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Structure–activity relationship of neomycin, paromomycin, and neamine–arginine conjugates, targeting HIV-1 gp120–CXCR4 binding step

Gadi Borkow, Veerappan Vijayabaskar, Humberto Herman Lara[1], Alexander Kalinkovich[2], Aviva Lapidot*

Department of Organic Chemistry, Weizmann Institute of Science, 76100 Rehovot, Israel

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Abstract

We have recently designed and synthesized aminoglycoside–arginine conjugates (AACs) as potential anti-HIV-1 agents. AACs exert a number of activities related to Tat antagonism. We here present a new set of AACs, conjugates of neomycin B, paromomycin, and neamine with different number of arginines (1–6), their (a) uptake by human T-cell lines, (b) antiviral activities, (c) competition with monoclonal antibody (mAb) 12G5 binding to CXCR4, (d) competition with stromal cell-derived factor-1 (SDF-1α) binding to CXCR4, and (e) competition with HIV-1 coat protein gp120 cell penetration. The appearance of mutations in HIV-1 gp120 gene in AACs resistant HIV-1 isolates, supports that AACs inhibit HIV-1 infectivity via interference of gp120–CXCR4 interaction. Our results point that the most potent AACs is the hexa-arginine–neomycin conjugate, the other multi-arginine–aminoglycoside conjugates are less active, and the mono-arginine conjugates display the lowest activity. Our studies demonstrate that, in addition to the core, the number of arginines attached to a specific aminoglycoside, are also important in the design of potent anti-HIV agents. The AACs play an important role, not only as HIV-1 RNA binders but also as inhibitors of viral entry into human cells.

Keywords: HIV-1; Aminoglycoside–arginine conjugates (AACs); Viral entry; CXCR4 blocking; gp120 gene mutations; HIV-1 therapeutic

1. Introduction

Despite intense progress that has been made in the past decade in anti-HIV-1 therapy, mainly by simultaneous administration of inhibitors of HIV-1 reverse transcriptase and protease, eradication of HIV-1 in infected individuals has not been achieved. Many of the available drugs have significant side effects, and in all cases low compliance of the patients results in the appearance of HIV-1 drug resistance (Michael and Moore, 1999). This emphasizes the urgent need for novel classes of anti-HIV drugs.

Significant advances in understanding the processes by which HIV-1 enters the host cells (Berger, 1997; Ugolini et al., 1997; Hung et al., 1999; Fernandez and Lolis, 2001) have brought into clear focus that HIV-1 receptors represent attractive targets for therapeutic intervention. Current models predict that HIV-1 entry is initiated by the interaction of the viral envelop protein gp120 with the host cells receptor CD4, which results in exposure of the gp120 domains. Although the interaction of gp120 with CD4 receptor is an obligatory step for efficient infection of cells by HIV-1, it is now known that an additional receptor(s) or coreceptor(s) is required for HIV-1 entry and infection. The identity of these coreceptors was elucidated only recently, being the chemokine receptors CXCR4 and CCR5 the main HIV-1 coreceptors (along with CD4). CXCR4 plays an important role in many biological functions, such as B-cell lymphopoiesis, neuronal cell migration, and vascular development (Nagasawa et al., 1996; Ma et al., 1998; Zou et al., 1998). The stromal cell-derived factor-1 (SDF-1α), the only known natural ligand of CXCR4 displays important roles in migration, proliferation, and differentiation of leukocytes (Oberlin et al., 1996; Bleul et al., 1996). CXCR4 belongs

* Corresponding author. Tel.: +972-8-934-3413; fax: +972-8-934-4142/2559.
E-mail address: aviva.lapidot@weizmann.ac.il (A. Lapidot).
[1] Present address: University of Monterey, Mexico.
[2] Present address: Department of Immunology, Weizmann Institute of Science, Rehovot, Israel.

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to the superfamily of G-protein-coupled receptors. These membrane proteins, which transmit signals from extracellular ligands to intracellular biological pathways, are a major class of therapeutic targets in a wide variety of human diseases (for review see Strader et al., 1994).

Several peptide-derived and other small molecule inhibitors of CXCR4-mediated HIV-1 infection have been described in the past few years (De Clercq, 2002; Schwarz and Wells, 2002). Interestingly, several of these molecules, e.g. ALX40-4C (O'Brien et al., 1996; Doranz et al., 1997) and CGP64222 (Hamy et al., 1997; Daelemans et al., 2000) were designed as HIV-1 transcription transactivator protein (Tat) basic domain mimetics that target HIV-1 transactivator responsive element (TAR) RNA in HIV-1 long terminal repeats (LTR). Interaction of HIV-1 Tat with TAR increases significantly the transactivation of the viral transcripts by cellular factors (Weeks et al., 1990; Karn, 1999). Tat-derived basic peptides and even nona-arginine peptide bind TAR RNA with high affinity in vitro (Hamy et al., 1997; Daelemans et al., 2000). Much less efficient than peptides, is the aminoglycoside antibiotic neomycin B that binds to TAR RNA at the lower stem and the bulge region (Wang et al., 1998; Faber et al., 2000).

Recently, based on peptide models of TAR RNA binding, NMR structures of TAR–ligand complexes and aminoglycoside–RNA interactions, we have designed and synthesized a set of novel peptidomimetic substances, conjugates of aminoglycoside antibiotics with arginine (Litovchick et al., 1999, 2000, 2001; Lapidot and Litovchick, 2001). These aminoglycoside–arginine conjugates (AACs), which include tetra-arginine–kanamycin A conjugate (R4K), tri-arginine–gentamicin C conjugate (R3G), and hexa-arginine–neomycin B conjugate (NeoR6), display high affinity to TAR RNA (Litovchick et al., 1999, 2000, 2001). The AACs, which have been found to efficiently penetrate cells, including neurons, and accumulate intracellularly (Litovchick et al., 1999, 2000, 2001), are expected to be resistant to enzymatic degradation, since AACs are not natural molecules and do not contain peptidase/protease recognition motifs. The AACs inhibit HIV-1 infection and proliferation in cultured human lymphocytes, displaying low cytotoxicity (Litovchick et al., 2000, 2001; Cabrera et al., 2000, 2002). Another important findings are that NeoR6 inhibits gp120-triggered death in human neuroblastoma cells, and crosses the blood–brain barrier (Catani et al., 2003).

Importantly, similarly to the Tat-mimetic peptide/peptoid compounds ALX40-4C and CGP64222 that target TAR RNA, but also inhibit HIV-1 T-tropic infection by blocking of CXCR4 coreceptor, we have also found that AACs, designed as Tat-mimetics, function also as CXCR4 antagonists (Litovchick et al., 2001; Cabrera et al., 2002). Our findings proved that NeoR6 competes with monoclonal antibodies binding to CXCR4 and inhibits raise of intracellular Ca^{2+} induced by SDF-1α (Litovchick et al., 2001; Cabrera et al., 2002). We also found that AACs antagonize some of the extracellular properties of HIV-1 Tat protein, such as increased viral production, induction of CXCR4 chemokine receptor expression, suppression of CD3-activated proliferation of lymphocytes, and upregulation of CD8 receptor (Litovchick et al., 2001), indicating that AACs and Tat bind to similar cellular targets. These results are in agreement with the finding of Xiao et al. (2000) that Tat protein behaves as specific CXCR4 antagonist which inhibits the entry and replication of X4 but not R5 viruses in PBMC.

