CAPÍTULO 4

INHIBICIÓN DE LA FUSIÓN DEL VIH CON LAS CÉLULAS DIANA: INTERACCIÓN CON EL RECEPTOR DE QUIMIOCINAS CXCR4

4.1.- ACTIVITY OF DIFFERENT BICYCLAM DERIVATIVES AGAINST HUMAN IMMUNODEFICIENCY VIRUS DEPENDS ON THEIR INTERACTION WITH THE CXCR4 CHEMOKINE RECEPTOR.

4.2.- ANTI-HUMAN IMMUNODEFICIENCY VIRUS ACTIVITY OF NOVEL AMINOGLYCOSIDE-ARGININE CONJUGATES AT EARLY STAGES OF INFECTION.

ACTIVITY OF DIFFERENT BICYCLAM DERIVATIVES AGAINST HUMAN IMMUNODEFICIENCY VIRUS DEPENDS ON THEIR INTERACTION WITH THE CXCR4 CHEMOKINE RECEPTOR

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ABSTRACT

Bicyclams represent a novel class of selective anti-HIV inhibitors with potent activity against T-cell tropic strains of HIV. The prototype compound, the bicyclam AMD3100, has an EC_{50} of 1 to 10 ng/ml against different strains of HIV-1, including clinical isolates. AMD3100 was shown to interact with the CXC-chemokine receptor CXCR4, the main coreceptor used by T-cell tropic strains of HIV. Here we describe the interaction of different bicyclam derivatives with CXCR4. A close correlation ($r^2 = 0.7$) was found between the anti-HIV potency of the bicyclams and their ability to inhibit the binding of anti-CXCR4 monoclonal antibody or the intracellular Ca^{2+} signal induced by the stromal cell-derived factor-1α, the natural ligand of CXCR4. These results indicate that the mechanism of action of the bicyclams is primarily mediated by their interaction with CXCR4. The most potent interaction with CXCR4, and thus anti-HIV activity was shown by bicyclam analogs with cyclam rings composed of fourteen members that are linked by an aromatic (phenyl) bridge. Elucidating the structural requirements for receptor interaction and the site(s) of interaction of bicyclams with CXCR4 will aid in the understanding of HIV-cell fusion.

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INTRODUCTION

The discovery of cellular cofactors involved in the entry of HIV into the host cell has renewed the interest in the early steps of virus replication as a target for therapeutic intervention (Cohen, 1997). These cofactors are selectively used by different HIV strains and belong to the family of G protein-coupled, seven-transmembrane proteins that function as receptors for chemokines (Deng et al., 1996, Feng et al., 1996).

Bicyclams are a class of antiviral compounds that act as potent and selective inhibitors of the replication HIV-1 and HIV-2. Bicyclams are known to inhibit an early event of HIV replication that follows adsorption to the CD4 receptor but precedes reverse transcription (De Clercq 1992). Thus, bicyclams were identified as HIV fusion/uncoating inhibitors (De Clercq et al., 1992). Recently AMD3100 [1,1´-(1,4-phenylenebis(methylene))-bis(1,4,8,11-tetrazacyclotetradecane) octahydrochloride dihydrate], the prototype of the bicyclams (De Clercq et al., 1994), has been shown to selectively interact with CXCR4 (Schols et al., 1997a, Schols et al., 1997b), the receptor for the CXC chemokine stromal cell-derived factor (SDF)-1 and also the main coreceptor used by T-tropic strains of HIV (referred as X4 strains, Berger et al., 1998) to enter their host cells (Feng et al., 1996, Oberlin et al., 1996). Small molecules such as AMD3100 that can be readily synthesized and easily administered may have a clear advantage for clinical development. Moreover, the understanding of the mode of action of AMD3100 and bicyclams in general may help to develop newer anti-HIV agents directed to CXCR4 or other chemokine coreceptors used by HIV to enter the cells.

Bridger et al., (1995) and Joao et al., (1995) have shown that the antiviral activity of bicyclam analogs is restricted to the presence of two macrocyclic structures of 12 to 14 members per cyclam ring although identical rings are not required. Furthermore, the distance between the two macrocyclic rings as reflected by the length of the linker and specific substitutions on the phenylenebis-(methylene) linker are important requirements for the anti-HIV potency of bicyclams. The structural requirements for the anti-HIV activity of the bicyclam analogs are summarised in Table 1.

In this study we investigate the previous structure-function relationship of the bicyclam analogs for their interaction with CXCR4. From a comparative analysis of the structure-function relationship of the bicyclams, for their interaction with CXCR4 and their anti-HIV activity, we conclude that the anti-HIV activity of the bicyclam derivatives primarily depends on their affinity for CXCR4.

Table 1. Structural requirements of antivirally active Bis-macrocycles

Requirements
• molecules require two chelating macrocyclic rings for high activity • distance between metal-binding centers must be 9.5 – 11.5 Å • plane torsion of –60 to 30º and 120-140º are allowed • plane angles of 40-70º and 110-140º are allowed • maximize metal affinity for each macrocyclic ring • optimum ring size for cyclam rings: 14 atoms
Features to avoid
• plane torsion of 70 to 110º • plane angles 0 to 35º and 160 to 180º

Adapted from Joao et al., (1995).

MATERIALS AND METHODS

Compounds. The bicyclam analogs described in Fig. 1a, 1b and Fig. 2 were synthesised at Johnson Matthey (West Chester, PA) as described previously (Bridger et al., 1995, Bridger et al., 1996). The chemokine SDF-1α was purchased from R&D Systems (Abingdon, UK).

Antiviral assay and cytotoxicity assay. 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 (MTT method) as described by Pauwels *et al.*, 1988. Anti-HIV activity in SUP-T1 cells (Smith et al., 1984) was based on inhibition of HIV-1 induced cytopathic effect observed microscopically. Anti-HIV activity in MAGI-CCR5 cells (Chackerian et al., 1997) was determined as follows: cells ($1x\ 10^5$/ml) were infected with 3000 ng/ml of

