from the cloning vector at polylinker restriction sites, 20 μg of *env* fragment DNA and 8 to 10 μg of restriction enzyme-digested NL4-3 wt DNA were used.

RESULTS

Generation of bicyclam-resistant HIV-1 variants. To rapidly and unambiguously identify genes and mutations associated with the development of resistance against the HIV-inhibitory bicyclams SID791 and JM2763, an HIV-1 molecular clone, NL4-3 (1), was used to generate resistant virus variants. A virus stock was prepared by transfecting CEM T cells with proviral DNA, and this virus was propagated in MT-4 cells. Virus replication was monitored microscopically by the appearance of syncytia and CPE and quantified by the MTT staining procedure as described previously (31). To allow the virus to replicate in the presence of bicyclam, a starting concentration of bicyclams which corresponds approximately to the fivefold EC_{50} was chosen. In fact, at concentrations 10-fold higher than the EC_{50}s, no virus breakthrough was observed. The replicating virus was passaged every 3 to 4 days by transferring an aliquot of the culture supernatant onto fresh cells. When a pronounced CPE was observed, the bicyclam concentration was raised two- to threefold. After 63 passages in the presence of SID791 or 25 passages in the presence of JM2763, virus variants that replicated in MT-4 cells at an actual bicyclam concentration of 500 μg/ml were obtained. The SID791^r strain was also resistant to JM2763, whereas the JM2763^r strain did not grow in the presence of 20 μg of SID791 per ml. These two strains were considered to represent the fully resistant phenotype. The EC_{50}s for these resistant strains were about 200- to 400-fold higher than those for the parental NL4-3 wt virus, passaged only once, or a strain passaged in the absence of compound in parallel to the selection of resistant strains (Table 2).

Marker rescue experiments. The marker rescue technique is a well-established method in genetic analyses of many DNA viruses (5, 28, 37, 40) to identify genes associated with a certain phenotype. The technique was modified to be useful in the analysis of the bicyclam-resistant HIV-1 NL4-3 variants generated in MT-4 cells. Different genes of the resistant strains were amplified by PCR using total cellular DNA isolated from infected MT-4 cells. The primer sequences used and their locations in the NL4-3 proviral genome are summarized in Table 1. Genome fragments of resistant mutants together with parental NL4-3 wt DNA, inactivated at the corresponding gene locations by restriction enzyme digestion, were used to transfect MT-4 cells by electroporation. To facilitate the formation of recombinants by homologous recombination, overlaps varying between 100 and 400 bp in length upstream and downstream of restriction sites were included in the PCR products. Usually, within 6 to 8 days after transfection, replication of recombinant virus was detectable by the appearance of syncytia and CPE. The bicyclam sensitivity of each of the recombinants was determined in the same experiment by splitting the cultures at day 2 after transfection and adding 20 μg of SID791 or JM2763 per ml to one half of the cultures. Growth of the recombinants at this bicyclam concentration is shown in Fig. 1. Quantification of drug sensitivity by EC_{50}s was performed with virus stocks prepared from supernatants of the transfected cultures showing complete CPE. In all recombination experiments, genome fragments of the MT-4 culture-adapted NL4-3 wt strain (passage 63), which had remained fully sensitive to bicyclams, were included as a control. In some control experiments in which only the restriction enzyme-digested proviral NL4-3 wt DNA was transfected at similar total DNA concentrations, virus replication was detectable, but not earlier than 2 to 3 weeks after transfection. Presumably, this virus originated from trace amounts of uncleaved proviral DNA present in the preparation. The various recombinants generated from PCR fragments are illustrated in Fig. 1, and their corresponding EC_{50}s are listed in Tables 2 and 4.

