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Human Immunodeficiency Virus Glycoprotein gp120 as the Primary Target for the Antiviral Action of AR177 (Zintevir)

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ABSTRACT

The human immunodeficiency virus (HIV) inhibitor AR177 (T30177, Zintevir) has been identified as a potent inhibitor of HIV integrase *in vitro*. The compound is currently the subject of clinical phase I/II trials. However, the primary target for the mechanism of action *in vivo* has not been identified unequivocally. We have found that AR177 inhibits syncytium formation between MOLT-4 cells and HUT-78 cells persistently infected with the HIV-1_{IIIB} or NL4–3 strain, at a 50% effective concentration of 3 μg/ml, roughly 3-fold higher than the concentration required to inhibit HIV replication. Furthermore, flow cytometric analysis has shown that AR177 at 25 μg/ml interferes with the binding of the monoclonal antibody 9284 (directed to the V3 loop of gp120) on HIV_{IIIB}-infected HUT-78 cells, pointing to inhibition of virus binding or virus fusion as the mechanism of action of AR177. To precisely characterize the site/target of intervention by AR177, we have selected HIV-1 (NL4–3) strains resistant to AR177. The binding of the AR177-resistant strain, unlike the parental HIV-1 NL4–3 strain, could not be inhibited by AR177. The resistant phenotype was associated with the emergence of mutations in the gp120 molecule. DNA sequence analysis revealed the presence of the K148E, Q278H, K290Q, and F391I mutations and a deletion of 5 amino acids (FNSTW) at positions 364–368 in the V4 region of the resistant strain but not of the wild-type HIV strain. Selection of resistant strains, although it takes a relatively long time to develop, may also select for strains with lower replicative capacity. No mutations were found in the integrase enzyme gene. Our data argue against HIV integrase being the primary target for the mechanism of anti-HIV action of AR177.

AR177 (T30177) is an oligonucleotide of 17 nucleotides in length, composed only of deoxyguanosine and thymidine, with single phosphorothioate internucleoside linkages at its 5′ and 3′ ends (Rando *et al.*, 1995). AR177 is a potent inhibitor of laboratory strains and clinical isolates of HIV-1 with 50% effective concentrations ranging between 0.025 and 3 μM in cell culture tests (Ojwang *et al.*, 1995).

AR177 has drawn a lot of attention because it has been shown to be one of the most potent inhibitors of the HIV integrase, prompting the development of this compound as a promising antiviral agent directed to a novel target of HIV replication. Indeed, we have shown that AR177 is able to inhibit integration of an oligonucleotide that mimics the integrase recognition sequence by inhibition of the first step of the integration reaction [that is, the formation of the initial stable complex between the integrase and the target DNA (Cherepanov *et al.*, 1997)]. However, it remains to be shown that the potent activity on the integrase is responsible for the observed antiviral effect seen in the cell culture assays. Other oligonucleotides have been described as specific inhibitors of HIV envelope-mediated cell fusion and virus binding (Buckheit *et al.*, 1994; Wyatt *et al.*, 1994). The polyanionic nature of these oligonucleotides, as in the case of dextran sulfate and heparin (Mitsuya *et al.*, 1988) must play an important role in their inhibitory effect on HIV replication. To decipher unequivocally the mode of action of AR177, we have studied its possible interaction during early events of HIV replication. We have also selected a strain resistant to AR177. A critical parameter in the determination of clinical efficacy of antiviral drugs may be the rate of resistance development which can be estimated from experiments *in vitro* (De Vreese *et al.*, 1996b). Also, the phenotype of the resistant strains may be different in infectivity and pathogenicity com-

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ABBREVIATIONS: HIV, human immunodeficiency virus; DS, dextran sulfate; AZT, 3-azido-3′-deoxythymidine; mAb, monoclonal antibody; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

pared with the parental wild-type strain, which has important implications for the clinical response of the patients.

Materials and Methods

Compounds. DS, (M_r 5000) and heparin were purchased from Sigma (St. Louis, MO). The bicyclam derivative AMD3100 was synthesized at Johnson Matthey (West Chester, PA), as described elsewhere (Bridger *et al.*, 1995). AZT was obtained from Wellcome (Greenford, UK). The oligonucleotides AR177 and ISIS5320 (Buckheit *et al.*, 1994) were provided by author R.F.R. (Ojwang *et al.*, 1995).

