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Bioorganic & Medicinal Chemistry Letters
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Bioorganic & Medicinal Chemistry Letters 14 (2004) 495–498

New bicyclam–GalCer analogue conjugates: synthesis and in vitro anti-HIV activity

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Received 24 April 2003; revised 30 May 2003; accepted 20 October 2003

Abstract—The synthesis of bipharmacophore anti-HIV compounds which, in a single molecule, combine two ligands, that is, the bicyclam AMD3100 and a GalCer analogue, that might inhibit several steps of the complex virus/cell cascade interactions has been performed. The 'double-drug' Gal-AMD3100 conjugates elicited inhibitory effects on T (or X4)-tropic HIV-1 replication in all CXCR4 expressing cell lines with EC_{50} values ranging from 0.25 to 6.0 μM which were however ~40- to 125-fold lower than that of AMD3100. Concerning the mechanism of inhibition of the Gal-AMD3100 conjugates, experiments performed with X4 or R5HIV-1 strains and GHOST cells genetically modified to express CD4 and CXCR4 or CCR5 indicated clearly that the conjugates interact with CXCR4 and not with CCR5.
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The human immunodeficiency virus (HIV) infects mainly CD4(+) lymphoid cells. Their recognition and infection are mediated by the binding of HIV, through its envelope glycoprotein gp120, to CD4 and then to a cellular co-receptor of the chemokine receptor family, such as mainly CCR5 or CXCR4, according to the cell type infected and the M (or R5)- or T (or X4)-tropism of the HIV strains.[1] When this ternary gp120/CD4/co-receptor complex has formed, the viral transmembrane gp41, through a fusion peptide located at its external N-terminal domain, anchors into the target cell membrane. This initiates the virus-cell fusion and the delivery of the viral nucleocapside into the cell. Intensive efforts were undertaken for the discovery of anti-HIV drugs that target two major viral proteins, that is, the reverse transcriptase and protease.[2] Recently, it has also been pointed out that virus-cell fusion was a major hallmark of HIV infection. This observation has led to the discovery of novel highly potent agents that interfere with the viral binding to the cellular co-receptors, such as the low molecular weight AMD3100 (Fig. 1) which is a CXCR4 antagonist,[3] or that interact with gp41, such as the most promising 36-amino acid peptide T20.[4] HIV can also infect many CD4(−) cells.[5] Their infection has been shown to occur through gp120 binding to galactosylceramide (GalCer, Fig. 1) they express. Very recently, GalCer was also found to interact with the HIV gp41.[6] Further studies suggested the involvement of GalCer-rich microdomains,[6,7] thus providing an attachment platform for the virus through its gp120 and/or gp41 onto the cell. These findings have urged the synthesis of various galactolipids and the examination of their anti-HIV activity.[8] Unfortunately, the most active derivatives were found to exhibit only a moderate in vitro anti-HIV activity (EC_{50} in the 1–20 μM range).[8]

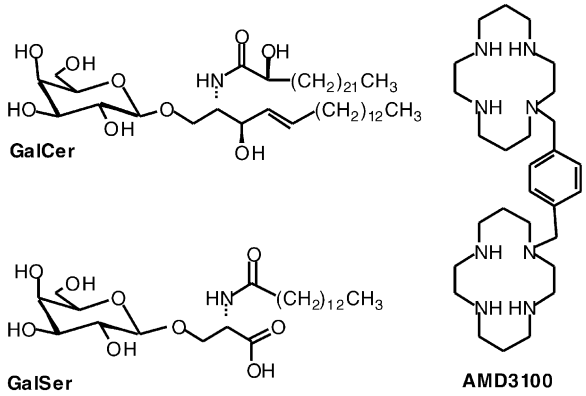

Figure 1. Chemical structure of galactosylceramide (GalCer), GalSer, an analogue of GalCer, and AMD3100.

