Synthetic Soluble Analogs of Galactosylceramide (GalCer) Bind to the V3 Domain of HIV-1 gp120 and Inhibit HIV-1-induced Fusion and Entry

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Galactosylceramide (GalCer) is an alternative receptor allowing human immunodeficiency virus (HIV)-1 entry into CD4-negative cells of neural and colonic origin. Several lines of evidence suggest that this glycosphingolipid recognizes the V3 region of HIV-1 surface envelope glycoprotein gp120. Since the V3 loop plays a key role in the fusion process driven by HIV-1, we decided to synthesize soluble analogs of GalCer with the aim to develop a new class of anti-HIV-1 agents that could neutralize HIV-1 infection through masking of the V3 loop. We describe a short route, in three steps, for the synthesis of soluble analogs of GalCer, using unprotected lactose as the starting sugar. The analogs were prescreened in an assay based on the interaction between a V3 loop-derived synthetic peptide and [3H]suramin, a polysulfonoyl compound displaying high affinity for the V3 loop. One of the soluble analogs, i.e. CA52(n15), strongly inhibited the binding of [3H]suramin to the V3 peptide, with an IC50 of 1.2 μM. This molecule was also able to inhibit [3H]suramin binding to recombinant gp120 with similar activity. Using a competition enzyme-linked immunosorbent assay with highly specific anti-gp120 monoclonal antibodies, the region recognized by CA52(n15) could be mapped to amino acids 318–323, i.e., corresponding to the highly conserved consensus motif GPGRAF. Interestingly, the region recognized by suramin, i.e., IQRGPF-R-F, was partially overlapping this motif. CA52(n15) was able to inhibit HIV-1-induced cell fusion as well as HIV-1 entry into both CD4 and CD4/GalCer+ cells. A structure-activity relationship study showed that: (i) the antiviral activity of soluble analogs of GalCer correlates with V3 loop binding, and (ii) the hydrophobic moiety of the molecule plays an important role in this activity. Taken together, these data show that synthetic analogs of GalCer can inhibit HIV-1 entry into both CD4 and CD4+ cells through masking of the V3 loop.

The third variable region of the HIV-1 surface glycoprotein gp120 (V3 loop) appears to play a key role in HIV-1 infection and pathogenesis (1, 2). This domain is the major immunodominant epitope for the generation of neutralizing antibodies (3) and is essential for virus infectivity and tropism (4–6). A current idea is that the V3 loop may be involved in the postbinding events necessary for viral entry into the cells (7, 8), probably by interacting with secondary receptors (2, 9). Due to the high variability of the V3 loop sequence, the neutralizing activity of anti-V3 antibodies is generally restricted to one or, in the best cases, to a few related HIV-1 isolates, which renders vaccine strategies particularly puzzling (10, 11). However, since the V3 domain of most HIV-1 strains contains several well conserved basic amino acid residues (12), it can bind to a wide variety of anionic compounds, including sulfated polysaccharides, heparin, and suramin, which are efficient inhibitors of HIV-1 infection in vitro (13–15). Moreover, the V3 loop is also involved in the recognition of galactosylceramide (GalCer), a glycosphingolipid allowing HIV-1 entry into CD4-negative cells of neural and colonic origin (16–19). These data prompted us to use soluble analogs of the GalCer receptor as potential inhibitors of HIV-1 infection. The synthetic scheme of these molecules was based on the original use of unprotected lactose, a low cost disaccharide, as the starting sugar (20).

We report here the synthesis and characterization of such soluble GalCer analogs that can block HIV-1-induced fusion as well as entry into both CD4 and CD4+ cells. These analogs have been first evaluated for their ability to inhibit the binding of [3H]suramin to SPC3, a synthetic peptide displaying eight V3 consensus motifs (GPGRAF) radially branched on uncharged poly-Lys core matrix (15). This prescreening assay proved useful to select those analogs that recognized the V3 loop, and a good correlation was found between the anti-HIV-1 activity of a given analog and its affinity for the V3 loop. These data show that synthetic soluble analogs of glycosphingolipids may represent a new class of anti-HIV-1 drugs that could be obtained at a large scale with a low cost.

EXPERIMENTAL PROCEDURES

Materials—SPC3, i.e. (GPGRAF)4-(K)4-(K)-K-βα, was generously provided by M. Mollard (Eurethics, Paris, France). The peptide was a generous gift from M. Mollard.