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 viability of cells that had been infected or not infected with HIV-1 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 [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium method] as described by Pauwels *et al.* (1988). The HIV-1 NL4–3 strain is a molecular clone obtained from the National Institutes of Health (Bethesda, MD).

Syncytium formation assay. HUT-78 cells (2×10^5 cells/ml) persistently infected with HIV-1_{IIIB} were cocultured with MOLT-4 (clone 8) cells (1.8×10^6 cells/ml) in the presence of AR177. After 24 hr, the number of giant cells (syncytia) was recorded microscopically as described previously (Witvrouw *et al.*, 1994).

Immunofluorescence binding assays. The glycoprotein gp120 immunofluorescence assay has been described by Schols *et al.* (1990a). Briefly, HUT-78 cells persistently infected with HIV-1_{IIIB} were incubated with AR177 or DS at the indicated concentrations for 20 min at room temperature. After incubation with anti-gp120 mAb (NEA 9284; DuPont De Nemours, Brussels, Belgium) for 50 min at 37° and subsequent staining with fluorescein isothiocyanate-conjugated F(ab)2 fragments of rabbit anti-mouse immunoglobulin antibody, the cells were fixed and analyzed by flow cytometry. For modulation of CD4 expression, MT-4 cells were incubated with 25 μg/ml AR177 or without compound and stained for flow cytometry analysis with mAb OKT4A (Ortho Diagnostics, Beerse, Belgium) directed to the CD4 receptor.

Interaction of AR177 with recombinant gp120. Interaction between AR177 and gp120 was analyzed by surface plasmon resonance technology (BIAcore; Pharmacia, Uppsala, Sweden), following the procedures described previously (Fischer *et al.*, 1995; Tamamura *et al.*, 1996). Briefly, recombinant gp120 from the HIV-1_{IIIB} strain (Intracell, London, UK) at 30 μg/ml was immobilized on the carboxymethylated surface of a sensor chip (CM5; Pharmacia), using a mixture of 400 mM *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide hydrochloride and 100 mM *N*-hydroxysuccinimide (Pharmacia). Excess, unreacted active groups on the surface were blocked using 1.0 M ethanolamine/HCl, pH 8.5, providing immobilized gp120 at 3097 response units (RU). Various concentrations of AR177 in 10 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20 (Pharmacia) were injected onto the gp120 sensor chip surface at a flow rate of 5 μl/min. At the end of each run, the surface was regenerated with 10 mM glycine, pH 2.0.

Selection of HIV-1 (NL4–3) mutant strains. MT-4 cells were infected with HIV-1 (NL4–3) in medium containing AR177 at 2 to 4 times the 50% effective concentration (EC_{50}). Cultures were incubated at 37° until an extensive cytopathic effect was present (5–6 days). The culture supernatants were used for further passage in MT-4 cells in the presence of 2- to 5-fold increasing concentrations of AR177.

Virus-binding assay. MT-4 cells (5×10^5) were infected with supernatant containing 1×10^5 pg of p24 antigen of either wild-type HIV-1 NL4–3 or AR177-resistant virus in the presence of different concentrations of the test compound. One hour after infection, cells were washed 3 times with PBS (Boehringer Mannheim, Barcelona, Spain) and p24 antigen bound to the cells was determined by a commercial test (Coulter, Barcelona, Spain).

DNA sequence analysis. MT-4 cells were infected with wild-type HIV-1 or AR177-resistant HIV-1 and incubated at 37° for 4 days. The cells were washed in PBS and total DNA was extracted with a QUIAquick blood kit (Westburg, Leusden, The Netherlands). PCR amplification was performed with ULTMA DNA polymerase with proof-reading capacity (Perkin Elmer, Nieuwerkerk, The Netherlands). Fragments of appropriate molecular size were excised from agarose gel and purified. DNA sequencing was performed directly on the purified PCR product following the protocol provided by the ABI PRISM dye terminator-cycle sequencing kit and analyzed on an ABI PRISM 310 genetic sequencer (Perkin Elmer). The primer sets used for PCR amplification and sequence analysis are summarized in Table 1.