In the present study, we demonstrate the structure–activity relationship of new AACs. We show the effects of the aminoglycoside core and the number of arginine residues conjugated to it, on the capacity of the AACs to compete with the binding of (a) mAb 12G5, (b) SDF-1α, or (c) the viral protein gp120, binding to CXCR4. Our results reveal significantly different affinity of the various AACs to CXCR4, as a result of different aminoglycosides and number of arginines conjugated to it. The hexa-arginine conjugate of neomycin B reveals the highest activity among the multi-arginine–aminoglycoside conjugates, while the mono-arginine derivatives of each one of the aminoglycoside used in this study, neomycin, paromomycin, and neamine exert the lowest activity. Since AACs were found to interfere with CXCR4 activities, as described before, it was of interest to test if resistance to AACs would involve mutations in the HIV-1 gp120 protein gene. Our results indeed show the appearance of several gene mutations in the HIV-1 gp120 protein of the AAC resistant HIV-1 isolates in comparison to the wild-type gp120.

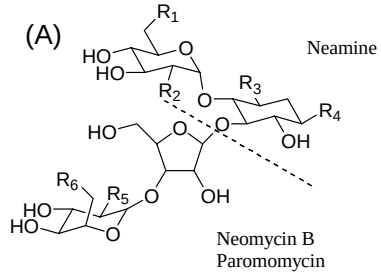
2. Materials and methods

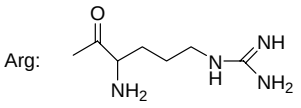
2.1. Synthesis of aminoglycoside–arginine conjugates

The procedures for the synthesis and purification of NeoR6 and R3G have been detailed (Litovchick et al., 1999, 2000, 2001). Improved synthesis of NeoR6 and synthesis, and chemical characterization of NeoR1, NeoR2, NeamR1, NeamR4, ParomR1 and ParomR5 (Fig. 1) will be published elsewhere. Neomycin B and paromomycin were purchased from Fluka. Neamine was prepared from neomycin as previously described (Park et al., 1996) with some modification.

2.2. AACs fluorescent derivatives

The acetate anions of all the AACs were removed by ion exchange chromatography by AG-MP 1 (converted from Cl to OH form, 100–20 mesh) using water as an eluent. The collected eluent was evaporated to dryness using Speedvac concentrator. AACs fluorescent derivatives (NeoR1-FITC, NeoR2-FITC, NeoR6-FITC, NeamR1-FITC, NeamR4-FITC, ParomR1-FITC, ParomR5-FITC, and R3G-FITC) were prepared by reacting AACs with fluorescent isothiocyanate (FITC, Sigma) in a 1:1 molar ratio in water:methanol:dioxane (1:1:1 v/v) mixture for 1 h at room





	R_1	R_2	R_3	R_4	R_5	R_6
Neomycin	NH2	NH_2	NH_2	NH_2	NH_2	NH_2
NeoR6	Arg	Arg	Arg	Arg	Arg	Arg
NeoR1	Arg	NH_2	NH_2	NH_2	NH_2	NH_2
NeoR1	NH_2	NH_2	NH_2	NH_2	NH_2	Arg
NeoR2	Arg	NH_2	NH_2	NH_2	NH_2	Arg
Neamine	NH_2	NH_2	NH_2	NH_2		
NeamR1	Arg	NH_2	NH_2	NH_2	--	--
NeamR4	Arg	Arg	Arg	Arg	--	--
Paromomycin	OH	NH_2	NH_2	NH_2	NH_2	NH_2
ParomR1	OH	NH2	NH_2	NH_2	NH_2	Arg
ParomR5	OH	Arg	Arg	Arg	Arg	Arg

	R_1	R_2	R_3
Gentamicin C1 R3G	NH_2 Arg	NH_2 Arg	NH_2 Arg

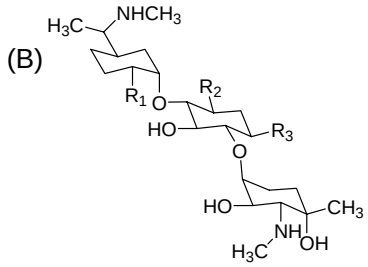


Fig. 1. Schematic representation of AACs and aminoglycosides used in this study. All AACs were prepared as acetate salts. (A) NeoR6, hexa-arginine–neomycin conjugate; NeoR1, a 1:1 mixture of two mono-arginine–neomycin conjugates; NeoR2, a di-arginine–neomycin conjugate; NeamR1, a mono-arginine–neamine conjugate; NeamR4, a tetra-arginine–neamine conjugate; ParomR1, a mono-arginine–paromomycin conjugate; and ParomR5, a penta-arginine–paromomycin conjugate. (B) R3G, a tri-arginine–gentamycin C1 conjugate.

temperature. The FITC–AAC conjugates were purified as described recently for NeoR6 (Litovchick et al., 2001).

2.3. Cell culture and inhibition of HIV-1 infection

Peripheral blood mononuclear cells (PBMC), PM1, MT2, and H9 (lymphocyte cell lines permissive to T-tropic HIV-1 isolates), U38 (a macrophage cell line permissive to M-tropic HIV-1 isolates), cMAGI (cMAGI cell line is permissive to both M- and T-tropic HIV-1 isolates as well as to primary and laboratory adopted HIV-1 isolates; Chackerian et al., 1997; Collins et al., 2000), and H9+ (H9 cells chronically infected with HIV-1_{IIIB}) cell lines were cultured in RPMI 1640 medium (GibcoBRL, Life Technologies, Paisley, UK) containing 10% fetal calf serum (FCS) and antibiotics. T-tropic HIV-1 isolates were propagated by subculture in MT2 as described previously (Litovchick et al., 2001). Aliquots of cell-free culture supernatants were used as viral inoculum. Each one of the AACs was dissolved in the RPMI 1640 medium. Cytotoxicity determinations were carried out in MT2 and H9 cells by trypan blue exclusion assay. Viral inhibition was determined by incubating cMAGI HIV-1 reporter cells with 0.2–0.5 multiplicity of infection of HIV-1_{IIIB} for 4 days at 37 °C in the presence or absence of various concentrations of AACs, before counting the number of HIV-1 infected cells (stained blue). The cytopathic effects of the viral infection of MT2 cells were also analyzed by microscopic assessment of syncytium formation. These latter data were obtained by analysis of duplicate samples by two independent observers.

2.4. Cellular uptake using AAC fluorescent probes

U38, PM1 or H9 cells, or PBMC (10^5 in 100 μl RPMI 1640 medium) were incubated for 20 min at room temperature with each one of the AACs–FITC derivatives at a final concentration of 0.5 μM, washed twice, resuspended in 100 μl medium and analyzed with a Zeiss Axiophot fluorescent microscope.

