

Anti-Human Immunodeficiency Virus Activity of Novel Aminoglycoside-Arginine Conjugates at Early Stages of Infection

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ABSTRACT

Conjugates of L-arginine with aminoglycosides have already been described as potent *in vitro* inhibitors of the HIV-1 Tat–trans-activation responsive element interaction. The polycationic nature of these agents leads us to suggest that they may be active against HIV-1 replication by inhibiting earlier stages of the virus life cycle. We have found that R4K and R3G, kanamycin A, and gentamicin C, conjugated with arginine, inhibited HIV-1 NL4-3 replication at EC₅₀ values of 15 and 30 μ M for R3G and R4K, respectively, without a detectable effect on MT-4 cells at concentrations higher than 4000 and about 1000 μ M, respectively. Both compounds inhibited the binding of a monoclonal antibody (12G5) directed to CXCR4 as well as the intracellular Ca²⁺ signal induced by the chemokine SDF-1 α on CXCR4⁺ cells, suggesting that aminoglycoside–arginine conjugates interact with CXCR4, the coreceptor used by T-tropic, X4 strains of HIV-1. On the other hand, CB4K, a conjugate of kanamycin A with γ -guanidinobutyric acid, structurally similar to R4K, failed to display any anti-HIV activity or CXCR4 antagonist activity. An HIV-1 strain that was made resistant to the known CXCR4 antagonist AMD3100 was cross-resistant to both R4K and R3G. However, unlike SDF-1 α and R4K, R3G inhibited the binding of HIV-1 to MT-4 cells. Aminoglycoside–arginine conjugates inhibit HIV replication by interrupting the early phase of the virus life cycle, namely virus binding to CD4 cells and interaction with CXCR4. R3G and R4K may serve as prototypes of novel anti-HIV agents and should be further studied.

INTRODUCTION

THE NEED FOR EFFECTIVE chemotherapeutic treatments of human immunodeficiency virus (HIV) infections has led to a search for and development of agents that target specific and critical events in the HIV replication cycle. The discovery of chemokine receptors as cofactors involved in the entry of HIV in the host cell¹ has renewed interest in the early steps of virus replication as a target for therapeutic intervention.^{2,3} A number of compounds have been described to interact with CCR5, the chemokine receptor used by macrophage-tropic (MT, R5) strains of HIV,⁴ including a nonpeptidic low molecular weight

molecule.⁵ In turn, the bicyclams were the first class of anti-HIV agents known to interact with CXCR4,^{6–9} the receptor used by T-tropic (TT, X4) strains of HIV-1. Two other groups have also described newly identified CXCR4 antagonists: ALX-40-4C, a polycationic, nonapeptide consisting solely of D-arginine residues¹⁰ and T22,¹¹ an octadecapeptide that has eight positive charges or its derivatives.¹² As bicyclams are also positively charged, it appears that the cationic nature of these compounds is necessary for their activity.

The conjugates of aminoglycoside antibiotics with L-arginine comprise a completely new class of peptidomimetic substances.^{13–15} The aminoglycoside–arginine conjugates resem-

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ble oligocationic peptides in terms of their chemical properties, including the capacity shown by other agents, such as ALX-40-4C, to bind to the HIV *trans*-activation responsive element (TAR) RNA *in vitro*.¹⁶ R4K is a tetra-L-arginine-kanamycin A conjugate, while R3G is a mixture of tri-L-arginine derivatives of gentamicin C1, C2, and C1a isomers, which differ by methylation of a single nitrogen and an adjacent CH₂ (Fig. 1 and Refs. 13 and 14). Both R4K and R3G display high affinity for TAR RNA *in vitro*. Dissociation constants (K_d), measured by the gel-shift technique, were found to be 416 nM for R4K and 83 nM for R3G.¹³ The conjugates caused pronounced inhibition of equine infectious anemia virus (EIAV, an equine lentivirus) proliferation in cell culture, with 90% effective concentration (EC₉₀) values of 25–50 μ M for R3G and 100 μ M

for R4K, without being toxic for the cells up to 1 mM concentrations.¹⁵ However, the potential anti-HIV activity of R4K and R3G and their mechanism of action in cell culture had not been described. We have found that R4K and R3G were able to inhibit HIV replication in a dose-dependent manner and to interact with CXCR4, thus impeding HIV-1 fusion and entry into CD4-positive cells.

