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Anti-HIV activity of a novel aminoglycoside-arginine conjugate

Cecilia Cabrera [a], Arantxa Gutiérrez [a], Jordi Barretina [a], Julià Blanco [a], Alexander Litovchick [b], Aviva Lapidot [b], Bonaventura Clotet [a], José A. Esté [a,*]

[a] *Retrovirology Laboratory, Fundació irsiCaixa, Hospital Universitari Germans Trias i Pujol, Universitat Autonoma de Barcelona, 08916 Badalona, Spain*

[b] *Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel*

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Abstract

We have previously described conjugates of L-arginine with aminoglycosides (AAC) that have shown anti-human immunodeficiency virus type 1 (HIV-1) activity in in vitro cell culture systems. Here, we extend our report to a novel neomycin B-arginine conjugate (NeoR) that has shown up to 30-fold increased potency over previous AAC compounds. NeoR inhibited the replication of both R5 and X4 strains of HIV-1 in cells expressing the appropriate coreceptor or peripheral blood mononuclear cells. In lymphoid tissue ex vivo, NeoR blocked the replication of the dualtropic strain 89.6 suggesting anti-HIV activity of AAC on the site of in vivo virus replication. NeoR blocked the binding of HIV particles to lymphoid cells and was also able to antagonize the activity of the CXCR4 receptor so it may prevent the emergence of X4 HIV-1 strains. Nevertheless, in a cellular assay, we were unable to detect anti-Tat dependent transactivation activity as previously suggested for this family of compounds.

Keywords: Anti-HIV; Chemokine receptors; Binding; Transactivation; Ex-vivo cultures

1. Introduction

The significant toxicity associated with the administration of approved antiviral drugs against human immunodeficiency virus (HIV) and the emergence of drug-resistant strains of HIV-1 severely diminish their therapeutic usefulness. The development of a number of agents with similar or greater potency than the ones currently used but with new targets for anti-HIV activity are needed to circumvent virus-drug resistance. These new drugs, when used in combination therapies, may help to prevent the emergence of drug-resistant HIV mutants and may also allow the use of individual drugs below their toxic concentrations. Furthermore, compounds that have more than one mechanism of anti-HIV activity may represent an important tool at preventing the emer-

* Corresponding author. Tel.: +34-93-46-56374; fax: +34-93-46-53968.

E-mail address: jaeste@ns.hugtip.scs.es (J.A. Esté).

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gence of HIV drug-resistant strains because multiple sites in the viral genome would have to mutate to generate drug-resistant strains.

Earlier, we reported the activity of a new class of anti-HIV agents. The conjugates of aminoglycoside antibiotics with L-arginine comprise a completely new class of peptidomimetic substances (Litovchick et al., 1999, 2000). The aminoglycoside-arginine conjugates resemble oligocationic peptides by their chemical properties including the capacity shown by other agents such as ALX-40-4C, to bind to the HIV transactivation responsive element (TAR) RNA in vitro (O'Brien et al., 1996). We have found that the lead compounds, tri-arginine-aminoglycoside conjugate (R3G) and tetra-arginine-aminoglycoside conjugate (R4K) reveal anti-HIV activity at the micromolar range (EC_{50} 24 μM for R3G and 31 μM for R4K against the HIV-1 NL4-3 strain), with undetected cytotoxicity at the active concentrations ($CC_{50} > 3940$ μM for R3G and 1130 μM for R4K) (Cabrera et al., 2000). In the present study we report on a new member of arginine aminoglycoside conjugates (AAC), neomycin B conjugated to arginine, NeoR (Fig. 1). The selection of neomycin B was based on the ability of neomycin B to bind directly to TAR RNA (Wang et al., 1998) in contrast to kanamycin A or gentamicin C. Here, we have evaluated the anti-HIV activity of NeoR and determined its potency and mode of action in vitro and in ex vivo cell culture systems.