2.5. Cellular uptake competition of AACs with NeoR6-FITC

Phosphate-buffered saline (PBS) containing 0.5 μM NeoR6-FITC only or mixed with 10-, 40- or 100-fold

higher concentrations of non-labeled AACs, was added to 10^5 MT2 cells (50 μl final volume). After 5 or 15 min of incubation at room temperature, the cells were washed twice with ice-cold PBS and fixed in PBS containing 1% paraformaldehyde. The fluorescence was then analyzed by flow cytometry (FACScan, Becton Dickinson Imunocytometry Systems, San Jose, CA). For each sample 10,000 events were acquired. Data were analyzed and processed using CellQuest™ software (Becton Dickinson).

2.6. Viral binding assay

To measure the ability of AACs to inhibit viral binding to cells, HIV-1 viral particles were radioactively labeled by endogenous reverse transcription (ERT), as previously described (Borkow et al., 1997; Litovchick et al., 2001). Briefly, 0.5 ml of HIV-1 particles (1.5–5 ng of p24) were added to 0.5 ml of ERT reaction mixture (final concentrations: 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 50 μM each dATP, dTTP, dCTP, and dGTP, as well as 50 μCi of [α-^{32}P]dATP). After 2 h of incubation at 37 °C the reaction mixture was diluted with PBS to 2 ml, and the viral particles were concentrated to final volume of 50 μl using Centricon YM-50 centrifugal concentrator (Millipore, Bedford, MA), according to the manufacturer's directions. This procedure was repeated four times in order to remove unbound radiolabel. The viral particles were incubated for 2 h at 37 °C with 200,000 MT2 or U937 cells in 0.5 ml of RPMI/10% FCS in the presence of various concentrations of AACs. Following the incubation, the cells were washed twice with PBS by centrifugation at 400 × *g* for 10 min. The pellets were resuspended in PBS and transferred onto glass-fiber filters (Millipore). The filters were washed twice with PBS, dried, and counted by a gamma-counter.

2.7. Interaction of AACs with CXCR4 receptor

Interaction of AACs with CXCR4 was determined by flow cytometry (FACScan, Becton Dickinson) as described recently (Litovchick et al., 2001). Briefly, 0.5×10^6 cells were washed in ice-cold PBS containing 0.1% sodium azide (wash buffer) and incubated at 4 °C with anti-CXCR4 mAb, 12G5, conjugated to phycoerithrine (PE), in the absence or presence of different concentrations of the AACs. After 30 min of incubation, the cells were washed with ice-cold wash buffer and fixed in PBS containing 1% paraformaldehyde. Non-specific fluorescence was assessed by using an isotype-matched control. For each sample 10,000 events were acquired. Data were analyzed and processed using CellQuest™ software (Becton Dickinson).

2.8. Competition between AACs fluorescent probes with CXCR4 natural ligand SDF-1α and HIV-1 gp120

Competition between FITC-labeled AACs and SDF-1α or HIV-1 gp120 was determined as follows: FITC-labeled AACs (1 μM) NeoR6, NeamR4, ParomR5, NeoR1, NeoR2, NeamR1, and ParomR1 were incubated at 4 °C with 2×10^5 PM1, MT2 or PBMC, with or without several concentrations of SDF-1α (R&D Systems, Minneapolis) or with 5 μM recombinant HIV-1_{IIIB} gp120 (NIH AIDS Research & Reference Reagent Program). After 30 min of incubation, the cells were washed with ice-cold wash buffer and analyzed by flow cytometry as described above.

2.9. Competition between AACs fluorescent probes with the CCR5 chemokine RANTES

Competition between FITC-labeled AACs (1 μM) and RANTES (Pepro Tech Inc., NJ) (2.5 μM) were determined in 2×10^5 cMAGI cells. After 30 min of incubation the cells were washed with ice-cold wash buffer and analyzed by flow cytometry as described above.

2.10. Selection of NeoR6 HIV-1 resistant isolates

MT2 cells (3×10^5 cells in 1 ml) were preincubated for 30 min with 1.9 μM of NeoR6 (the EC_{50} value) and then infected with HIV-1_{IIIB} (5×10^5 $TCID_{50}$). Culture fluids were replaced twice weekly with fresh medium containing an appropriate drug concentration. During the propagation of the virus, when at each cycle (at certain NeoR6 concentration) about 70% syncytium appeared, 250 μl of undiluted clarified culture supernatant, obtained from the HIV-infected cells, were added to 3×10^5 fresh MT2 cells in 1 ml final volume, containing two times higher NeoR6 concentration. From the final cycle of each experiment, the resistant virus was propagated as described above. The EC_{50} of the AACs against the resistant NeoR6 isolates was examined and compared to the wild-type (wt) virus. After 24–26 cycles of selection of NeoR6 HIV-1 resistant isolates, genomic DNA was purified from the infected MT2 cells according to Sambrook et al. (1989). A fragment of 648 bp of proviral HIV-1 DNA corresponding to the HIV-1 gp120 sequence were amplified by PCR with Taq DNA polymerase (Sigma, Rehovot, Israel) and the following forward and reverse primers, respectively: 5′-CACTTCTCCAATTGTCCCTCA-3′ and 5′-TGTTAAATGGCAGCCTAGCA-3′ (Biological Services, Weizmann Institute of Science). Amplified products were purified by gel electrophoresis on 2% agarose gels. Sequencing was carried out by using the forward primer with an ABI Prism, 3700 DNA Analyzer, PE, Applied Biosystems, Hitachi.

3. Results

A series of AACs based on the aminoglycosides neomycin, gentamicin, neamine, and paromomycin, recently prepared in our laboratory (Vijayabaskar et al., submitted) were used in the current study. NeoR6 (previously described as NeoR; Lapidot and Litovchick, 2001; Litovchick

et al., 2001), contains six arginine groups conjugated to the three pyranoside rings of neomycin B (Fig. 1A). NeoR1 is 1:1 mixture of two mono-arginine substituted neomycin isoforms, in which either ring I or IV contains an arginine residue (Fig. 1A). NeoR2 is a di-arginine–neomycin derivative in which both rings I and IV contain an arginine residue (Fig. 1A). NeamR1 and NeamR4 contain a neamine core with either one or four arginine groups, respectively, attached to amino groups present on the two pyranoside rings of the aminoglycoside (Fig. 1A). ParomR1 and ParomR5 contain a paromomycin core with 1 or 5 arginine groups, respectively, attached to amino groups present on three pyranoside rings of paromomycin (Fig. 1A). R3G contains three arginine groups conjugated to two of the three gentamicin C1 pyranoside rings (Fig. 1B). These compounds are peptidomimetic and share features of relative rigidity of the pyranoside sugar rings with relative flexibility of inter-ring and side chain links. The arginine groups are important for high affinity AACs–RNA interaction, at least with respect to interaction with the HIV-1 TAR element and HIV-1 Rev Response Element (RRE) (Litovchick et al., 2000, 2001).