MATERIALS AND METHODS

Compounds, viruses, and cells

The synthesis, purification, and chemical characterization of the L-arginine-aminoglycoside conjugates (Fig. 1) have been

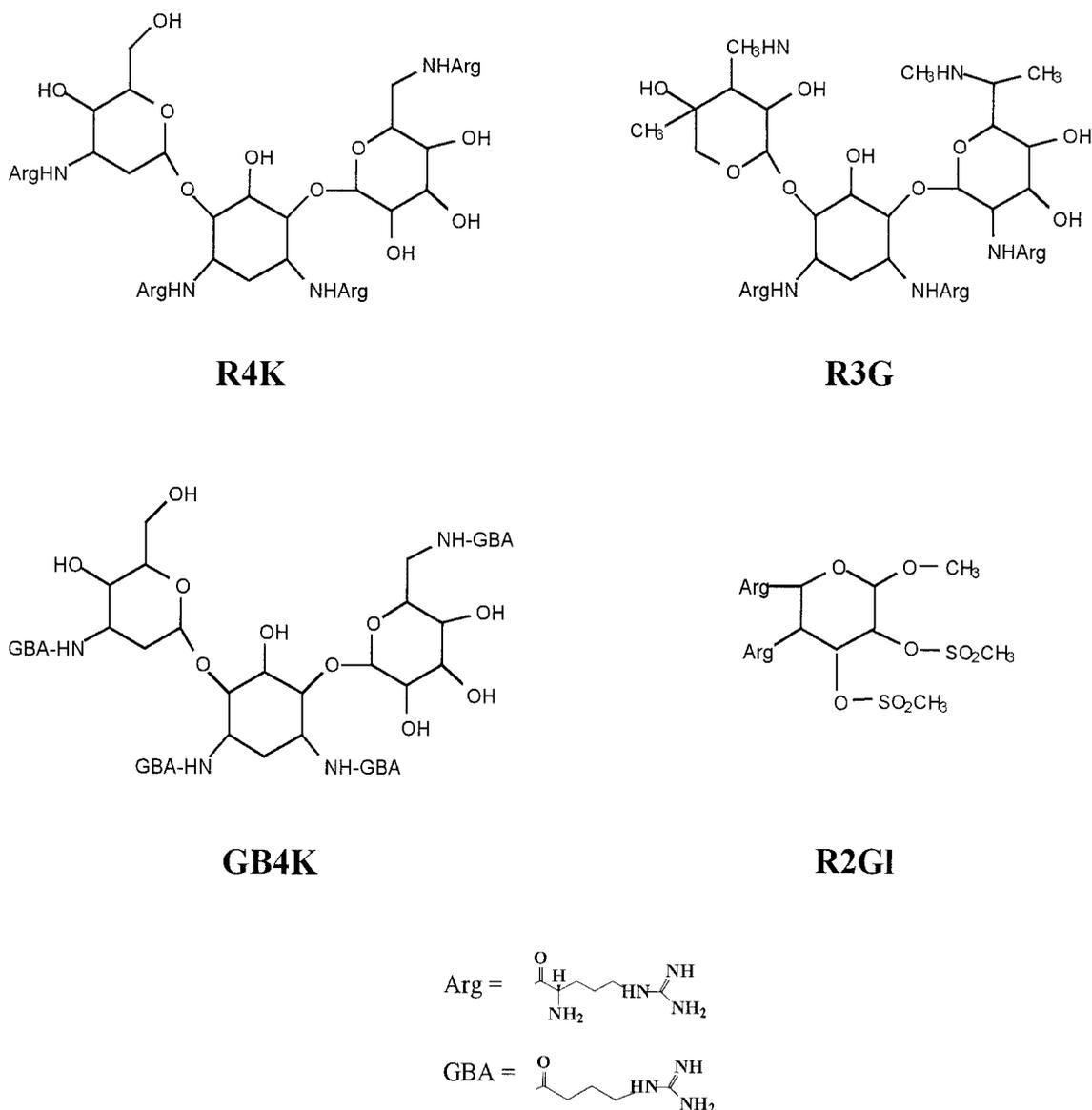


FIG. 1. The structure of kanamycin and gentamicin C conjugates: arginine R4K is a tetrasubstituted derivative of kanamycin; R3G is a mixture of trisubstituted derivatives of gentamicin; GB4K is equivalent to R4K, but has guanidinobutyric acid (GBA) residues instead of L-arginine residues. Arg, Arginine residues.