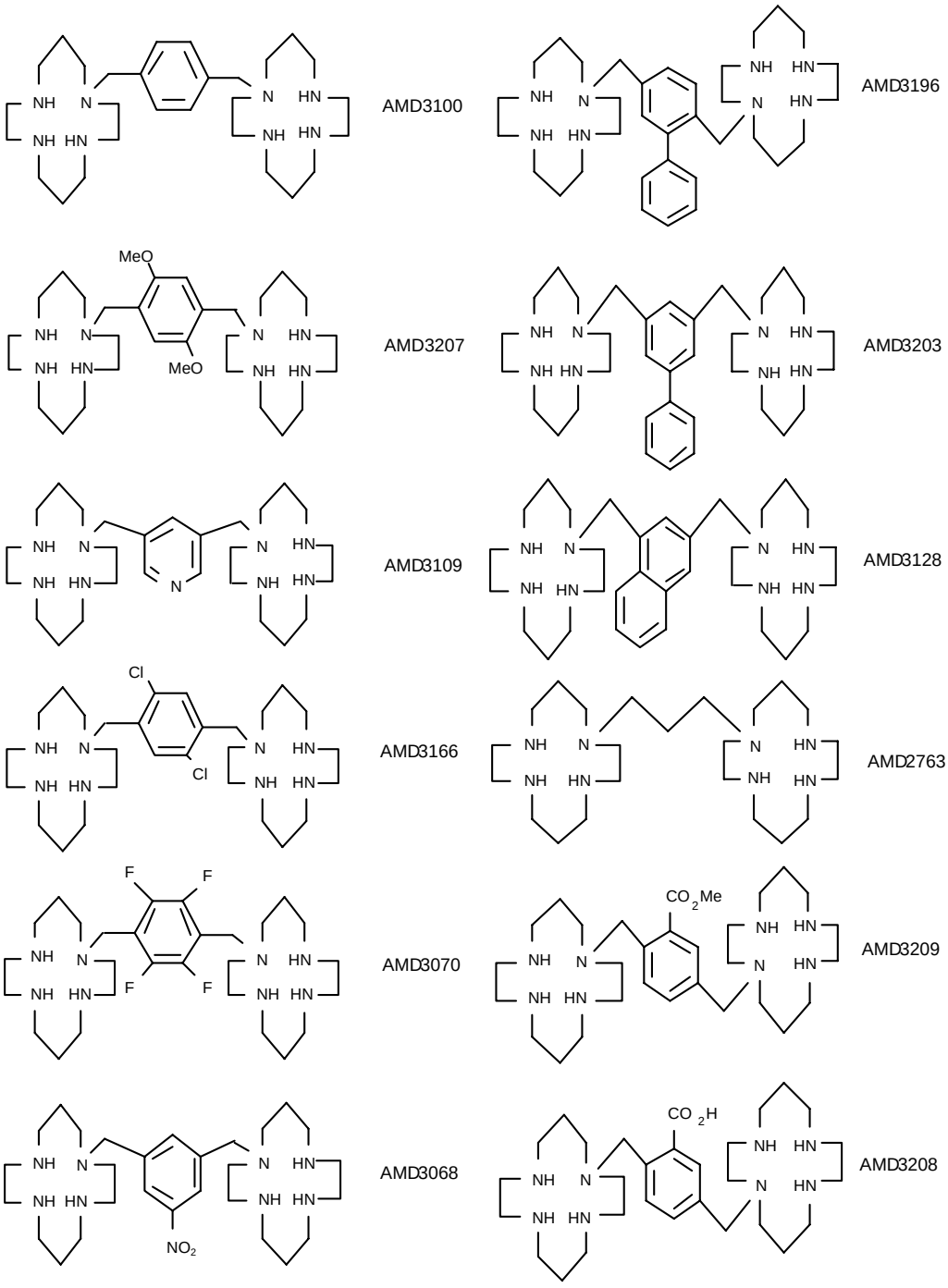


Figure 1a . Structures of Bis-macrocyclic (bicyclam) analogs.

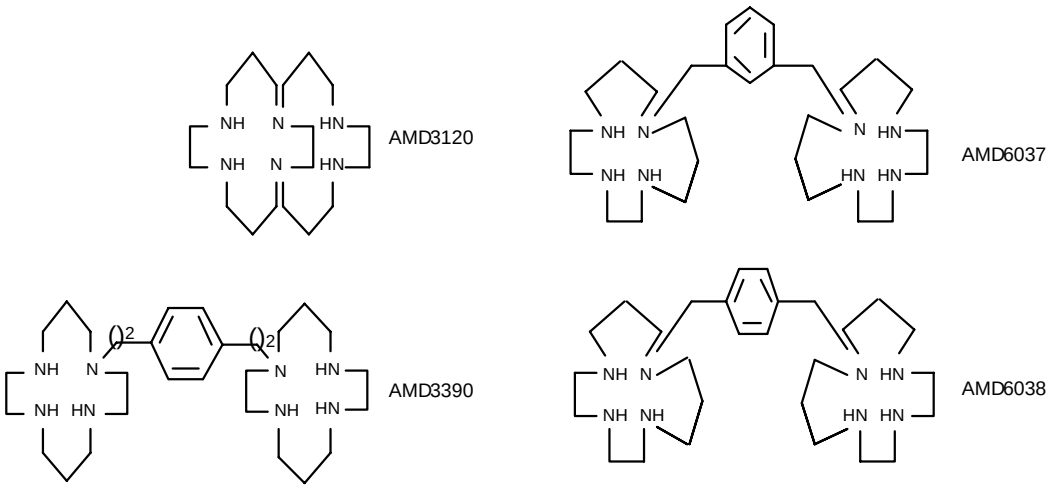
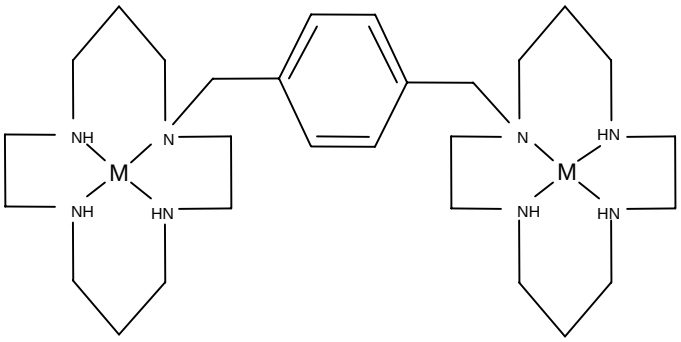


Figure 1b. Structures of Bis-macrocyclic (bicyclam) analogs (continued).



M = free	**AMD3100**
M= Zn $(Cl_2O4)_2$	**AMD3479**
M= Ni $(Cl_2O4)_2$	**AMD3462**
M= Co (Cl_2)	**AMD3461**
M= Cu $(Cl_2O4)_2$	**AMD3469**
M= Pd $(Cl_2O4)_2$	**AMD3158**

Figure 2. Structure of transition metal complex analogs of AMD3100.

p24 antigen of HIV-1 BaL in the presence of varying concentrations of the test compound. Five days after infection, the cells were washed with phosphate-buffered saline (PBS) and evaluated for β-galactosidase activity as described earlier (Esté, et al., 1995).

The HIV-1 NL4-3 virus (Adachi et al., 1986), is a molecular clone obtained from the National Institutes of Health (Bethesda, MD). The R5X4 HIV-1 RF strain (Alkhatib et al., 1996, Doms and Moore, 1997) was obtained from the Medical Research Council (London, UK) through the AIDS Reagent Project. The AMD3100-resistant strain (De Vreese et al., 1996a, De Vreese et al., 1996b) was derived after sequential passage of the NL4-3 virus in the presence of increasing concentrations of AMD3100 in MT-4 cells. The X4 HIV-1 strain AOM is a low-passage clinical isolate from our cohort of HIV positive patients. HIV-1 AOM was able to induce syncytium formation in MT-2 cells and uses CXCR4 as its main entry coreceptor. HIV-1 168.10 (de Jong et al., 1992) is a molecular clone virus that uses CXCR4 and CCR5 as entry coreceptors. HIV-1 BaL (Gartner et al., 1986) is a macrophage tropic strain of R5 phenotype (Doms et al., 1997).

Flow cytometric analyses. SUP-T1 cells were incubated with the anti-CXCR4 monoclonal antibody (12G5 mAb) (R&D Systems) for 45 min at 4ºC in the presence or absence of 0.5 μg/ml of test compound. Then the cells were washed with PBS and incubated with fluorescein isothiocyanate-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, San Jose, CA). Data were acquired and analysed with CellQuest software (Becton Dickinson) on an Apple Macintosh computer.

Correlation between the EC_{50} of each drug in the MTT assay and the IC_{50} of each drug of 12G5-labelled cells was evaluated using a simple linear regression model with $IC_{50\text{-}12G5}$ as the dependent variable. The slope (β), the 95% confidence interval of the slope (95% CI), the Pearson correlation coefficient (r^2) and their statistical significance (p) were calculated.

Measurement of intracellular calcium concentrations. The intracellular calcium concentrations $[Ca^{2+}]_i$ were determined as described previously (Wuyts et al., 1997). Briefly, SUP-T1 cells were loaded with Fura-2 (Molecular Probes, Leiden, The Netherlands) or Fluo-3 (Sigma, St. Louis, MO). Fluorescence was measured in a

luminescence spectrophotometer fitted with a water-thermostable, stirred 4-position cuvette holder (Perkin-Elmer, Norwalk, CT) or a Fluoroskan Ascent fluorometer (Labsystems, Helsinki, Finland). Cells were first stimulated with dilution buffer (control) or test compound at different concentrations. SDF-1α was used as a second stimulus to induce $[Ca^{2+}]_i$ increase, it was added 100 sec after the first stimulus. The compound concentration required to inhibit the $[Ca^{2+}]_i$ increase by 50% ($IC_{50\ [Ca2+]i}$) was calculated.

RESULTS

Antiviral activity of bicyclams against HIV-1 strains. The antiviral activity, as measured by the MTT method (Pauwels et al, 1988) is shown for a series of bicyclam analogs (Table 2). The prototype compound AMD3100 proved to be the most potent inhibitor of HIV-1 NL4-3 replication. If the bridge between the two cyclam rings was eliminated as in compound AMD3120, or if the cyclam rings were linked by an aliphatic bridge as in compound AMD2763, instead of an aromatic [phenylenebis(methylene)] bridge, the anti-HIV activity was markedly reduced (1436- and 70-fold respectively). The distance, as measured by the number of atoms in the bridge between the two cyclam rings also had an influence on the anti-HIV activity even if the aromatic linker was maintained; compound AMD3390 was >6000-fold less active than AMD3100.