The abbreviations used are: HIV, human immunodeficiency virus; GalCer, galactosylceramide; PBS, phosphate-buffered saline; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBMC, peripheral blood mononuclear cells; n.o.i., multiplicity of infection; XTT, sodium 3′-1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate; PMS, phenazine methosulfate.
solved analogs of GalCer inhibit HIV-1 infection.

Synthesis of Soluble GalCer Analog CA52(n15)—The synthetic scheme of analog CA52(n15) is illustrated in Fig. 4. The intermediate 2 is obtained in two stages from lactose and the corresponding α-amino acid, after reduction with sodium borohydride according to a procedure described elsewhere (20, 23). Briefly, to a solution of 11-aminoundecanoic sodium carboxylate (22 mmol) in methanol (60 ml) is added 13.7 mmol of lactose monohydrate in 30 ml of water. After 1 h at 37°C, the plates were washed five times with 200 ml of PBS, 1% gelatin for 90 min at 37°C. [3H]Suramin (1 Ci/ml) was then added in either the absence or presence of synthetic GalCer analogs to compete with [3H]suramin for binding to SPC3. In the prescreening assay based on the potential ability of synthetic \( \text{GalCer analogs} \) to inhibit HIV-1 infection in two cell targets: the CD4/GalCer human colon epithelial cell line HT-29 and normal human PBMC. In both cases, HIV-1 was preincubated with the indicated concentration of the synthetic analog for 30 min at 37°C. The mixture was then either used in six-well plates onto exponentially growing HT-29 cells (m.o.i. of 0.1 TCID50 per cell) or used to resuspend a cell pellet of PBMC (m.o.i. of 0.01 TCID50 per cell). The level of infection was analyzed 7 days postinfection by measuring the concentration of p24 antigen in the cell-free culture supernatants as described elsewhere (15, 24). An antigen capture assay (DuPont, Les Ulis, France) was used for p24 determinations. The viruses used in these experiments were the prototype HIV-1(LAI) or the African HIV-1(NDK) isolates, as described previously (18, 19, 22, 24).

Toxicity Assay—The effects of synthetic analogs of GalCer on the proliferation and viability of PBMC were studied in a colorimetric assay utilizing the tetrazolium salt XTT, \( \text{i.e.} \) sodium 3’-1-(phenylamino)-carbonyl)-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (25). In these experiments, the cells were cultured in phenol red-free RPMI 1640 to reduce the blank values. Briefly, XTT was dissolved in prewarmed medium at a concentration of 1 mg/ml. Immediately before use, phenazinemethosulfate (PMS) was added to the XTT solution (final concentration of PMS, 125 \( \mu \text{g/ml} \)). 25 \( \mu \text{g/ml} \) of XTT/PMS solution was added to 100 \( \mu \text{l} \) of culture giving a final concentration of 0.2 mg/ml XTT and 25 \( \mu \text{g/ml} \) PMS. After incubation at 37°C for 4–8 h, the optical density was determined using a test wavelength of 450 nm and a reference wavelength of 650 nm, subtracting blank control values with medium alone.

RESULTS

Recognition of SPC3 by an Anti-gp120 mAb Directed to the V3 Loop—A first set of experiments was designed with the aim to assess that the multibranched peptide SPC3 was representative of the tip of the V3 loop as exposed in native gp120. Using an ELISA detection method, we analyzed the reactivity of SPC3 with F5, an anti-gp120 mAb directed to the GPGRAFVT motif of the HIV-1(LAI) isolate (15). As shown in Fig. 1, this anti-V3 mAb bound to SPC3 with high specificity. In contrast, the F5 mAb did not recognize the monomeric GPPGRAF peptide.
motif nor SPC3 derivatives with lower valencies, i.e. the four-branched (GPGRAF)4-[K]2-K-βA and the 2-branched (GPGRAF)2-K-βA synthetic peptides. These data suggested that the presentation of the GPGRAF motif in the eight-branched peptide SPC3, but not in its derivatives with lower valencies, was reminiscent of the physiological situation. Thus, SPC3 was used throughout this study as a model for the tip of the V3 loop.

Specificity of [3H]Suramin Binding to SPC3—The binding of [3H]suramin to the multibranched V3 peptide SPC3 was analyzed in a solid phase radioassay. In this assay, the peptide was coated on polyvinyl chloride 96-well plates, and [3H]suramin was used as a revealing agent. As shown in Fig. 2, the binding of [3H]suramin to SPC3 was dose-dependent and saturable. The binding specificity was further demonstrated by using SPC3 derivatives with either a shorter or a different motif (GPGR, GPGRA, GPGKTL, or GPGQAF). [3H]Suramin did not bind to any of these peptides (Ref. 15 and data not shown), indicating that [3H]suramin recognized the entire V3 consensus motif GPGRAF, with special emphasis to the Arg (R) and Phe (F) residues. Moreover, [3H]suramin could not detect SPC3 derivatives with lower valencies, in agreement with the data of Fig. 1. This raised the interesting possibility to use the [3H]suramin solid phase radioassay as a prescreening test for a rapid and low-cost identification of a new class of anti-HIV drugs susceptible to bind to the consensus V3 loop motif.