TABLE 1. ANTI-HIV-1 ACTIVITY OF VARIOUS COMPOUNDS AND CXC-CHEMOKINE SDF-1 α

Compound	<i>EC</i> ₅₀ ^a (μ M)					
	HIV-1 NL4-3	HIV-1 RF	HIV-1 AOM	HIV-1 AMD3100-resistant	HIV-1 Ba-L ^b	<i>CC</i> ₅₀ ^c (μ M)
R4K	31	>113	67	>113	>113	>1130
R3G	15	35	16	>133	29	>3940
R2G1	>200	—	—	>203	—	>200
GB4K	>200	—	—	>203	—	>200
AMD3100	0.01	0.02	0.5	>100	>100	—
SDF-1	0.04	—	—	>1	—	>0.1
AZT	0.01	0.02	3.7	0.005	0.2	>7.5

^a*EC*₅₀: 50% effective concentration, or concentration of the compound required to inhibit HIV-1 replication by 50%, as measured by the MTT assay.

^b*EC*₅₀: 50% effective concentration, or concentration of the compound required to inhibit HIV-1 replication by 50%, as measured by β -galactosidase activity in P4-CCR5 cells infected with the HIV-1 Ba-L strain.

^c*CC*₅₀: 50% cytotoxic concentration, or concentration of the compound required to reduce the viability of MT-4 cells, as by the MTT assay.

described.^{13–15} R4K is an arginine–kanamycin A conjugate containing four arginine residues per molecule. The arginine–gentamicin conjugate mixture (R3G) consists of triarginine-substituted gentamicin C isomers. GB4K is structurally similar to R4K, but has γ -guanidinobutyric acid residues instead of L-arginine conjugated to a kanamycin A core. SDF-1 α was purchased from Peprotech (London, UK). Azidothymidine (AZT) was purchased from Sigma (St. Louis, MO). The HIV-1 strains NL4-3 and Ba-L; the CD4⁺ lymphocytic cell lines SUP-T1, MT-4, and MT-2; and P4-CCR5 MAGI¹⁷ cells were obtained from the Medical Research Council (MRC) AIDS Reagent Program (London, UK) or the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Bethesda, MD).

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 by Pauwels *et al.*¹⁸ Anti-HIV activity in P4-CCR5 MAGI cells was done as follows: cells (1×10^5 /ml) were infected with p24 antigen of HIV-1 Ba-L (10 ng/ml) in the presence of varying concentrations of the test compound. Twenty-four hours postinfection, cells were washed twice with phosphate-buffered saline (PBS) and resuspended in the medium containing the appropriate drug concentration. Five days after infection the cells were washed with PBS and evaluated for β -galactosidase activity as described previously.¹⁹

Flow cytometry analyses

Measurement of chemokine receptors CXCR4 and CCR5, and of the CD4 receptor on SUP-T1 cells or peripheral blood mononuclear cells (PBMCs) was performed by flow cytometry analysis as previously reported.²⁰ Briefly, 0.5×10^6 cells were washed in ice-cold PBS and incubated for 30 min at 4°C with monoclonal antibodies (MAbs) 12G5, 2D7, and Leu3a (Beckton

Dickinson, San Jose, CA) conjugated to phycoerythrin (PE), fluorescein isothiocyanate (FITC), and peridinin chlorophyll protein (PerCP), respectively, or with isotype control MAbs in the presence or absence of test compound. The cells were then washed with ice-cold PBS and were fixed in PBS containing 1% formaldehyde. For each sample 10,000 events were analyzed in a FACScalibur system (Becton Dickinson). Data were acquired and analyzed with CellQuest software (Becton Dickinson).