Identification of gp120^{env} as a target associated with drug resistance. In two independent marker rescue experiments, we generated recombinant viruses in which the entire *gag* gene and the upstream packaging signal sequence psi of the NL4-3 parental proviral genome were replaced by corresponding sequences of the bicyclam-resistant strains. No virus replication was observed in transfected cultures containing bicyclam-supplemented media. Determination of the drug sensitivities of

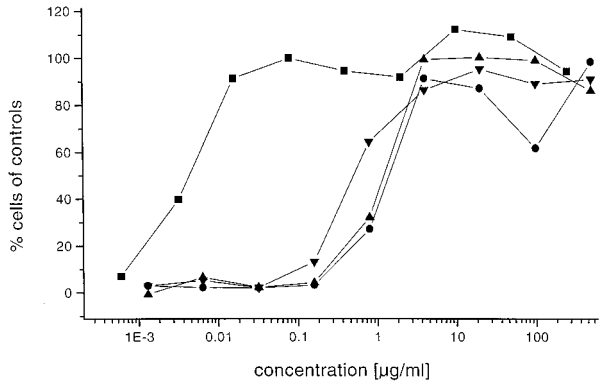

FIG. 2. Inhibition of NL4-3 strains and recombinant viruses by SID791. Dose-dependent inhibition of virus replication of NL4-3 wt (■), the SID791^r strain (●), and the recombinant viruses SID791^r-gp120 (▲) and SID791^r-gp120-V2V5 (▼) in MT-4 cells by SID791 is illustrated by the percentage of viable MT-4 cells at a given compound concentration (infective virus dose of 100 50% cell culture infective doses) as determined in MTT assays. The values represent the optical densities at 450 nm of untreated and uninfected control cultures, which are taken as 100%.

TABLE 3. Mutations in gp120 of bicyclam-resistant NL4-3 strains

Amino acid position[a] (region)	NL-43 wt (proviral DNA)		SID791^r strain		JM2763^r strain	
	Codon[b]	Amino acid[c]	Codon	Amino acid	Codon	Amino acid
145 (V2)	UUC	F	UU**A**	L (4)	UUC	F
268 (V3)	**U**AC (AAC)	Y (N)	AAC, **UAC**	N,Y(ins)	AAC	N
269 (V3)	AA**A** (AAC)	K (N)	AAC	N	AAC	N
270 (V3)	AAU	N	A**GU**	S	AAU	N
272 (V3)	AGA	R	A**C**A	T	AGA	R
274 (V3)	AGU	S	AG**A**	R	AG**A**	R
278 (V3)	CAG	Q	CA**C**	H	CA**U**	H
288 (V3)	AUA	I	**G**UA	V	**G**UA	V
293 (V3)	AAU	N	**C**AU	H	AAU	N
297 (V3)	GCA	A	**A**CA	T	**A**CA	T
385 (V4)	CCA	P (2)	C**U**A	L (8)	C**U**A	L (4)
410 (C4)	CAA	Q (2)	**G**AA	E (9)	CAA	Q (4)
433 (V5)	UCC	S (2)	**C**CC	P (10)	UCC	S (4)
457 (C5)	GUA	V (2)	**A**UA	I (10)	GUA	V (4)

[a] Numbering of amino acids and domain classification are according to the published secondary structure of the mature HIV-1 (human T-cell leukemia virus IIIb strain) gp120 protein (9).

[b] Nucleotides which are mutated in comparison with the NL4-3 sequence published in GenBank database accession number M19921 are in boldface.

[c] Unless otherwise indicated (numbers in parentheses), the following numbers of clones have been sequenced and were identical at the positions shown: 4 of NL4-3 wt (passage 63), 16 (two independent PCR amplifications) of the SID791^r strain (passage 63), and 6 of the JM2763^r strain (passage 34). Both bicyclam-resistant strains, but not NL4-3 wt, carry an in-frame deletion in the N-terminal V4 loop sequence comprising the amino acids FNSTW (364 to 368), which are encoded by the direct repeat UUUAAUAGUACUUGG.

the recombinant viruses which replicated in substance-free transfected cultures, SID791^r-*gag* and JM2763^r-*gag*, revealed EC_{50}s for both bicyclams identical to that of NL4-3 wt (Table 2). Sequencing of the genome segment transferred into the NL4-3 wt genetic background confirmed the evidence of the biological experiments that this part of the genome does not contribute to the development of resistance. That is, either silent mutations or a few leading to conservative amino acid changes were identified (data not shown).

