CAPÍTULO 2

OBJETIVOS

Una prometedora aproximación para el desarrollo de nuevos fármacos anti-VIH consiste en interferir con la entrada viral. Esta aproximación ofrece la capacidad de atacar un proceso que implica tanto a receptores celulares como a ligandos virales, pudiéndose llegar, hipotéticamente, a detener la infección y muerte de células no infectadas. Nosotros queríamos determinar si se podría bloquear eficientemente la entrada viral y de ser así cual sería la respuesta del virus frente a este tipo de presión selectiva.

Para intentar dar respuesta a estas cuestiones, en este estudio se plantearon tres objetivos principales:

1.- Determinación de la eficacia *in vitro* de nuevos agentes antivirales dirigidos a las etapas tempranas del ciclo de replicación del VIH: evaluación de la actividad antiviral, frente a varias cepas de VIH-1 de diferentes compuestos aniónicos y catiónicos.

2.- Identificación del mecanismo de acción de dichos agentes antivirales: una vez demostrada la actividad antiviral de los diferentes compuestos, determinación de su mecanismo de acción. Para intentar dilucidar este mecanismo de acción se utilizará, entre otras técnicas, la creación de virus resistentes a dichos fármacos, como un método eficaz de distinción de la diana molecular de un agente antiviral.

3.- Establecimiento del efecto que estos nuevos agentes antivirales presentan sobre la patogénia viral. Determinación del efecto que ejerce la presión selectiva de un inhibidor de la entrada viral en la evolución del virus.

Los resultados en este trabajo están divididos en tres capítulos. En el primer capítulo se presentan los resultados de la evaluación de dos clases de compuestos que actúan en el primer paso del proceso de la entrada viral, en la unión de la glucoproteína de la envuelta viral con el receptor CD4. En el segundo capítulo se estudian compuestos que interactúan con el segundo paso de este proceso de entrada, la unión del complejo Env/CD4 a los receptores de quimiocinas. El tercer capítulo está dedicado a la evaluación del efecto que esta nueva estrategia de inhibición de la entrada del VIH presenta sobre la evolución viral.

Este trabajo, por tanto, está centrado en el estudio del proceso de entrada del VIH y más concretamente en el estudio del modo de acción de nuevos agentes que bloqueen este mecanismo de entrada. Esto nos permitirá obtener un mayor conocimiento del mecanismo de entrada en sí y a la vez su aplicación en la búsqueda de nuevos agentes antivirales, enfocando el mecanismo de entrada viral como una posible, nueva y eficaz estrategia terapéutica.

CAPÍTULO 3

INHIBICIÓN DE LA UNIÓN DEL VIRUS A CÉLULAS $CD4^+$: INTERACCIÓN CON gp120/CD4

3.1.- RESISTANCE OF HUMAN IMMUNODEFICIENCY VIRUS TO THE INHIBITORY ACTION OF NEGATIVELY CHARGED ALBUMINS ON VIRUS BINDING TO CD4

3.2.- HUMAN IMMUNODEFICIENCY VIRUS GLYCOPROTEIN gp120 AS THE PRIMARY TARGET FOR THE ANTIVIRAL ACTION OF AR177 (ZINTEVIR)

RESISTANCE OF THE HUMAN IMMUNODEFICIENCY VIRUS TO THE INHIBITORY ACTION OF NEGATIVELY CHARGED ALBUMINS ON VIRUS BINDING TO CD4

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ABSTRACT

Negatively charged albumins (NCAs) have been identified as potent inhibitors of HIV-1 replication *in vitro*. Time of addition studies suggests that succinylated and aconitylated human serum albumin (Suc-HSA and Aco-HSA) act at an early stage of the virus life-cycle, and surface plasmon resonance (BIAcore) experiments have confirmed a direct interaction of NCAs with the HIV-1 gp120. Resistance to Suc-HSA and Aco-HSA was analyzed by characterizing HIV-1 variants that were selected in the cell culture after serial passage of the NL4-3 strain in the presence of the compounds. After 24 passages (126 days) we isolated variants that were resistant to Suc-HSA (>27-fold) and Aco-HSA (37-fold), as compared to the wild-type NL4-3 virus. The binding of the NCA-resistant HIV strains to $CD4^+$ MT-4 cells could no longer be inhibited by either Suc- or Aco-HSA. The emergence of mutations in the envelope gp120 of the resistant virus paralleled the emergence of the resistant phenotype. The Suc-HSA-resistant strain was 100-fold cross-resistant to the G quartet-containing oligonucleotide AR177 (Zintevir, an HIV-binding inhibitor), and partially cross-resistant to dextran sulfate, but remained sensitive to the bicyclam AMD3100 and the chemokine SDF-1α, which block HIV replication by interaction with the chemokine receptor CXCR4. Furthermore, neither Suc-HSA nor Aco-HSA inhibited the binding of monoclonal antibodies 12G5 and 2D7 (directed to CXCR4 and CCR5, respectively) in SUPT-1 cells or THP-1 cells. These results confirm that NCAs bind primarily to gp120 and do not interact directly with the HIV chemokine receptor but block the binding of the virus particles (through the gp120) with $CD4^+$ cells.

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INTRODUCTION

Polyanions have been known for their potent antihuman immunodeficiency virus activity *in vitro* (1,2); however, their strong anticoagulant activity and acute toxicity *in vivo* (3, 4) have deterred their use as therapeutic agents against HIV and AIDS. Another class of polyanions with potent anti-HIV activity, the negatively charged albumins (NCAs), which are active in the nanomolar range but do not interfere with blood coagulation, have already been described (5). Modifications of human serum albumin (HSA) by succinylation or aconitylation introduce one (Suc-HSA) or two (Aco-HSA) COOH groups per reacted amino group in the protein, resulting in two or three extra anionic charges per reacted NH_2 group, respectively (5, 6).

The anti-HIV activity of NCAs, like that of other polyanionic compounds, could be ascribed to their electrostatic interaction with positively charged amino acids of HIV-1 gp120 and, in particular, with the gp120 variable loop 3 (the V3 loop). Indeed, the interaction of NCAs with the V3 loop has been proposed (7). The V3 loop appears to mediate virus-cell binding (8), virus-cell fusion (9) and virus-coreceptor interaction (10).

The bicyclams, a novel class of anti-HIV agents (11), have been identified as potent and selective antagonists of CXCR4, the natural receptor of the SDF-1α chemokine and the coreceptor used by T cell-tropic strains of HIV (12-14). HIV-1 resistance to AMD3100 or to SDF-1α leads to genotypic changes in the V3 loop of the resistant strains, which in turn induce cross-resistance to polyanions, such as dextran sulfate (DS), and heparin (15). While at first glance these results indicate that polyanions may be involved in postbinding events, the cross-resistance observed could alternatively be explained by an indirect effect on virus binding to $CD4^+$ cells as a consequence of the virus escaping the blockade of CXCR4 by AMD3100 through mutations in the gp120.

Although the mechanism of action of NCAs has been previously examined (5, 6, 7, 27, 37), it is not yet clear if these compounds interact during any postbinding events. We have now further characterized the inhibitory effect of NCAs on HIV-1 replication: we studied the direct interaction of this class of proteins with gp120, investigated their possible intervention in postbinding events, and confirmed gp120-mediated virus-cell binding as the mode of action of these anti-HIV agents.

