# Chemotherapeutic Interventions Targeting HIV Interactions with Host-Associated Carbohydrates

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## *Contents*



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## *1. Introduction*

### 1.1. Scope of Review

This review focuses on host-associated carbohydrate-mediated entry processes of HIV-1. It begins with an overview of HIV-1 entry mechanisms, with a particular emphasis on viral and host cell receptor structural features and what is known regarding regions important for binding interactions between the virus and the host cell. The HIV-associated glycoprotein, gp120, is of particular importance during viral fusion and entry, as it serves as the first point of contact with the host cell. Information concerning what is known of the three-dimensional structure of this protein, in addition to specific regions where contacts are made with the host cell, is presented. CD4 serves as the main host-associated receptor in the infection of helper T-cells and macrophages; however, in the past decade, it has been discovered that other host cell receptors are also important for viral infection, namely the entry coreceptors, CCR5 and CXCR4, and the attachment receptors, heparan sulfate proteoglycans (HSPGs) and, in CD4-negative cells, galactosylceramide (GalCer). Entry receptors mediate fusion between the host cell and viral membranes, while attachment receptors aid in the capture and uptake of the virus but do not facilitate membrane fusion. The role of each of these receptors during the HIV entry process is delineated.

The remainder of the review focuses on what is known with regard to carbohydrates as inhibitors of the entry process. Carbohydrates have long been recognized as significant and potent inhibitors of HIV-1 entry *in vitro*; however, to date, none have been approved for use in the treatment of HIV-1. The structural diversity of this class of inhibitors encompasses natural polysaccharides, chemically modified/derived oligo- and polysaccharides, polymer-bound oligosaccharides, glycosphingolipid derivatives, and multivalent sugar conjugates. These compounds, and their mech-

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Katherine McReynolds received a B.S. in biochemistry from California Polytechnic State University at San Luis Obispo in 1994, followed by a Ph.D. in chemistry, under the direction of Jacquelyn Gervay-Hague, investigating the anti-HIV properties of synthetic glycosphingolipids. Upon completion of her Ph.D. in 1999, she pursued postdoctoral research in the laboratory of Frederic A. Troy at The University of California, Davis, and was engaged in the study of the biosynthesis of polysialic acids and their implications in development. In 2001, she joined the faculty at California State University, Sacramento. She is currently interested in the design, synthesis, and biological properties of glycodendrimers, and she involves predominantly undergraduate researchers in her investigations.

Jacquelyn Gervay-Hague received a B.S. degree from The University of California, Los Angeles, in 1985, where she also earned a Ph.D. in 1990 under the direction of Professor Michael E. Jung. In 1990 she moved to Yale University as a National Institutes of Health Postdoctoral Fellow with Professor Samuel J. Danishefsky. In 1992, Professor Gervay-Hague joined the faculty in the Department of Chemistry at the University of Arizona, and she was promoted to Associate Professor in 1998. In 2001, she was appointed Professor of Chemistry at University of California, Davis. Professor Gervay-Hague's research interests are in the area of carbohydrate chemistry directed toward the design and synthesis of chemotherapeutics targeting HIV infection and cancer. Although the disease states differ, the methods of drug development employed by her research group consistently involve understanding the disease processes at a molecular level. This approach has naturally led to studies at the interface of chemistry and biology. In these investigations, the development of new synthetic methods has provided access to compounds that uniquely serve as biological probes to study structure/activity relationships. Development of NMR techniques for solution-phase structure determination, and novel biological assays for testing interactions between small molecules and proteins have also been a focus of her research efforts. More recently, solid-phase synthesis of amide-linked carbohydrates has led to the production of novel materials with stable secondary structure in aqueous solution, providing a foundation for future investigations in artificial protein engineering. Together, these endeavors serve as a platform for drug discovery.

anism of action, are presented. A summary of the key structural features that lend to the function of the polyanionic inhibitors will also be given. Recent strides made in the development of new carbohydrate anti-HIV agents, with improved biological activity, suggest that these compounds may play increasingly important roles in the treatment or prevention of this disease in the not-too-distant future. In all likelihood, the clinical area where these compounds will have the most impact will be in infection prevention, in instances of viral exposure. In fact, some of the most current reports regarding anti-HIV polyanionic carbohydrates indicate that these compounds work well in topical preparations and may prove to be effective prophylactics in sexual transmission.

## 1.2. Current HIV/AIDS Treatments and Limitations

Today there are approximately 37.8 million people worldwide infected with HIV-1 (hereafter referred to as simply HIV).[1] In the United States, it is estimated that 850,000−950,000 people are infected, including 180,000−280,000 people who are unaware of their HIV-positive status.[2] The current cumulative estimate of the number of AIDS-related deaths in the United States through 2003 stands at 524,060.[3] The Food and Drug Administration (FDA) has approved 24 drugs/drug combinations to battle HIV infection, falling into four main categories: non-nucleoside reverse transcriptase inhibitors (NNRTI's), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PI), and fusion inhibitors.[4] These drugs can be combined in HAART (highly active antiretroviral therapy) regimens to suppress viral replication and progression of HIV infection into AIDS, and they may result in undetectable viral loads in the patient's blood. There are, however, a wide variety of side effects experienced by patients on HAART that range in severity from skin rashes and gastrointestinal intolerance to coronary artery disease, nephrotoxicity, and bone marrow suppression. For the more severe conditions, discontinuation of the causative antiretroviral agent is recommended.[5]