3.1. Anti-HIV activity of AACs

The AACs described above inhibited a variety of HIV-1 isolates, including HIV-1_{IIIB} laboratory adapted T-tropic isolate, clade C HIV-1 clinical isolate, as well as AZT, 3TC, and UC781 resistant HIV-1_{IIIB} strains, with no significant differences in the concentrations that caused 50% inhibition of viral production (EC_{50} of 1.7–6.2 μM for the multi-arginine conjugates and >10 μM for the mono-arginine conjugates). In contrast, the aminoglycoside antibiotics: neamine, gentamicin, neomycin, and paromomycin did not reveal antiviral activity. The concentration of AACs that caused cytotoxicity in 50% of the cells (CC_{50}) was higher than 250 μM. The in vitro 50% therapeutic index (TI_{50} = ratio CC_{50}/EC_{50}) is therefore at least 40 for most AACs. Importantly, as shown in two representative examples for R3G and NeoR6 in Fig. 2, the presence of the AACs compounds only during the first 2 h of infection, inhibited viral proliferation, indicating that the AACs can inhibit the first stages of HIV-1 infectivity, and/or that the AACs are taken readily into the cells, and inhibit subsequent viral infectivity steps.

3.2. Effect of AACs on viral binding to cells

In order to test if the AACs may interfere with the binding of the virus to the cells, we studied the competition of NeoR6 and R3G with the binding of ^{32}P-labeled (see Section 2; Borkow et al., 1997) HIV-1 particles to MT2 cells (CD4+ T-lymphocytes) or to U937 cells (CD4+ monocytes). Both compounds efficiently inhibited in a dose-dependant manner the binding of HIV-1_{IIIB} to MT2 cells or HIV-1 clinical isolate clade C to U937 cells (Fig. 3).

3.3. Cellular uptake of AACs

Fluorescent-labeling studies have shown that R4K, R3G, and NeoR6 accumulate in different cell types, including lymphocytes, PBMC, and neurons (Litovchick et al., 2000, 2001; Catani et al., 2003). Similarly, as shown in Fig. 4A for ParomR5 and NeamR4 labeled with FITC, as well as the mono- and di-arginine conjugates of neomycin B, mono-arginine-paramomycin and mono-arginine–neamine conjugates labeled with FITC, also penetrate and accumulate

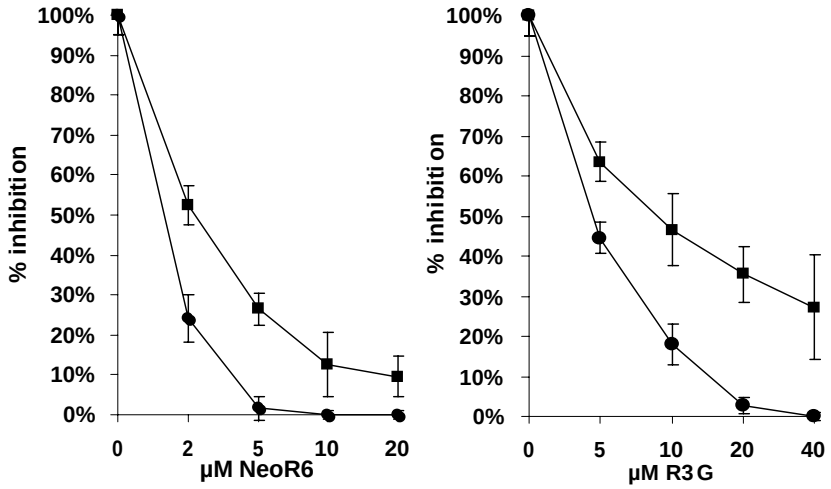


Fig. 2. Inhibitory effect of NeoR6 and R3G on the replication of HIV-1 clade C infection in MT2 cells. MT2 cells were infected for 2 h at 37 °C in the absence or presence of 2–20 μM NeoR6 or 5–40 μM R3G followed by cell wash. About 5×10^4 infected cells were seeded per well in 96-well plate and were incubated for 4 days in the absence or presence of the appropriate drug concentrations, until syncytia were observed (>25% cpe). Cell viability was measured by tetrazolium-based colorimetric method. The results shown are mean ± S.D. of triplicates. (●) The AACs were present during the infection step and after the cells were washed; (■) the AAC were present only during the first 2 h, before the cells were washed.

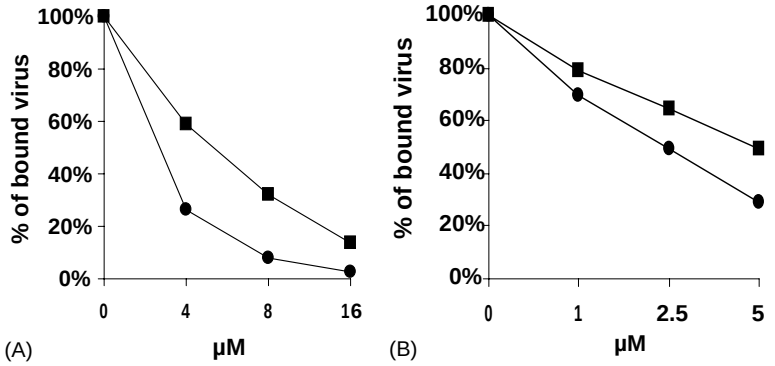


Fig. 3. Inhibitory effect of NeoR6 and R3G on binding of HIV-1_{IIIB} to MT2 cells (A) and HIV-1 clade C to U937 cells (B). The viral particles of HIV-1_{IIIB} or HIV-1 clade C clinical isolate were radioactively labeled by ERT, as described in Section 2. After excess of radiolabel was washed out, these particles were used to infect MT2 (A) or U397 (B) cells. The infection was performed for 2 h at 37 °C in the presence of various concentrations of NeoR (●) or R3G (■). Following the infection, the cells were washed thoroughly and the radioactivity of the cell pellet was determined.

in less than 10 min of incubation in macrophages (U38) and lymphocytes (H9 and PM1 cells).

In order to test if the AACs may compete for cell uptake, we examined the uptake of NeoR6-FITC (0.5 μM) in the absence and presence of excess of AACs by flow cytometry after 5 or 15 min of incubation in MT2 cells (Fig. 4B). At 100-fold excess of the non-labeled AACs than NeoR6-FITC, the uptake of NeoR6-FITC was inhibited by ParomR5, NeamR4, and NeoR2, about 50% less efficient than by NeoR6. Whereas, the mono-arginine derivatives of NeoR1, NeamR1, and ParomR1 have not exert a significant competition with NeoR6-FITC. At 40-fold excess of the AACs than NeoR6-FITC, only ParomR5 inhibited cellular uptake of NeoR6-FITC 50% less efficient than NeoR6 (Fig. 4B).

3.4. Inhibition of anti-CXCR4 mAb binding to cells by AACs

The inhibition of T-tropic HIV-1 isolates binding to cells, together with our previous observations, showing that NeoR6 and R3G interact with CXCR4, but not with CD4 or CCR5 (Cabrera et al., 2000, 2002; Litovchick et al., 2001), led us to further investigate the interaction of the new AACs with CXCR4. The capacity of the various AACs to block the binding of the PE-labeled 12G5 monoclonal antibody (mAb) to CXCR4 in PM1 cells and in PBMC was examined and is summarized in Table 1 as percentage of inhibition. As shown in one representative experiment in Fig. 5, the median fluorescence intensity (MFI) of 12G5 mAb binding to PM1 cells was 451, while of the isotype control was 30. In the presence of 10 μM of NeoR6, NeoR2, NeoR1, ParomR5, ParomR1, NeamR4, NeamR1, and R3G, the MFI of the mAb binding to the cells was reduced to 76, 237, 231, 118, 385, 189, 429, and 89, respectively. In contrast, no inhibition of the 12G5 mAb binding to the cells was noted even in the presence of 20 μM of neomycin, paromomycin, and neamine (Table 1).