MATERIALS AND METHODS

Compounds. Derivatization of HSA with succinic anhydride or cis-aconitic anhydride was performed as previously described by Jansen et al (5). The bicyclam AMD3100 was synthesized as described previously (16) and was obtained from AnorMED (Langley, British Columbia, Canada) The chemokine SDF-1α was purchased from R&D Systems (Abingdon, UK). Dextran sulfate (MW 5000) was purchased from SIGMA (St. Louis, MO).

Time of addition experiment. MT-4 cells (17) were infected with HIV-1 NL4-3 at a multiplicity of infection (MOI) of 1, and the test compounds were added at various times after infection. Viral p24 antigen production was determined at 30 h post infection by a commercial enzyme-linked immunosorbent assay (ELISA) (NEN Life Science Products, Brussels, Belgium). The compounds were added to MT-4 cells at a standardized concentration, that is, 100 times their 50% effective concentration (EC_{50}) in the anti-HIV test.

Antiviral assay and cytotoxicity assay. Anti-HIV activity and cytotoxicity measurements in MT-4 cells 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 (MTT method) as described previously (18).

Interaction of NCAs with recombinant gp120. Interaction between NCAs and gp120 was analyzed by surface plasmon resonance technology (BIAcore; Pharmacia, Piscataway, NJ), following previously described procedures (19,20). Briefly, recombinant gp120 from the HIV-1 IIIB strain (Intracell, Cambridge, MA) 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 with 1.0 M ethanolamine/HCl (pH 8.5) providing immobilized gp120 at 3097 response units (RU). Various concentrations of compounds 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. Association (K_a) and dissociation (K_d) constants were

calculated with the BIAcore curve fitter software, based on the association model AB = A+B.

Selection of HIV-1 (NL4-3) mutant strains. MT-4 cells were infected with HIV-1 NL4-3 (21) in medium containing Suc-HSA or Aco-HSA at two to four times the 50% effective concentration (EC_{50}). Cultures were incubated at 37°C until an extensive cytopathic effect was present (5 to 6 days). The culture supernatants were used for further passage in MT-4 cells in the presence of two- to five-fold increasing concentrations of the respective compound.

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, Suc-HSA-resistant virus or Aco-HSA-resistant virus in the presence of various concentrations of the test compound. One hour after infection cells were washed three times with phosphate-buffered (PBS) and p24 antigen bound to the cells was determined by a commercial ELISA test (Coulter, Madrid, Spain).

Inhibition of gp120-CD4 interaction. To evaluate the activity of negatively charged albumins in inhibiting the interaction between gp120 and CD4, 5×10^5 persistently HIV-1 IIIB-infected H9 cells, which express gp120 on their surface, were incubated with soluble CD4 (sCD4, 1 μg/ml; Intracell) in the presence or absence of either Aco-HSA or Suc-HSA, DS or anti-CD4 monoclonal antibody (MAb) Q4120 known to block the binding of CD4 to gp120, for 30 min at 37ºC. Cells were then washed and incubated with fluorescein isothiocyanate (FITC)-labeled OKT4 MAb (Ortho Diagnostics, Raritean, NJ), which recognizes CD4 without affecting the gp120-biding site. Cells were then washed and resuspended in PBS and analyzed by flow citometry

DNA sequence analysis. MT-4 cells were infected with wild-type HIV-1 or Suc-HSA- or Aco-HSA-resistant HIV-1 and incubated at 37°C for 4 days. The cells were washed in PBS and total DNA was extracted with a QUIAquick blood kit (Qiagen, Westburg, The Netherlands). Polymerase Chain Reaction (PCR) amplification was performed with ULTMA DNA polymerase with proof-reading capacity (Perkin-Elmer, Norwalk, CT). DNA sequencing was performed directly on the purified PCR product, following the protocol provided by the ABI PRISM dye terminator-cycle sequencing kit and analysed on an ABI PRISM 310 genetic sequencer (Perkin-Elmer). The primer sets used for PCR amplification and sequence analysis are summarized in Table 1.

Flow cytometric analyses of chemokine receptor interactions . CD4-positive, SUP-T1 (22) cells or THP-1 (23) cells were incubated with the anti-CXCR4 monoclonal antibody (12G5 MAb) (R&D Systems) (24) or the anti-CCR5 monoclonal antibody (2D7 MAb) (Pharmingen, San Diego, CA) (24) for 45 min at 4ºC, in the presence or absence of test compound (0.5 μg/ml). The cells were then washed with PBS and incubated with FITC-conjugated goat-anti-mouse antibody (GaM-IgG-FITC) (Becton Dickinson, San Jose, CA) for 30 min. The cells were washed with PBS and analyzed by flow cytometry in a FACScalibur system (Becton Dickinson). Data were acquired and analysed with CellQuest software (Becton Dickinson), on an Apple Macintosh computer. For interaction with CD4, cells were labeled with Leu3a (Becton Dickinson) which recognizes the HIV-binding site in the CD4 molecule.

Table 1. Primers used to amplify and sequence genes of the wild-type and drug-resistant HIV-1 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]
gp120-gp41	GGAGGAGGCGATATGAGGGACAATTGG (7631-7657)	CTCTGTCCCACTCCATCCAGGT (8099-8120)[b]
	CGCAAAACCAGCAAGAAAAG (8166-8185)[b]	TGGGAGGTGGGTCTGAAACG (8366-8385)[b]
		GCTGGCTCAGCTCGTCTCATTCTTTCC (8843-8869)

[a]Numbering of nucleotides is according to the NL4-3 sequence in the GeneBank database, accession number M19921.

[b]Used only for DNA sequencing.

RESULTS

Time (site) of intervention. Time of addition experiments were performed to pinpoint the possible step in the HIV replicative cycle that was inhibited by NCAs. HIV-1 replication, as measured by p24 antigen production, could be inhibited by DS, Suc-HSA or Aco-HSA only if they were added at the time of infection. In contrast, addition of the reverse transcriptase inhibitor AZT (zidovudine) could be delayed for up to 4 hours and still to obtain complete inhibition of virus replication (Fig.1). These results confirm those reported by Kuipers et al (15b) for Suc-HSA but highlight the similarity in the mode of action of NCAs to DS.