Measurement of intracellular calcium concentrations

The intracellular calcium concentrations [Ca²⁺]_i were determined as described previously.⁹ Briefly, SUP-T1 or THP-1 cells were loaded with Fluo-3 (Sigma). Fluorescence was measured in a Fluoroskan ascent fluorometer (Labsystems, Helsinki, Finland). Cells were first stimulated with dilution buffer (control) or test compound at various concentrations. As a second stimulus, SDF-1 α (20 ng/ml) or RANTES (1 μ g/ml) was used to induce [Ca²⁺]_i increase. The second stimulus was added 10 sec after the first stimulus. The compound concentration required to inhibit the [Ca²⁺]_i increase by 50% (*IC*_{50[Ca²⁺]_i) was calculated.}

TABLE 2. INHIBITION OF ANTI-CXCR4 MAb (12G5) BINDING TO CXCR4⁺ CELLS

Compound	<i>IC</i> _{50-12G5} ^a (μ M)	
	SUP-T1 cells	PBMCs
R4K	3.7	2.2
R3G	7.7	2.7
R2G1	>40	25
GB4K	>40	>40
AMD3100	0.01	0.001
SDF-1 α	0.013	— ^b

^a*IC*_{50-12G5}: 50% inhibitory concentration, or concentration of the compound required to inhibit by 50% the binding of 12G5 MAb to CXCR4⁺ cells.

^b—, Not tested.

Virus-binding assay

MT-4 cells (5×10^5) were incubated with supernatant containing 1×10^5 pg of p24 antigen of wild-type HIV-1 in the presence of various concentrations of the test compound. One hour after infection, cells were washed three times with PBS and p24 antigen bound to the cells was determined by a commercial enzyme-linked immunosorbent assay (ELISA) test (Coulter, Hialeah, FL).

RESULTS

Anti-HIV activity of *L*-arginine–aminoglycoside conjugates

The comparative effects of seven compounds against HIV-1 NL4-3 are presented in Table 1. Compounds R3G and R4K

were both active at 50% effective concentrations (EC_{50}) of 15 and 31 μ M, respectively. The 50% cytotoxic concentrations (CC_{50}) were calculated to be >1130 and >3940 μ M for R4K and R3G, respectively. The chemokine SDF-1 α was active at an EC_{50} of 0.04 μ M while the CXCR4 antagonist AMD3100 was active at an EC_{50} of 0.01 μ M. Conversely, compounds R2G1, which contains two *L*-arginine residues conjugated to a monosaccharide core, and GB4K, which contains four γ -guanidinobutyric residues, did not show any significant anti-HIV activity. R3G, the most active in the aminoglycoside–arginine conjugate series, was likewise active against the X4 HIV-1 RF; the X4 HIV-1 clinical isolate AOM, which is resistant to AZT; and the R5 HIV-1 Ba-L strain. AMD3100 was inactive against HIV-1 Ba-L, while the reverse transcriptase inhibitor AZT was active against all HIV-1 strains tested except against HIV-1 AOM.