3.5. Inhibition of FITC-labeled AAC uptake by stromal cells-derived factor 1α (SDF-1α)

Based on the above results, and since SDF-1α is the natural ligand of CXCR4, we examined if the cellular uptake of the various AACs would be reduced in the presence of SDF-1α. For control, we present the capacity of SDF-1α to inhibit the binding of PE-labeled anti-CXCR4 mAb 12G5 to PM1 cells. As shown in Fig. 6, 62.5 and 125 nM of SDF-1α inhibited significantly the binding of the anti-CXCR4 mAb 12G5 to cells. Similar results were obtained when PBMC were used. As shown in one representative experiments using PM1 cells (Fig. 6), SDF-1α decreased NeoR6-FITC, ParomR5-FITC, and NeamR4-FITC (1 μM each) cell uptake in a dose-dependant manner. While 62.5 nM of SDF-1α caused minor inhibition of NeoR6 (∼10%), 125, and 250 nM of SDF-1α inhibited 30 and 40% cell uptake of NeoR6, respectively (from MFI of 61 to 54, 46 and 42, for 62.5, 125, and 250 nM SDF-1α, respectively, and a background MFI of 10). Cell uptake inhibition by SDF-1α at concentration 125 nM of ParomR5 and NeamR4, were similar (∼50%; from MFI of 104 to 83, 59 and 53, for 62.5, 125, and 250 nM SDF-1α, respectively for ParomR5; and from a MFI of 96 to 56, 52 and 50, for 62.5, 125, and 250 nM SDF-1α, respectively, both with a background MFI of 10). No significant increased effects could be detected in the presence of the higher dose of SDF-1α (250 nM) or even in the presence of 1 μM SDF-1α (not shown). Similar effects were found by using MT2 cells. In contrast, no effect of SDF-1α on mono-, di-arginine derivatives of neomycin or mono-arginine of paromomycin and neamine conjugates uptake, either by 250 nM or 1 μM of SDF-1α could be observed in PM1 or MT2 cells. It is worth noting that the anti-HIV-1 EC_{50} of SDF-1α is ∼70-fold lower than that of NeoR6, whereas the inhibition (IC_{50}) of 12G5 mAb binding to CXCR4 is only 2.6 times lower than of NeoR6 (Cabrera et al., 2002).

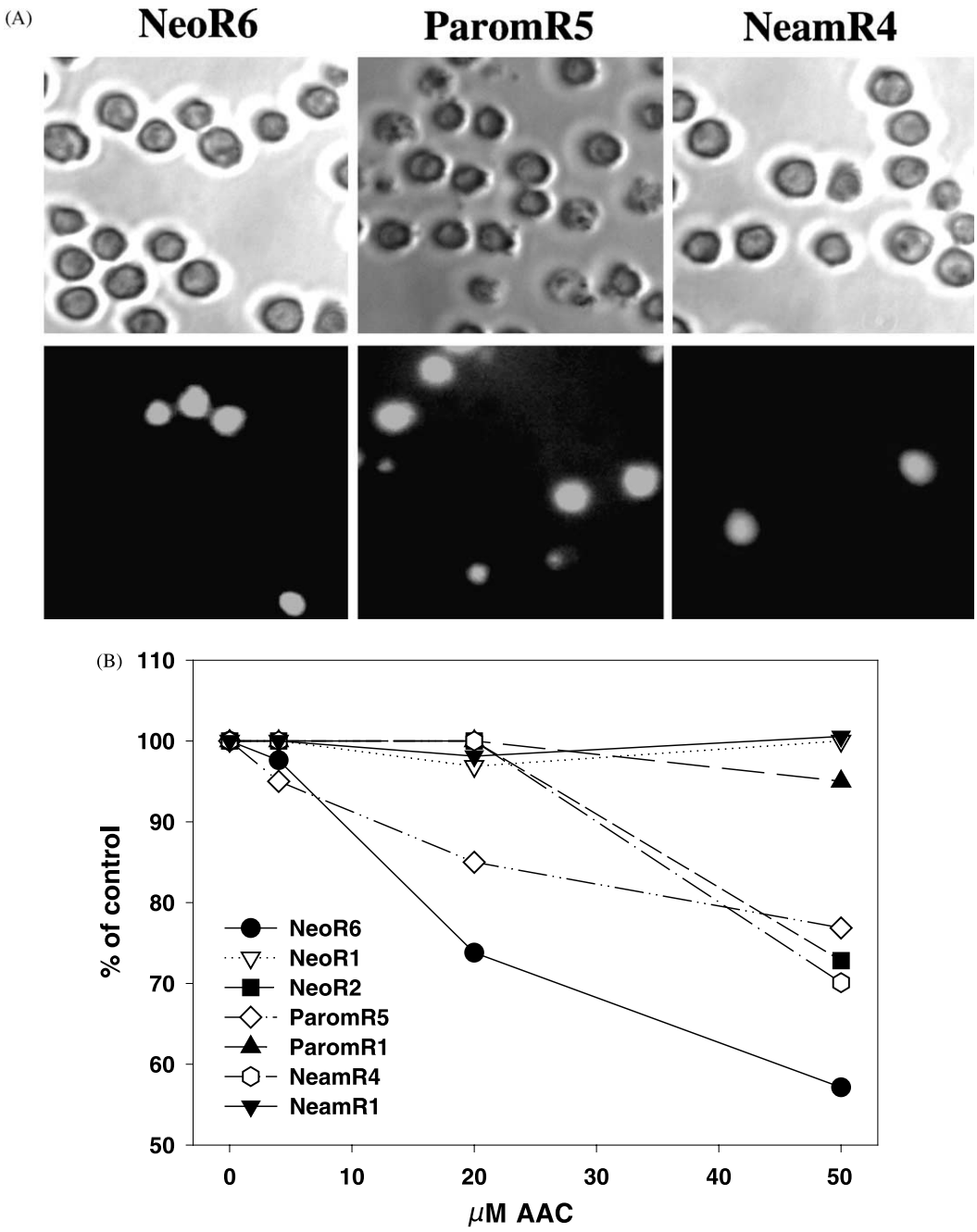


Fig. 4. Cellular uptake of AACs. (A) Zeiss Axiophot microscopy images of live PM1 cells incubated for 10 min at room temperature with 0.5 μM of NeoR6-FITC, ParomR5-FITC or NeamR4-FITC. Upper panels: optical microscopy of the cells. Lower panel: the same fields as in the upper panels with a FITC filter. (B) Cellular uptake competition of AACs with NeoR6-FITC. NeoR6-FITC (0.5 μM, final concentration) alone or mixed with 5, 20 or 50 μM non-labeled AACs, was added to 10^5 MT2 cells (50 μl final volume). After 15 min of incubation at room temperature, the cells were washed twice with ice-cold PBS and fixed in PBS containing 1% paraformaldehyde. The fluorescence was then analyzed by flow cytometry as described in Section 2. The MFI of FITC-NeoR6 alone was 62. Similar results were obtained by incubating the cells with the AACs for 5 min only.