Modifications of the phenyl linker of AMD3100 had various effects on the anti-HIV activity against the NL4-3 strain of HIV; inclusion of substituents attached to the aromatic ring in the linker (compounds AMD3068, AMD3196, AMD3128, AMD3203, AMD3207, AMD3208 and AMD3209) resulted in reduced or no antiviral activity. This effect was less if at least two substituents (compounds AMD3207, AMD3166 were 4-fold and 7-fold less active than AMD3100, respectively) or four substituents (compounds AMD3070 was 18-fold less active than AMD3100) were included in the phenyl linker. The reason for reduced activity of these bicyclam analogs appears to result purely from steric hindrance effects and restricted rotation of the macrocyclic rings upon the size of the substituent as demonstrated previously (Bridger et al., 1995).

Alteration of the disposition of nitrogen atoms in the macrocycles also had a detrimental effect on the anti-HIV potency of the compounds: AMD6037 and AMD6038 were less active than AMD3100 (40-fold and 8-fold, respectively).

Table 2. Anti-HIV-1 activity of the different bicyclam analogs and the CXC-chemokine SDF-1α.

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Compound	EC_{50}[a] (μg/ml)						CC_{50}[b] (μg/ml)
	NL4-3	AOM	RF	168.10[c]	BaL[d]	AMD3100-resistant NL4-3	
AMD2763	0.35	0.19	1.26	0.2	>25	>25	>250
AMD3068	0.134	0.65	0.07	0.02	-	3.00	>250
AMD3070	0.090	0.067	0.08	0.02	15	0.48	>250
AMD3100	0.005	0.05	0.13	0.002	>25	0.41	>250
AMD3109	0.100	0.05	0.08	0.24	>25	5.00	>250
AMD3120	7.18	86.7	>250	6.0	>25	>50	>250
AMD3128	0.20	0.22	0.36	0.24	>25	>25	>250
AMD3158	25.8	23.4	125	2.0	-	>100	>250
AMD3166	0.036	0.086	0.12	0.048	-	0.68	>250
AMD3196	0.134	0.34	0.39	0.24	>25	12.10	>250
AMD3203	0.20	0.08	0.1	0.12	-	0.42	>250
AMD3207	0.020	0.03	0.07	0.01	-	0.24	185
AMD3208	>250	>10.0	>10	-	-	>250	>250
AMD3209	0.81	0.87	1.4	1.2	-	>25	>250
AMD3390	30.4	5.6	>250	1.2	-	>5	>250
AMD3461	11.1	7.98	>250	6.0	-	>50	>250
AMD3462	0.008	0.028	0.08	0.048	-	0.26	>250
AMD3469	0.023	0.074	0.27	-	-	>1	>250
AMD3479	0.007	0.015	0.023	<0.08	7	0.30	>250
AMD6037	0.20	0.56	1.94	0.24	>25	>25	>250
AMD6038	0.04	0.14	0.22	0.04	>25	2.65	>250
SDF-1α	0.30	1.06	1.36	0.2	-	>2.00	>5

[a] EC_{50}: as measured by the MTT assay.
[b] CC_{50}: 50% cytotoxic concentration, or concentration of the compound required to reduce the viability of MT-4 cells, as measured by the MTT assay.
[c] EC_{50}: in SUP-T1 cells.
[d] EC_{50}: based on the inhibition of HIV-1-induced β-galactosidase activity in MAGI-CD4-CCR5 cells.
-: not tested.

There was a strong correlation between the antiviral activity of the bicyclam analogs tested against HIV-1 NL4-3 and their activity against the low-passage clinical isolate HIV-1 AOM ($r^2 = 0.8$), and the HIV-1 strains 168.10 ($r^2 = 0.7$) or HIV-1 RF ($r^2 = 0.7$); therefore, subsequent experiments were correlated to the anti-HIV activity of compounds against HIV-1 NL4-3. Compounds that were highly potent against NL4-3 were evaluated against the R5 strain BaL. Only AMD3479 (the zinc complex of

AMD3100) was active against HIV-1 BaL albeit at a 1000-fold higher EC_{50} of that required to inhibit NL4-3 replication (Table 2).

Antiviral activity of bicyclams against the AMD3100-resistant NL4-3 strain. The AMD3100-resistant NL4-3 strain showed reduced sensitivity to AMD3100 (82-fold resistance as compared with the wild type strain) and was insensitive to SDF-1α at its highest concentration tested (2 μg/ml). The resistant virus was also cross-resistant to all the bicyclam analogs tested, although with different magnitudes. The AMD3100-resistant strain could replicate in the presence of the most potent compounds, as it showed reduced sensitivity to the drugs (43-fold, 33-fold, 12-fold, 19-fold, and 66-fold, against AMD3479, AMD3462, AMD3207, AMD3166 and AMD6038, respectively) (Table 2). Compounds with lesser activity against the wild type strain were also less active (AMD3109, AMD3070, AMD3068, and AMD3196 were 50-, 5-, 21- and 89-fold less active, respectively), or became completely inactive (AMD3128, AMD2763, AMD3209, AMD3120, AMD3208, AMD3158, AMD3469, AMD3390, AMD3461 and AMD6037) against the AMD3100-resistant strain. Notably, compound AMD3203 was only 2-fold less active against the AMD3100-resistant strain.

Interaction with CXCR4 receptor. To elucidate whether the anti-HIV activity of bicyclam analogs is due to their interaction with CXCR4, we tested the capacity to inhibit the binding of a mAb to CXCR4 of different bicyclam analogs with anti-HIV-1 activity ranging from highly active (EC_{50} values in the ng/ml range as for AMD3100) to analogs selected because of their marginal or no anti-HIV activity (EC_{50} values greater than 10 μg/ml or not active even at 250 μg/ml). SDF-1α, the natural ligand of CXCR4, and active as an HIV-1 inhibitor, was included for comparison to the activity of the bicyclam analogs. Figure 3 shows the correlation for twenty-one bicyclam derivatives between the antiviral activity for HIV-1 NL4-3 (EC_{50}) and the interaction with CXCR4 as measured by the $IC_{50\text{-}12G5}$. Compounds showing high affinity for CXCR4 (as measured by the inhibition of 12G5 binding to SUP-T1 cells) exhibited potent anti-HIV activity . A clear correlation was seen between the anti-HIV potency expressed as $\log_{10}$ EC_{50} and the $IC_{50\text{-}12G5}$. The correlation coefficient was 0.8 and the calculated r^2 value was 0.7 ($p<0.01$). Compared with the bicyclam analogs, SDF-1α at 0.5 μg/ml (roughly the same concentration as its EC_{50} for anti-HIV activity) inhibited by 50% the binding of 12G5 mAb to SUP-T1 cells. Similarly, a close correlation was found between the

anti-HIV potency of the compounds tested and their capacity to inhibit the intracellular Ca^{++} signal induced by SDF-1α in SUP-T1 cells ($r^2 = 0.7$) (Fig. 4).