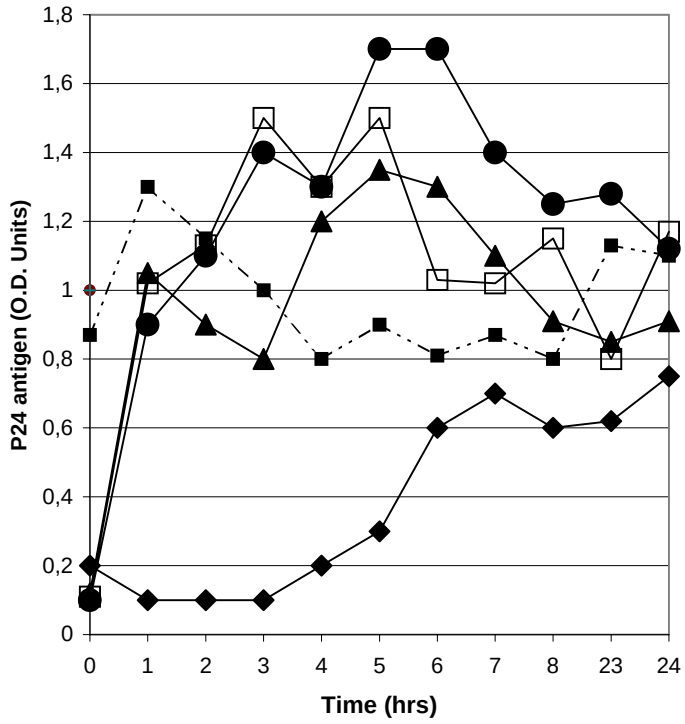

Figure 1. Time of addition experiment. MT-4 cells were infected with HIV-1 IIIB at an moi of >1 and the test compounds were added at 100 times their 50% effective concentration (EC_{50}) at various times postinfection: DS (●), Suc-HSA (▲), Aco-HSA (□), AZT(◆) or PBS (■) were added. Viral p24 antigen production was determined at 30 h postinfection.

Interaction of NCAs with recombinant gp120. To test the interaction of NCAs with gp120, various concentrations of Aco-HSA were injected onto the gp120-immobilized sensor chip. From the sensorgrams (Fig. 2) it is clear that Aco-HSA showed a dose-dependent interaction with the immobilized gp120. Association with

gp120 was 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 $K_a = 6.8 \times 10^2$ ($M^{-1}sec^{-1}$) and $K_d = 6.5 \times 10^{-4}$ (sec^{-1}). In a similar experiment the bicyclam AMD3100 (125 µg/ml) did not bind to the recombinant gp120 (data not shown).

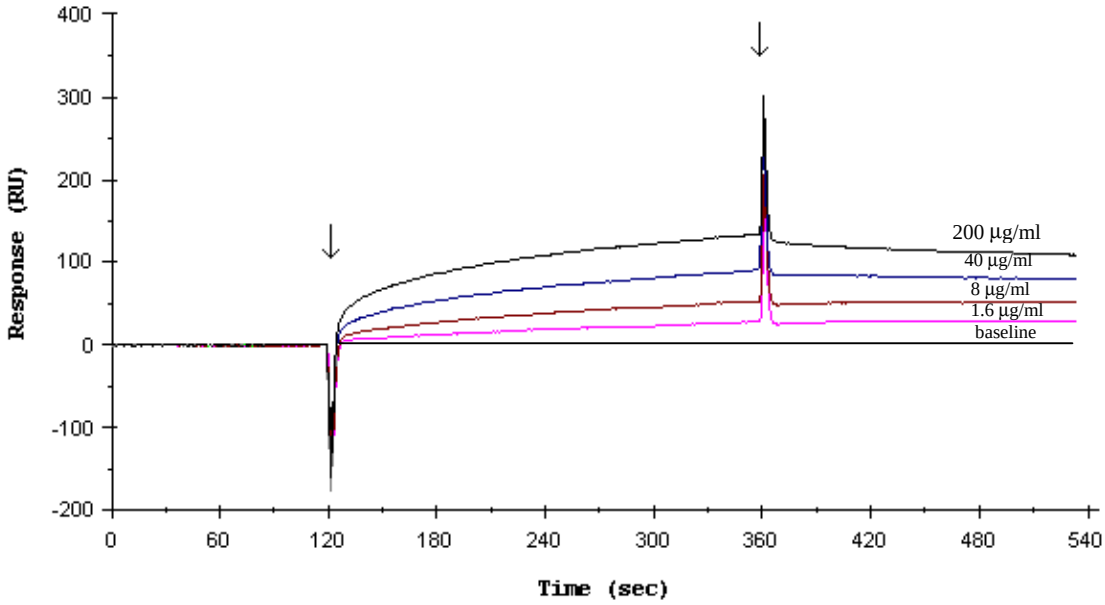

Figure 2. Sensorgrams obtained after injection of Aco-HSA at 200, 40, 8, and 1.6 µg/ml, onto immobilized gp120. Arrows indicate the start and end of the injection.

Development of HIV-1 NL4-3 resistant mutants. HIV-1 NL4-3 strains resistant to Suc-HSA and Aco-HSA were raised in MT-4 cells. After 24 passages (126 days), we isolated variants that were resistant to Suc-HSA (EC_{50} >125 µg/ml) and partially resistant to Aco-HSA (EC_{50} 56 µg/ml) while the wild-type NL4-3 strain that was passaged in parallel but without drug treatment remained sensitive to both compounds (Suc-HSA, EC_{50} 4.7 µg/ml; Aco-HSA, EC_{50} 1.5 µg/ml). The Suc-HSA-resistant strain was 100-fold cross-resistant to the G quartet-containing oligonucleotide AR177 (zintevir); 8-fold cross-resistant to DS; but not cross-resistant to the bicyclam AMD3100, the chemokine SDF-1α or AZT. The Aco-HSA-resistant virus was cross-resistant to Suc-HSA (15-fold), AR177 (100-fold), but only 3-fold resistant to DS (Table 2). The AMD3100-resistant virus previously reported (25) was cross-resistant to Suc-HSA (7-fold), Aco-HSA (17-fold), AR177 (155-fold), DS (>30-fold) and the

chemokine SDF-1α (>10-fold), but was not resistant to the reverse transcriptase inhibitor AZT.

Table 2. Antiviral activity of different compounds against Wild-type, Suc-HSA-resistant and Aco-HSA resistant HIV-1 (NL4-3) virus strains.

Compound	EC_{50} (μg/ml)[a]			
	Wild type NL4-3	Suc-HSA-resistant NL4-3	Aco-HSA-resistant NL4-3	AMD3100-resistant NL4-3
Suc-HSA	4.7	>125	71.7	31.1
Aco-HSA	1.5	71.7	55.9	26.1
AR177	0.2	20.4	20.4	31.0
DS (MW 5000)	0.8	6.1	2.5	>25
AMD3100	0.003	0.01	0.01	0.1
SDF-1α	0.5	0.42	0.5	>5
AZT	0.004	0.007	0.01	0.005

[a]EC_{50} : 50% effective concentration based on the inhibition of HIV-1-induced cytopathicity in MT-4 cells, as determined by the MTT method (18).

DNA sequence analysis. We have identified several mutations in the *env* gene sequence of Suc-HSA- and Aco-HSA-resistant HIV-1 strains that were not present in the wild-type HIV NL4-3 strain (Table 3) (26). The Suc-HSA-resistant strain showed seven mutations in the gp120 gene, two of which were located in the V3 loop region (S306R and A316S), and a deletion of five amino acids in the V4 loop that was also found to ocurr in the strains made resistant to AMD3100 (25), AMD2763 (25), DS (27) and AR177 (19). The Aco-HSA-resistant strain showed two mutations in the gp120 gene, at positions 164 (S to G), adjacent to the I165T mutation found in the Suc-HSA resistant virus, and at position 279 (D to N) which was common to both Aco-HSA- and Suc-HSA-resistant viruses. Surprisingly, we were able to detect the emergence of mutations in the gp41 gene of Suc-HSA-resistant virus (I515M, N674D) and Aco-HSA-resistant virus (Q548H, N637K and I704V) that were not present in the wild type strain when passaged in parallel in untreated cells. The distinct pattern of mutations found in

both Aco-HSA- and Suc-HSA-resistant viruses may indicate that HIV resistance to polyanions may not depend solely on the substitution of one or several specific amino acids.