Site-directed mutagenesis was performed using the Altered Sites II *in vitro* mutagenesis system (Promega, The Netherlands). The V2-V5 region of gp120 was cloned in the pALTER-1 vector. An oligonucleotide (GACCCTTCAGTACTCCA-AGTACTATTAAACAG-CTGTGTTGAATTAC) was used to introduce a deletion of 5 amino acids (FNSTW) in the V4 region of the cloned gp120. The mutated gp120 was recombined with the HIV-1 clone pNL4–3 by the marker rescue technique as described before (De Vreese *et al.*, 1996a).

Results

Syncytium formation assay. AR177 inhibited giant cell (syncytium) formation between MOLT-4 cells and HUT-78 cells persistently infected with the HIV-1_{IIIB} or NL4–3 at an EC_{50} of 3.0 μg/ml.

Immunofluorescence binding assays. AR177 had no effect on the binding of anti-CD4 mAb (OKT4A) to the CD4 cell receptor (Fig. 1A). However, AR177 at 25 μg/ml inhibited the binding of the mAb 9284 (directed to an epitope in the V3 loop region) to HUT-78 cells persistently infected with HIV-1 IIIB (Fig. 1B).

TABLE 1
Primers used to amplify and sequence genes of the resistant NL4-3 virus strains

Gene amplified/sequenced	Sequence	
	5′ primer	3′ primer
gp120-V1-V3	AATTAACCCCACTCTGTGTTAGTTTA (6587–6612)[a]	GGTCTCCCCTGGTCCCTCTCA (7147–7167)
gp120-V1-V3	AGGTATCCTTTGAGCCAATTCC (6840–6861)[b]	TGATACTACTGGCCTAGTTCCA (6967–6988)[b]
gp120-V3-V5	CTGCCAATTTCACAGACAATGCT (7041–7062)	TCTTTGCCTTGGTGGGTGCTA (7707–7727)
gp120-V3-V5	AATCTTTAAGCAATCCTCAG (7397–7316)[b]	CCCCTCCACAATTAAAACTG (7342–7361)[b]

[a] Numbering of nucleotides is according to the NL4-3 sequence in the GeneBank database, accession number M19921.
[b] Used only for DNA sequencing.