On the contrary, in recombination experiments with genome segments including *vpr*, *vpu*, and gp120-encoding *env* of bicyclam-resistant strains, the fully resistant phenotype of the parental strains was rescued (Table 2). Specifically, the recombinant viruses SID791^r-gp120 and JM2763^r-gp120, in which the *Sal*I-*Bsa*BI region of the NL4-3 wt proviral genome (Fig. 1) was replaced by the corresponding part of resistant viruses, showed EC_{50}s for the bicyclams very similar to that of the parental resistant virus strains. This is further illustrated by the titration curves shown in Fig. 2. Moreover, the gp120 recombinants and the parental bicyclam-resistant strains showed similar cross-resistances to dextran sulfate (Table 2). The JM2763^r-gp120 recombinants were resistant to JM2763 concentrations 200- to 300-fold higher than the EC_{50}s for NL4-3 wt but remained sensitive to SID791, thus behaving exactly like the parental JM2763^r virus strain.

Mutations identified in *env* genes of bicyclam-resistant virus strains. PCR fragments used in the *Sal*I-*Bsa*BI marker rescue experiments were cloned, and several clones were sequenced. No differences in the *vpr* and *vpu* genes between resistant and wild-type viruses were identified. However, many mutations were found in the gp120 gene sequence of the bicyclam-resistant strains, predominantly in the region encoding the V3 loop, but also in V2, V4, and V5 as well as C4 and C5. The gp120 sequences of the passaged NL4-3 wt strain differed at only two positions, in the V3 loop (N268Y and N269K), from the original proviral sequence. Mutations identical in all clones of the bicyclam-resistant gp120 genes and the corresponding amino acid substitutions are listed in Table 3. All amino acid substitutions found in the gp120 sequence of JM2763-resistant viruses are also present in the SID791^r NL4-3 strain. Seven additional mutations were exclusively found in the gp120 sequence of the SID791^r strain, including a tyrosine codon insertion after amino acid 268 in the V3 loop. None of these mutations were introduced by PCR, as they were consistently found in all clones of two independent PCR amplifications. Additional mutations, which were uniquely found in one or another clone, led to conservative amino acid substitutions. They are not shown, as they probably do not contribute to the resistance phenotype. This was indicated by recombination experiments with individual, cloned fragments leading to recombinant viruses with phenotypes identical to those of the parental strains or the recombinants generated from the original total PCR DNA. All bicyclam-resistant viruses displayed a deletion spanning the codons for amino acids 364 to 368. This sequence, encoding FNSTW, is part of a tandem direct repeat in the N-terminus of the V4 loop of NL4-3.

Contributions of different mutations to the resistant phenotype. To determine which mutations are important in the development of the resistant phenotype, marker rescue experi-

TABLE 4. Inhibition of *env* recombinant viruses by bicyclams[a]

Recombinant virus (recombination sites)	EC_{50} (μg/ml)	
	SID791	JM2763
SID791^r-gp120-V1V2 (*Sal*I-*Stu*I)	0.002	0.163
JM2763^r-gp120-V1V2 (*Sal*I-*Stu*I)	0.002	0.14
NL4-3 wt-gp120-V1V2 (*Sal*I-*Stu*I)	0.003	0.16
SID791^r-gp120-V2V5 (*Stu*I-*Bsa*BI)	0.23	>250
JM2763^r-gp120-V2V5 (*Stu*I-*Bsa*BI)	0.061	42.8
NL4-3 wt-gp120-V2V5 (*Stu*I-*Bsa*BI)	0.006	0.45
SID791^r-gp120-V4gp41 (*Bsa*BI-*Xho*I)	0.014	0.52
JM2763^r-gp120-V4gp41 (*Bsa*BI-*Xho*I)	0.009	0.35
NL4-3 wt-gp120-V4gp41 (*Bsa*BI-*Xho*I)	0.006	0.36
SID791^r-gp120-V2gp41 (*Stu*I-*Bam*HI)	0.60	>170
JM2763^r-gp120-V2gp41 (*Stu*I-*Bam*HI)	0.074	36.8
NL4-3 wt-gp120-V2gp41 (*Stu*I-*Bam*HI)	0.006	0.36

[a] Recombinant viruses harboring genome segments of bicyclam-resistant strains as illustrated in Fig. 1 were analyzed for drug sensitivity as described in the footnotes to Table 2.