Inhibition of virus binding to CD4$^+$ cells. DS, Aco-HSA and Suc-HSA inhibited the detection of p24 antigen bound to MT-4 cells after one hour incubation of cells with wild-type virus supernatant. The 50% inhibitory concentrations were 0.5, 3 and 39 µg/ml, respectively. P24 antigen bound to MT-4 cells was detected in cells

Table 3. Mutations found in the *env* gene of Suc-HSA and Aco-HSA-resistant virus.

Amino acid position[a] (region)	Wild type NL4-3	Suc-HSA-resistant NL4-3	Aco-HSA-resistant NL4-3
164 (V2)	S	S	**G**
165 (V2)	I	**T**	I
279 (C2)	D	**N**	**N**
306 (V3)	S	**R**	S
316 (V3)	A	**S**	A
345 (C3)	I	**M**	I
396 – 400 (V4)	FNSTW	**Deletion**	FNSTW
409 (V4)	T	T	**A**
513 (gp41 ectodomain)	I	**M**	I
548 (gp41 ectodomain)	Q	Q	**H**
635 (gp41 ectodomain)	N	N	**K**
672 (gp41 ectodomain)	N	**D**	N
702 (gp41 ectodomain)	I	I	**V**

[a]Numbering of amino acids according to the HIV gene and protein numbering scheme described in the Los Alamos database web site (26) for HIV-1 HXB2 gp160 protein (GenBank accession number K03455). Mutations indicated in boldface.

cultured with Suc-HSA-resistant and Aco-HSA-resistant supernatant. However, neither Suc-HSA nor Aco-HSA significantly inhibited virus binding (measured as bound p24 antigen) (Fig. 3). In fact, the binding of Suc-HSA-resistant virus to cells appeared to be enhanced by the presence of Suc-HSA in a dose-dependent manner. The reverse transcriptase inhibitor AZT (1.0 µg/ml), the chemokine SDF-1α (0.5 µg/ml), and the

fusion inhibitor AMD3100 (5 μg/ml) did no inhibit the binding of HIV to MT-4 cells when wild-type or NCA-resistant virus were used (data not shown).

Inhibition of gp120-CD4 interaction. Our results show that NCAs interact with gp120 (Fig. 2) and inhibited wild-type virus binding to $CD4^+$ cells but not NCA-resistant strains binding (Fig. 3). Furthermore, resistance to NCAs is accompanied by cross-resistance to dextran sulfate and zintevir but not to the chemokine blockers SDF-1α and AMD3100 (Table 2). To substantiate more rigorously the proposed mode of action of NCAs on HIV-1 replication, that is, through inhibition of gp120-CD4 interaction, the binding of sCD4 to cells expressing gp120 was evaluated by flow cytometry. Both Aco-HSA and Suc-HSA inhibited the binding of sCD4 to persistently infected H9 cells in a dose-dependent manner. MAb Q4120 at 20 μg/ml completely inhibited the binding of sCD4 to gp120-expressing cells but DS was less effective than either Aco-HSA or Suc-HSA (Fig. 4).

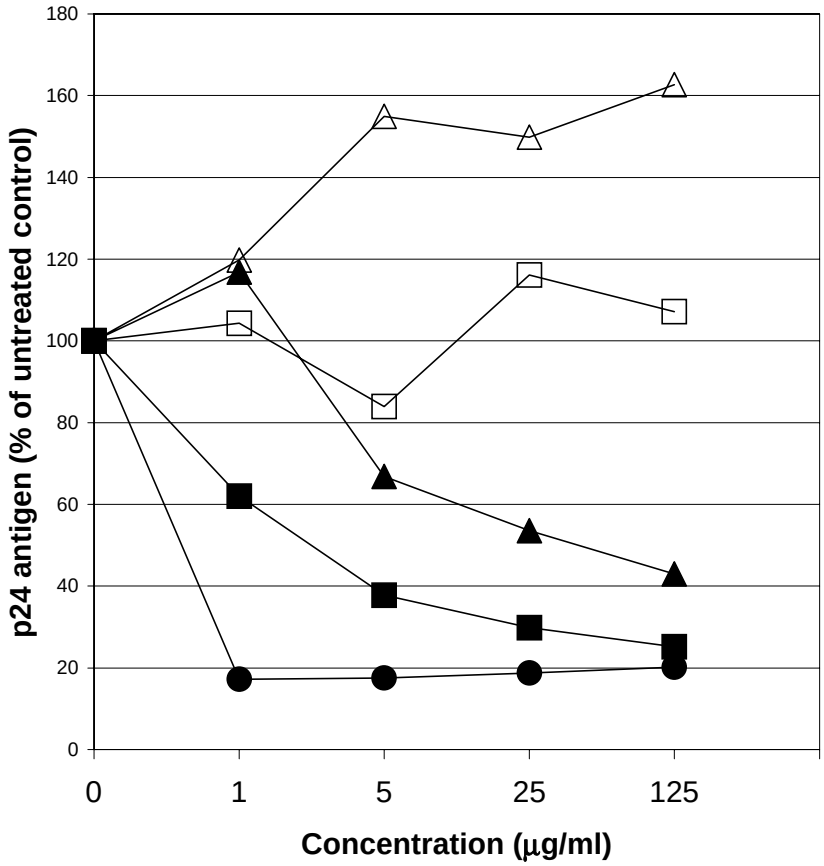

Figure 3. Inhibition of virus binding to MT-4 cells. The cells were incubated with 1 x 10^5 pg of p24 antigen of wild type HIV-1 NL4-3 in the presence of dextran sulfate (●), Suc-HSA (▲), or Aco-HSA (■), with Suc-HSA-resistant HIV-1 in the presence of Suc-HSA (△), or Aco-HSA-resistant HIV-1 in the presence of Aco-HSA (□), at various concentrations. After one hour incubation, cells were washed in PBS an p24 antigen bound to the cells was determined by a commercial test.

Interaction with chemokine receptors. Neither Suc-HSA (not shown) nor Aco-HSA at 25 µg/ml inhibited the binding of monoclonal antibody 12G5, which recognizes CXCR4 in a number of lymphoid cell lines and peripheral blood mononuclear cells (PBMCs) (23) (Fig. 5), whereas SDF-1α effectively blocked 12G5 binding. Similarly, neither Suc-HSA nor Aco-HSA inhibited the binding of MAb 2D7 to CCR5 in THP-1 cells (data not shown) or the binding of Leu3a to CD4 as previously reported (5).