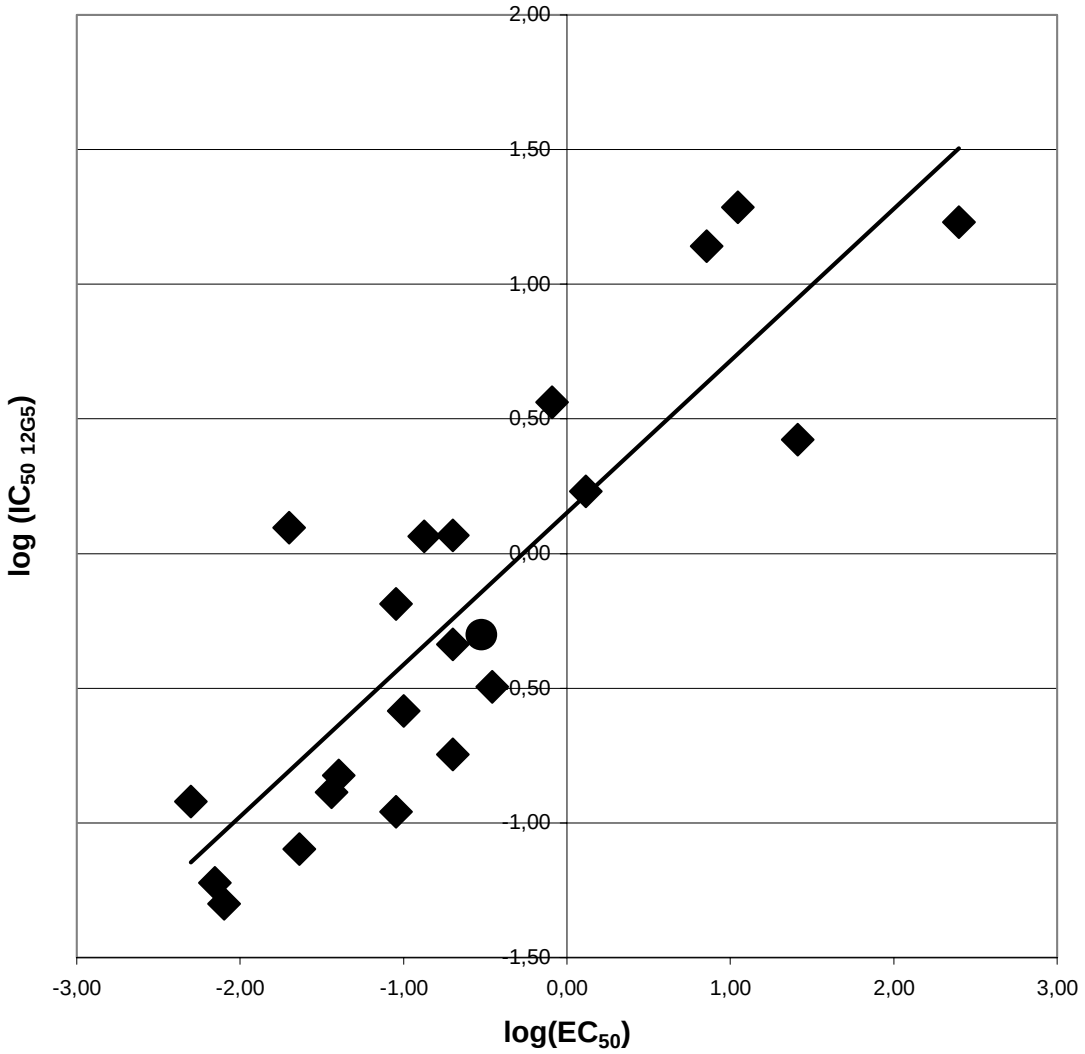

Figure 3. Correlation of the anti-HIV-1 (NL4-3) activity and interaction with the CXCR4 receptor of the different bicyclam analogs as assessed by linear regression analysis. Anti-HIV-1 activity [$\log(EC_{50})$] is plotted against the mean fluorescence intensity of SUP-T1 cells labeled with the CXCR4 mAb (12G5) and FITC-conjugated anti-mouse antibody in the presence of 0.5 μg/ml of SDF-1α (•)or each of the bicyclam analogs presented in Table 2 (♦).

Effect of metal complexes to AMD3100. For transition metal complexes of the prototype AMD3100, the anti-HIV-1 activity depended on the bound metal (Table 3). The Zn^{2+} complex (AMD3479) was slightly more active (10-fold) than AMD3100; and

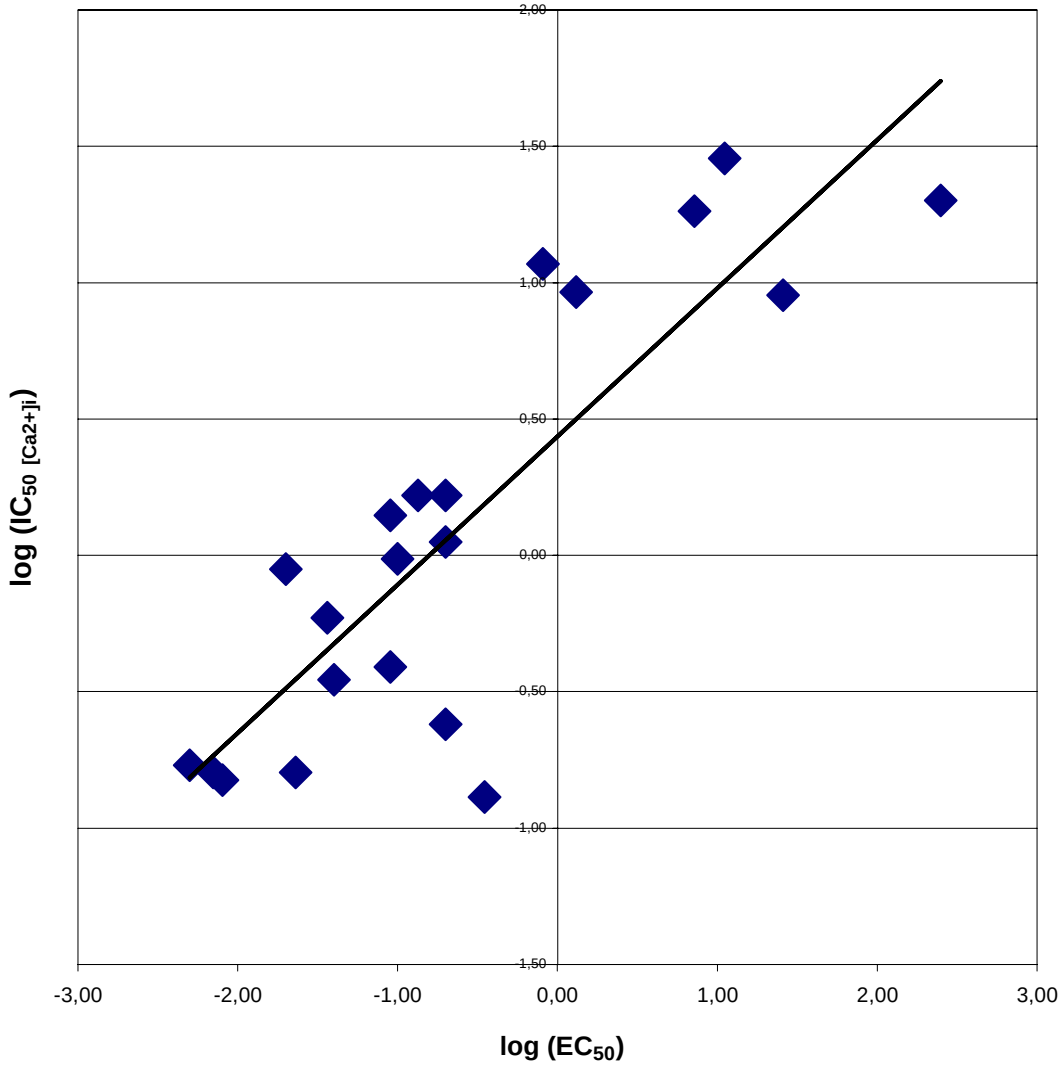

Figure 4. Correlation of the anti-HIV-1 (NL4-3) activity and interaction with the CXCR4 receptor of different bicyclam analogs as assessed by linear regression analysis. Anti-HIV-1 activity [log(EC_{50})] is plotted against the inhibition of SDF-1α-dependent intracellular Ca^{2+} mobilization [log ($[Ca^{2+}]_i$)] in SUP-T1 cells.

the Ni^{2+} complex (AMD3462) was as active as AMD3100 in their capacity to inhibit 12G5 binding. The Cu^{2+} (AMD3469) and Co^{3+} (AMD3461) complexes were 5-fold and 2220-fold less active, respectively than AMD3100. The Pd^{2+} complex (AMD3158) was virtually inactive. Similar differential inhibitory effects were noted for the metal complexes on the binding of the mAb with CXCR4; the IC_{50} for 12G5 binding to SUP-T1 cells closely paralleled the EC_{50} for anti-HIV activity ($r^2 = 0.8$). Similarly, the EC_{50} for the antiviral activity of the metal complexes correlated with the IC_{50} for inhibition of

the Ca^{2+} flux induced by SDF-1α indicating the dependence on the interaction of metal-complexed bicyclams with CXCR4 for their respective anti-HIV activity.