Further complicating HAART therapy are issues of viral resistance. All four classes of anti-viral agents have reported viral mutations that lend themselves to decreased effectiveness. HIV reverse transcriptase is error-prone, as it does not proofread nucleotide sequences during replication. This, coupled with a high rate of replication, can lead to even more genetic variation in the virus. Thus, the virus can develop multiple drug resistance during the course of HAART therapy, decreasing the usefulness of the corresponding drugs. These multi-drug-resistant viruses can then be transmitted to other individuals, making it more difficult to treat subsequent generations of HIV-infected individuals.[6]

As it is likely that the generation of a viable vaccine to prevent HIV is still years away, new drugs are needed as alternatives when resistance to one therapy occurs. HIV inhibitors based upon blocking viral fusion/entry are particularly attractive candidates. To date, only one drug that fits this category, the peptide-based drug Fuzeon, has gained FDA approval (*vide infra*). Entry/fusion inhibitors, therefore, represent a relatively unexploited area of research. The compounds have a distinct advantage over other current approved therapies in that they do not require cellular uptake in order to carry out their prescribed activity. Additionally, as their mode of action is distinct from those of the other three classes of drugs, there is the possibility for generating compounds that will act synergistically with existing drugs. Therefore, there is much potential for the development of new and potent entries to this drug category, including carbohydrate-based drugs designed using host-associated sugars as a template.

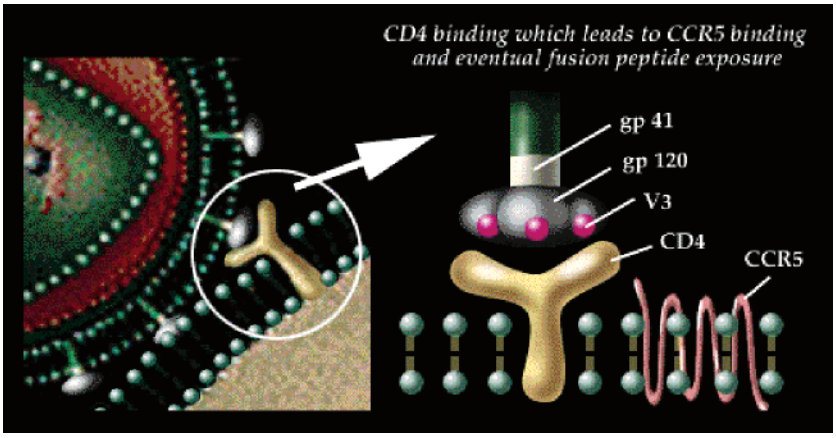



**Figure 1.** Binding of HIV viral gp120/gp41 to host cell CD4 and coreceptor CCR5. (Reproduced with permission from Toronto General Hospital University Health Network (http://www.tthhivclinic.com/lifecycle1.htm).)

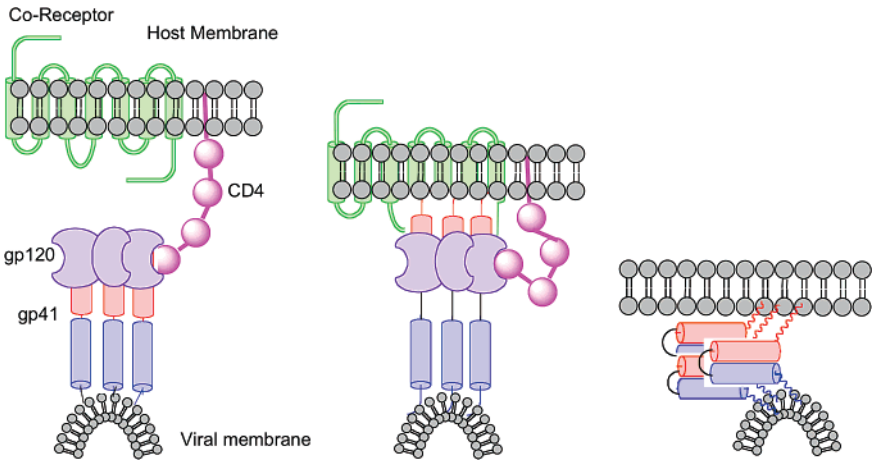



**Figure 2.** The process of HIV infection of CD4-positive cells involves initial contact between gp120 and CD4, which causes a conformational change allowing coreceptor interactions with gp120 and gp41 and subsequent membrane fusion.

## 2. HIV-1 Surface Protein Gp120 and Its Role in the Viral Entry of CD4-Positive T-Cells via Fusion

HIV gains access to several human cell types, most notably CD4-positive helper T-cells, macrophages, and dendritic cells. There are two known mechanisms of HIV-1 entry into host cells: fusion and uptake. HIV-1 fusion occurs primarily in CD4+ T-cells and macrophages, and it is mediated by interactions between HIV-env and host-associated receptors including the chemokine coreceptors CCR5 (R5) and CXCR4 (X4) (Figure 1).[7] Env is composed of two subunits corresponding to a 41 kDa transmembrane glycoprotein (gp41) and a noncovalently associated 120 kDa glycoprotein (gp120). In native virus, env is trimeric with gp120 noncovalently capping the surface-exposed regions of gp41.[8] The mechanism of uptake, which occurs in dendritic cells, is less well understood, but cell surface glycolipids appear to play a critical role in viral adhesion and transport during this process (sections 4 through 4.5).