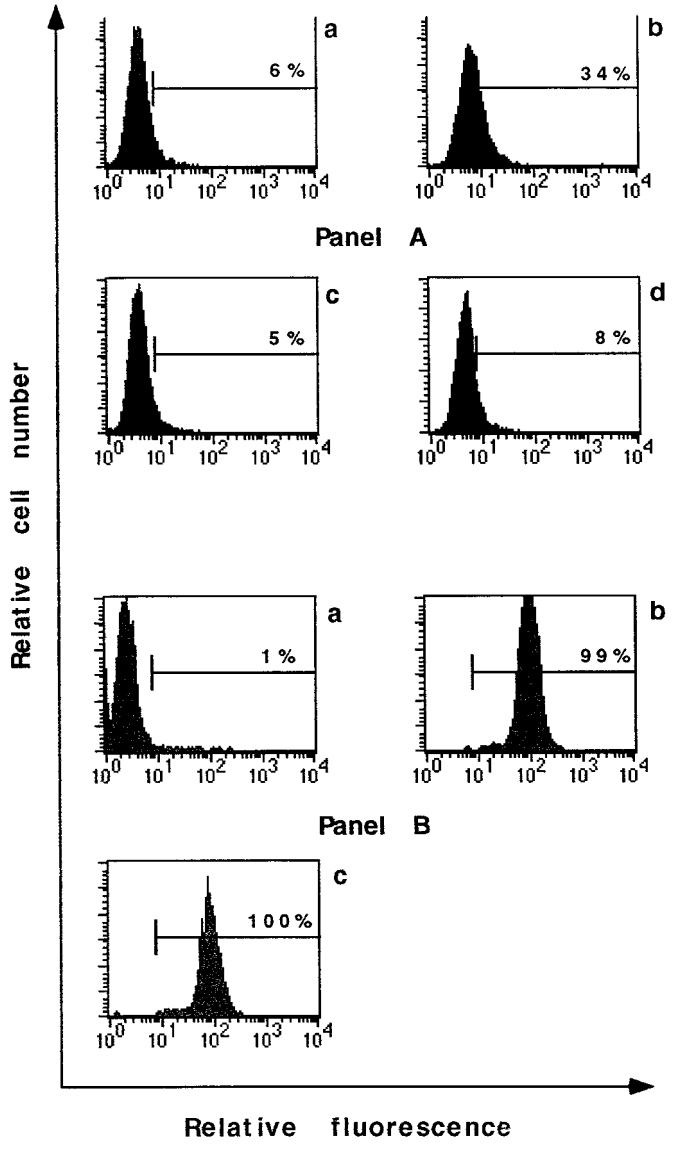

Fig. 1. A, Flow cytometric histograms of the binding of anti-gp120 mAb (9284) on HUT-78 cells persistently infected with HIV-1 IIIB. Histograms: *a*, fluorescence of infected cells incubated only with RaM-IgG-F(ab')$_2$-fluorescein isothiocyanate; *b*, specific fluorescence of the infected cells; *c* and *d* present the infected cells incubated with DS (25 μg/ml) and AR177 (25 μg/ml), respectively. B, Binding of OKT4A mAb to MT-4 cells in the absence (*b*) or presence of AR177 (25 μg/ml) (*c*). Histogram *a*, control fluorescence of the isotype mAb.

Interaction of AR177 with recombinant gp120. After various concentrations of AR177 were injected onto the gp120-immobilized sensor chip, we obtained the sensorgrams shown in Fig. 2. AR177 showed a dose-dependent interaction with the immobilized gp120. Association with the gp120 was rapid, followed by a plateau (equilibrium) and then a gradual decrease (dissociation) after the end of the injection. The association (K_a) and dissociation (K_d) constants were calculated to be K_a = 1.65 [M^{-1} s^{-1}] and K_d = 0.0156 [s^{-1}]. In a similar experiment, the bicyclam AMD3100 (125 μg/ml) did not bind to the recombinant gp120.

Selection of HIV-1 (NL4–3) mutant strains. HIV-1 strains resistant to AR177 were raised in MT-4 cells. HIV-1 (NL4–3) was able to grow in the presence of AR177 at 300 μg/ml after 33 passages (182 days). The EC_{50} of AR177 for this resistant strain was >125 μg/ml. The HIV-1 (NL4–3) wild-type strain that was passaged in MT-4 cells for the same period of time remained sensitive to AR177 (EC_{50} = 0.6 μg/ml). The AR177- resistant virus was partially cross-resistant to DS5000 (34-fold, EC_{50} = 23.8 μg/ml), heparin (20-fold, EC_{50}=3.9 μg/ml), and the oligonucleotide ISIS5320 (Buckheit *et al.*, 1994) (>50-fold) but remained as sensitive to the bicyclam AMD3100 as the wild-type strain (Table 2).

DNA sequence analysis. We have identified several mutations in the gp120 gene sequence of the AR177-resistant strain that were not present in the wild-type strain (Table 3). Two of these mutations are located in the V3 loop region: Q278H and K290Q. The first one corresponds to a mutation also found in the HIV-1 NL4–3 strains resistant to DS (17) and the bicyclam AMD3100 (De Vreese *et al.*, 1996a). Also, a deletion of 5 amino acids at position 364 to 368 (in the V4 loop) was detected in the AR177-resistant strain (Fig. 3). No mutations were found in the sequence of the integrase gene of the AR177-resistant strain when compared with the wild-type strain (Cherepanov *et al.*, 1997).

Virus-binding assay. AR177 and DS inhibited the detection of p24 antigen bound to MT-4 cells after 1 hr incubation of cells with wild-type virus supernatant. The 50% inhibitory concentrations (IC_{50}) were 2.4 μg/ml and 0.8 μg/ml for AR177 and DS, respectively. P24 antigen bound to MT-4 cells was detected in cells cultured with AR177-resistant supernatant; however, AR177 or DS did not significantly inhibit virus binding (as measured as bound p24 antigen) (Fig. 4). Similarly, the reverse transcriptase inhibitor AZT (1.0 μg/ml) or the fusion inhibitor AMD3100 (125 μg/ml) did not inhibit the