The NL4-3 AMD3100-resistant virus was cross-resistant to

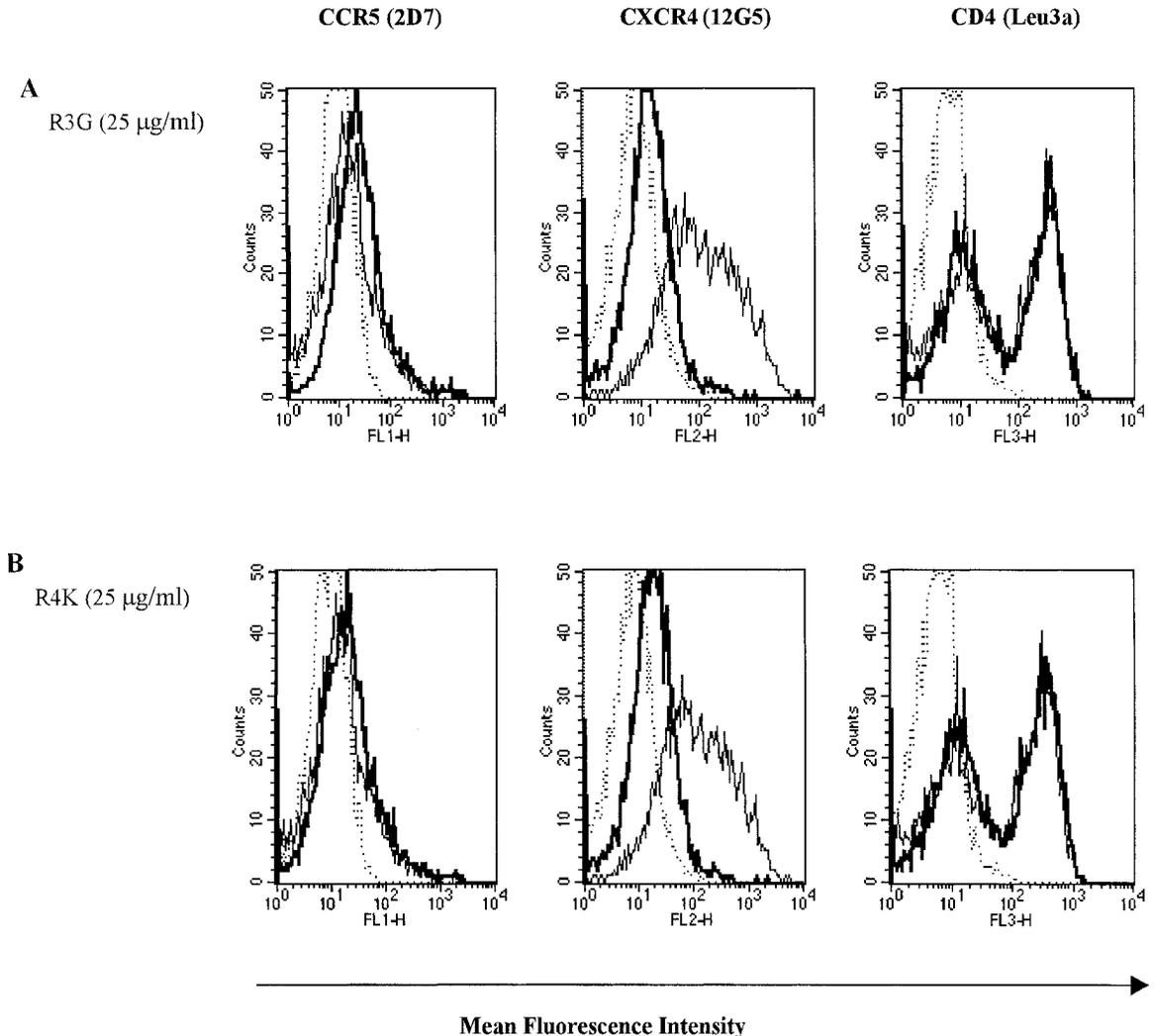


FIG. 2. Effect of R3G (25 μ g/ml) and R4K (25 μ g/ml) on binding of 2D7 MAb to CCR5, 12G5 MAb to CXCR4, and Leu3a MAb to CD4 in stimulated peripheral blood mononuclear cells. For each experiment cells were incubated with MAbs 2D7, 12G5, and Leu3a conjugated to FITC, PE, and PerCP, respectively, or isotype control MAb (dotted line) in the presence (thick line) or absence (thin line) of the corresponding compound (25 μ g/ml). After a 30-min incubation at 4°C, cells were washed with PBS and analyzed by flow cytometry.

SDF-1 α (>25-fold), R4K (>4-fold), and R3G (>9-fold), suggesting that these compounds share a similar mode of action.

Interaction with CXCR4 receptor

To elucidate whether the anti-HIV activity of aminoglycoside-arginine conjugates is due to their interaction with CXCR4, we tested the capacity of the various analogs to inhibit the binding of an MAb to CXCR4 (12G5). SDF-1 α , the natural ligand of CXCR4, and AMD3100, a CXCR4 antagonist that is active as an HIV-1 inhibitor, were used for the comparison. Table 2 shows the concentrations of 50% inhibition (IC₅₀) of 12G5 MAb binding (IC_{50-12G5}) by R3G, R4K, GB4K, R2G1, AMD3100, and SDF-1 α . The conjugates R3G and R4K showed

high affinity for CXCR4 (as measured by the inhibition of 12G5 binding to SUP-T1 cells), which is consistent with their anti-HIV activity. Neither compound inhibited the binding of 2D7, a monoclonal antibody directed to CCR5, or of an anti-CD4 antibody (Leu3a) in interleukin 2 (IL-2)/phytohemagglutinin (PHA)-stimulated PBMCs (Fig. 2).