Table 3. Anti-HIV-1 activity, inhibition of 12G5 mAb binding and inhibition of $[Ca^{2+}]_i$ flux of AMD3100 and its different transition metal complexes.

Compound (bound metal)	EC_{50}[a] for HIV-1 (IIIB) (μg/ml)	IC_{50}[b] 12G5 binding (μg/ml)	$IC_{50\ [Ca2+]i}$[c] (μg/ml)
AMD3100 (free)	0.009	0.01	0.005
AMD3479 (Zn)	0.008	0.001	0.003
AMD3462 (Ni)	0.008	0.016	0.002
AMD3469 (Cu)	0.048	0.2	0.05
AMD3461 (Co)	0.74	0.5	0.6
AMD3158 (Pd)	68.62	12.5	70

[a] EC_{50}: as measured by the MTT assay.
[b] IC_{50}: 50% inhibitory concentration, or concentration of the compound required to inhibit by 50% the binding of 12G5 mAb to $CXCR4^+$ SUP-T1 cells.
[c] $IC_{50\ [Ca2+]i}$: by SDF-1α in SUP-T1 cells.

DISCUSSION

Earlier studies have shown that bicyclams, while being very potent inhibitors of HIV-1 replication, fail to inhibit virus-cell binding and are ineffective in blocking the viral reverse transcriptase or protease in cell-free systems. (De Clercq et al., 1992, De Clercq et al., 1994). From this earlier work it was suggested that bicyclams must interfere with a postbinding event coinciding with the virus fusion/uncoating process. De Vreese et al., 1996a; De Vreese et al., 1996b selected, after prolonged passage of the HIV-1 NL4-3 strain in MT-4 cells in the presence of increasing concentrations of AMD3100 a mutant strain that was aproximately 100-fold resistant to the compound. Resistance to AMD3100 was mapped to the envelope gp120 molecule. Several mutations leading to amino acid substitutions were found in the V3-V5 domain; they appeared to be particularly clustered at or near the V3 loop (De Vreese et al., 1996b). Thus, the HIV glycoprotein gp120 was suggested as the target of the bicyclams, but the specific site and mode of interaction with gp120 remained elusive.

With the discovery of the chemokine receptors as cofactors for the entry of HIV into $CD4^+$ cells, the mode of action of bicyclams has become clearer. We have shown

that AMD3100 selectively interacts with CXCR4 (Schols et al., 1997a, Schols et al., 1997b) which pointed to the direct interaction of CXCR4 with bicyclams as the mode of action for this class of compounds. The correlation shown here between the anti-HIV activity of the different bicyclam analogs and their interaction with the CXCR4 receptor (as monitored by inhibition of mAb 12G5 binding to cells and inhibition of SDF-1α-dependent intracellular Ca^{2+} flux) strongly suggests that blockade of the interaction between HIV and CXCR4 is the primary site of intervention of the bicyclams.

All the bicyclam analogs that showed activity against HIV-1 NL4-3 were also active against an X4 HIV-1 clinical isolate AOM and the HIV-1 RF and 168.10 strains that primarily use CXCR4 as coreceptor but can enter cells by using CCR5 as coreceptor (Alkhatib et al., 1996, data not shown). There was a close correlation between the antiviral activity of the different compounds against these three HIV-1 strains. However, the most active compounds (AMD3100, AMD3462, AMD3479, AMD3207 and AMD3469) were slightly less active against HIV-1 AOM and RF. Clinical isolates of HIV are composed of a heterogeneous population whereas the RF strain may use CCR5 (although inefficiently) to enter cells (Alkhatib et al., 1996; Doms et al., 1997). The anti-HIV activity of bicyclams would be attenuated by the ability of HIV-1 to use other coreceptors; nevertheless, the correlation found between the anti-HIV activity against NL4-3, AOM, RF, and 168.10 reiterates that bicyclams interfere with HIV replication through a similar mode of action. Furthermore, the lack of activity shown by different bicyclam analogs against the R5 strain BaL indicates that bicyclams are only active against those strains that use CXCR4 as entry coreceptors.

We have clearly shown that the interaction of bicyclams with CXCR4 (monitored by inhibition of 12G5 mAb binding), follows a similar structure-activity relationship as found earlier for inhibition of HIV-1 replication (Bridger et al.; 1995, Bridger et al., 1996). The interaction with the CXCR4 receptor appears to depend on the size of the tetraazamacrocyclic rings (which should be restricted to no more than fourteen members) and the linker [preferably phenylenebis(methylene)]. Also, for the metal-AMD3100 complexes, a close correlation was found between the anti-HIV activity and CXCR4 interaction, the order of decreasing activity being Zn > Ni > Cu >Co>Pd. Furthermore, the anti-HIV activity of bicyclams also parallels their capacity to inhibit the intracellular Ca^{2+} signal induced by SDF-1α, suggesting that bicyclams inhibit HIV-1 replication through a similar mode of action as SDF-1.

We have shown that HIV binding inhibitors such as dextran sulfate (DS) and the oligonucleotide AR177 (Zintevir) are no longer able to inhibit the binding of DS-resistant and AR177-resistant viruses (Esté et al., 1997, Esté et al., 1998), thus confirming the mode of action of these compounds (i.e. inhibition of virus adsorption to the cells). Mutations required to generate partial resistance to AMD3100 (De Vreese et al., 1996b) also lead to cross-resistance to polyanions such as DS (Esté et al., 1996) and to the chemokine SDF-1α (Schols et al., 1998). DS-resistant NL4-3 (Esté et al., 1997), AR177-resistant NL4-3 (Esté et al., 1998) and SDF-1α-resistant NL4-3 (Schols et al., 1998) show mutations in the gp120 that are also present in the AMD3100-resistant strain. At first glance these results suggest that polyanions may share similarities in their mode of action to bicyclams, that is, polyanions such as DS or AR177 could interact with postbinding events. However, the cross-resistance observed could be explained by an indirect effect on virus binding to $CD4^+$ cells as an consequence of the virus escaping the antagonism of AMD3100 on CXCR4 through mutations in the gp120 glycoprotein.

To escape the antiviral activity of bicyclams, the AMD3100-resistant strains could have switched coreceptors use or (as it has been demonstrated with different HIV-1 and HIV-2 strains) it could be using CXCR4 differently than the parental NL4-3 virus (Brelot et al., 1997). The results presented here do not address this issue; however, the AMD3100-resistant strain was cross-resistant to all the bicyclam analogs tested, This indicates that all the bicyclams share the same mode of action with AMD3100, that is, they “see” CXCR4 in a similar fashion. If different virus strains interact with CXCR4 in a different fashion (i.e. the AMD3100-resistant virus as opposed to the NL4-3 virus) that allows them to escape the anti-HIV activity of AMD3100, then all bicyclam analogs will show a reduced inhibitory capacity because they all appear to interact in a similar fashion. Nevertheless, their specific activity against 12G5 binding and SDF-1α-induced intracellular Ca^{++} flux points to their inhibition of the HIV-fusion process through interaction with the HIV cofactor CXCR4.

The bicyclam derivatives exhibit a mode of anti-HIV activity that is clearly different from that of the other anti-HIV agents presently used or considered for use in the treatment of HIV infection. However, after the report by Schols et al. (1997a) on the AMD3100-CXCR4 interaction, two other groups described newly identified CXCR4 antagonists: 1) ALX40-4C, a polycationic, nonapeptide solely existing of arginine residues (Doranz et al., 1997) and 2) T22 (Murukami et al., 1997), an 18-residue peptide

which has eight positive charges. As AMD3100 is also positively charged, it appears that the cationic nature of these compounds is necessary for their activity. Furthermore, the restriction on the number and position of amino groups in the bicyclam structure suggests that specific disposition of positive charges is required for strong interaction with CXCR4. Furthermore, T22, like AMD3100, may form a Zn^{2+}complex that is 4-fold more active than T22 itself (Tamamura et al., 1996). Because the Zn^{2+}complex of AMD3100 was 10-times more potent than AMD3100 in its interaction with CXCR4, it is possible that Zn^{2+}complex formation of these antagonist of CXCR4 may be of importance.