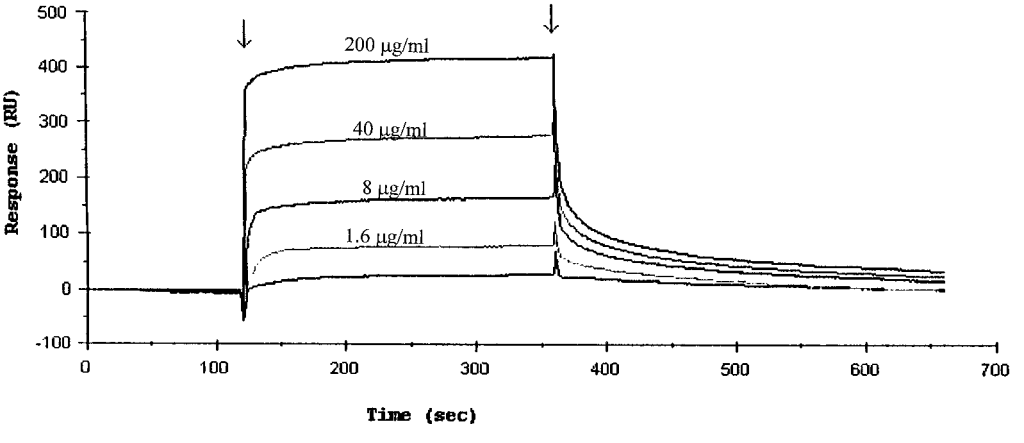

Fig. 2. Sensorgrams obtained after injection of AR177 onto immobilized gp120. The concentrations of AR177 are 1.6, 8, 40, and 200 μg/ml. *Arrows,* start and end of the injection.

TABLE 2

Anti-HIV activity of different compounds against wild-type HIV-1 and AR177-resistant HIV-1.

EC_{50} based on the inhibition of HIV-induced cytopathicity in MT-4 cells, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium method. CC_{50} based on the viability of MT-4 cells, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium method.

Compound	EC_{50}		CC_{50}
	Wild-type NL4-3	$AR177^r$ NL4-3	
		$\mu g/ml$	
AR177	0.6	>125	>125
DS (M_r 5000)	0.7	23.8	>125
Heparin	0.2	3.9	>125
ISI5320	0.2	>10	>10
AMD3100	0.003	0.004	>125

TABLE 3

Mutations in the gp120 of AR177-resistant NL4-3 strain

Amino acid position[a]	NL4-3 wild-type strain		NL4-3 $AR177^r$ strain	
(Region)	Codon	Amino acid	Codon	Amino acid
148 (V2)	AAA	K	GAA	E
278 (V3)	CAG	Q	CAU	H
290(V3)	AAA	K	CAA	Q
Δ364-368 (V4)	UUU AAU AGU ACU UGG	FNSTW	deletion	
391 (CD4BD)[b]	UUU	F	AUU	I

[a] Numbering of amino acid and domain classification according to the published secondary structure of the mature HIV-1 gp120 protein (De Vreese *et al.*, 1996a).
[b] CD4BD: CD4 binding domain of the gp120 molecule as in Gallagher *et al.*, 1995.

binding of HIV to MT-4 cells when wild-type or AR177-resistant virus were used (data not shown).

Mutagenesis of the V4 loop. Recombinant virus that carries the deletion in the V4 loop corresponding to amino acids FNSTW was recovered after transfection of the HIV-1/NL4–3/DV4 proviral DNA into MT-4 cells. The EC_{50} of AR177 for the recombinant virus was 0.4 μg/ml, roughly the same as the EC_{50} required for the wild-type HIV-1 NL4–3 strain. The recombinant virus was sensitive to AMD3100 (EC_{50} = 0.003 μg/ml) but partially cross-resistant to DS (25-fold).

Discussion

AR177 is a potent inhibitor of laboratory strains and clinical isolates of HIV-1 (Ojwang *et al.*, 1995). The mode of action of this compound, which forms a guanosine-quartet structure, has been attributed in part to its anti-integrase activity. Mazumder *et al.* (1996) have postulated that "G-quartet structures may act as negative regulators of autointegration *in vivo* and that novel AIDS therapies could be based upon G-quartets as inhibitors of HIV integrase" We have shown that the bases for the anti-integrase activity of AR177 may be different from those of such polyanionic compounds as DS, prompting the use of AR177 as a tool to study the fundamental aspects of HIV integration and the molecular structure that determines the effect of AR177 on HIV integrase (Cherepanov *et al.*, 1997).