3.6. No inhibition of multi-arginines–aminoglycoside conjugates cell uptake by CCR5 ligand RANTES

We have recently shown that neither R3G nor R4K or NeoR6 inhibited the binding of 2D7, a mAb directed to CCR5 or of an anti CD4 antibody (Leu 3a) in PHA stimulated peripheral blood lymphocytes (PBL) (Litovchick et al., 2001; Cabrera et al., 2002). In the present study, we found that 2.5 μM RANTES did not affect uptake of FITC derivatives of NeoR6, ParomR5 or NeamR4 by cMAGI cells (not shown), supporting our previous studies implying that AACs do not interact with the HIV-1 coreceptor CCR5.

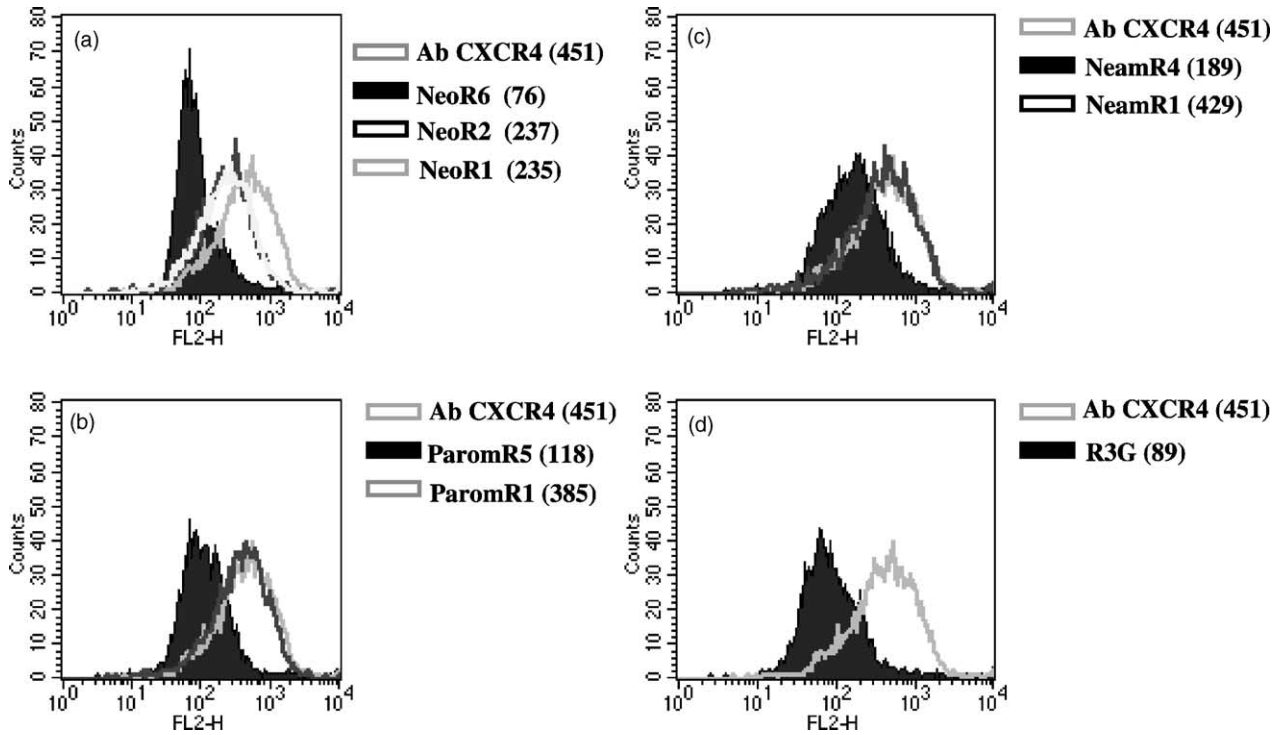


Fig. 5. Competition of AACs and 12G5 mAb binding to CXCR4 on PM1 cells. Cells were incubated with PE-conjugated isotype control mAb, PE-anti CXCR4 conjugated mAb (12G5) alone or in the presence of 10 μM AACs for 30 min at 4 °C. The cells were then washed twice with PBS and analyzed by flow cytometry. The MFI are shown in parenthesis.

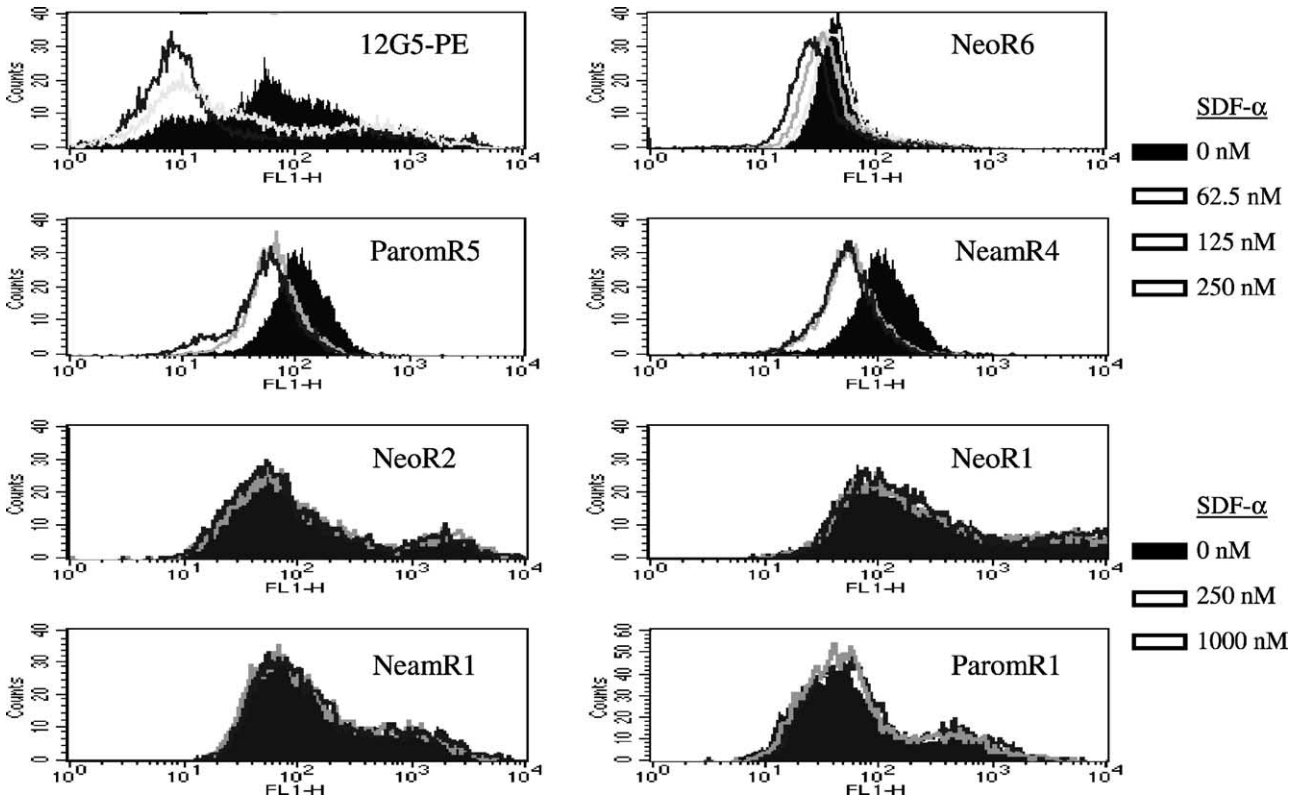


Fig. 6. Inhibition of anti-CXCR4 mAb binding and FITC-labeled AACs entry to PM1 cells by SDF-1α. PM1 cells (10^5) were incubated with (i) PE-anti CXCR4 conjugated mAb (12G5), (ii) 0.5 μM NeoR6-FITC, ParomR5-FITC or NeamR4-FITC in the presence of 0, 62.5, 125 or 250 nM SDF-1α or (iii) 0.5 μM NeoR2-FITC, NeoR1-FITC, NeamR1-FITC or ParomR1-FITC, in the presence of 250 or 1000 nM SDF-1α. After 20 min of incubation at room temperature the cells were washed twice with PBS and analyzed by flow cytometry.