ments with various overlapping parts of the *env* gene were performed. As shown in Table 4, transfer of the V1 and V2 loop sequences of the bicyclam-resistant strains in the NL4-3 wt proviral genome did not alter the drug sensitivity of the resulting recombinant viruses, SID791^r-gp120-V1V2 and JM2763^r-gp120-V1V2. The gp120-V4gp41 recombinant viruses, which harbor sequences of the resistant strains including the V4 loop and downstream parts of *env* up to the *Bam*HI site in the gp41 gene or to the *Xho*I site in the *nef* gene, did not replicate at a 10-fold EC_{50} of SID791 but grew at 20 μg of JM2763 per ml. The JM2763^r-gp120-V4gp41 recombinant viruses replicated in JM2763-supplemented cultures with delay of 1 to 2 days in comparison with the corresponding SID791^r recombinants. Nevertheless, all of these recombinant viruses displayed EC_{50}s that were not significantly different from those of NL4-3 wt. The gp120-V2gp41 recombinant viruses containing the *env* sequences of the resistant strains downstream of *Stu*I showed EC_{50}s for both bicyclams similar to those of the parental resistant virus strains.

SID791^r-gp120-V2V5 recombinants generated at the *Stu*I-*Bsa*BI sites produced viruses which were repeatedly found to have EC_{50}s somewhat lower (two- to threefold) than those of the SID791^r-gp120 recombinant viruses (Fig. 2). Because the full resistant phenotype was rescued by the gp120 recombinants (*Sal*I-*Bsa*BI) as well as by the gp120-V2gp41 recombinants, it is evident that only mutations of the genome segment identical in both experiments, i.e., sequences between nucleotides 6588 and 7748, can contribute to resistance. The difference in sensitivity to SID791 between the fully resistant gp120 recombinants and the less resistant gp120-V2V5 recombinants, which both had been generated from PCR fragments carrying all of the relevant gp120 mutations (Table 3), could then be based on differences in the genome parts of the overlap regions transferred to the wt genome during homologous recombination. Therefore, the actual sequences of recombinant viruses were analyzed by cloning reverse transcription-coupled PCR fragments of the overlap regions. It was found that all five clones of the SID791^r-gp120 recombinant viruses (*Sal*I-*Bsa*BI) contained the V457I mutation, whereas the population of gp120-V2V5 recombinant viruses was heterogeneous at this position, with 40% (two of five) being wt. Both additional upstream mutations at this 3′ recombination site, i.e., Q410E and S433P, were present in all clones sequenced. On the other hand, all clones of the SID795^r-gp120-V2V5 recombinant viruses displayed the F145L mutation in the V2 loop sequence, which is the only mutation located at the 5′ recombination site. Hence, the rather conservative amino acid substitution V457I in C5 seems also to contribute to the SID791^r phenotype.

DISCUSSION

The bicyclams are a novel class of potent antiviral compounds which selectively inhibit the replication of HIV-1 and HIV-2 (6, 14). So-called time-of-addition experiments (15) had indicated that they block an early step in the viral replication cycle. Binding studies and PCR analyses confined the inhibitory effect on an event following adsorption but preceding reverse transcription (16), i.e., viral entry or fusion. Inhibition of entry or fusion was implicated by the finding that SID791 blocks envelope-mediated syncytium formation, albeit at concentrations considerably higher than that needed to inhibit de novo virus infection (15). The fact that [^{3}H]uridine-labeled RNA of virions used to infect cell cultures in the presence of SID791 or JM2763 was found to be protected from RNase A degradation shortly after adsorption supported inhibition of the fusion/uncoating process as a potential mode of action and the envelope or capsid proteins as the possible target proteins (15). Our studies described here point to the envelope gp120 glycoprotein as the major target molecule for this class of compounds.