In cell culture, however, the primary target for AR177 seemed to be the envelope gp120 protein of HIV-1. The polyanionic nature of AR177 correlates well with that of other polyanionic compounds, such as DS and heparin. We have shown that AR177 interacts in a concentration-dependent manner with recombinant gp120. Furthermore, AR177 inhibits the binding of recombinant gp120 to $CD4^+$ cells (Fig. 1) and inhibits syncytium formation (which can be interpreted as HIV-1 fusion with the cell membrane) at roughly the same concentration as needed for its HIV replication inhibitory activity. Thus, AR177 has a bimodal mechanism of action, namely inhibition of virus binding and inhibition of virus fusion, which both seem to be mediated by gp120 but may depend on distinct viral and cellular determinants.

It is possible, however, that AR177 may be active at later step(s) of the virus replication cycle. One way to clearly identify the viral proteins targeted by an antiviral compound is the development of resistance to the compound *in vitro*. We have shown previously that HIV-1 strains that are resistant to inhibitors targeted at the binding/fusion step of replication can emerge after sequential passaging of the virions in cell culture in the presence of the antiviral compound (De Vreese *et al.*, 1996a; Esté *et al.*, 1997; Esté *et al.*, 1996). Resistance to the activity of such compounds as dextran sulfate will arise if the virus is given sufficient time and enough selective pressure for the resistant virus to emerge. Resistance to the bicyclam AMD3100 and to dextran sulfate appear to be mediated by a combination of specific mutations in the gp120 molecule of the resistant HIV strains (De Vreese *et al.*, 1996a; Esté *et al.*, 1997).

Here, we show that resistance to AR177 develops after 33 passages of HIV-1/NL4–3 in MT-4 cells in the presence of increasing concentrations of the compound. The resistant virus was able to replicate in the presence of AR177 at concentrations up to 125 μg/ml. DNA sequence analysis of the gp120 coding region showed the emergence of mutations in the AR177-resistant strain that were not present in the wild-type strain. No mutations were detected in the DNA coding region of the integrase.

Although indirect, the data presented here represents strong evidence of gp120 as the molecular target of AR177, and it becomes clear that AR177 binds and interacts with the gp120 molecule. However, to directly challenge the functional importance of this interaction, we tested the binding of wild-type and AR177-resistant virus to CD4 positive cells in the absence or presence of compound. AR177 inhibited the binding of wild-type virus at roughly the same concentrations required for inhibition of syncytium formation or virus replication (4-fold higher) but was not able to significantly inhibit virus binding when AR177-resistant virus was used, even in the presence of concentrations up to 125 μg/ml. Agents that do not interact with the gp120 molecule, such as the reverse transcriptase inhibitor AZT or the CXCR-4 antagonist AMD3100 (Schols *et al.*, 1997) did not inhibit the binding of either wild-type or AR177-resistant strains to CD -positive cells. We show that the gp120, which mediates the binding to the CD4 positive cells (Mitsuya *et al.*, 1988; Gallagher *et al.*, 1995), is indeed a binding target of AR177. The AR177-resistant strain, which expresses the mutant gp120, can now bind to CD4 positive cells in the presence of the compound, which suggests that the relevant mutant gp120 is not a functional binding target of AR177.

It is noteworthy that the DNA sequence of the AR177-resistant strain revealed the emergence of mutations that appear also in the AMD3100-resistant and DS-resistant strains, namely mutation Q278H in the V3 loop and a deletion of 5 amino acids (FNSTW) at positions 364 to 368 in the