2. Methods

2.1. Compounds, viruses and cells

The synthesis, purification, and chemical characterization of arginine aminoglycoside conjugates (AAC) and the new compound NeoR have been described (Litovchick et al., 1999, 2000). NeoR is a neomycin B conjugated to arginine (Fig. 1). The CCR5 ligand AOP-RANTES was a kind gift of Dr Robin Offord (Department of Medical Biochemistry, University of Geneva, Geneva, Switzerland). SDF-1α was purchased from Peprotech (London, UK). Azidothymidine (AZT) was purchased from Sigma (St. Louis, MO). The X4 HIV-1 AZT-resistant is a low-passage clinical isolate from our cohort of HIV positive patients. The X4 HIV-1 strain NL4-3, the X4/R5 HIV-1 89.6 and RF and the R5 HIV-1 BaL viruses; the CD4+ lymphocytic cell lines SUPT-1, MT-4 and P4-CCR5-MAGI 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.

2.2. 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 at multiplicity of infection (moi or the ratio of $CCID_{50}$ per cell) of 0.003 and exposed to various concentrations of test compound. After 5 days of infection, the number of viable cells was quantified by a tetrazolium-based colorimetric method (MTT method) as described (Pauwels et

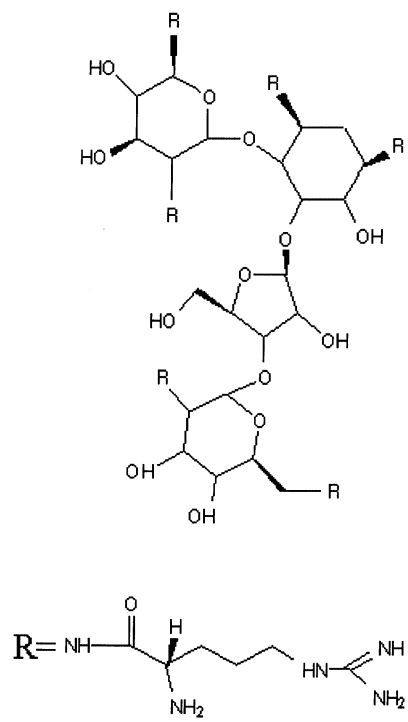

Fig. 1. The structure of neomycin B-arginine conjugate NeoR.

al., 1988). Anti-HIV activity in P4-CCR5-MAGI cells (Chackerian et al., 1997) was done as follows: cells (1×10^5/ml) were infected with HIV-1 BaL or NL4-3, in the presence of varying concentrations of the test compound. Twenty-four hours postinfection, cells were washed twice with PBS and resuspended in fresh medium containing the appropriate drug concentration. Five days after infection the cells were washed with PBS and evaluated for β-galactosidase activity as previously described (Esté et al., 1995).

Virus input corresponded to 3000 pg/ml of p24 antigen containing supernatant, or a moi of 0.06, necessary to induce a significant (10-fold) increase in β-galactosidase activity after 5 day incubation of cells at 37 °C.

2.3. Flow cytometry analysis

Measurement of chemokine receptor CXCR4 and CCR5, and the CD4 receptor on SUP-T1 or peripheral blood mononuclear cells (PBMCs) was performed by flow cytometry analysis as previously reported (Blanco et al., 1999). 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 (anti-CXCR4), 2D7 (anti-CCR5) and Leu3a (anti-CD4) (BD, San Jose, CA) conjugated to phycoerythrin (PE), fluorescein isothiocyanate (FITC), and peridin chlorophyll protein (PerCP), respectively. The cells were then washed with ice-cold PBS and were fixed in PBS containing 1% formaldehyde and analyzed by flow cytometry in a FACSscalibur system (BD, San Jose, CA). Data were acquired and analyzed with CELLQUEST software (BD).