The current model for viral entry via fusion begins with each gp41 strand initially in an extended conformation consisting of an amino terminus fusion peptide linked to a helix (N-helix), which in turn is linked to a carboxyl terminus helix (C-helix) via a hydrophobic loop region. Gp120 first binds to cell surface CD4 (binding strength of approximately 10 nM) (Figure 2).[9] The viral gp120/gp41 complex then undergoes a conformational change, exposing the N-terminal heptad repeat region of gp41. At this stage, the major chemokine coreceptors, CXCR4 or CCR5 on the host cell surface, are recruited to the CD4−gp120 binding site. This step may occur with the aid of cholesterol-rich glycosphingolipid rafts; however, this remains controversial at present.[10] The binding affinity for gp120−CCR5 interactions is in the range of 4−15 nM; interactions between gp120 and CXCR4 have proven more difficult to measure, but they are estimated to be 200 nM, or 100-fold lower than gp120−CCR5 interactions.[11] Once gp120 binds the appropriate host cell coreceptor, gp120 and gp41 dissociate, and a six-helix bundle is formed. Finally, the fusion peptide of gp41 is inserted into the host cell membrane and pore expansion occurs, allowing the contents of the virus to be introduced into the host cell.[12] Binding of the gp120/gp41 complex to CD4/coreceptors is cooperative, requiring six coreceptors, three CD4 binding events, and multiple clusters of gp41/gp120 to effectively form a fusion pore, analogous to influenza hemagglutinin membrane fusion.[11b,13]

As mentioned in the previous section, there is currently only one compound with FDA approval that acts as an entry/fusion inhibitor. T-20, (aka Fuzeon) is a peptide derived from the C-helix of gp41. It associates with the N-helix, preventing hairpin formation required for membrane fusion. T-20 must be administered through injection, which, in combination with the expense of production ($10,000−15,000 annually per patient), limits its widespread use. Nevertheless, T-20 is a viable option for individuals who have developed resistance to NRTI, NNRTI, and PIs.[14]

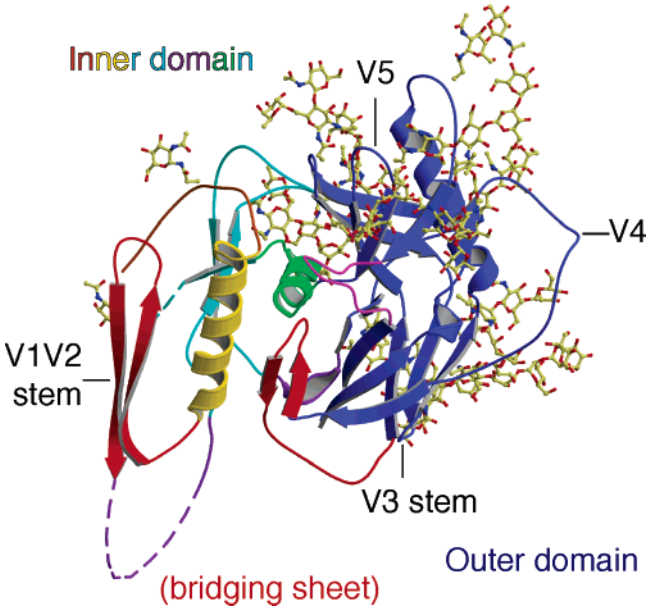



**Figure 3.** Structure of the unliganded SIV gp120 core. The protein is shown in ribbons, and the carbohydrates are shown in stick models. (Reprinted by permission from Macmillan Publishers Ltd: *Nature* **2005**, *433*, 834−841, copyright 2005.)

Structurally, there are two main questions that researchers have focused on with regard to viral env proteins, gp120 and gp41. First, what are the finer structural details of these proteins in native virus, and how do they interact with the host? It has long been suspected that gp120/gp41 is multimeric on the viral surface. A recent research report showed by cryoelectron microscopy (cryo-EM) tomography that HIV had an average of 14 ± 7 trimeric env spikes per virion.[15] These spikes were found in clusters with separation distances of less than the predicted 23 nm for randomly distributed spikes. Clustering may increase fusion efficiency or antibody neutralization, as noted by the authors. Studies by Zhu et al. also included the dimensions and shape characteristics of the env spikes from SIV (simian immunodeficiency virus). SIV has a higher number of spikes (73 ± 25) on the viral surface, with the following overall dimensions: spike height 13.7 nm, head diameter 10.5 nm, and stalk height 1.9 nm. Each SIV env spike contains three gp120 and three gp41 oriented in a triangular fashion, such that the three gp120 lobes rest on a tripod of transmembrane gp41. Comparisons of HIV and SIV env structures are considered valid due to strong sequence similarity.[16]

X-ray crystallography, combined with molecular modeling, has been used to further elucidate structural and electrostatic information pertaining to gp120 binding to host cell receptors, as well as some of the finer structural characteristics not discernible with techniques such as cryo-EM.[8,17] Early studies provided only a partial picture of the gp120 structure because high levels of glycosylation (∼50% total mass) and the presence of numerous variable regions made it necessary to truncate the protein structure through either deglycosylation or variable region removal. Newly reported X-ray crystallographic studies, however, have provided a more complete picture of the gp120 structure. A fully glycosylated unliganded gp120 protein core structure isolated from SIV, in which the V1−V2 and V3 loops were removed, was reported at 4 Å resolution by Chen et al. (Figure 3).[16] This represented a significant advance, as it was the first structure with all 13 N-linked glycan chains left intact. The glycan chains form clusters on the protein surface; however, further