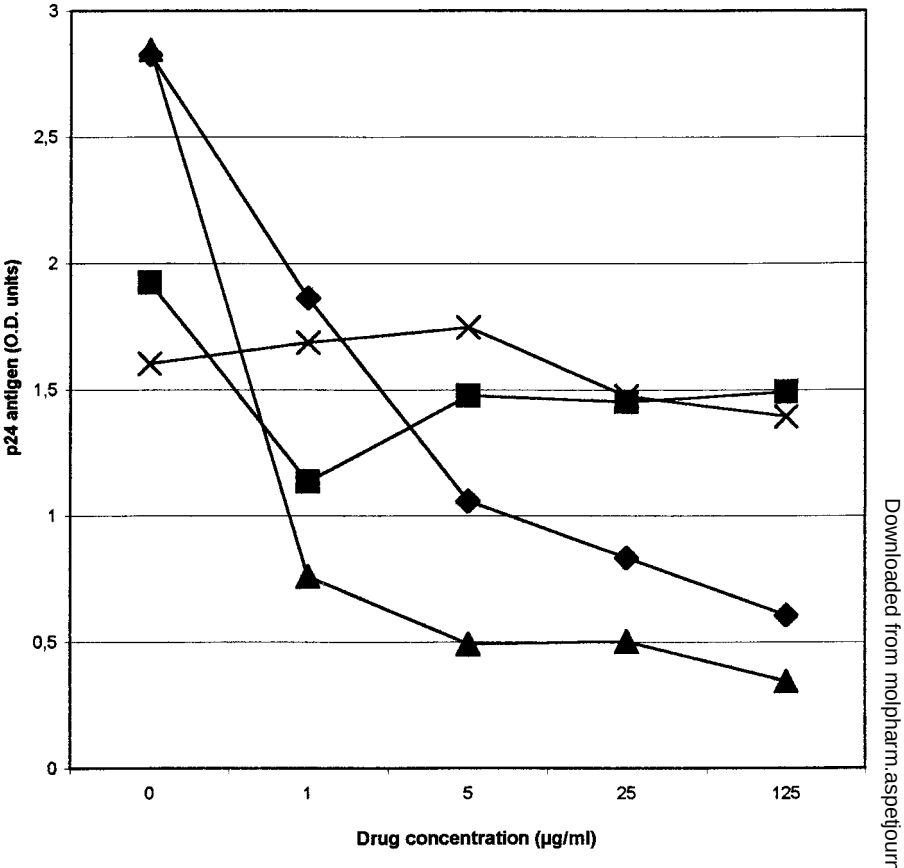

Fig. 3. Inhibition of virus binding to MT-4 cells. The cells were incubated with 1×10^5 pg of p24 antigen of wild-type HIV-1 NL4–3 in the presence of varying concentrations of AR177 (◆), DS (▲), or AR177-resistant HIV-1 in the presence of AR177 (■) or DS (×). After 1 hr incubation, cells were washed in PBS an p24 antigen bound to the cells was determined by a commercial test.