To evaluate further the interaction of aminoglycoside-arginine conjugates with CXCR4, we tested the capacity of SDF-1 α to induce an intracellular Ca²⁺ signal in the presence of these conjugates. Both R3G and R4K inhibited the SDF-1 α -dependent Ca²⁺ signal in a dose-dependent manner, which is similar to the effect of bicyclam AMD3100. Compounds R2G1 and GB4K did not significantly inhibit SDF-1 α -dependent signal (Fig. 3). R3G and R4K did not inhibit the intracellular Ca²⁺

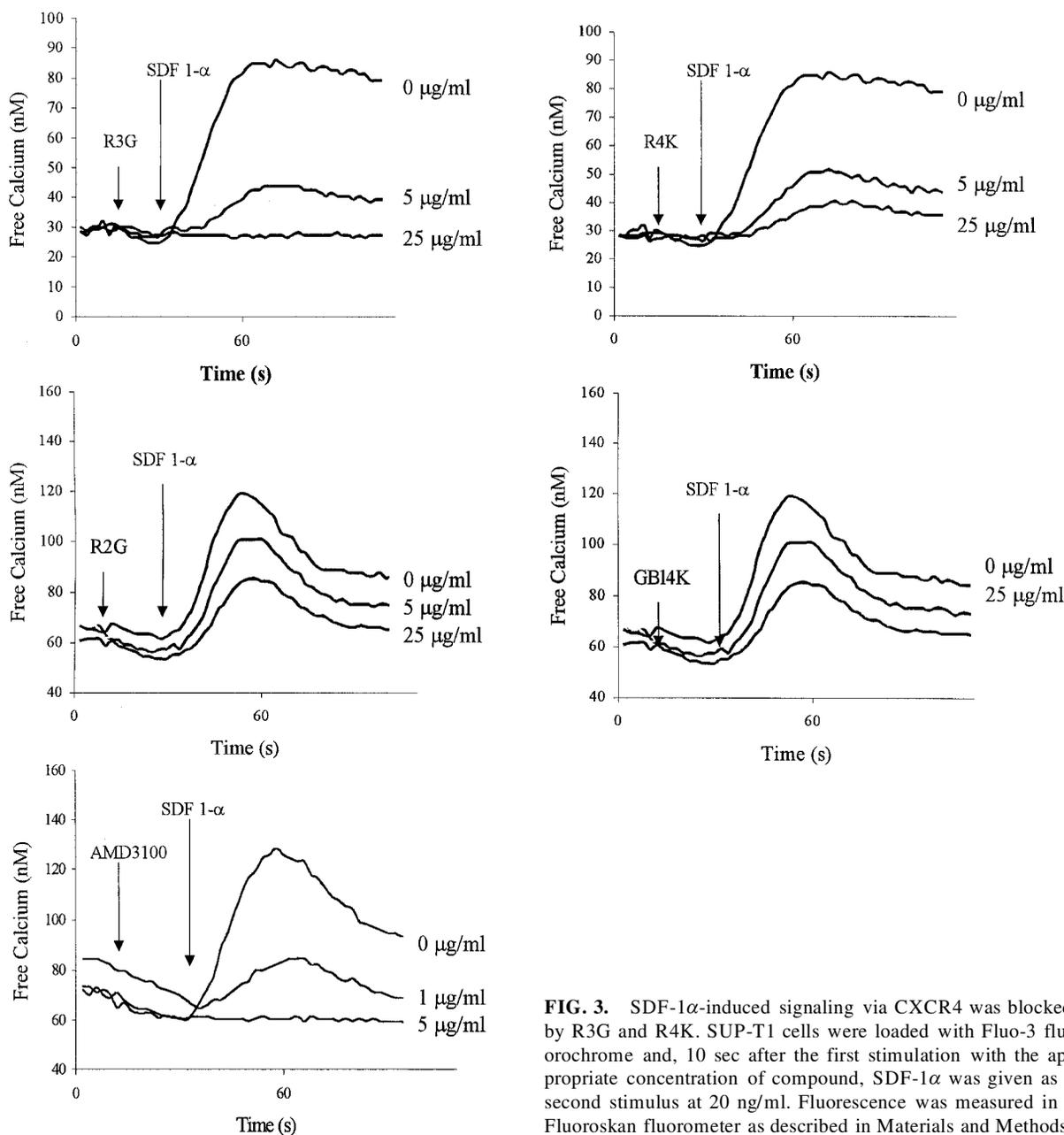


FIG. 3. SDF-1 α -induced signaling via CXCR4 was blocked by R3G and R4K. SUP-T1 cells were loaded with Fluo-3 fluorochrome and, 10 sec after the first stimulation with the appropriate concentration of compound, SDF-1 α was given as a second stimulus at 20 ng/ml. Fluorescence was measured in a Fluoroskan fluorometer as described in Materials and Methods.

signal induced by RANTES in THP-1 (CCR5⁺) cells (data not shown).