Keywords: HIV; CXCR4; AMD3100; Galactosylceramide.
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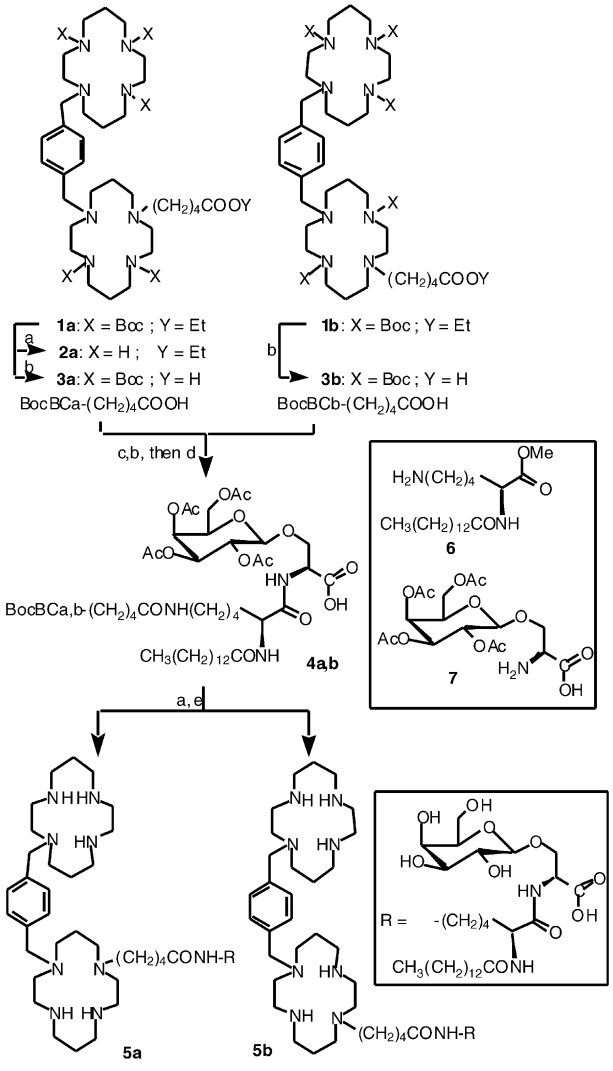
doi:10.1016/j.bmcl.2003.10.036

Our interests were to explore the development of bipharmacophore anti-HIV compounds which, in a single molecule, combine two ligands, that is, the bicyclam AMD3100 and a GalCer analogue, that might inhibit several steps of the virus/cell complex cascade interactions. It was indeed of great interest to establish whether such 'double-drug' conjugates could interfere synergistically (with respect to AMD3100 and the GalCer analogue) with both the gp120/CD4 and gp120/CD4/CXCR4 interactions, and/or with gp41, and, consequently, could constitute highly potent inhibitors of the viral entry into cells.

To our knowledge, such a 'double-drug' approach focusing on the close cascade virus/cell adsorption/fusion processes has not been investigated. 'Double-drug' prototypes associating in a single molecule a nucleoside reverse transcriptase inhibitor with a CXCR4 antagonist (e.g., AMD3100)[9,10] or a virus–cell interaction inhibitor,[11] or a protease inhibitor[12] have indeed been reported. However, these 'double-drug' prodrugs target two distinct but remote steps of the viral replicative cycle.



Scheme 1. Synthetic pathway to the bipharmacophore derivatives **5a**,**b**. Reagents and conditions: (a) (1:1) TFA/CH_2Cl_2, quantitative; (b) 5N NaOH, (1:1) THF/H_2O; (c) **6**, HOBt, EDC, CH_2Cl_2, 90%; (d) *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-serine **7** (1 equiv), HOSu, DCC, CH_2Cl_2, 33% for **a** and 53% for **b**; (e) (1:1:2) Et_3N/MeOH/H_2O, quantitative.

Herein, we report on the synthesis of conjugates **5a**,**b** (Scheme 1) deriving formally from GalSer (which is a GalCer analogue, Fig. 1)[8a] and AMD3100, and on their in vitro anti-HIV activities that were established with X4- and dual (X4 and R5)-tropic HIV-1 strains on various CXCR4 expressing cells. To have a further insight into their inhibition mechanism, tests were also performed with a R5-tropic HIV-1 strain and CCR5 expressing cells. It should be emphasized that neither the 3-D structure of native gp120, gp120/gp41, nor that of the gp120/CD4/co-receptor complexes are known. Therefore, it is most difficult to rationally design bifunctional inhibitors that act simultaneously on two distinct sites and interfere with the most complex cascade HIV-cell adsorption-fusion events. In order to circumvent these drawbacks, we adopted a modular design for the chemical structure of the Gal-AMD3100 conjugates **5** wherein the length of the spacer arm linking the quintessential galactosylated and bicyclam units can be varied. The synthesis of the Gal-AMD3100 conjugates **5a**,**b** is mostly divergent (Scheme 1). The two regioisomers **3a**,**b** which contain the bicyclam moiety conjugated to the $(CH_2)_4COOH$ spacer at different nitrogen positions constitute the key synthons. Their time consuming syntheses and purifications were performed following already published procedures.[9,13] These two **3a**,**b** regioisomers were then coupled using conventional peptide coupling reagents to the *N*-tetradecanoyl L-lysine methylester derivative **6** obtained from commercially available Fmoc-L-lysine through a standard two-step procedure. Hydrolysis of the obtained methyl esters under basic conditions followed by the activation of the resulting acids with HOSu/DCC then action of the *O*-Ac protected galactosylated serine derivative **7**[8a] led to the Gal- and AMD3100-protected conjugates **4a**,**b**. Quantitative *N*-Boc deprotection using an excess of TFA, then quantitative *O*-Ac deprotection with a Et_3N/MeOH/H_2O mixture led to the target compounds **5a**,**b**.[14]