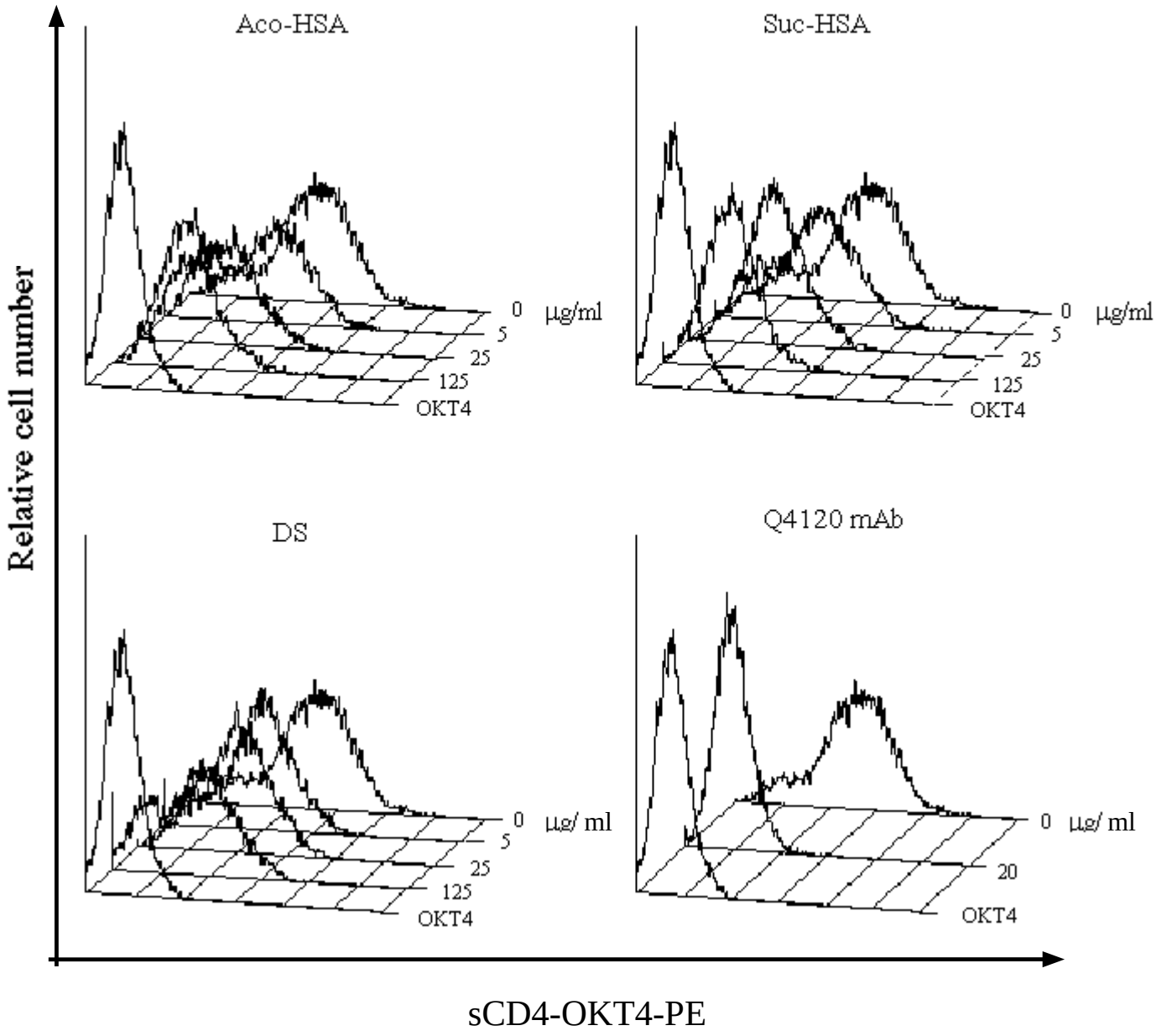

Figure 4. Interaction of gp120 with soluble CD4 (sCD4). Persistently HIV-1 IIIB-infected H9 cells were incubated with sCD4 (1 µg/ml) in the presence or absence of Aco-HSA, Suc-HAS or dextran sulfate (DS) at 0, 5, 25 and 25 µg/ml; or with Q4120 MAb (20 µg/ml), known to block the gp120-CD4 interaction. After 30 min, cells were washed and further incubated with PE-labeled anti-CD4 antibody OKT4 and analyzed by flow cytometry.

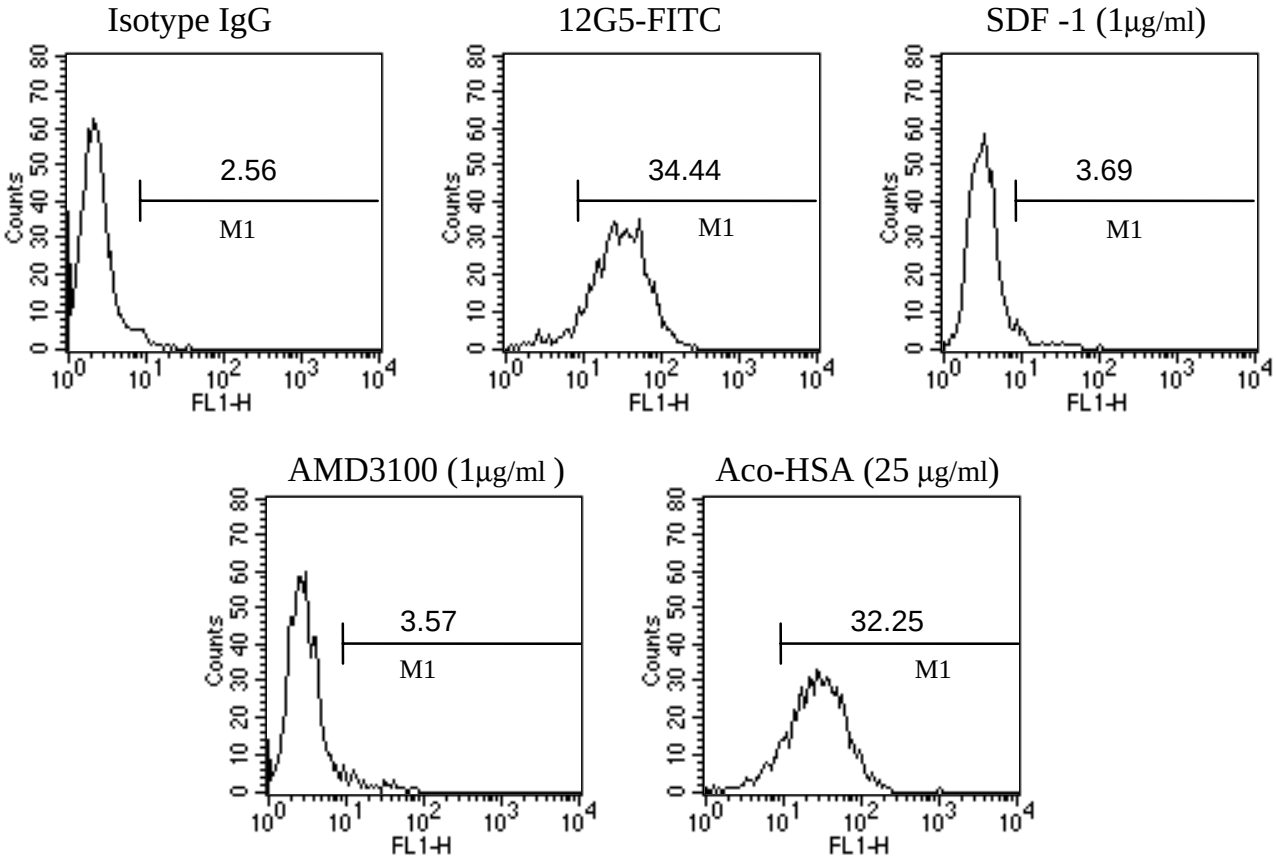

Figure 5. Flow cytometric histograms of the binding of anti-CXCR4 MAb (12G5) to SUPT-1 cells in the presence of Aco-HSA (25 µg/ml), AMD3100 (1 µg/ml) or SDF-1α (1 µg/ml) and a control RaM-IgGF(ab')$_2$-FITC (Isotype IgG). The mean fluorescence intensity values (M1) are shown.