The recent studies by Tachibana et al., (1998) and Zou et al., (1998) have revealed that CXCR4 and SDF-1 are important in embryonic development and could have non-redundant functions in adults. This poses serious concerns on the use of CXCR4 antagonists as therapeutic agents against HIV. A possible toxic effect was not reported after administration of AMD3100 (10 mg/kg/day b.i.d.) to SCID-hu Thy/Liv mice for 28 days, in spite of a significant decrease in HIV viral load in the infected mice (Datema et al., 1996). Furthermore, homozygosity for an SDF-1 gene variant that has been associated with a delayed progression to AIDS is found in about 3% of healthy individuals studied (Winkler et al., 1998). Althought this finding appears to be controversial (Mummidi et al., 1998), alterations of the SDF/CXCR4 systems may not necessarily induce an adverse condition in healthy individuals. In turn, low but significant levels of CXCR4 antagonist could block the development of X4 strains that are clearly associated with disease progresion (Fauci et al., 1996, Glushakova et al., 1998)

Bicyclams not only demonstrate the feasibility of developing non-peptidic, small-molecule antagonists to the chemokine receptors but may serve, through the understanding of the structural components that are required for coreceptor interaction, for the development of new compounds against a broader spectrum of HIV-1 strains.

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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 HIV-1 Tat-transactivation responsive element interaction. The polycationic nature of these agents lead us to suggests 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, conjugates with arginine, inhibited HIV-1 NL4-3 replication at EC_{50} values of 15 and 30 μM for R3G and R4K, respectively, without a detectable toxic effect on MT-4 cells at concentrations higher than 4000 and around 1000 μM, respectively. Both compounds inhibited the binding of a monoclonal antibody (12G5) directed to CXCR4 as well as the intracellular Ca^{2+} signal induced by the chemokine SDF-1α 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, GB4K, 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-1α 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.

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INTRODUCTION

The need for effective chemotherapeutic treatments of human immunodeficiency virus (HIV) infections has led to search 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 cell (1) has renewed the 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 small 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 residues (10) 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 resemble 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 CH_2 (Fig. 1 and Refs 13, 14). Both R4K and R3G display high affinity to 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 equine infectious anemia virus (EIAV, an equine lentivirus) proliferation in cell culture with 90% effective concentration (EC_{90}) values of 25-50 μM of R3G and 100 μM 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 recently described (13-15). R4K is an arginine-kanamycin A conjugate containing four arginine residues per molecule. The arginine-gentamicin conjugate mixture (R3G) consists of tri-arginine 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-1α was purchased from Peprotech (London, UK). Azidothymidine (AZT) was purchased from Sigma (St. Louis, MO). The HIV-1 strains NL4-3 and Ba-L and the $CD4^+$ lymphocytic cell lines SUP-T1, MT-4 and MT-2 and P4-CCR5 MAGI (17) cells were obtained from the Medical Research Council (MRC) AIDS reagent program (London, UK) or the National Intitute 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 ($1x\ 10^5$/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 before (19).

Flow cytometry analyses. Measurement of chemokine receptors CXCR4, CCR5 and the CD4 receptor on SUP-T1 cells or peripheral blood mononuclear cells (PBMC) 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 minutes at 4ºC with monoclonal antibodies (Mab) 12G5, 2D7 and Leu3a (Beckton Dickinson, San Jose, CA) conjugated to phycoerytrin (PE), flourescein isothiocyanate (FITC) or peridin chlorophyll (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

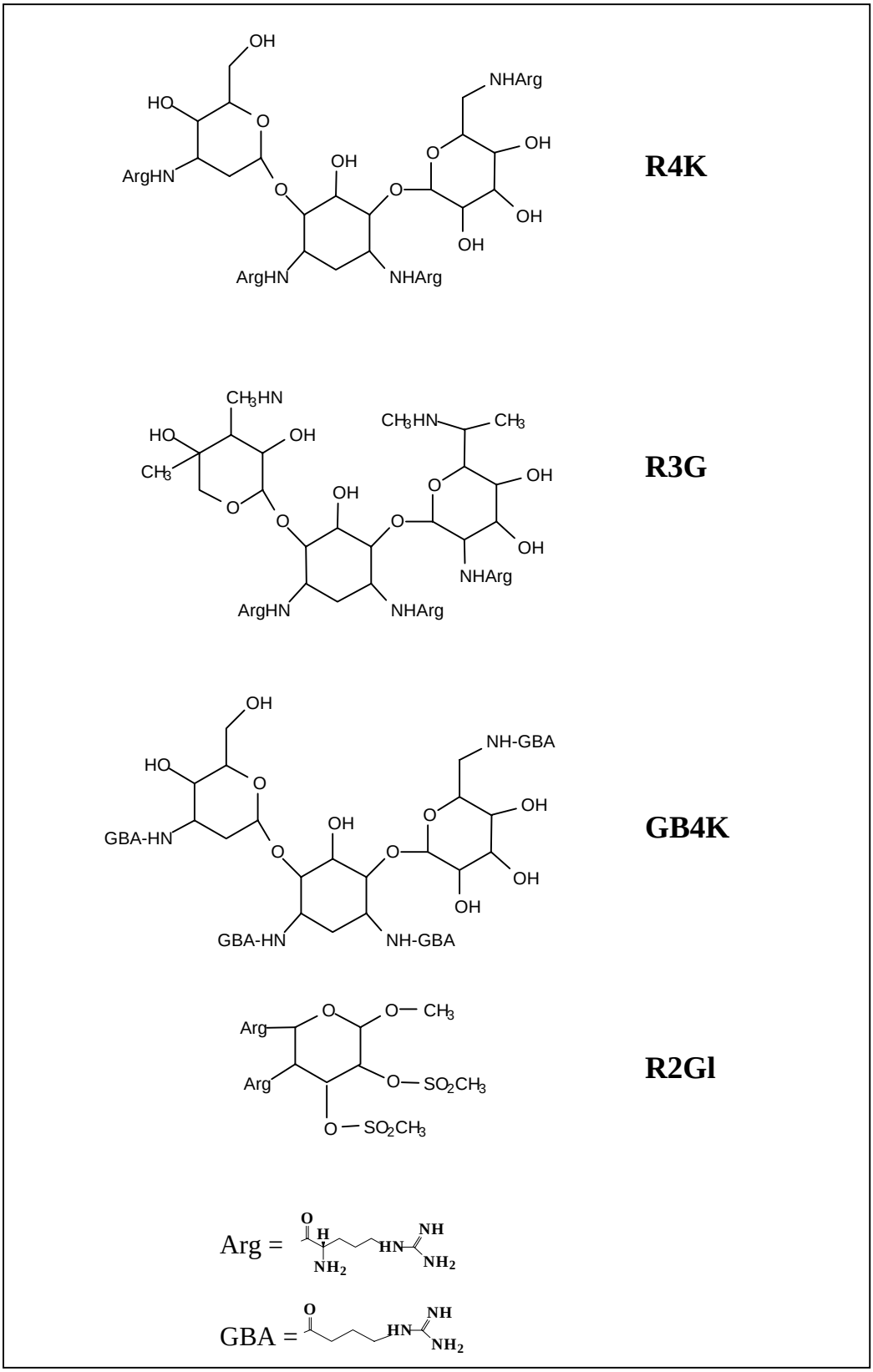

Figure 1. The structure of kanamycin and gentamicin C conjugates: arginine R4K is a tetrasubstituted derivative of kanamycin; R3G is a mixture of trisubstituted derivatives of gentamicin; GB4K is equivalent to R4K, but has guanidinobutyric acid (GBA) residues instead of L-arginine reidues. Arg, Arginine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

fixed in PBS containing 1% formaldehyde. For each sample 10.000 events were analysed in aFACScalibur system (Becton Dickinson). Data were acquired and analysed with CellQuest as software (Becton Dickinson).

Measurement of intracellular calcium concentrations. The intracellular calcium concentrations $[Ca^{2+}]_i$ were determined as described previously (9). Briefly, SUP-T1 or THP-1 cells were loaded with Fluo-3 (Sigma, St. Louis, MO). 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-1α (20 ng/ml) or RANTES (1 μg/ml) was used to induce $[Ca^{2+}]_i$ increase. The second stimulus was added 10 sec after the first stimulus. The compound concentration required to inhibit the $[Ca^{2+}]_i$ increase by 50% ($IC_{50\ [Ca2+]i}$) was calculated.