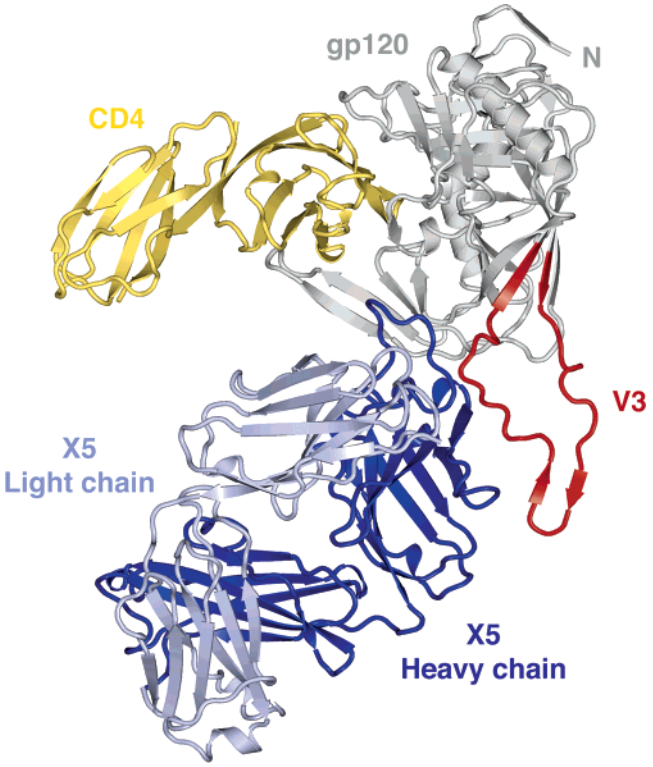



**Figure 4.** Structure of HIV-1 gp120, complete with V3 loop. The gp120 core is shown in gray, the V3 loop in red, the two membrane distal CD4 domains in yellow, and the Fab portion of the X5 antibody in dark and light blue. As depicted, the viral surface would be at the top, and the target cell at the bottom of the figure. (Reprinted with permission from Huang, C. C.; et al. *Science* **2005**, *310*, 1025−1028 (http://www.sciencemag.org). Copyright 2005, AAAS.)

resolution is required to identify specific interactions within the clusters and the amino acids in the protein, as well as to identify specific sugars in the glycan chains.

Another paper reported a structure of HIV-gp120, complexed to CD4 and the X5 antibody, whereby the V3 loop structure was maintained (Figure 4).[18] Complexation of gp120 with CD4 projected the V3 loop toward the target cell by 30 Å, due to conformational changes in gp120 that allow for coreceptor binding. This was an important study, as it demonstrated the importance of the V3 loop in HIV entry, providing critical structural information about a region that is prone to mutation.

The structure of gp41 has proven difficult to study, likely due to the membrane-bound nature of the protein; thus, only limited crystallographic data has been reported for fragments of the ectodomain region, comprising the six-helix bundle.[12a,19]

Much remains to be learned about the molecular structures of both gp120 and gp41, and it is hoped that as experimental methods improve, it will be possible someday to have structures of the complete heterotrimer complex of gp120/gp41. This will aid researchers greatly in the understanding of viral infection, and it will facilitate drug discovery.

## 2.1. Chemokine Coreceptors CCR5 and CXCR4 and Their Roles in HIV Entry

As previously noted, the binding of gp120 to CD4 alone is not sufficient to mediate viral entry and replication.[20] Chemokine coreceptors found on the host cell surface play an important role in the viral fusion/entry process. Chemokine receptors are G protein-coupled receptors (GPCRs) with

seven transmembrane domains, four of which are extracellular: three extracellular loops (ECLs) and the N-terminus.[9b,21] There have been approximately a dozen coreceptors identified in addition to CCR5 and CXCR4; however, these alternate receptors do not appear to efficiently mediate viral infection. Thus, CCR5 and CXCR4 are known as the primary chemokine coreceptors.[11b,21] After CD4 has bound to gp120, a conformational shift in the V1 and V2 loops, as well as the conserved core of gp120, reveals the coreceptor binding site.[22] All HIV-1 isolates use CCR5 (R5 strains), CXCR4 (X4 strains), or both coreceptors (R5X4 strains) to gain entry into host cells, even in the absence of CD4. The nomenclature for the coreceptors refers to the location of the first cysteines in the receptors and the order of discovery: CCR5 has adjacent Cys residues and was the fifth chemokine coreceptor of this type identified, while CXCR4 has an intervening amino acid between the Cys residues and was the fourth chemokine coreceptor of this type elucidated.[20] R5 viral isolates primarily infect macrophages and are typically the isolates transmitted between individuals early in infection, while X4 viral strains predominantly infect primary and transformed T-cell lines and evolve over several years as a consequence of viral mutation in env.[11b,] X4 viral strains are more cytopathic than R5, and it is believed the targeting of T-cells by X4 viruses is responsible for the decline of viable T-cells that defines the transformation from HIV-infected to AIDS status in patients. Accordingly, R5X4 strains are known as dual-tropic viruses, because of their ability to infect both cell types.[20]

### 2.1.1. Chemokine Coreceptor CCR5

CCR5 was identified as one of the primary chemokine coreceptors involved in HIV fusion and entry in 1996.[23] The gp120 CCR5 binding site has been identified through mutagenesis of gp120 and antibody binding. The receptor site is adjacent to the V3 loop and is dependent on CD4 binding, which involves movement of the V1 and V2 loops to expose the CCR5 binding site. Monoclonal antibody 17b is a CD4-induced (CD4i) antibody that recognizes a region on gp120 overlapping the CCR5 binding site, and it was used to inhibit binding of wild type and mutant gp120 to CCR5-presenting cell lines.[24] These studies identified several gp120 amino acid residues critical to gp120−CCR5 binding, notably Thr 123, Lys 121, Lys 207, Glu 381, Pro 438, Arg 440, Gly 441, Gln 422, Lys 421, and Ile 420. Mutation of these residues led to a ≥90% decrease in CCR5−gp120 binding. The authors also noted the overall basicity of the amino acids presented by gp120 in this region and implicated electrostatics as a factor facilitating the binding of gp120 to CCR5 via the sulfated tyrosines in the extracellular N-terminal domain of CCR5. Work by Dragic and co-workers confirmed the importance of the polyanionic CCR5 N-terminus, whereby they postulated that gp120 initially contacts CCR5 through this region and then more tightly binds CCR5 through other extracellular domains.[25] Another study implicated the importance of an N-linked glycosylation site in the V1/V2 stem region of gp120, through mutation of Asn 197 to Gln, Ser, or Lys.[26] Elimination of this glycosylation site was found to be sufficient to render the virus capable of binding to CCR5+/CD4− cells. The authors summarized that the CCR5 binding region is covered by the V1/V2 loops and that the glycan modulates the positioning of the V1/V2 loops, influencing the CCR5 binding process and subsequent viral infection. This same effect was observed for viruses with deleted V1/V2 loops, whereby CD4-independent CCR5 binding of gp120 was observed.