DISCUSSION

Human serum albumins can be modified to become polyanions (negatively charged albumins, NCAs) with potent anti-HIV activity. Indeed, NCAs have been demonstrated as active agents against laboratory strains and clinical isolates of HIV, regardless of their syncytium-inducing capacity or cell tropism (27). These modified proteins are devoid of anticoagulant activity which is a common feature of other negatively charged substances with anti-HIV activity. The interaction of the HIV gp120 with the CD4 molecule plays a key role in the replication cycle of HIV and it is considered a potential target for therapeutic intervention (28).

We have found that the prototype NCA, Aco-HSA, binds to recombinant gp120 in a dose-dependent manner. The calculated kinetic constants for the interaction between gp120 and Aco-HSA were $K_a = 6.8 \times 10^2$ ($M^{-1}sec^{-1}$) and $K_d = 6.5 \times 10^{-4}$ (sec^{-1}). This is direct evidence that NCAs interact with the gp120 molecule. Time of addition experiments, in which cells infected at a high multiplicity of infection are treated at various time intervals after infection, have confirmed that NCAs, need to be added at the time of infection; they lose their inhibitory activity if they are not already present during the infection. The compound AZT, which works at a later step (reverse transcriptase), lost its protective capacity when added later than 4 hours postinfection. Furthermore, NCAs were unable to inhibit the binding of monoclonal antibodies directed to CXCR4 or CCR5 (12G5 and 2D7, respectively); these MAbs can inhibit HIV entry into cells expressing these receptors (24). Moreover, Aco-HSA-resistant and Suc-HSA-resistant HIV-1 strains remained sensitive to CXCR4 blockers such as SDF-1α and AMD3100. NCAs appear to interact with the immunogenic amino acid region GPGRAT, which is located in the gp120 V3 loop region of many laboratory-adapted strains of HIV (15b). Although this result suggests that NCAs could prevent the positively charged V3 loop from interacting with chemokine receptors such as CXCR4, our results indicate that NCAs do not interact with HIV-1 fusion coreceptors. Furthermore, we have found that, similar to the QS4120 antibody directed to the V1 region of CD4, the putative binding region of gp120, both Aco-HAS, and Suc-HSA effectively blocked the binding of soluble CD4 to gp120-expressing cells. Taken together, these results point to NCAs interaction with gp120 and interference with virus binding to $CD4^+$ cells as the mode of action of NCAs.

One way to identify the viral proteins targeted by an antiviral compound is through the development of resistance to the compound *in vitro*. We have previously shown that HIV-1 strains that are resistant to inhibitors targeted at the binding/fusion step of replication can emerge after sequential passaging of the virus in cell culture in the presence of the antiviral compound (19,25,27). Full resistance to the activity of compounds such as dextran sulfate will arise if the virus is given sufficient time and enough selective pressure for resistant virus to emerge. Resistance to the bicyclam AMD3100, the G quartet-forming oligonucleotide AR177, the chemokine SDF-1α and dextran sulfate appear to be mediated by a combination of specific mutations in the gp120 molecule of the resistant HIV strains (15a, 18, 27-29). Here, we show that resistance to NCAs develops after 24 passages of HIV-1 NL4-3 in MT-4 cells in the

presence of increasing concentrations of Suc-HSA or Aco-HSA. DNA sequence analysis of the *env* coding region showed in the resistant strains the emergence of mutations that were not present in the wild-type strain. The number of mutations required for partial resistance to NCAs could indicate that resistance to Aco-HSA or Suc-HSA may not be easily acquired *in vivo*.

To challenge directly the functional importance of the mutations found in the *env* gene of NCA-resistant virus, we tested the binding of wild-type, Suc-HSA-resistant- and Aco-HSA-resistant-virus to $CD4^+$ cells in the absence or presence of compound. Suc-HSA and Aco-HSA efficiently inhibited the binding of wild-type virus but they were less efficient in inhibiting virus binding when Suc-HSA- or Aco-HSA-resistant virus was used. Furthermore, Suc-HSA appeared to enhance the binding of the Suc-HSA-resistant strain to MT-4 cells. Although this phenomenon is not well understood, it could help to explain why certain polyanions of known anti-HIV activity *in vitro* have been shown to potentiate HIV replication *in vivo* (3, 30). Patients are commonly infected with a heterogeneous population of HIV strains, the so-called HIV quasispecies, that may differ in biological properties such as replication rate, cell tropism, and sensitivity to neutralizing agents (30-32). It is possible that naturally occurring strains with enhanced capacitiy to bind to $CD4^+$ cells are favored in the presence of polyanions such as Suc-HSA. The NCA-resistant strains, which express the mutant envelope glycoproteins, can now bind to CD4-positive cells in the presence of the compound, suggesting that the relevant mutant gp120 is no longer an efficient functional binding target of NCAs. The greater potency of Aco-HSA as an anti-HIV agent could explain the slower emergence of resistance to this compound than to Suc-HSA. It could also explain why the enhancement of virus binding observed with Suc-HSA was not observed with Aco-HSA. In view of our results we postulate that Aco-HSA should be preferred to Suc-HSA for development as a potential therapeutic agent.

Both NCA-resistant strains showed mutations in the DNA region coding the ectodomain of gp41. This protein is associated with postbinding, virus-cell membrane fusion. However, it has been shown that mutations in the gp41-coding region confer neutralization resistance to anti-V3 antibodies, indicating previously unrecognized interactions between regions of the two envelope proteins (33, 34). Resistance to neutralization by NCAs, which are directed to the gp120, may be determined by other regions of the envelope proteins. Although the role of each specific mutation needs to

be evaluated further, the results presented here could provide evidence of the functional interaction between gp120 and gp41.

Resistance to compounds such as the bicyclam AMD3100 or the chemokine SDF-1α, which block HIV-1 replication at the point of virus-chemokine receptor interaction, appear to modify the sensitivity of HIV to binding inhibitors such as NCAs. Indeed, the AMD3100-resistant virus previously reported (25, 35) appeared to be cross-resistant to polyanions such as DS and heparin (15,35) and cross-resistant to both NCAs (Table 2). However, DS-resistant virus or, as presented here, NCA-resistant viruses are not cross-resistant to the bicyclam AMD3100 or SDF-1α, and the pattern of mutations of the resistant viruses, including those of Suc-HSA and Aco-HSA are different. These results suggest that resistance to polyanions or to compounds that inhibit binding or fusion of HIV with $CD4^+$ cells may not depend solely on the substitution of one or several specific amino acids but rather depend on the overall change in the conformation of the gp120 molecule. Further studies of the mutations involved in HIV resistance to NCAs may provide important information on the mechanisms of virus escape from neutralizing agents directed to early stages of virus replication and on the interaction between different regions of HIV envelope proteins.