Development of bicyclam resistance of NL4-3 requires long-term passaging. Using the HIV-1 molecular clone NL4-3 to infect MT-4 cells, we found that resistant variants arose only after prolonged passaging in the presence of gradually increasing compound concentrations. Development of full resistance against SID791 compared with JM2763 required much longer passaging. The JM2763^r viruses were still sensitive to inhibition by SID791. That the JM2763^r strain is selected much faster than the SID791^r virus population in this system may simply be attributed to the much weaker antiviral efficacy of JM2763 (EC_{50} = 0.5 μg/ml) than of SID791 (EC_{50} = 0.005 μg/ml). The lower antiviral potency of JM2763 may be the result of a lower binding affinity for the target protein. Escape from inhibition by mutating the binding site may, therefore, be easier for the virus. More detailed analyses of the biological phenotypes of these resistant strains are reported elsewhere (16).

The *gag* genes do not contribute to resistance. The nucleocapsid protein nc-p7 was considered to be a plausible target for bicyclams. Because of their high chelating potential, bicyclams could be hypothesized to bind or extract the zinc ions of the two zinc fingers in nc-p7 (18, 32, 35). Evidence supporting nc-p7 as a target was obtained from in vitro binding experiments with psi RNA and recombinant nc-p7 protein (unpublished data). However, we repeatedly isolated recombinant viruses harboring *psi* and *gag* of resistant strains that were indistinguishable from the wt strain in their susceptibility to inhibition by bicyclams. Therefore, we conclude that Gag proteins of infectious virus particles do not serve as a target for the antiviral action of bicyclams.

Resistance of NL4-3 to bicyclams is based exclusively on mutations in gp120. The bicyclam-resistant phenotype of the NL4-3 variants was rescued entirely by recombinants in which the gp120 sequences of the envelope genes were exchanged. The SID791^r-gp120 and JM2763^r-gp120 recombinant viruses showed EC_{50}s similar to those of the corresponding resistant NL4-3 strains (Table 2). Obviously, the gp41 transmembrane protein is not involved in the development of bicyclam resistance, nor are the N-terminal domains of gp120, including V1, V2, and C2, as indicated by the phenotypes of the gp120-V1V2 and the gp120-V2V5 recombinants (Fig. 1 and Table 4). In addition, the biological and sequencing data obtained with these recombinants exclude a role of *vpr* and *vpu* in bicyclam resistance.

The recombinants SID791^r-V4gp41 and JM2763^r-V4gp41 harbor the V4 loop and downstream sequences including gp41. In two independent experiments, in which recombinations at the *Bsa*BI site and either the *Bam*HI or the *Xho*I site were forced, the recombinants did not grow in the presence of 10-fold EC_{50}s of SID791. Further, in the MTT assay, we obtained EC_{50}s for both bicyclams similar to those found with NL4-3 wt (Table 4). These recombinants, however, replicated in transfected cultures and after de novo infection of MT-4 cells with undiluted supernatants of transfected cultures in the presence of 20 μg of JM2763 per ml, as detected by syncytium formation and appearance of CPE (Fig. 1). The JM2763^r-V4gp41 recombinant viruses induced a complete CPE with a delay of 1 to 2 days compared with the corresponding SID791^r recombinants. This apparent discrepancy with the EC_{50} determinations may be explained by the different experimental settings. The EC_{50} determinations are performed at a rather low multiplicity of infection. In the transfected cultures, however, virus particles gradually accumulate in supernatants and may have a long