### 2.1.2. Chemokine Coreceptor CXCR4

Earlier in the same year in which coreceptor CCR5 was identified, cells with CXCR4 were also found to be susceptible to infection by HIV.[27] CXCR4 was identified by cDNA cloning and the ability of CD4-positive nonprimate-derived cells to fuse with, and be infected by, TCL-adapted env-expressing cells, and was thusly dubbed "fusin".[27a] Further studies with fusin have revealed more about its structure−function activity relating to gp120 binding and viral fusion.

In one study, it was found that the CXCR4-specific antibody, 12G5, was only able to inhibit gp120−CD4 interactions by about 50%.[28] The authors suggested that the gp120 binding site of CXCR4 was occupied either by other chemokine coreceptors or by heparan sulfate proteoglycans (HSPGs), as noted previously by Roderiquez and co-workers (*vide infra*),[29] and consistent with the low binding affinity between gp120 and CXCR4. Another group found that oligomerization of gp160 increased the valency of CXCR4 binding sites, yielding higher affinity interactions between the two proteins.[30] Mondor and co-workers suggested that the V3 loop on gp120 is involved in CXCR4 binding and that the binding of gp120−CXCR4 is analogous to the binding mechanism observed for gp120−CCR5 with one important difference:[28] CCR5 binding requires CD4 to bind before the CCR5 binding site is revealed, whereas CXCR4 binding of env occurs independent of CD4 binding.[31] In subsequent studies, deletion of critical amino acids in the C1 region of gp120 (amino acids 61−85) rendered the resultant mutant gp120 incapable of binding CD4, but it maintained affinity for CXCR4.[31b] Additional studies have been undertaken to determine the specific structural elements and key amino acids involved in interactions between CXCR4 and gp120. Several negatively charged amino acids in the N-terminal and extracellular loop 1 (ecl1) regions of CXCR4 were found to be important for binding gp120 (Glu14, Glu15, Glu32, and Asp97) using Ala walk mutation studies.[32] Other Ala substitutions in the amino terminus (Asn11 and Arg30) and in ecl2 (Asp187 and Asp193) amplified the ability of CXCR4 to serve as a coreceptor for otherwise R5-specific viruses while maintaining activity to X4 and R5X4 strains. While it was noted that electrostatic interactions with the V3 loop in gp120 were of importance for these amino acids, substitution of a CCR5 amino acid (Ser179 to Asp) led to a decrease in coreceptor activity with R5 and R5X4 isolates. Further incorporation of acidic amino acids in ecl2 of CXCR4 led to increased binding activity. The authors noted that a conserved element in ecl2 for both CCR5 and CXCR4 was important in defining the type of viral isolates that would be bound by the coreceptors.[33]

Mutational analysis was also used to examine the role of posttranslational tyrosine sulfation and glycosylation in modulation of the binding activity of CXCR4 to gp120, similar to studies undertaken with CCR5 (*vide supra*).[34] Farzan and co-workers found that sulfation of Tyr21 only played a minor role in HIV entry by X4 strains.[34b] This contrasts with what this same group found for CCR5, whereby the sulfated tyrosine residues at the N-terminus helped to modulate binding to gp120, and, ultimately, viral entry. Glycosylation of CXCR4 can occur at Asn 11 in the N-terminus, as noted by Chabot and co-workers in 2000.[35] Wang and co-workers studied the effects of the *N*-glycosy-

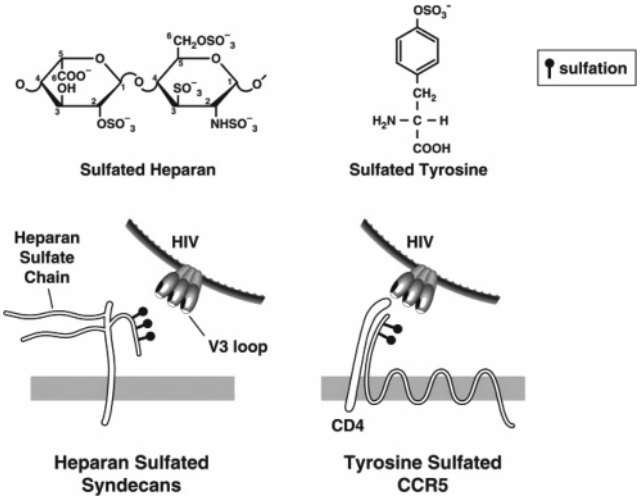



**Figure 5.** Schematic of HIV-1/syndecan and HIV-1/CCR5 interactions. (Reprinted with permission from de Parseval, A.; et al. *J. Biol. Chem.* **2005**, *280*, 39493. Copyright 2005 ASBMB.)

lation on viral binding and entry and found that glycosylation of Asn 11 prevents binding of CXCR4 to gp120 and subsequent infection.[34a] They also discovered that *N*-deglycosylated Asn 11 led to recognition of both R5 and X4 viral isolates, as compared to the wild type protein. The authors proposed that glycan removal reveals a shared conserved structure in both CXCR4 and CCR5. The *N*-glycan was, however, found to be inhibitory in the binding event between CXCR4 and gp120. The authors proposed that this inhibitory effect of the glycan on viral binding and infection was mediated through steric and electrostatic effects and that the deglycosylated form of the protein made CXCR4 more accessible to gp120 and led to a 2−3.5-fold increase in viral entry as compared to the wild type protein.