2.4. Measurement of intracellular calcium concentrations

The intracellular calcium concentrations [Ca^{2+}] were determined as described previously (Esté et al., 1999b). Briefly, SUP-T1 cells or PBMCs were loaded with Fluo-3 (Sigma, Badalona, Spain). 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 was used to induce [Ca^{2+}] increase. The second stimulus was added 10 sec after the first stimulus. The compound concentration required to inhibit the [Ca^{2+}] increase by 50% ($IC_{50\ [Ca2+]}$) was calculated.

2.5. Virus-binding assay

Virus-binding to lymphoid cells was evaluated as previously described (Witvrouw et al., 1994). Briefly, MT-4 cells (5×10^5) were incubated with supernatant containing 1×10^5 pg of p24 antigen of wild type HIV-1 (NL4-3 strain) in the presence of different concentrations of the test compound. One hour after infection, cells were washed 3 times with PBS and p24 antigen bound to the cells was determined by a commercial ELISA test (Innogenetics, Barcelona, Spain).

2.6. Tat-dependent transactivation assay

Tat-dependent transactivation of the HIV long terminal repeat (LTR) was evaluated as reported (Esté et al., 1995). Briefly, P4-CCR5-MAGI cells that expressed the β-galactosidase gene driven by the HIV LTR were transiently transfected with a Tat expression plasmid and the cells were incubated in the presence or absence of different drug concentrations. After 48 h incubation, cells were lysed and evaluated for β-galactosidase activity.

2.7. Lymphoid tissue cultures

Anti-HIV activity in lymphoid tissue was evaluated as described before (Glushakova et al., 1998; Grivel and Margolis, 1999). Briefly, tonsils from healthy individuals that were removed during therapeutic tonsillectomy and maintained in phosphate-buffered saline (PBS) were dissected into 2–3-mm blocks and placed on top of collagen sponge gels. HIV-1 infection was carried out with 3 μl of the dual tropic HIV-1 89.6 p24-antigen supernatant in the absence or presence of the test compound. Twelve days after infection, the concentration of p24 antigen in the supernatant was evaluated by a commercial ELISA test (Innogenetics, Barcelona, Spain).

Table 1
Anti-HIV-1 activity of the different compounds including the CXC-chemokine SDF-1α

Compound	EC_{50}[a] (μM) HIV-1 strain					CC_{50}[c] (μM)
	NL4-3	RF	AZT-resistant	BaL[b]	AMD3100-resistant NL4-3	
NeoR	0.73	1.4	0.8	4.1	1.2	>55
R3G	24	30	22	156	60	>90
Alx-40-4C	0.7	1.8	1.7	1.6	1.8	80
AMD3100	0.005	0.06	0.02	>40	0.45	>200
AOP-RANTES	>0.06	–	–	0.0004	–	>0.06
AZT	0.004	0.002	>7	0.07	0.008	>15
Dextran sulfate	0.01	0.4	–	0.012	0.018	50
SDF-1α	0.01	–	0.01	>0.2	0.2	>0.2

[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] 50% effective concentration based on the inhibition of HIV-1-induced β-galactosidase activity in P4-CCR5-MAGI cells.
[c] 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.
–, not tested. There were no significant intra-assay differences in the EC_{50} or CC_{50} values.

3. Results

3.1. Anti-HIV activity of NeoR

Results on the anti-HIV activity of NeoR are shown in Table 1 and Fig. 2. NeoR proved more than 30-fold more active than the gentamicin AAC, R3G. The 50% effective concentration (EC_{50}) of NeoR ranged between 0.7 and 4 μM depending on the HIV-1 strains (including an AZT-resistant HIV-1). Notably, NeoR was also active against a HIV-1 NL4-3-derived strain that was made resistant to the CXCR4 antagonist AMD3100 (De Vreese et al., 1996a) and to the macrophage-tropic, CCR5-using strain BaL, albeit at a higher EC_{50} than for X4 HIV-1 NL4-3 strain.