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 comercial 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 μM and 31 μM, respectively. The 50 % cytotoxic concentrations (CC_{50}) were calculated to be >1130 μM and >3940 μM for R4K and R3G respectively. The chemokine SDF-1α was active at EC_{50} of 0.04 μM while the CXCR4 antagonist AMD3100 was active at EC_{50} of 0.01 μM. Conversely, compounds R2Gl, which contains two L-arginine residues conjugates 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.

Table 1. Anti-HIV-1 activity of the various compounds and CXC-chemokine SDF-1α.

Compound	EC_{50}[a] (μM)					CC_{50}[b] (μM)
	HIV-1 NL4-3	HIV-1 RF	HIV-1 AOM	HIV-1 AMD3100res	HIV-1 Ba-L[c]	
R4K	31	>113	67	>113	>113	1130
R3G	15	35	16	>133	29	> 3940
R2GL	>200	-	-	>203	-	>200
GB4K	>200	-	-	>203	-	>200
AMD3100	0.01	0.02	0.02	0.5	>100	>100
SDF-1α	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] CC_{50}: 50% cytotoxic concentration, or concentration of the compound required to reduce the viability of MT-4 cells, as measured by the MTT assay.
[c] EC_{50}: 50% effective concentration, or concentration of the compound required to inhibit HIV-1 replication by 50%, as measured by ß-galactosidase activity in P4-CCR5 cells infected with the HIV-1 Ba-L strain.

The NL4-3 AMD3100-resistant virus was cross-resistant to SDF-1α (>25-fold) to 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-1α, 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 (IC_{50}) of 12G5 MAb binding ($IC_{50\text{-}12G5}$) by R3G, R4K, GB4K, R2Gl, AMD3100 and SDF-1α. 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)/phytohemoagglutinin (PHA)-stimulated PBMC (Fig.2).

Table 2. Inhibition of anti-CXCR4 MAb (12G5) binding to $CXCR4^+$ cells

	$IC_{50\text{-}12G5}$[a] (μM)	
Compound	SUP-T1 cells	PMBC
R4K	3.7	2.2
R3G	7.7	2.7
R2Gl	>40	25
GB4K	>40	>40
AMD3100	0.01	0.001
SDF-1α	0.013	-[b]

[a] $IC_{50\text{-}12G5}$: 50% inhibitory concentration, or concentration of the compound required to inhibit by 50% the binding of 12G5 MAb to $CXCR4^+$ cells.
[b] -:Not tested.

To evaluate further the interaction of aminoglycoside-arginine conjugates with CXCR4, we tested the capacity of SDF-1α to induce an intracellular Ca^{2+} signal in the presence of these conjugates. Both R3G and R4K inhibited the SDF-1α-dependent Ca^{2+} signal in a dose-dependent manner, which is similar to the effect of bicyclam AMD3100. Compounds R2Gl and GB4K did not significantly inhibit SDF-1α-dependent signal (Fig. 3). R3G and R4K did not inhibit the intracellular Ca^{2+} 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-1α at a concentration of 0.5 μg/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).

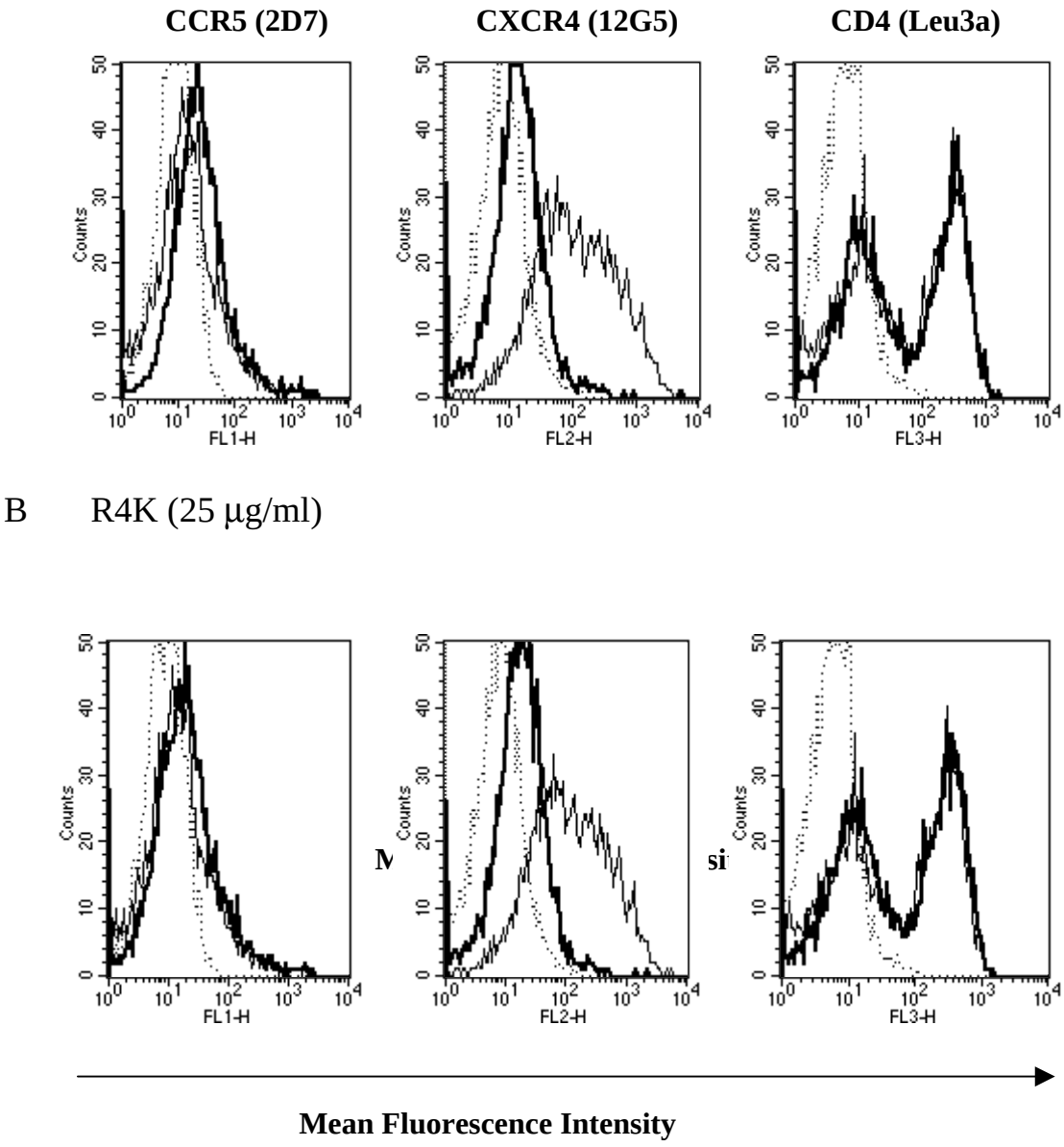

Figure 2. Effect of R3G (25 µg/ml) and R4K (25 µg/ml) on binding of 2D7 MAb to CCR5, 12G5 MAb to CXCR4 and Leu3a MAb to CD4 in stimulatedperipheral blood mononuclear cells. For each experiment cells were incubated with MAbs 2D7, 12G5 and Leu3a conjugated to FITC, PE or PerCP, respectivelly or isotype control MAb (doted line) in the presence (thick line) or absence (thin line) of the corresponding compound (25 µg/ml). After a 30 min incubation at 4º C, cells were washed with PBS and analysed by flow cytometry.