## 2.2. Heparan Sulfate Proteoglycans and Their Role in HIV Entry

### *2.2.1. Interactions with HIV-1 gp120*

Besides CD4 and the coreceptors CCR5 and CXCR4, other cell surface structures can also play a role in viral contact and entry. Specifically, there have been several reports of the involvement of heparan sulfate proteoglycans (HSPGs), also known as syndecans, in the early stages of viral entry. HSPGs are part of the glycosaminoglycan (GAG) family of cell-surface structures. HSPGs are glycoproteins, containing a protein core with linear polyanionic GAG carbohydrate branches. The GAG content is up to 95% of the total molecular composition. Heparan sulfate is structurally similar to heparin sulfate (see Figure 6, structure **1**), with the exception that it has lower sulfate content. In 1995, it was proposed that HIV could bind to cell surface HSPGs via the polybasic V3 loop of gp120 (Figure 5).[29] This is a sequential process in which HSPG first binds through a high-affinity, selective interaction with the V3 loop on gp120, followed by a second, lower affinity interaction with the conserved chemokine coreceptor region of gp120.[36] Other studies have shown that HSPG can compensate for low levels of CD4 in macrophages, thus regulating HIV infection in these cells.[37] HSPG is also a key player in the infection of CD4− brain endothelial cells, as noted by Argyris and co-workers.[10] It

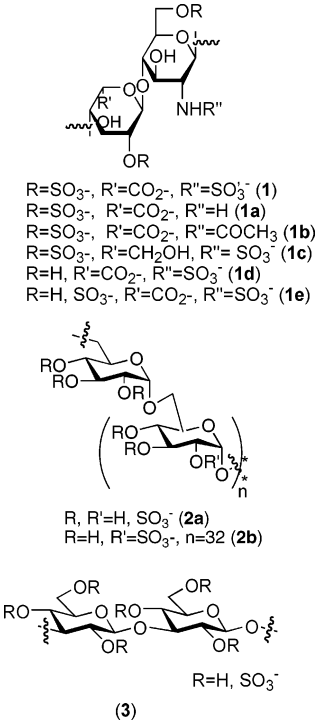



**Figure 6.** Chemical structures of HS (**1**), chemically modified heparins (**1a**−**e**), DS, D2S (**2a**, **2b**), and Curdlan sulfate (**3**).

has been reported that binding between gp120 and polyanions occurs without disrupting gp120−CD4 interactions, further strengthening the argument that the polybasic regions of gp120 bind the chemokine coreceptor.[29,36,38] In a more recent report, it was noted that the binding of HSPG to recombinant monovalent gp120 (rgp120) was enhanced if CD4 had bound to gp120 prior to HSPG, compared to free gp120 binding HSPG.[39] The authors used a combination of SPR (surface plasmon resonance) analysis, molecular modeling, and antibody binding studies to show that the HSPG binding site on gp120 consists of the V3 loop and the chemokine receptor binding region. The amino acids in the V3 loop purported to be involved in this binding event were identified as Arg304, Arg306, Arg308, and Arg327. For the CD4i region, molecular modeling revealed that amino acids Lys121, Arg419, Lys421, and Lys432, clustered between the V1/V2 loop stems and the V3 loop and oriented linearly in a 25 Å space, have the potential of binding a polyanionic octasaccharide. Antibody binding studies confirmed that an octasaccharide of heparin sulfate (HS) was the smallest oligosaccharide capable of inhibiting the interaction between antibody 17b and gp120 in the presence of CD4. This finding supports the hypothesis that binding of HSPG to gp120 is a CD4-induced event, as antibody 17b is a CD4i antibody.

Further studies by de Parseval et al. highlighted a single highly conserved amino acid in the V3 loop, Arg 298, and its importance in binding to both HSPGs and CCR5 (Figure 5).[40] This study involved the use of whole virus with trimeric gp120, which is important given that carbohydrate interactions within biological systems are typically multivalent in nature. The interesting, and perhaps most significant, aspect of the findings in this study was that a specific sulfation motif was preferred by the virus, 6-*O*-sulfation, indicating that random sulfation or negative charges were not sufficient to yield a strong binding event between the virus and the host cell and that the virus can use the single Arg 298 residue to

mediate binding to the host either through HSPG or through CCR5 as needed.

#### 2.2.2. Interactions with HIV-1 Tat Exogenous Transcription Factor

In addition to the importance of interactions of gp120 with CD4, coreceptors, and HSPG on the host cell surface, there are reports of yet another type of viral−host interaction involving the HIV-1 viral polypeptide, tat. Tat is a transcription factor protein that binds to RNA at the 5′ end of viral transcripts and leads to an increase in the rate of transcription initiation and elongation from the LTR (long terminal repeat) promoter.[41] Tat can be released exogenously from an infected cell, bind to another infected cell, enter, and translocate to the nucleus of the cell, where it stimulates transcription of HIV-LTR and transactivates cellular genes.[42] Tat can also enter uninfected cells and transactivate endogenous genes, which can lead to increased production of cytokines and cytokine receptors, in addition to influencing many other cellular functions responsible for cell activity and survival, implying that tat may be significant in the promotion of HIV pathogenesis.[41]