3.2. Interaction with CXCR4 receptor

CXCR4 antagonists are selective inhibitors of the replication of HIV strains that use CXCR4 as entry coreceptor. To elucidate whether the anti-HIV activity of NeoR is due to their interaction with CXCR4, we tested the capacity to inhibit the binding of a mAb to CXCR4 (12G5). SDF-1α, the natural ligand of CXCR4 and the CXCR4 antagonists AMD3100 and Allelix-40-4C (Schols et al., 1997a,b; Doranz et al., 1997; Donzella et al. 1998) completely block the binding of the 12G5 mAb to CXCR4-expressing cells. Table 2 shows the concentrations of each compound that is required to block the binding of 12G5 to CXCR4+ cells by 50% inhibition ($IC_{50\text{-}12G5}$). NeoR showed high affinity for CXCR4 (as measured by the inhibition of 12G5 binding to SUP-T1), which is consistent with its anti-HIV activity. NeoR also inhibited the binding of 12G5 to PBMCs. Neither compound inhibited the binding of 2D7 (a monoclonal antibody directed to CCR5) to PBMCs or an anti-CD4 antibody (Leu3a) in SUP-T1 or stimulated PBMCs (data not shown).

To evaluate further the interaction of NeoR with CXCR4 we tested its capacity to block the intracellular Ca^{2+} signal induced by SDF-1α in CXCR4+, SUP-T1 cells. NeoR inhibited the SDF-1α-induced intracellular signal in a dose-dependent manner with an IC_{50} that is similar to that required for anti-HIV-1 activity (Table 2). These results suggest that NeoR acted as a CXCR4 antagonist and with greater potency than R3G but lower potency than AMD3100. However, these results could not explain why NeoR may block the replication of R5 viruses such as BaL or the AMD3100-resistant strain.

3.3. Inhibition of virus binding

We then tested the ability of NeoR to block an early step of virus replication. As expected, the bicyclam AMD3100, a CXCR4 antagonist that has been shown not to block HIV-1 binding to CD4+ cells (De Vreese et al., 1996b), and the reverse transcriptase inhibitor AZT, did not have an inhibitory effect on HIV binding to cells (Table 2). Like dextran sulfate (DS), NeoR was able to efficiently block the binding of HIV-1 to lymphoid cells at roughly the same concentrations as needed to block HIV-1 replication. Inhibition of virus binding, as evaluated by the detection of bound p24 antigen to lymphoid cells, was dose-dependent and was completely blocked by dextran sulfate at 5 μM) and NeoR at 55 μM.

3.4. Anti-HIV activity in lymphoid tissue cultures

Primary cultures and co-cultures of isolated cells mimic neither the full cellular repertoire within lymph tissue nor the functional relationship of HIV to lymphoid tissue structure (Glushakova et al., 1995). Lymphoid tissue cultures ex vivo may provide the means to evaluate HIV replication in a system that retains the complex three-dimensional cellular organization found in vivo. Similarly, the evaluation of antiviral agents in lymphoid tissue ex vivo may better represent the potential of the test agent as a candidate therapeutic drug. CXCR4 antagonists such as the bicyclam AMD3100 have been tested for their capacity to block the replication of HIV-1 strains in tonsil lymphoid tissue cultures.