Table 1
Percent of inhibition of 12G5 mAb binding to CXCR4 by (A) AACs and (B) aminoglycosides

AACs (10 μM)	NeoR6	ParomR5	R3G	NeamR4	NeoR2	NeoR1	ParomR1	NeamR1
(A)								
Inhibition (%)	90	85	84	60	50	50	16	5
Aminoglycosides (20 μM)	Neomycin	Paromomycin	Neamine					
(B)								
Inhibition (%)	0	0	0					

The data shown are the mean of three separate experiments with PM1 cells. The standard deviation was not greater than 5%. Similar results were obtained with PBMC cells.

3.7. Inhibition of FITC-labeled AAC uptake by gp120

HIV-1 gp120, capable of binding to CXCR4 via CD4 (Ugolini et al., 1997; Staudinger et al., 2001), reduced the uptake of NeoR6-FITC and R3G-FITC (Fig. 7), while it did not inhibit significantly the uptake of ParomR5-FITC or NeamR4-FITC (not shown). As shown in a representative experiment in Fig. 7, the presence of 5 μM HIV-1_{IIIB} gp120 reduced the uptake of 0.5 μM NeoR6-FITC or R3G-FITC by ~40%, from a MFI of 92 to 60, and from 74 to 49 (with a MFI background of 10) for NeoR6-FITC and R3G-FITC, respectively.

3.8. Mutations in gp120 glycoprotein in NeoR6 resistant HIV-1 isolate

Based on the results described above, indicating that the antiviral mechanism of the AACs involves an interaction with CXCR4, it was interesting to test if resistance to AACs would emerge mutations in the gp120 gene. Three sets of

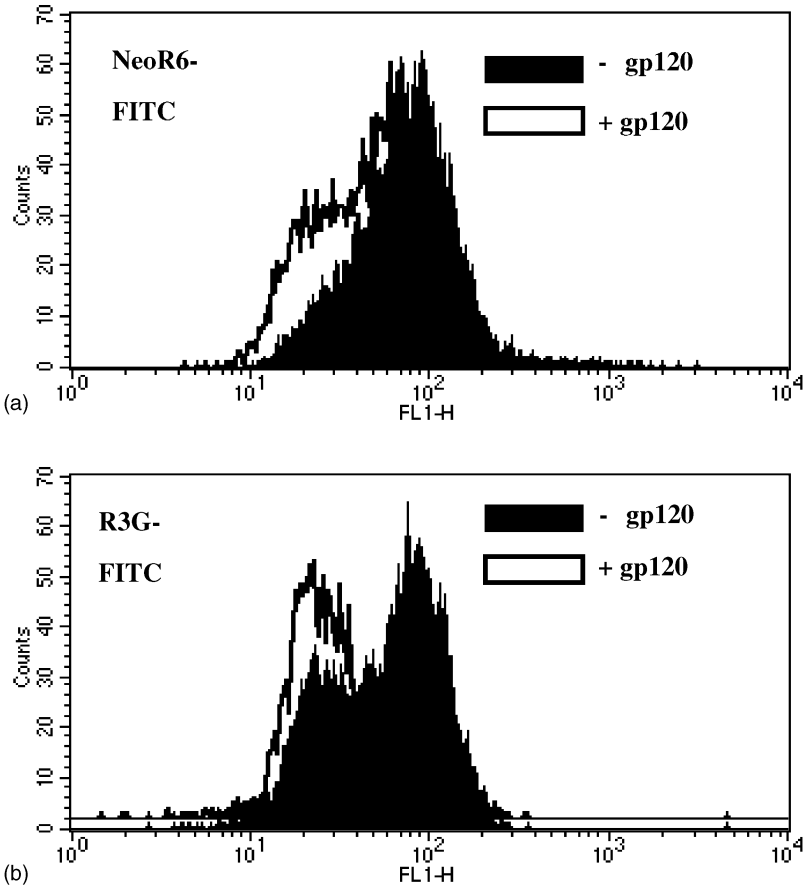


Fig. 7. Inhibition of (a) NeoR6-FITC and (b) R3G-FITC entry to PM1 cells by HIV-1_{IIIB} gp120. The cells were incubated with (a) 0.5 μM NeoR6-FITC or (b) 0.5 μM R3G-FITC in the absence or presence of 5 μM HIV-1_{IIIB} gp120 for 20 min at room temperature. The cells were then washed twice with PBS and analyzed by flow cytometry.

Table 2
Mutations in gp120 of the NeoR6^{res} virus

Amino acid position (region)	Wild-type virus		NeoR6^{res} virus codon Amino acid	
	Codon	Amino acid	Codon	Amino acid
249 (C3)	ATT	I	ACT	T
283 (V4)	TCA	S	TTA	L
306 (C4)	CAG	Q	AAG	L

selection of HIV-1 resistant isolates to NeoR6 were carried out, as described in Section 2, by gradually increasing the concentration of NeoR6 during cell culture from 1.9 μM (∼EC_{50}) to 150 μM. After 25 passages, the NeoR6 resistant isolates were ∼46 times more resistant than the wt virus to NeoR6, i.e. the EC_{50} to NeoR6 increased from 1.9 ± 0.96 μM to 87 ± 34 μM. The NeoR6 resistant isolates were also approximately five times more resistant to R3G (EC_{50} increased from 4.1 ± 2.4 μM for the wt virus to 21 ± 8.8 μM for the NeoR6 resistant isolates). In contrast, the NeoR6 resistant isolates were not resistant to SDF-1α (EC_{50} = 1 μg/ml for both wt and NeoR6 resistant HIV-1 isolates). Analysis of the gp120 gene of the NeoR6 resistant isolates, revealed the appearances of the following three mutations, as compared to the sequence found in the wt HIV-1_{IIIB} isolates used to develop resistance: In four out of four of the resistant isolates, there were changes from T to C at position 746, corresponding to the C3 region; and in three out of the four isolates, there were also changes from C to T and from C to A at positions 848 and 916, respectively, corresponding to the V4 and C4 regions, respectively (Table 2). Similarly, in vitro development of resistance in HIV-1 isolates to SDF-1α, the natural ligand of CXCR4, resulted in the appearance of mutations in the gp120 gene (de Vreese et al., 1996; Schols et al., 1998).