First two stages of the synthesis leads to the GalCer-like unit. Another point is the terminal carboxylate function of this new compound leading to the good solubility of the substrate in aqueous media. For this reason, the molecules synthesized according to this scheme can be considered as true soluble analogs of GalCer. Indeed, the synthetic analog CA52(n15) was fully recognized by the anti-GalCer R-mAb in an ELISA assay (Fig. 5).

Synthesis of GalCer Analogs—The synthetic analogs of GalCer developed in this study were obtained in only three stages from lactose. The chemical structure of CA52(n15), the prototype of this new series of analogs, is shown in Fig. 3. Other analogs were obtained by varying the number of carbon atoms in the hydrophobic moiety of the synthetic scheme of CA52(n15) is illustrated in Fig. 4. The feature of note is the use of unprotected lactose as starting sugar, a low cost disaccharide consisting of a galactose and glucose units linked by a 1–4 junction in the β configuration.

Thus, opening of the glucose ring by reductive amination in the first two stages of the synthesis leads to the GalCer-like unit. Another point is the terminal carboxylate function of this new compound leading to the good solubility of the substrate in aqueous media. For this reason, the molecules synthesized according to this scheme can be considered as true soluble analogs of GalCer. Indeed, the synthetic analog CA52(n15) was fully recognized by the anti-GalCer R-mAb in an ELISA assay (Fig. 5).
was the only analog able to totally prevent the binding of [3H]suramin. This derivative interfered with SPC3 recognition in a dose-dependent manner, with a 50% inhibitory concentration (IC50) of 0.7 μM (0.6 μg/ml). The activity of CA52(n15) was highly specific, since a more polar derivative with only 8 carbon atoms in the hydrophobic chain, i.e. CA50(n8) did not affect the binding of [3H]suramin to SPC3. Moreover, when the length of the carbon chain was intermediary between the fully active CA52(n15) and the nonactive CA50(n8) analogs, the corresponding molecule (i.e. CA49(n11), with 11 carbon atoms) showed a weak, yet nonnegligible activity, with an IC50 of 9.4 μM (7 μg/ml). These data underscored the high specificity of the [3H]suramin solid phase assay, which could discriminate between closely related GalCer analogs.

Inhibition of [3H]Suramin Binding to gp120 by CA52(n15)—Two experiments were conducted in order to ensure that CA52(n15) actually recognized the V3 loop motif GPGRAF. In the first one, CA52(n15) was preincubated with SPC3 before the plate was rinsed and then exposed to [3H]suramin. Under these conditions, the IC50 of CA52(n15) was not changed, which strongly suggests that the analog binds to SPC3 and not to
The results in Fig. 7 show that CA52(n15) was indeed able to inhibit the binding of mAb 110-H (IC50 47 μg/ml) and mAb 110-A (IC50 2.4 μg/ml) to recombinant gp120 in a dose-dependent fashion, with an IC50 of 2.4 μM (38 μg/ml). Thus, CA52(n15) is an inhibitor of the GalCer-dependent pathway of infection.

Since the V3 loop is also involved in the fusion process between the HIV-1 particle and the plasma membrane of CD4+ cells, the activity of CA52(n15) as a fusion inhibitor was evaluated in a syncytium-forming assay. In this test, human T-lymphoblastoid cell were acutely infected with HIV-1(LAI) and cultured for 7 days. At this time, the formation of syncytia was evaluated in a syncytium-forming assay. In this test, human T-lymphoblastoid cell were acutely infected with HIV-1(LAI) and cultured for 7 days. At this time, the formation of syncytia was

Table I). The putative binding sites for suramin and CA52(n15) at the tip of the V3 loop are indicated in Fig. 8 (see “Discussion” for comments).