The two Gal-AMD3100 conjugates **5a**,**b**, as well as the different moieties composing these conjugates [that is, the AMD3100-linker derivative **2a** (Scheme 1),[15] and AMD3100], GalSer, and AZT as control, were evaluated for their inhibitory effects on T-tropic HIV-1 replication in CXCR4 expressing cells (CEM-SS, MT4) and on dual-tropic HIV-1 replication in PBMC cells (Table 1). Under assay conditions, the 'double-drug' Gal–AMD3100 conjugates **5a**,**b** elicited anti-HIV activities in all CXCR4 expressing cell lines with EC_{50} values ranging from 0.25 to 6.0 μM. In the same testing conditions and for a given CXCR4 expressing cell line, the AMD3100 'single drug' and even its *N*-alkylated derivative **2a** inhibited HIV replication much more efficiently (see Table 1). By contrast, the lipidic GalSer 'single drug' displayed a lower anti-HIV activity in CEM-SS cells than the Gal–AMD3100 conjugates **5a**,**b**, and was found to be inactive in MT4 cells. These testings show that conjugation of the linker to AMD3100

Table 1. Antiviral activity and cytotoxicity data of the AMD3100 conjugates determined in various cells and with various HIV strains (X4 strain: HIV-1 LAI and HIV-1 IIIB; R5 strain: HIV-1 Bal; dual X4/R5 strain: HIV-1 89.6)

Compd	EC_{50}[a] in μM [Relative potency/ AMD3100][e]	CC_{50}[b] (μM)	EC_{50}[c] in μM [Relative potency/ AMD3100[e]]	CC_{50}[b] (μM)	EC_{50}[c] in μM [Relative potency/ AMD3100[e]]	CC_{50}[b] (μM)	IC_{50}[d] in μM [Relative potency/ AMD3100[e]]	IC_{50}[d] (μM)
	CEM-SS (HIV-1 LAI)		MT4 (HIV-1 IIIB)		PBMC (HIV-1 89.6)		GHOST CXCR4 (HIV-1 LAI)	GHOST CCR5 (HIV-1 Bal)
2a	0.91 [∼7]	>100	0.69 [∼10]	>100	0.025 [∼7]	>100	0.0071 [∼7]	>50
5a	5.2 [∼40]	>50	2.8 [∼45]	>50	0.47 [∼125]	28	0.086 [∼90]	>50
5b	6.0 [∼50]	>100	2.9 [45]	>100	0.25 [∼65]	>10	0.021 [∼20]	>10
AMD3100	0.127	>10	0.065 0.010[f]	>10 1000[f]	0.0038	>10	0.00095	>5
GalSer[g]	10	>100	>100	>100	nt		nt	nt
AZT	0.0063		0.0099		0.024			

[a] EC_{50}: concentration required to inhibit 50% of virus replication by reverse transcriptase activity measurement on CEM-SS cells.
[b] CC_{50}: concentration required to cause 50% death of uninfected cells.
[c] EC_{50}: concentration required to protect 50% of the virus-infected MT4 or PBMC cells against virus cytopathogenicity.
[d] See ref [16].
[e] EC_{50} (IC_{50}) ratio of conjugate versus AMD3100.
[f] From ref 3.
[g] From ref 8a. nt = not tested.

as in **2a** induces a substantial (∼7- to 10-fold) decrease of the antiviral activity of AMD3100. Unfortunately, this decrease is not compensated but even enhanced by the attachment of the anti-HIV galactosylated-serine moiety as in the target derivatives **5a**,**b** which display a ∼40- to 125-fold lower antiviral activity than AMD3100. However, when compared with GalSer, conjugation of GalSer to the highly anti-HIV active AMD3100 increases the antiviral activity of GalSer, though moderately, in CEM-SS cells.