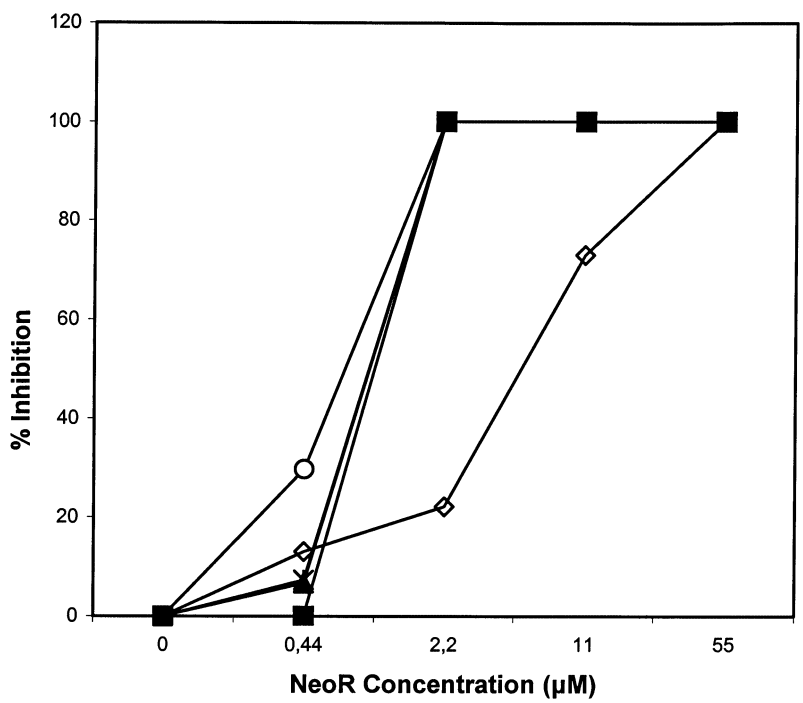

Fig. 2. Inhibition of virus-induced cytotoxicity by NeoR. MT-4 cells were infected with the HIV-1 strains NL4-3 (○), RF (■), AZT-resistant (*) and AMD3100-resistant (▲), 5 days post infection cell viability was measured by the MTT assay. For the evaluation of HIV-1 BaL (◇), P4-CCR5-MAGI cells were infected with HIV-1 BaL, 5 days post infection β-galactosidase activity was measured. A representative of at least three experiments is shown.

Table 2
Inhibition of NeoR and control compounds on different parameters

Compound	IC_{50}[a] (μM)			
	12G5 binding[a]	[Ca2+]i[b]	HIV binding[c]	HIV-1 Tat transactivation[d]
NeoR	1.3	2.7	0.44	>110
R3G	7.2	2.0	17.4	–
Alx-40-4C	0.4	–	–	0.5
AMD3100	0.005	0.03	>8	>8
Dextran sulfate	–	–	<0.2	–
SDF-1α	0.5	–	–	–

[a] 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. 12G5 mAb binding was evaluated by flow cytometry as described in Section 2.

[b] Concentration of the compound required to inhibit by 50% the intracellular Ca^{2+} concentration induced by SDF-1α (10 ng/ml) in SUP-T1 cells.

[c] Concentration of the compound required to reduce by 50% the detection of p24 antigen in MT-4 cells that were incubated with HIV-1 (NL4-3) for 1 h.

[d] IC_{50} values represent the concentration required to inhibit 50% of the β-galactosidase activity of untreated P4-CCR5-MAGI cells transfected with a Tat expression plasmid.

–, not tested. Values represent the mean of at least 3 assays. There were no significant intra-assay differences in IC_{50} calculations.

AMD3100, a selective CXCR4 antagonist was able to block the replication of dualtropic (R5/X4) strains of HIV-1 with similar potency as X4 variants (Glushakova et al., 1999), suggesting that dualtropic HIV-1 preferentially use CXCR4. Therefore, we wanted to evaluate the efficacy of NeoR against a virus that may alternatively use both CCR5 or CXCR4.

Similarly to AMD3100 and AZT, NeoR inhibited the replication of HIV-1 89.6 (Fig. 3) as measured by the detection of p24 antigen in the supernatant of infected tissue blocks. Thus, NeoR appeared to be active in lymphoid tissue that is the site of active viral replication.