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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 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 for 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 relative 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.

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INTRODUCTION

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

MATERIALS AND METHODS

Compounds. Dextran sulfate (DS, molecular weight 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). 3'-azido-3'-deoxythymidine (AZT) was obtained from Wellcome (Greenford

UK). The oligonucleotides AR177 and ISIS5320 (Buckheit et al., 1994) were provided by author RFR, Aronex Pharmaceutical, Texas (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)-2,5diphenyltetrazolium 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 x 10^5 cells/ml) persistently infected with HIV-1 IIIB were cocultured with MOLT-4 (clone 8) cells (1.8 x 10^6 cells/ml) in the presence of AR177. After 24 h, 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°C and subsequent staining with fluorescein isothiocyanate-conjugated F(ab)2 fragments of rabbit anti-mouse immunoglobulin antibody, the cells were fixed and analysed 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 previously described (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°C 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 comercial 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°C 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 analysed 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-AGTACTATTAAACAGCTGTGTTGAATTAC) 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).

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.

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).

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.

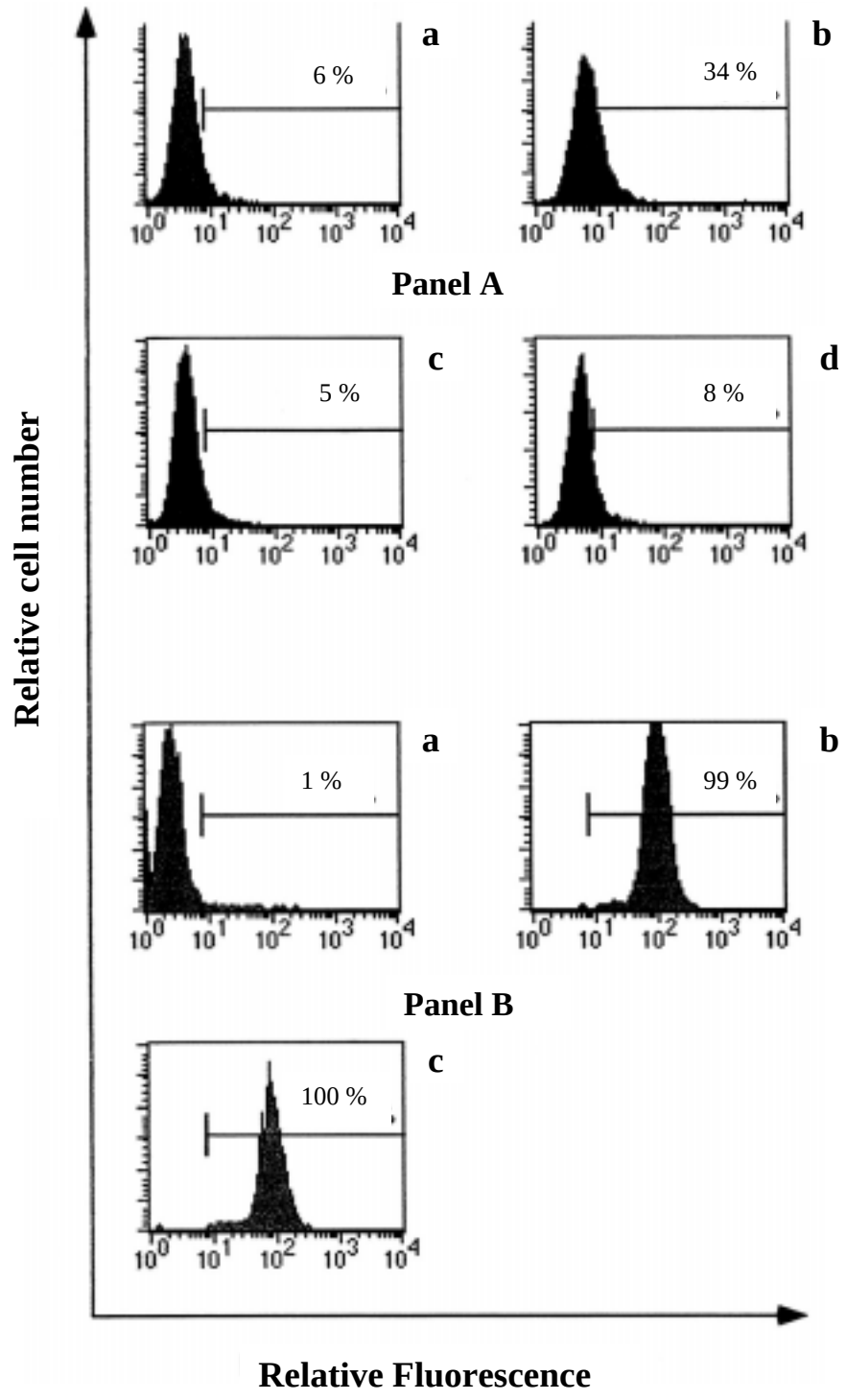

Figure 1. A, Flow cytometric histograms of the biding of anti-gp120 mAb (9284) on HUT-78 cells persistently infected with HIV-1 IIIB. Histogram: a, fluorescence of infected cells incubated only with RaM-IgG-F(ab')$_2$-fluorescein isothiocyanate; b, specific fluorescence of the infected cells; c and d, 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.

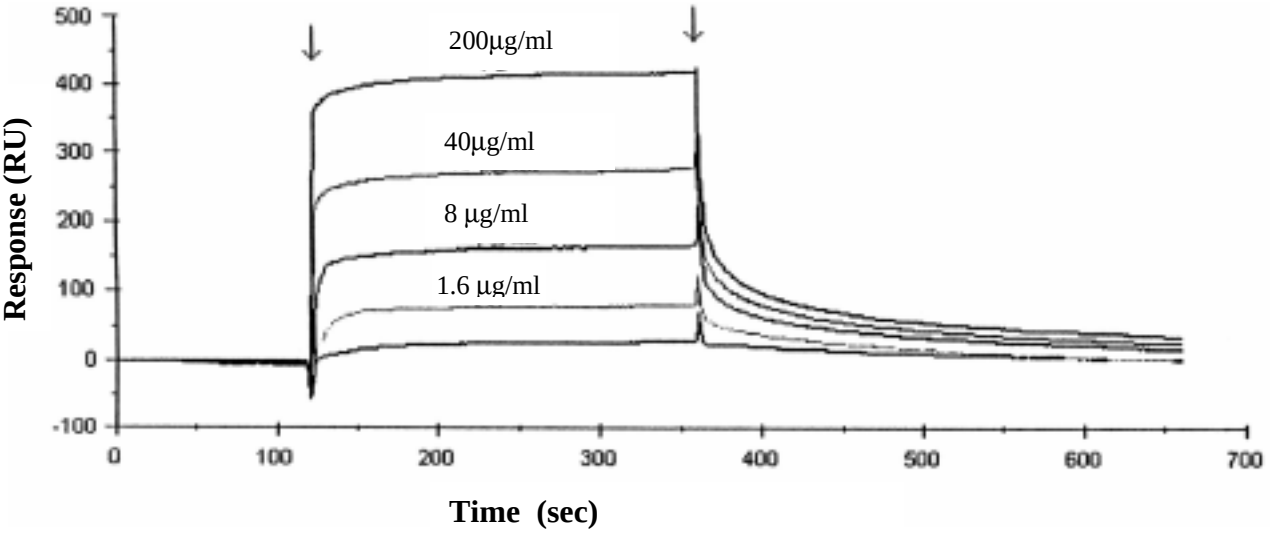

Figure 2. Sensorgrams obtained after injection of AR177 onto immobilized gp120. The concentrations of AR177 are 1.6, 8, 40 and 200 µg/ml. Arrows, the start and end of the injection.