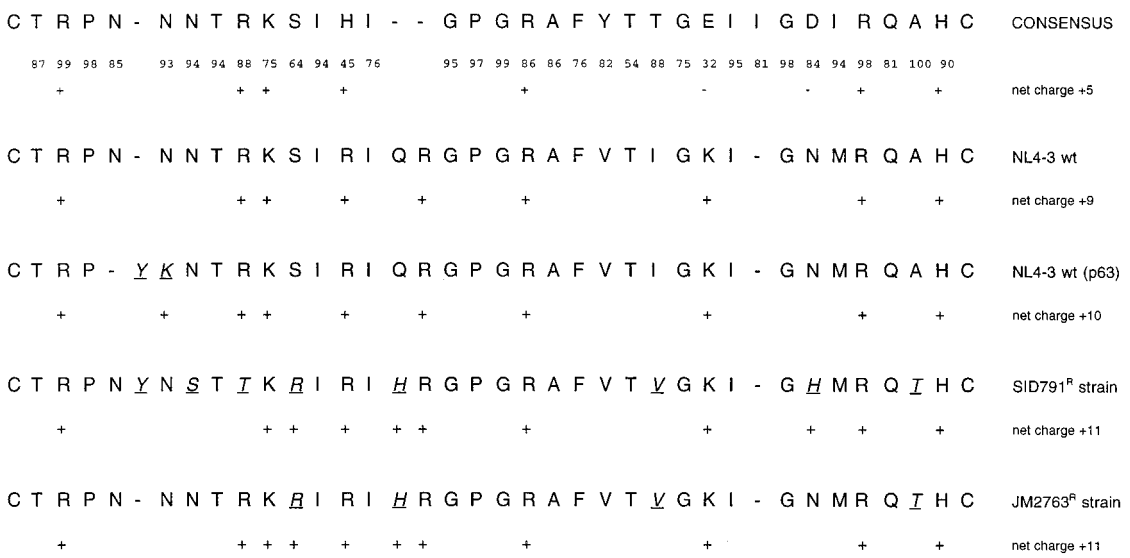

FIG. 3. Amino acid substitutions in the V3 loops of bicyclam-resistant NL4-3 strains and resulting changes in net charge in comparison with the consensus sequence. On the top row, the V3 loop consensus sequence of North American HIV-1 isolates is shown. Numbers represent the degree of conservation (in percentage) within the V3 loop amino acid sequences of these HIV-1 isolates as published recently (10). Amino acids which differ in the bicyclam-resistant NL4-3 strains and in the long-term-passaged, bicyclam-sensitive NL4-3 wt (p63) strain from the NL4-3 wt proviral sequence are underlined.

half-life, while the effective concentration of free bicyclam may decrease, e.g., through uptake by replicating cells. Nevertheless, virus breakthrough was never observed with the recombinant viruses obtained from NL4-3 wt. Therefore, it can be concluded that the gp120 mutations carried by the SID791^r-V4gp41 recombinants (Δ364-368, P385L, Q410E, S433P, and V457I) or the JM2763^r-V4gp41 recombinants (Δ364-386 and P385L) induce a certain degree of resistance to the less potent bicyclam JM2763, whereas they cannot significantly counteract inhibition by SID791. As the SID791^r and JM2763^r recombinants have the P385L mutation as well as the deletion Δ364–368 in common, these changes seem to be necessary but not sufficient for resistance to develop.

Importance of V3 loop mutations for the resistance phenotype. A major contribution to the development of resistance undoubtedly stems from the mutations in the V3 loop. The V3 loop mutations found in the JM2763^r strain, which all are also present in SID791^r viruses, lead to only a slight decrease in sensitivity to SID791 (Table 4). The overall high number of positively charged amino acids in the V3 loop of the NL4-3 strain is further increased in the resistant strains by the substitutions S274R and Q278H, present in both SID791^r and JM2763^r viruses, and the additional N293H mutation unique to SID791^r viruses (Fig. 3). The A297T and P385L mutations involve amino acids in close proximity to the disulfide bridges of the V3 and V4 loops, respectively. The alanine in position 297 of gp120, according to a sequence comparison (24) (Fig. 3), is strictly conserved among all HIV-1 and HIV-2 isolates and even among simian immunodeficiency virus V3 loop sequences. Although the A297T substitution is semiconservative, it may lead to a conformational change at the base of the V3 loop. A similar interpretation is certainly valid for the P385L mutation in V4, as this substitution is clearly nonconservative. All other mutations in V3 as well as the deletion in V4 may further contribute to conformational changes. This idea is supported by the finding of cross-resistance of the bicyclam-resistant strains to dextran sulfate (Table 2). Sulfated polysaccharides inhibit entry and fusion of HIV most likely by binding to the positively charged amino acids of the V3 loop (2, 4, 7, 27, 30). Although the overall positive charge of the V3 loop of the bicyclam-resistant viruses is even increased (Fig. 3), these viruses are either fully (SID791^r strains) or partially (JM2763^r strains) resistant to inhibition by dextran sulfate. This finding points to an altered conformation of the V3 loop in which the postulated interaction with its as yet unknown natural cellular target (8, 12, 19, 39) is no longer affected by the binding of dextran sulfate molecules.