**Anti-HIV-1 Activity of CA52(n15)**—The antiviral activity of CA52(n15) was first evaluated in HT-29, a CD4+ cell line expressing high levels of the GalCer receptor. In these experiments, the virus (HIV-1(NDK) at a m.o.i. of 0.1 TCID50 per cell) was preincubated with various concentrations of CA52(n15) and then exposed to exponentially growing HT-29 cells cultures in six-well plates. After 2 h of infection, the cells were treated with trypsin to remove excess inoculum, split in 25-cm2 flasks, and analyzed for p24 production at day 7 postinfection. The results are expressed as the mean of three separate experiments (±S.D.).
evident in the infected cultures (Fig. 10A), especially when compared with noninfected ones (Fig. 10B). Pretreatment of the virus with 123 µM CA52(n15) (100 µg/ml) was sufficient to abrogate the formation of syncytia, as shown in Fig. 10C. In contrast, the GalCer analog CA50(n8), which did not recognize the V3 loop (Fig. 6), had no activity in this test, even when present overall the culture time at a concentration of 284 µM (200 µg/ml) (Fig. 10D). Finally, analog CA49(n11), which displayed some activity in the [3H]suramin solid phase radioassay (Fig. 6), could block the formation of syncytia at a concentration of 201 µM (150 µg/ml) (Fig. 10E), but was not active at lower concentrations (Fig. 10F). Indeed, some syncytia could still be observed in cultures treated with 167 µM (125 µg/ml) CA49(n11) (not shown).

Then, the anti-HIV-1 activity of CA52(n15) was studied in normal human PBMC, which represent the natural cellular targets for HIV-1. As shown in Fig. 11, the soluble GalCer analog was able to block the infection of PBMC at 246 µM (200 µg/ml), and this inhibition was not associated with any toxicity as evidenced by the XTT assay. The IC50 was 108 µM (88 µg/ml), in agreement with the data obtained with HT-29 and C8166 cells.

**DISCUSSION**

The aim of the present study was to synthesize soluble analogs of GalCer that were expected to bind to the V3 loop of HIV-1 gp120 and thus to block HIV-1 infection. The rationale for this strategy was based on previous observations suggesting that the GalCer receptor, putatively used by HIV-1 to infect neural cells (16, 17) and colon cells (18, 19), was recognized by the V3 domain of gp120: (i) anti-V3 antibodies block gp120 binding to GalCer (26); (ii) these antibodies inhibit HIV-1 infection of CD4+ /GalCer1 human colon epithelial HT-29 cells (15, 26); (iii) synthetic multimeric peptide constructs of the V3 consensus sequence (GPGRAF) bind to GalCer and prevent HIV-1 entry into HT-29 cells (24); (iv) the V3 loop is a common genetic determinant controlling HIV-1 tropism for neural SKNMC cells (17, 28) and HT-29 cells (29), as demonstrated by using chimeric proviral clones. Although these data strongly suggested the involvement of the V3 loop in GalCer recognition, direct evidences for a physical interaction between GalCer and the V3 loop of gp120 were lacking. Thus, special attention was devoted to the demonstration that the soluble GalCer analogs described in this study actually bound to the V3 loop. This was done by two distinct methods. First, we developed a quantitative V3 binding assay based on the interaction between suramin and the V3 loop of gp120 (15). In this assay, the V3 loop was mimicked by SPC3, a synthetic multibranched V3 peptide that has been recently characterized by our group as an inhibitor of HIV-1 infection in both CD4+ and CD4+ cells (30). Synthetic analog CA52(n15) was a potent inhibitor of [3H]suramin binding to SPC3. This GalCer analog acted through interaction with SPC3, since it worked with equal efficiency in competition or in preincubation with the peptide. CA52(n15) also inhibited the binding of [3H]suramin to whole
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recombinant gp120, which occurs in the V3 region (31). Based on these data, one could conclude that CA52(n15) binds to the V3 motif GPGRAF harbored by both SPC3 and gp120 and thus competitively inhibits the binding of \(^{3}H\)suramin to the same domain. To refine this mapping, we used two anti-V3 mAbs recognizing closely related and partially overlapping epitopes of the V3 loop: IQRGP (mAb 110-A) and GPGRAFVTI (110-H). CA52(n15) inhibited the binding of both antibodies to recombinant gp120, while suramin interfered with mAb 110-A binding exclusively. However, one cannot simply conclude from these data that suramin binds preferentially to the IQRGP linear sequence, based on: (i) the antigenic mapping of suramin and CA52(n15) to partially overlap- ping binding sites on gp120, while suramin interfered with mAb 110-A binding exclusively. However, one cannot simply conclude from these data that suramin binds preferentially to the IQRGP sequence and CA52(n15) to GPGRAFVTI. Indeed, our results demonstrate that both suramin and CA52(n15) bind to the GPGRAF-bearing multibranched peptide SPC3. Moreover, recent data showed that \(^{3}H\)suramin did not bind to SPC3 derivatives exhibiting a shorter motif (i.e. GPGR and GPGRA), which underscores the importance of the C-terminal Phe residue (F) in the GPGRAF motif of SPC3. In addition, acetylation of the N-terminal Gly residue (G) of the motif abolished the binding of \(^{3}H\)suramin (data not shown). Thus, it is likely that the suramin binding site on the V3 loop involves the Gly-Pro doublet (GP) and the downstream Phe (F), although it is probable that the two Arg residues (R) may stabilize the interaction with negatively charged sulfonyl groups of suramin. Thus we propose that suramin binds essentially to the discontinuous motif IQRGP-R-F (Fig. 8). As for CA52(n15), its binding site was assigned to the GPGRAF linear sequence, based on: (i) the ability of both CA52 (this study) and natural GalCer (15) to bind to SPC3 and (ii) the fact that CA52(n15) competes with the binding of mAbs 110-H and 110-A to recombinant gp120 (binding to the GPGRAF motif should totally or partially mask the epitopes of 110-H and 110-A mAbs, respectively). This tentative mapping of suramin and CA52(n15) to partially overlapping binding sites on gp120 could conciliate the finding that both drugs bind to SPC3 while recognizing slightly different regions of the V3 loop as revealed by competition ELISA.