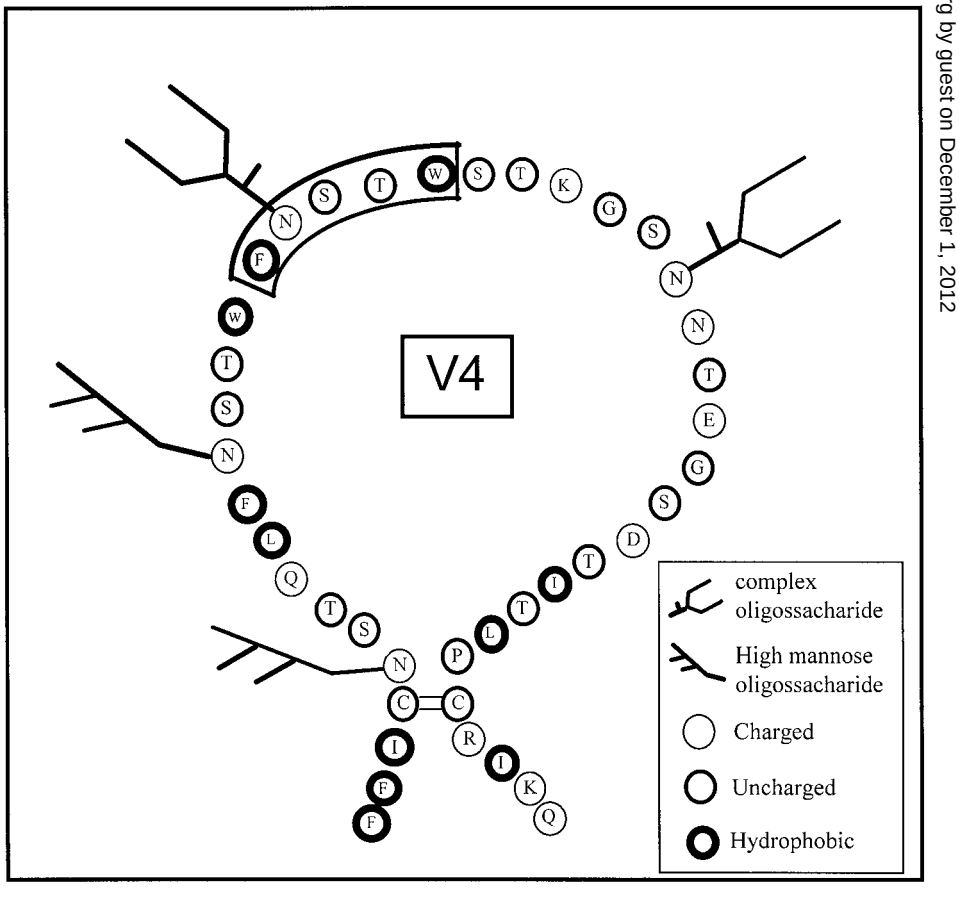

Fig. 4. Amino acid sequence of the V4 loop of HIV-1 (Gallagher *et al*, 1995). *Boxed amino acids* (FNSTW), deletion found in the AR177-resistant strain.

V4 loop (Fig. 4). The AR177-resistant strain is partially cross-resistant to DS but not to AMD3100. This apparent discrepancy can be explained by (i) the fact that the deletion in the V4 loop, as seen in the mutagenesis experiments, is not sufficient to generate resistance to either AR177 or AMD3100 but confers partial (25-fold) resistance to DS; (ii) a longer time (up to 60 passages) and more mutations (up to 12) are required for resistance to AMD3100 to develop than for either AR177 or DS resistance; (iii) if each one of the mutations found in the AMD3100 strain are introduced in the wild-type strain by site-directed mutagenesis, the recovered virus remains sensitive to AMD3100 (De Vreese K, manuscript in preparation), Thus, while the Q278H mutation and the 364FNSTW368 deletion may not to be sufficient to induce resistance to either AR177 or AMD3100, they may be a necessary requirement for resistance to occur for binding and/or fusion inhibitors such as the bicyclams, sulfated polysaccharides, and polyanions (such as AR177) in general.

Another interesting aspect of the resistance development to AR177 is that although the resistant virus was able to induce a clear cytopathic effect on MT-4 cells, the amount of p24 antigen required to induce the same cytopathic effect as the wild-type strain was up to 6-fold higher (data not shown). Similarly, the amount of bound virus, as measured as p24 antigen bound to MT-4 cells, was lower when AR177-resistant virus was used instead of wild-type virus. Although this phenomenon must be studied further, the data indicates that AR177 may select for strains with a lower replicative capacity or with lower pathogenicity.

It is not impossible that further passage of the AR177-resistant strain in ever-increasing concentrations of AR177 could eventually lead to the emergence of mutations in the integrase gene. However, it should be pointed out that concentrations of AR177 higher than the ones used in our experiments could be toxic to the cell. Although G-quartet structures seem to be highly stable, an oligomer of this relatively high molecular mass (5400 Da) may not be readily internalized by the cell. If it were internalized, we imagine that not only might the integrase activity but also the reverse transcriptase activity serve as targets for the action of AR177, because AR177 seems to also inhibit reverse transcriptase *in vitro* (Ojwang *et al.*, 1995).

The HIV envelope gp120 has gained renewed interest as a potential target for chemotherapeutic intervention in view of recent reports that this molecule mediates the interaction of the virus, not only with its primary receptor, CD4, but also with the β-chemokine receptor, HIV's second receptor (Cocchi *et al.*, 1995; Feng *et al.*, 1996; Dragic *et al.*, 1996). Both binding and fusion of HIV to the cell seem to be mediated in part by the gp120 molecule. Polyanions, such as DS or AR177, interfere with both processes, which are clearly interrelated but may have different molecular determinants within the gp120 molecule. Further insight into the mechanism of action of such compounds as AR177 will aid our understanding of the molecular determinants of virus binding and virus fusion and of the role of the cellular receptors that permit this binding/fusion.

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