4. Discussion

The interaction of gp120 with the chemokine receptors, CXCR4 and CCR5, is a critical step that allows the fusion of the viral membrane with the cellular membrane and the penetration of the viral genetic material into the cytoplasm. Thus, inhibition of any of these first critical stages of HIV-1 infectivity comprises an important direction in anti Acquired Immunodeficiency Syndrome (AIDS) drug discovery.

The AACs are a new family of inhibitors, which obstruct HIV-1 infectivity as well as antagonize extracellular HIV-1 Tat detrimental activities (Litovchick et al., 1999, 2000, 2001; Lapidot and Litovchick, 2001). Although the AACs were designed as HIV-1 Tat-mimetics, it was recently demonstrated that their mechanism of inhibition of T-tropic HIV-1 infectivity includes interacting with CXCR4 (Cabrera et al., 2000, 2002; Litovchick et al., 2001).

In order to further elucidate the mode of antiviral activity of the AACs, we have designed novel AACs in which we modified both the core (e.g. from neomycin B to paromomycin and neamine) and the number or arginine moeties (1–6 arginine groups). We have investigated the effect of the AACs structure on (i) their interaction with HIV-1 TAR and RRE RNA (Vijayabaskar et al., submitted) and (ii) their interaction with CXCR4 and competition with SDF-1α and gp120 to bind CXCR4 (present study).

Dissociation constants of AACs binding to TAR and RRE revealed structure dependence on the core aminoglycoside and number of arginine groups conjugated to it. Neomycin–hexa-arginine exerted the highest affinity to both TAR and RRE_{IIIB} with K_D = 5 and 23 nM, respectively. ParomR5 and NeamR4 were less active by three-fold and six-fold, respectively, in comparison to NeoR6. The mono-arginine derivatives showed the lowest activity (K_D = 200–500 nM) (Vijayabaskar et al., submitted). Even though AACs binding to HIV-1 RRE RNA in cell culture has not been studied, we hypothesize that once AACs are taken up by the cells, they may interfere with Rev–RRE interaction, similarly to their interference with Tat–TAR interaction (Litovchick et al., 2001). The fact, that in cell culture higher concentrations of AACs are needed to inhibit viral proliferation, indicate that the actual concentrations that may interfere with Rev–RRE and Tat–TAR and other viral molecules within the cells is significantly lower than those added extracellularly. One possible explanation is that the AACs also interact with cellular factors, lowering the actual "available" number of AACs capable of interfering with intracellular viral functions. This is supported by the finding that AACs inhibit translation (Carriere et al., 2002).

The inhibition of binding of mAb 12G5 to CXCR4 by each one of the new AACs presented in this current study, supports the notion that the AACs interact with CXCR4, being the interaction of NeoR6 the most potent. ParomR5, NeamR4, and NeoR2 or NeoR1 exert 90, 70, and 55% of that of NeoR6, respectively. The mono-arginine derivatives of paramomycin and neamine inhibit the binding of the mAb to CXCR4 only by 20 and 5.5%, respectively, in comparison to NeoR6. Interestingly, R3G, which has only three arginines, exerted 96% inhibition, indicating that the core, in addition to the number of arginines found in the AACs, is important in the interaction with CXCR4. Our findings reveal a decreased binding to CXCR4 and reduced efficiency to compete with SDF-1α or gp120 binding to CXCR4 by ParomR5 and NeamR4 in comparison to NeoR6, and even lower efficiencies have been found for the mono-arginine–aminoglycoside conjugates. This may be due to a weaker binding efficiency to CXCR4 of the AACs with less arginines than six and different "cores" or that the AACs enter to cells via another site of the HIV-1 coreceptor, not specific to the binding site(s) of SDF-1α or gp120. This hypothesis should be further investigated. In accordance with these findings, SDF-1α seems to have a multiple site interaction with CXCR4 (Gupta et al., 2001). Additionally,

the possibility that the part of the antiviral activity of the AACs is mediated by downregulation of CXCR4 should be examined.

Additional support to the idea that AACs act also at the entry step of HIV-1 to target cells are our findings showing that (i) the presence of NeoR6 and R3G only during the first 2 h of cell exposure to HIV-1_{IIIB} was sufficient to inhibit viral production; (ii) NeoR6 and R3G inhibit viral binding to cells (Fig. 3); (iii) HIV-1 viral envelope glycoprotein 120 (gp120), known to bind to CXCR4, inhibits NeoR6-FITC and R3G-FITC entry into cells (Fig. 7); (iv) appearance of mutations in the gp120 gene of NeoR6 resistant HIV-1 isolates; and (v) protective anti gp120-triggered death exerted by NeoR6 in human neuroblastoma cells seems related to the ability of NeoR6 to interact with CXCR4 coreceptors (Catani et al., 2003). In contrast to the above, no one of the AACs competed with RANTES, the natural ligand for the CCR5 coreceptor, in accordance with our finding that neither R3G nor R4K or NeoR6 inhibit a monoclonal antibody directed to CCR5 (Litovchick et al., 2001; Cabrera et al., 2002).

The apparent anti-HIV-1 activity of the AACs presented in the current study is a combination of targeting several steps in the viral life cycle, including cell entry and intracellular functions. It may be that the AACs exert other antiviral activities that mask the differences between them. For example, AACs may also interact with other intracellular factors, such as with HIV-1 dimerization initiation site (DIS), as was recently demonstrated for aminoglycosides (Ennifar et al., 2003). We are currently exploring this possibility. Recently, we have also shown that inhibition of translation by AACs is directly related to the number of arginine groups linked to the aminoglycoside backbone and on the nature of the aminoglycoside (Carriere et al., 2002).

The appearance of mutations in the gp120 gene of the NeoR6 resistant HIV-1 isolates did not appear at the V3 loop of gp120, as was found for other HIV-1 inhibitors of gp120–CXCR4 interaction (de Vreese et al., 1996; Schols et al., 1998), but at the constant regions C3 and C4, and in the variable region V4. Although the V3 domain of gp120 was found to be necessary for interaction with the HIV-1 chemokine coreceptors, other domains of gp120 were also found to play a role in the interaction of gp120 with CCR5 (Wu et al., 1996; Trkola et al., 1996). The role of the V3 domain in the interaction of gp120 with CXCR4 has not been directly demonstrated; variable domains such as V1/V2 and V3 of gp120 can both contribute to the interaction with CXCR4 coreceptor (Mondor et al., 1998). Therefore, it cannot be excluded that the mutations that we found in the NeoR6 resistant isolates confer resistance by allowing the interaction of gp120 with CXCR4 in such a way that NeoR6 interference would be minimized. Such possibility is now being studied by us. In addition, we have examined other mechanisms of resistance to AACs, such as the appearance of mutations in the TAR RNA, RRE RNA, DIS RNA, and Tat gene, but found no mutations.

In conclusion, our study demonstrates that the neomycin, paromomycin, and neamine–arginine conjugates, exert their antiviral activity not only via obstruction of key HIV-1 transcriptional events, but also by hampering early steps in the viral cycle of T-tropic HIV-1, including interference of the interaction of gp120 with CXCR4. In addition, our results point that NeoR6 is the most active AAC, indicating that the number of arginines attached, as well as the core itself are important for their capacity in interfering also with gp120–CXCR4 interaction.

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