Inhibition of virus binding to CD4⁺ cells

It has been shown that cationic peptides may inhibit the adsorption of HIV-1 to the cell surface.²¹ We have found that aminoglycoside-arginine conjugates R4K and R3G inhibited the binding of HIV-1 NL4-3 to MT-4 cells in a dose-dependent manner (Fig. 4). Dextran sulfate was also active, as reported,²² while SDF-1 α at a concentration of 0.5 μ g/ml did not inhibit the binding of HIV-1 to MT-4 cells (data not shown). Similarly, R4K and R3G inhibited the binding of the R5 strain Ba-L to MT-4 cells in a dose-dependent manner (data not shown).

DISCUSSION

Important efforts are being made to identify and evaluate water-soluble compounds with the capacity to bind coreceptors and inhibit HIV envelope interactions. The small-molecule inhibitor TAK779⁵ has been shown to antagonize CCR5 and bicyclams (i.e., AMD3100), and T22 and ALX-40-4C have been identified as antagonists of CXCR4. These compounds interact with CXCR4 presumably because their cationic nature leads to electrostatic interactions with negatively charged residues of CXCR4.²³ Nevertheless, polycationic compounds have also been shown to inhibit the binding of HIV to CD4⁺ cells^{24,25}

and syncytium formation.²⁶ Thus, polycations may be designed to exert an anti-HIV activity through distinct mechanisms of action of early stages of infection.

Aminoglycoside-arginine conjugates were designed as specific TAR RNA binders, on the basis of the idea that a combination of two RNA-binding patterns, aminoglycoside and peptide, might be effective. These conjugates displayed high affinity and specific TAR RNA binding *in vitro*, along with low cytotoxicity and antiliviral potency in the model system of EIAV (Malmquist)-infected equine fibroblasts.^{14,15} We have found that the lead compounds R3G and R4K have anti-HIV activity at the micromolar range (EC₅₀ 15 μ M for R3G and 31 μ M for R4K against the HIV-1 NL4-3 strain), with undetected cytotoxicity at the active concentrations (CC₅₀ was >3940 μ M for R3G and >1130 μ M for R4K). Here, we suggest that the inhibitory capacity of these compounds is due to their cationic nature. Still unclear is why the cationic compounds R2G1 (containing two arginine residues) and GB4K (containing four γ -guanidinobutyric residues and structurally similar to R4K) are devoid of the antiviral effect observed with R4K and R3G. It is possible that the number and position of nitrogen residues in the molecule affects the antiviral activity.

The anti-HIV active compounds inhibited the binding of a monoclonal antibody directed to CXCR4 and blocked the intracellular Ca²⁺ signal induced by SDF-1 α in CXCR4⁺ cells, without affecting the intracellular Ca²⁺ signal induced by RANTES in CCR5⁺ cells. The preceding suggests that antagonism to CXCR4 may be, in part, the mode of anti-HIV action