Concerning the mechanism of inhibition of the Gal–AMD3100 conjugates **5a**,**b**, the experiments performed with X4 or R5HIV-1 strains and GHOST cells genetically modified to express CD4 and CXCR4 or CCR5 indicate clearly that the Gal–AMD3100 conjugates interact with CXCR4, blocking viral entry. Indeed, these compounds, as well as AMD3100 and its *N*-alkylated derivative **2a**, inhibited viral replication of only the X4HIV-1 strain in the CXCR4 (+) GHOST cells while they had no effect on viral replication of the R5HIV-1 strain in the CCR5(+) GHOST cells (Table 1). The Gal–AMD3100 conjugates **5a**,**b** were further found to be less (∼20- and 90-fold) active than AMD3100 on the CXCR4(+) GHOST cells, in line with the results obtained with the other CXCR4(+) cells investigated here. To further confirm their interactions with the CXCR4 co-receptors, it would be interesting to investigate by FACS whether these conjugates can abolish the binding of the natural SDF-1α ligand to CXCR4.

Although the remainder of our initial goal (i.e., development of highly potent HIV inhibitors that block viral entry into cells) was not reached, Gal–AMD3100 conjugates such as **5a**,**b** constitute nevertheless 'double-drug' type molecules worth to be extended. The synthesis of conjugates with longer spacers between the two pharmacophore units is currently under progress in our laboratory. These efforts are intended to show if the length of the spacer between these two units is indeed critical for a synergistic antiviral effect.

Acknowledgements

We are grateful to the CNRS, the Agence Nationale de Recherche sur le SIDA (ANRS) and Sidaction for financial support.

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14. The chemical structure of compounds **1a**,**b**, **2a**, **3a**,**b**, **4a**,**b**, and **5a**,**b**, was unambiguously established by ^{1}H, ^{13}C-DEPT NMR, ESI-MS, and by comparison with the published NMR data of the known compounds (i.e., **1a**, **3a**). The regioisomer **a** of compounds **3** to **5** displays identical NMR data to that of its respective regioisomer **b**. **5a**,**b** are isolated as TFA salts: ESI-MS $(M+H)^+$: 1190.9 (calcd for $C_{62}H_{115}N_{11}O_{11}+H$: 1190.9). Compound **2a** is isolated as its hydrochloride salt: ESI-MS $(M+Na)^+$: 653.4 (calcd for $C_{35}H_{66}N_8O_2+Na=653.4$).
15. The in vitro antiviral activity and cytotoxicity (as estimated by the EC_{50} and CC_{50}, respectively) of the compounds in CEMS-SS, MT4 and PBMC cells were determined according to published procedures.[16] For the CEM-SS and PBMC cells, the growing of HIV-1 (LAI and 89.6 strain, respectively) was evaluated by measuring the reverse transcriptase (RT). The growing of HIV-1 [HTLV-I (IIIB)] in MT4 cells was followed by the cytopathogenic effect induced by the virus. The EC_{50} and CC_{50} are defined in Table 1. GHOST cells (human osteogenic sarcoma) transformed to express human CD4, CXCR4 or CCR5, and the green fluorescent protein (GFP) under the control of an HIV-2 LTR, were cultured in Dulbecco medium supplemented with 10% FCS. Cells were distributed in 24-well plates at a density of 2.5 10^5 cells per well in 500 μL medium. Twenty four hours later, the medium was eliminated and cells were pretreated with different concentrations of the compounds diluted in medium for 1 h at 37 °C, then infected in presence of the drugs with HIV-1 Lai (GHOST-CXCR4) or HIV-1 Bal (GHOST-CCR5). Untreated cells were infected in parallel. After 2 h adsorption, cells were washed and cultured in the presence of the same concentrations of drugs for 48 h. At the end of the incubation period, cells were treated with trypsin, washed, suspended in 700 μL 1.5% paraformaldehyde and analyzed by flow cytometry to detect the presence of GFP resulting from LTR transactivation mediated by Tat which is produced upon cell infection by HIV. The percentage of infected cells expressing GFP in the absence or in the presence of different concentrations of antivirals was measured and the 50% inhibitory concentration of cell infection (IC_{50}) was derived from the computer-generated median effect plot of the dose–effect data.
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