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. 4). No mutations were found in the sequence of the integrase gene of the AR177-resistant strain when compared to the wild-type strain (Cherepanov et al., 1997).

Table 2. Anti-HIV activity of different compounds against wild-type HIV-1 and AR177-resistant HIV-1

Compound	EC_{50} (μg/ml) wild-type NL4-3	EC_{50} (μg/ml) $AR177^r$ NL4-3	CC_{50} (μg/ml)
AR177	0.6	>125	> 125
DS (Mr. 5000)	0.7	23.8	> 125
Heparin	0.2	3.9	> 125
ISI5320	0.2	>10	> 10
AMD3100	0.003	0.004	> 125

EC_{50} based on the inhibition of HIV-induced cytopathicity in MT-4 cells, as determined by the MTT method.
CC_{50} based on the viability of MT-4 cells, as determined by the MTT method.

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

Amino acid position[a] (region)	NL4-3 wild-type strain		NL4-3 $AR177^r$ strain	
	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).

Virus-binding assay. AR177 and DS inhibited the detection of p24 antigen bound to MT-4 cells after one hour incubation of cells with wild type virus supernatant. The 50% inhibitory concentration (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 meassure as bound p24 antigen) (Fig. 3). Similarly, the reverse transcriptase inhibitor AZT (1.0 μg/ml) or the fusion inhibitor AMD3100 (125 μg/ml) did no inhibit the binding of HIV to MT-4 cells when wild-type or AR177-resistant virus were used (data not shown)

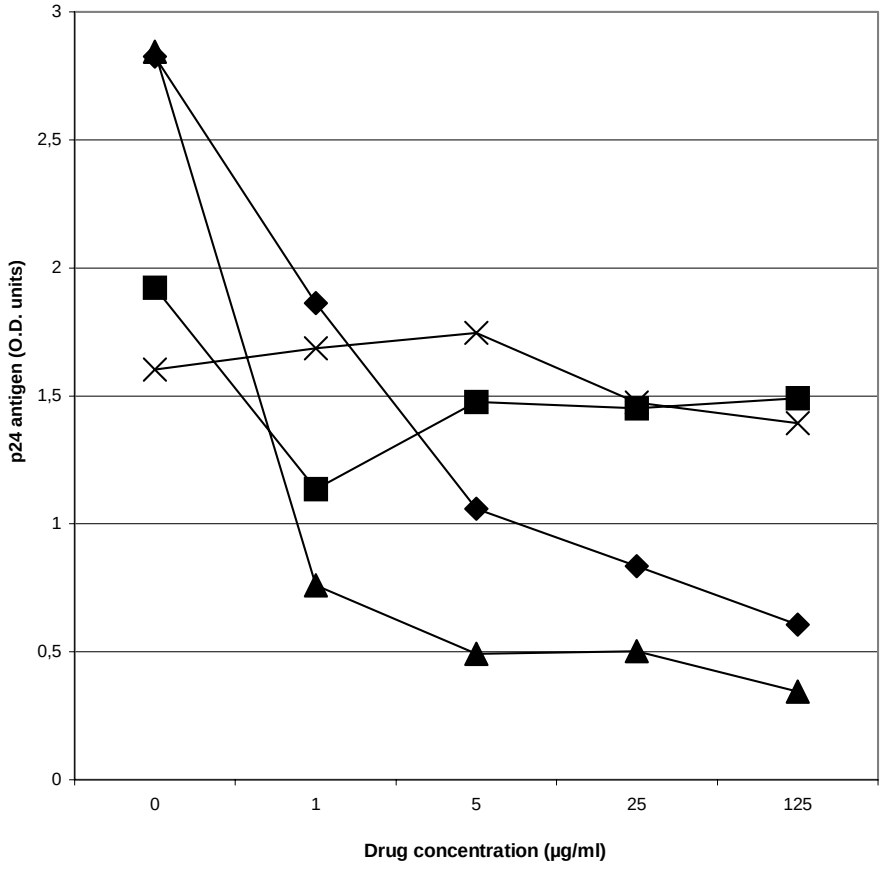

Figure 3. Inhibition of virus binding to MT-4 cells. The cells were incubated with 1 x 10^5 pg of p24 antigen of wild-type HIV-1 NL4-3 in the presence of varying concentrations of AR177 (♦) or DS (▲) or AR177-resistant HIV-1 in the presence of AR177 (■) or DS (x). After one hour incubation, cells were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

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 partiallly cross-resistant to DS (25-fold).

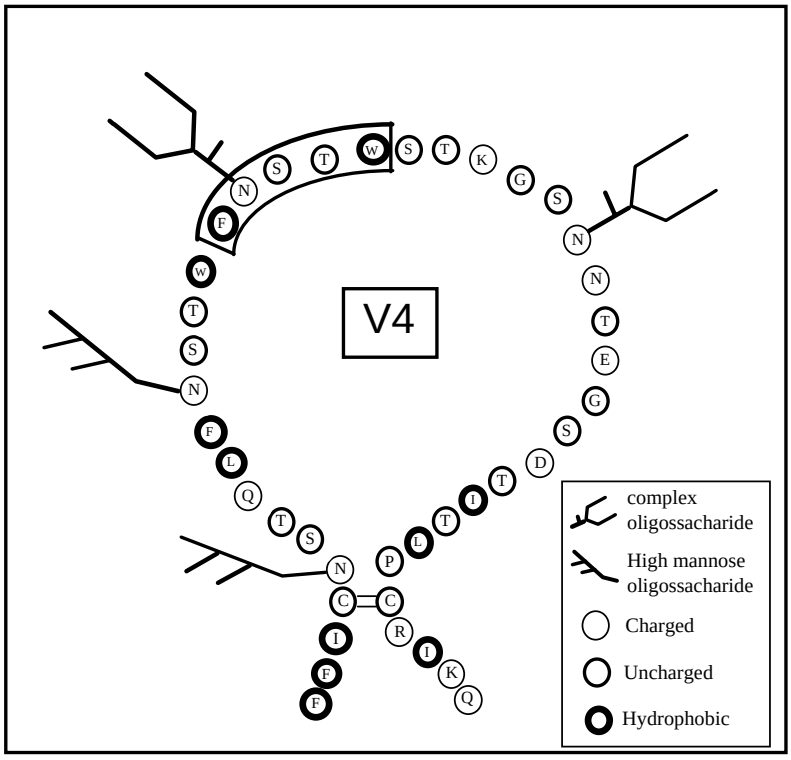

Figure 4. Amino acid sequence of the V4 loop of HIV-1 (Gallagher et al., 1995). Boxed amino acids (FNSTW), deletion found in the AR177-resistant strain.

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 appear both 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 replicative 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 transciptase 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 CD4-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 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

measure 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 appears 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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