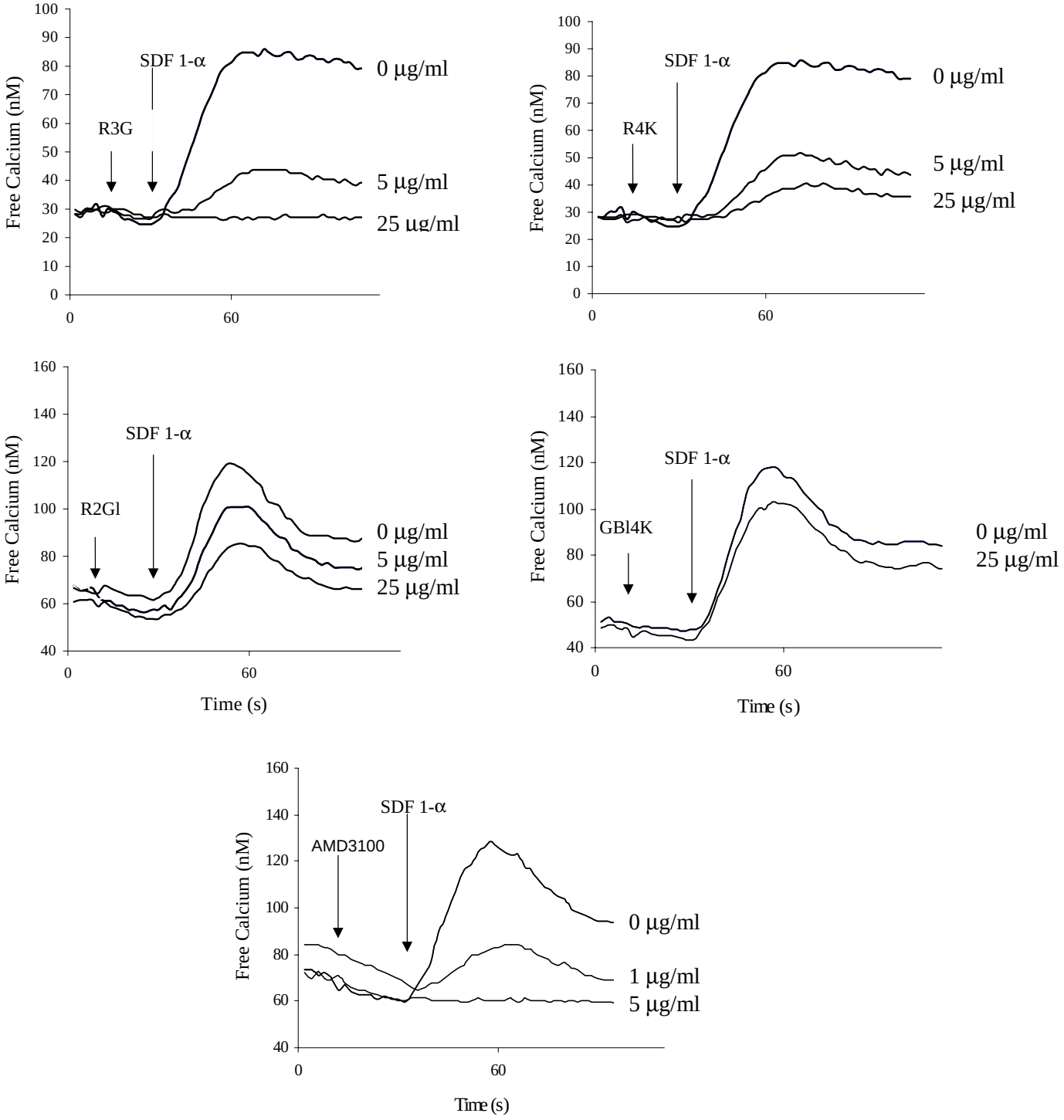

Figure 3. SDF-1α-induced signaling via CXCR4 was blocked by R3G and R4K. SUP-T1 cells were loaded with Fluo-3 fluorochrome and,10 sec after the first stimulation with the appropriate concentration of compound, SDF-1α was given as a second stimulus at 20 ng/ml. Fluorescence was measured in a Fluoroskan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

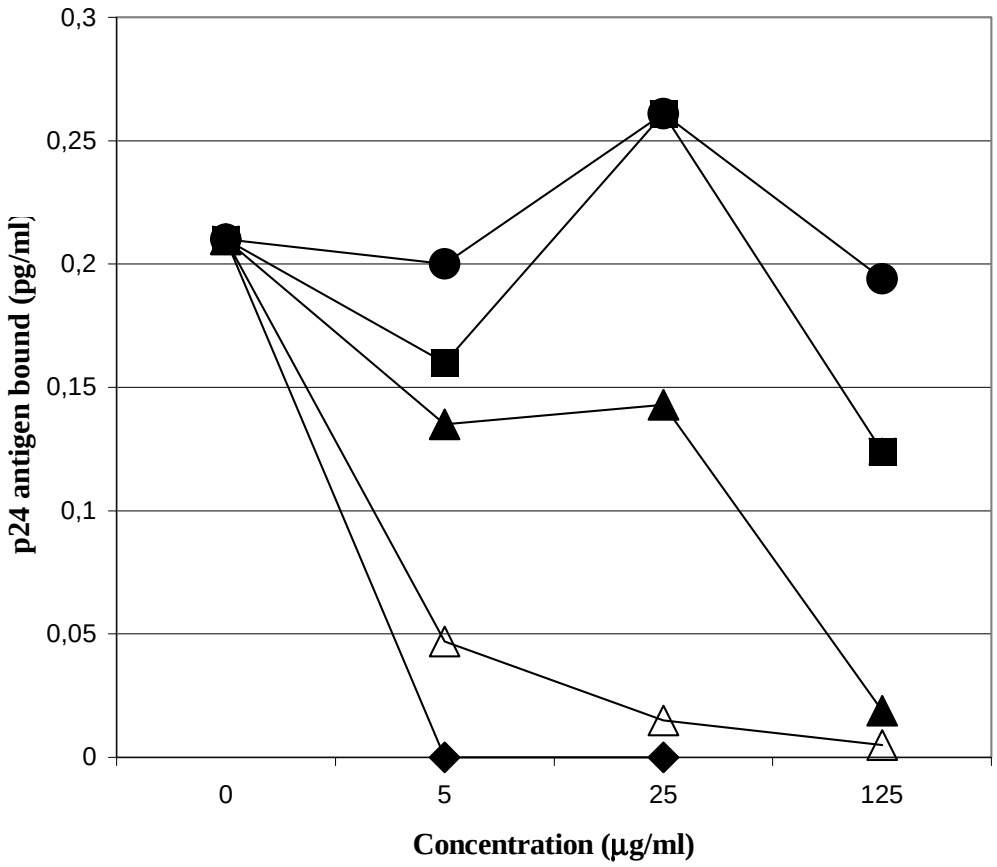

Figure 4. R3G and R4K inhibited the binding of HIV-1 to $CD4^+$ cells. MT-4 cells were infected with $1x10^5$ pg of p24 antigen of HIV-1 (NL4-3) in the presence of various concentrations of the corresponding compound. After 1 hour incubation a 37ºC, cells were washed 3 times in PBS and p24 antigen bound to cells was determined by an ELISA test. R4K (▲), R3G (△), R2Gl (■), GB4K (●), dextran sulfate (◆).

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 TAK779 (5) 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 at early stages of infection.

Aminoglycoside-arginine conjugates were designed as specific TAR RNA binders, based on the idea that a combination of two RNA-binding patterns, aminoglycoside and peptide may be effective. These conjugates displayed high affinity and specificity TAR RNA binding *in vitro,* along with low cytotoxicity and antilentiviral 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 (EC_{50} 15 μM for R3G and 31 μM for R4K against the HIV-1 NL4-3 strain), with undetected cytotoxicity at the active concentrations (CC_{50} was >3940 μM for R3G and 1130 μM 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 R2Gl (containing two arginine residues) and GB4K (containing four γ-guanidinobutyric residues and is structurally similar to R4K) are devoid of the antiviral effect observed with R4K and R3G. It is possible that the number a 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 Ca^{2+} signal induced by SDF-1α in $CXCR4^{+}$ cells, without affecting the intracellular Ca^{2+} signal induced by RANTES in $CCR5^{+}$ cells. The preceding suggests that antagonism to CXCR4 may be, in part, the mode of anti-HIV action of these compounds. The AMD3100-resistant HIV-1 strain (27) 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 transcriptsase 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 of 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 *trans*-ativation 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: blockade 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 Wyoming strain used as a model lentivirus is avirulent and is incapable to infect directly equine fibroblasts (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 modifyed 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 for $CD4^+$ 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 potently inhibited by CXCR4 antagonists in *ex vivo* cultures (33). Furthermore, blockade of CXCR4 prevented the evolution of HIV clinical isolates into CXCR4-using strains (34) or gp120-mediated apoptosis (35, 36). These outstanding findings warrant the examination of CXCR4 antagonists as anti-HIV agents.

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