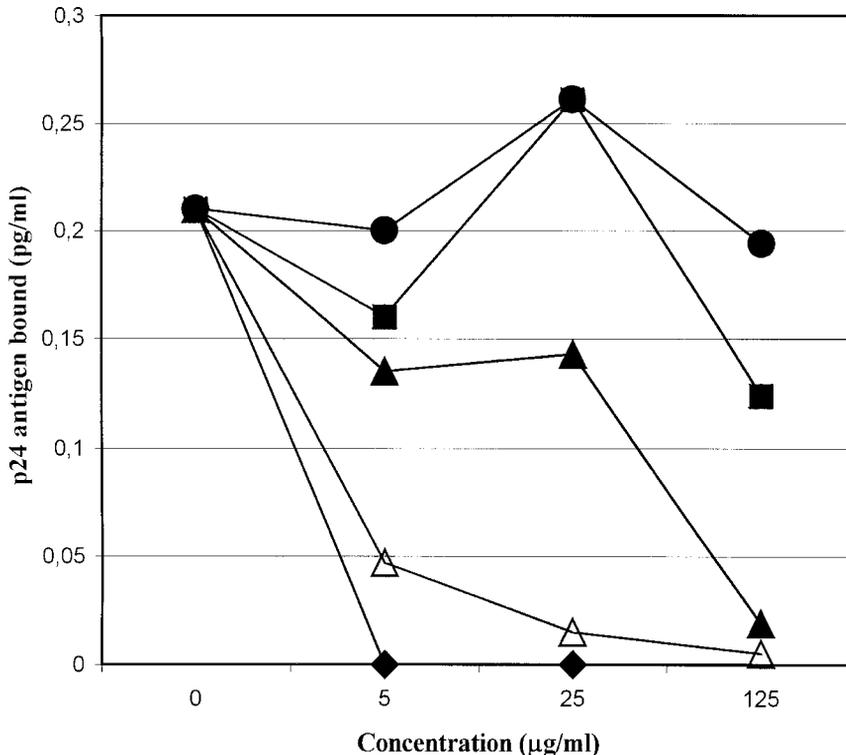


FIG. 4. R3G and R4K inhibited the binding of HIV-1 to CD4⁺ cells. MT-4 cells were infected with 1×10^5 pg of p24 antigen of HIV-1 (NL4-3) in the presence of various concentrations of the corresponding compound. After a 1-hr incubation at 37°C, cells were washed three times in PBS and p24 antigen bound to cells was determined by an ELISA test. R4K (▲); R3G (△); R2G1 (■); GB4K (●); dextran sulfate (◆).

of these compounds. The AMD3100-resistant HIV-1 strain²⁷ was cross-resistant to R3G and R4K. AMD3100 is a specific antagonist of CXCR4 and it has no anti-HIV activity against virus strains that do not use CXCR4. However, the AMD3100-resistant strain was also cross-resistant to the binding inhibitors dextran sulfate and AR177,²⁵ presumably because the amino acid changes in the gp120 protein that confer resistance to AMD3100 also alter the capacity of inhibitors of virus binding to function effectively.²⁵ In turn, virus strains that are resistant to anti-HIV agents that act at later stages of infection (i.e., reverse transcriptase or later) are sensitive to AMD3100.²⁸ Thus, the early stages of infection appear to be the time/site of action of aminoglycoside–arginine conjugates. This is further confirmed by the capacity of R3G and R4K to inhibit the binding of HIV particles to CD4⁺ cells, regardless of the coreceptor usage of the HIV strains used. The inhibitory activity on virus binding explains the anti-HIV activity of the most potent anti-HIV agent, R3G, against the R5 HIV-1 Ba-L strain (Table 1) despite its lack of interaction with CCR5 (Fig. 2). Although aminoglycoside–arginine conjugates are proven to be efficient and specific TAR RNA binders *in vitro* and possess an anti-EIAV activity in cell culture,^{14,15} HIV transactivation inhibition by the conjugates *in vivo* must be the subject for further studies. Here, we can state another mechanism of their antiviral activity through a bimodal action: blockage of CXCR4 and inhibition of virus binding to cells. A similar type of action could not be assigned to their anti-EIAV activity, since the Malmquist derivative of the Wyoming strain used as a model lentivirus is avirulent and is incapable of infecting equine fibroblasts directly.¹⁵

In conclusion, our results support the idea that the cationic nature of known CXCR4 antagonists is necessary for their biological effect. Aminoglycoside–arginine conjugates R3G and R4K are novel lead compounds with moderate anti-HIV activity and low cytotoxicity. Future studies would be directed to design modified aminoglycoside–arginine conjugates with increased anti-HIV potency relative to that of R3G and R4K. In turn, it will be necessary to demonstrate that blockade of CXCR4 is a valid therapeutic strategy without significant adverse effects.^{29,30} CXCR4 use by HIV-1 appears to be a causal factor in CD4⁺ cell depletion.^{31,32} Coreceptor-dependent tropism of HIV-1 appears to be even more restricted *in vivo* than may be predicted from *in vitro* testing since dual-tropic (R5X4) strains such as 89.6 behave like X4 strains and may be potentially inhibited by CXCR4 antagonists in *ex vivo* cultures.³³ Furthermore, blockade of CXCR4 prevented the evolution of HIV clinical isolates into CXCR4-using strains³⁴ or gp120-mediated apoptosis.^{35,36} These outstanding findings warrant the examination of CXCR4 antagonists as anti-HIV agents.

ACKNOWLEDGMENTS

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