Cell surface HSPGs appear to be important in the internalization of tat.[41] Tat has an Arg-rich basic region (amino acids 49−57), and as such, it has long been known that tat can interact with polyanions such as heparin and dextran sulfate.[42,43] In a study by Tyagi and co-workers, it was found that tat uptake could be blocked by soluble heparin, but not other soluble GAGs, and that cells with defects in HSPG production were incapable of internalization of tat. The same research group had previously found that mutation of the tat Arg-rich region prevented both heparin binding to tat and tat internalization.[44] Structurally, it was determined that, at a minimum, a hexasaccharide of heparin was required to bind to tat and that *N*- or *O*-desulfation of heparin led to a dramatic loss of binding capability.[43c,45] The authors of the tat studies concluded from this that the interaction between HSPG and tat is likely defined by the size, polysaccharide composition, and degree of sulfation.[41] As many oligo-/polysaccharide structures are known to possess secondary structural domains, it is also likely that the overall shape and conformation of the HSPG and oligo-/polysaccharide antagonists of the HSPG−tat interaction play an important role in the uptake of tat. HIV-1 tat, therefore, represents an attractive target for the design of a unique class of polyanionic inhibitors.

## 3. Polyanionic Polysaccharides as Anti-HIV Agents in CD4-Positive Cells

The findings of the past decade relating to the determination that CD4 alone is not sufficient for allowing HIV entry into host cells and that coreceptors and HSPGs on the host cell are utilized for viral fusion highlights the importance of electrostatic interactions between gp120, tat, and the host cell surface. Gp120 has several highly basic regions, most notably, the V3 loop, while host cell surface structures are now known to have polyanionic regions that can interact with the basic regions of gp120. HIV-1 tat has an Arg-rich region that interacts with host cell surface polyanionic structures to gain entrance, and perhaps make cells more vulnerable to infection. These charge−charge interactions can occur either between the chemokine coreceptors CXCR4 or CCR5 or also through other polyanionic structures on the host cell surface, such as HSPGs. It should therefore not be a surprise that there have been a large number of anionic polysaccharides of both natural and semisynthetic nature reported to have anti-HIV activities relating to early stages of infection, namely viral fusion and entry. The earliest reports outlined the anti-HIV activity of two common sulfated polysaccharides, heparin sulfate and dextran sulfate, long before the discovery of the roles of the chemokine coreceptors and HSPGs on the host cell surface.

### 3.1. Heparin Sulfate and Dextran Sulfate

Sulfated polysaccharides have been recognized as having potent *in vitro* anti-HIV activity since the late 1980s. Several naturally occurring sulfated polysaccharides, such as heparin sulfate (HS, **1**) and dextran sulfate (DS, **2a**), have been shown to inhibit the binding of HIV to CD4-positive cells *in vitro* in the microgram per milliliter or micromolar range (Figure 6).[46] The mechanism by which polyanionic polysaccharides inhibit HIV has been actively studied. There are regions in gp120 containing multiple basic amino acids, namely the V3 loop (amino acids 303−338), the C-terminal region (amino acids 495−516), and a conserved region involved in chemokine coreceptor binding (discontinuous amino acids in regions 117−123, 207, and 419−444)[24,38,47] These regions interact with polyanions such as HS and DS, preventing binding to complementary antibodies.[36,47b] Callahan and co-workers showed that gp120 recognition of CD4 was not impeded by the presence of DS, indicating that DS binds to gp120 remote from the CD4 binding site, subsequently identified as the V3 loop.[38] Studies have also been dedicated to defining the minimum structural requirements to achieve anti-HIV activity. One group reported neutral and non-sulfated polysaccharides lacked activity, while many different polysulfated polysaccharides had inhibitory activity against HIV.[48] Another group reported that, for chemically modified heparins, *N*-desulfation led to reduced activity (Figure 6, **1a)**, while *N*-acetylation restored the activity (**1b**).[49] Reduction of carboxyl groups in the polysaccharide had little effect (**1c**), while complete *O*-desulfation abolished the ability of the polysaccharide to bind the V3 loop and abolished *in vitro* anti-viral activity (**1d**). Further, selective *O*-desulfation of some of the 2-*O*-sulfates and all of the 6-*O*-sulfates of heparin led to a substantial loss of activity (**1e**). In a study by Jagodzinski et al. it was suggested that polysaccharide chain length and glycosidic linkage type could influence the ability of sulfated polysaccharides to affect the conformations of the V2, V3, and C4 (constant region 4) regions of gp120 and ultimately affect the ability of the virus to infect macrophages.[50] The viral strain used was an X4 isolate with the V3 loop swapped. This was done to try to elucidate the observed differences in mediating infection of macrophages by three sulfated polysaccharides: high molecular weight DS (HMDS, 500 kDa), low molecular weight DS (LMDS, 8 kDa), or curdlan sulfate (CRDS, ∼79 kDa). Curdlan sulfate is a $\beta$-1→3-linked glucose polymer (**3**), while DS is an α-1→6-linked glucose polymer (**2a**) (Figure 6). The mutant virus was monitored for conformational changes in the V2, V3, and C4 regions of gp120 by flow cytometry with anti-V2, V3, and C4 antibodies in the presence of HMDS, LMDS, and CRDS. The authors noted that only HMDS increased HIV binding and infection of the macrophages, and they attributed this to conformational changes in gp120 induced by HMDS. They suggested that the binding of HMDS to gp120 revealed a conformationally dependent

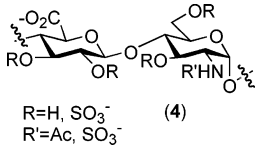



**Figure 7.** Structure of *E. coli* K5 capsular polysaccharide.

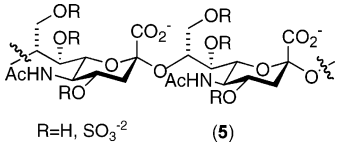



**Figure 8.** Structure of colominic acid.

epitope in the V2 region, leading to a higher affinity binding event between gp120 and CCR5. One can surmise from the results that LMDS and CRDS did not possess the right conformations to achieve this, either by virtue of not being large enough, by not having the correct secondary structure, or in not having the correct shape, as in CRDS, due to the $\beta$-glycosidic linkages. The authors suggested size and glycosidic linkages as potential explanations for the different effects observed.