The validation of the \(^{3}H\)suramin solid phase radioassay as a prescreening test for V3 loop-targeted anti-HIV-1 compounds is emphasized by the virological data obtained with the soluble GalCer analogs. Indeed, CA52(n15), which was the more potent inhibitor in the prescreening assay, was also the most potent inhibitor of HIV-1 infection. CA52(n15) inhibited the formation of syncytia induced by HIV-1 in C8166 cultures, in agreement with the well-established role of the V3 loop in HIV-1 fusion (7, 8). This analog also inhibited the formation of HT-29 cells and PBMC through the GalCer and CD4 pathways, respectively, with similar IC\(_{50}\) values. Taken together, these data are consistent with the putative mechanism of action of such compounds, i.e., neutralization of the virion through masking of the V3 loop of gp120. In addition, the synthetic analog CA52(n15) appeared to be a more potent inhibitor of HIV-1 infection than the authentic soluble form of GalCer, i.e., 3'-sulfo-GalCer (sulfadote). Indeed, recent data from our laboratory showed that sulfadote could inhibit the GalCer-dependent pathway of HIV-1 entry into HT-29 cells, but not the CD4-dependent pathway in PBMC. The lower efficiency of sulfadote may be related to a decrease of concentration following the spontaneous transfer of this natural glycolipid from the aqueous medium to the plasma membrane (32).

Interestingly, the IC\(_{50}\) of the antiviral effect of CA52(n15), i.e., 100 μg/ml, was superior to the IC\(_{50}\) determined by biochemical means (i.e., 1 μg/ml), in agreement with the notion that the exposure of the V3 loop on the virion spikes is different from the one of monomeric gp120 (33). Similar data were obtained with anti-V3 mAbs (data not shown). However, the difference of activity between CA52(n15) and CA49(n11) in the biochemical assays was also found in the antiviral assays, especially when one compares the ability of each analog to inhibit the formation of syncytia in our fusion assay (Fig. 10). Finally, CA50(n8), which did not bind to the V3 loop, was also devoid of antiviral activity, at least over the range of concentration tested (up to 200 μg/ml). In conclusion, the antiviral activity of the soluble analogs of GalCer was consistent with their activity in the prescreening assay.

The difference between the fully active CA52(n15), the non-active CA50(n8), and the intermediary active CA49(n11) correlated with the level of hydrophobicity of the analog: the longer the hydrophobic moiety, the higher the biological activity. Yet analogs with more than 14 methylene residues were not active, suggesting that a narrow range of hydrophobicity is necessary to achieve the antiviral activity. The lack of activity of those analogs with a high degree of hydrophobicity could be tentatively explained by their ability to be incorporated in the plasma membrane, resulting in a significant decrease of concentration. This phenomenon has been reported previously for various glycolipids, including sulfatide (see above). Further studies will help to clarify this point.

In conclusion, we describe the synthesis of a new class of low-cost anti-HIV-1 compound that neutralize HIV-1 infection through masking of a highly conserved motif of the V3 loop. The soluble analogs of GalCer characterized in this study will constitute the basis for the design of second generations molecules, which will be selected for improved anti-HIV-1 activity. The prescreening assay described in this report will help to evaluate a wide number of molecules for their ability to bind to the V3 loop.

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