Potential binding sites for bicyclams in gp120. None of the amino acids mutated in gp120 of bicyclam-resistant strains are candidate binding partners for bicyclams. The strong basic character of bicyclams at physiological pH as well as their high capacity for chelating divalent cations make a direct binding to positively charged amino acids unlikely under physiological conditions. This finding is in line with the increase in the number of positively charged amino acids in the V3 loop of bicyclam-resistant strains, which is thought to impede the interaction of bicyclams with the V3 loop. Further indications that bicyclams do not directly bind to the native V3 loop are coming from binding studies with monoclonal antibodies recognizing different V3 loop epitopes as published elsewhere (16). The binding of these antibodies to gp120, exposed either on membranes of infected cells or on virus particles after adsorption to $CD4^+$ T cells, was not blocked by high concentrations of bicyclams as analyzed by fluorescence-activated cell sorting. Remarkably, one of these antibodies recognizes an epitope at the N terminus of the V3 loop (NTRKSIRIQRG) which has been found to be mutated in the bicyclam-resistant NL4-3 strains (Fig. 3). In line with the assumption that the amino acids mutated in the resistant strains do not directly interact with bicyclams, this antibody does not recognize this site in the resistant strains (16).

More likely, binding partners of bicyclams are carboxylate or sulfide anions. The A297T and P385L mutations, which are in close proximity to the disulfide bridges of the V3 and V4 loops, may indicate the cysteines as potential binding sites. Recently, inhibitors of protein disulfide isomerases have been found to inhibit an early, postbinding step of HIV replication (33). It was proposed that the reduction of disulfide bridges by a mem-

brane-bound protein disulfide isomerase may be the first and necessary step in a cascade of conformational changes in gp120 initiated after binding to CD4. These events should ultimately lead to an unfolding of the hydrophobic N-terminal extracellular domain in gp41, which is thought to mediate fusion of the viral membrane with the host cell membrane to initiate entry of the viral capsid (26, 34, 36). We suggest that bicyclams interfere with fusion and entry of HIV by interacting with sulfide anions of the V3 and V4 loops that are formed upon reduction of the disulfide bridges during enzyme-catalyzed isomerization. In CD4-bound gp120, the two loops may be in close proximity. Bicyclams building a bracket between both loops would preclude unfolding of gp120. A conformational change at the base of the loops of bicyclam-resistant strains could hinder the bracket formation or decelerate the kinetics of its formation, which must counteract the dynamics of the unfolding steps. The cysteines, and consequently the disulfide bridges in gp120, are highly conserved among all HIV strains, and their mutation has been shown to affect assembly and infectivity of the virus particles (11). Hence, it is not surprising that in all of the bicyclam-resistant, infectious HIV-1 variants, these cysteines are conserved.

Obviously, the two substitutions of the conserved amino acids A297T and P385L are not sufficient to confer resistance to SID791, nor are the S274R, Q278H, and I288V substitutions, since they are also present in the JM2763^r strain, which is still inhibited by SID791. On the other hand, the finding that all of these mutations lead to identical amino acid substitutions, although they have been selected in independent experiments with structurally different bicyclams, points strongly to their involvement in the development of the bicyclam-resistant phenotype. Furthermore, it can be concluded that the additional mutations present only in the SID791^r strains, i.e., the tyrosine insertion after N268 and the substitutions N270S, R272T, and N293H in V3 and probably the other mutations in C4, V5, and C5 as well, are necessary to develop full resistance against this compound. These amino acids may influence the binding of SID791 but not of JM2763 to gp120.

Taken together, the many mutations accumulating in the gp120 genes of fully bicyclam resistant HIV-1 NL4-3 strains explain the fact that so many passages with moderate increases of the compound added to the culture medium were necessary to select this phenotype. This observation could indicate that resistance to SID791 is also not easily acquired in vivo, since this compound is very potent in all in vitro HIV infection models and well tolerated in laboratory animals (unpublished data). Importantly, SID791 is broadly active against all HIV strains and primary isolates tested (15, 17).

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