4. Discussion

The ability of NeoR to inhibit the binding of HIV particles to CD4 may be the principal mode of action of this family of compounds. In addition, NeoR also interferes with CXCR4. NeoR blocked mAb binding to the chemokine receptor and blocked SDF-1α-induced intracellular signal at similar concentrations as those required to inhibit virus replication. These results suggest that antagonism to CXCR4 may, in part, explain the mode of anti-HIV action of these class of compounds. Other compounds such as polycationic peptides (T22 and ALX-40-4C) and bicyclams (AMD3100) have been identified as antagonist of CXCR4 (Donzella et al. 1998; Doranz et al., 1997; Esté et al., 1999b; Murakami et al., 1997; Schols et al., 1997a,b). These compounds interact with CXCR4 presumably because their cationic nature leads to electrostatic interactions with negatively charged residues of CXCR4 (Labrosse et al., 1998). We have shown that blockade of CXCR4 may prevent the emergence of CXCR4-using virus strains from clinical isolates. Furthermore, if clinical isolates with the X4 phenotype are grown in the presence of AMD3100, resistance to the compound will develop but with a concomitant switch to the R5 phenotype (Esté et al., 1999a). In turn, X4 strains have been associated with faster CD4+ cell decline and accelerated progression to AIDS (Fauci, 1996; Penn et al., 1999). Our results suggest that inhibition of HIV-1 replication with compounds such as NeoR, will block X4 and dualtropic (R5X4) strains of HIV, and thus limit the emergence of the X4 strains.

In conclusion, we extend the observation made with arginine-conjugated aminoglycosides that block HIV replication to a new member with

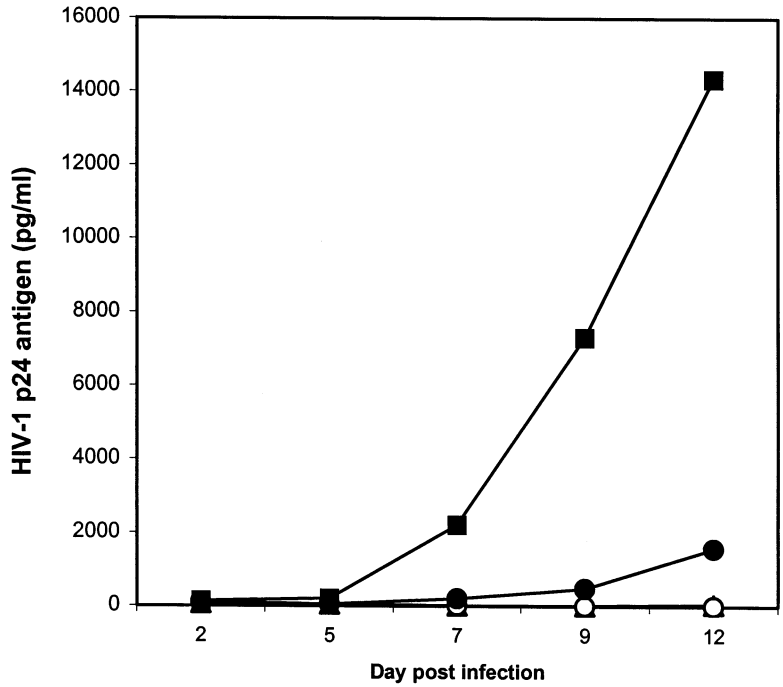

Fig. 3. Replication of HIV-1 89.6 in human tonsil histoculture in the absence (■) or presence of anti-HIV agents: NeoR 20 μM (●), AZT 0.8 μM (○) and AMD3100 12 μM (▲). Tissue blocks from a healthy donors were infected as indicated in Section 2 and viral replication was monitored. For each experiment medium bathing six blocks of tissue was pooled and p24 antigen was evaluated by a commercial ELISA test. A representative of two experiments is shown.

improved potency, and show that NeoR may block HIV replication both in vitro and in ex vivo with low cytotoxicity. Ongoing research is aimed at generating chemical variants with improved anti-HIV activity in the search for a potential candidate for clinical evaluation.

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