Other research in this area has focused on the determination of complementary protein valency issues relating to the mechanism of the anti-HIV activity of polysulfated polysaccharides. Surface plasmon resonance studies revealed strong polyanion−gp120 binding with immobilized HS and monomeric gp120, yielding an affinity constant of 220 nM. These studies also determined that an average of 4.4 gp120 molecules bind each chain of heparin, indicating that even higher avidity binding would be possible in a multivalent sense.[36,51]

While many of the studies in the literature reporting the anti-HIV activity of DS and HS have focused on gp120 binding, there have also been reports of the ability of both HS and DS to bind to the HIV-1 tat protein and prevent interactions with HSPGs on the cell surfaces. In one study, a polydisperse derivative of DS was prepared (Figure 6, compound **2b**), known as D2S (the sulfate ester is located at position 2).[42] The median molecular mass was 9 kDa. The authors found that D2S bound tightly to tat ($EC_{50} = 0.1$ $\mu$g/mL; the $EC_{50}$ is the effective concentration needed for 50% activity) and inhibited HIV-1 tat transactivation dose-dependently ($IC_{50} = 0.5$ $\mu$g/mL, the inhibitory concentration required to prevent 50% of the process under study, as compared to an untreated control), while the non-sulfated dextrin polysaccharide parent compound was found to have no anti-HIV properties. The study concluded that polysulfated polysaccharides such as D2S could serve as potential inhibitors of HIV-1 infection by virtue of the fact that they can bind tat and ultimately inhibit the viral replication process.

HS and DS, while having strong affinity to the basic regions of gp120, are also anticoagulants, making it difficult to achieve therapeutic anti-HIV levels of the drugs without compromising blood clotting time.[52] Furthermore, in phase I/II clinical trials, DS was found to be poorly absorbed orally, and when given intravenously, it resulted in toxic side effects such as reversible thrombocytopenia and alopecia in the study subjects and did not yield a therapeutic effect based on HIV marker levels such as p24.[53] However, a more recent clinical study of DS that employed different analytical techniques found that DS was absorbed into the blood stream after oral administration, indicating that DS has therapeutic potential and merits further study.[54]

In addition to toxicity issues, HIV resistance has also been noted for polysulfated polysaccharides. In one report, a DS-resistant laboratory strain of HIV-1 was generated by incubating cells with HIV-1 in the presence of LMDS (5 kDa).[55] This resistant virus did not recognize an anti-V3 loop antibody. The authors also noted several mutations in this virus in the V1, V2, and V3 loops, in the C3 region, and in the CD4 binding domain. The V4 region also contained a five amino acid deletion mutation, suggesting that resistance of this viral isolate was also responsible for cross-resistance against other similar polyanionic inhibitors.

## 3.2. Bacterial Polysaccharides

### *3.2.1. Escherichia coli K5*

The bacterium *Escherichia coli* K5 possesses a capsular polysaccharide containing repeating GlcUA (glucuronic acid) and GlcNAc (*N*-acetyl glucosamine) disaccharide units, similar to that of heparin (**4**) (Figure 7). When the polysaccharide is *N*- and *O*-sulfated,[56] the resultant polysulfated polysaccharide was found to possess significant anti-HIV activities. In one study, it was determined that two sulfated derivatives of the K5 polysaccharide (from hereon referred to as K5), namely the *N*-acetylated, *O*-sulfated K5 (K5-OS(H)) and the *N*- and *O*-sulfated K5 (K5-N,OS(H)), were found to inhibit replication in both X4 and R5 HIV-1 viral strains with $IC_{50}$ values falling between 0.07 and 0.46 $\mu$M.[57] These compounds were also determined to be nontoxic to cells up to the maximum concentration tested of 9 $\mu$M. Finally, the authors noted that the K5-N,OS(H) derivative possessed greater activity against R5X4 HIV-1 strains than did the K5-OS(H) polysaccharide. Further studies indicated that anti-HIV-1 activity stemmed from K5-N,OS(H) binding to extracellular tat and subsequent inhibition of tat binding to host cell receptors such as HSPGs, ultimately preventing tat uptake and HIV-LTR transactivation.[58] The authors noted that the degree of sulfation of the polysaccharide, as well as the location of the sulfate esters (*N*- or *O*-sulfates), was responsible for the modulation of the anti-HIV activity of these compounds.

### *3.2.2. Colominic Acid*

Colominic acid (CA) is a homopolymer of sialic acid linked via α-2→8 glycosidic bonds (**5**) (Figure 8). It is a polydisperse polymer, isolated from bacterial fermentation broths as a secondary metabolite that averages 100 sialic residues in length.[59] Yang and co-workers reported that a randomly sulfated analogue of colominic acid (SCA, sulfated colominic acid) yielded $EC_{50}$ values as low as 0.06 $\mu$g/mL for the larger molecular weight, more highly sulfated derivatives (MW ranging from 8 to 16 kDa, sulfation 8−12%) in MT-4 and C8166 cell lines infected with HIV in the presence of these molecules.[52] Their assay control, DS, yielded $EC_{50}$ values of 0.5 and 2.51 $\mu$g/mL in the same cell lines, respectively. The SCA compounds inhibited syncytium (giant cell) and abolished HIV-1 p24 antigen production. With regard to the side effect issues that have prevented the clinical usage of other polyanionic polysaccharides such as HS and DS, dosing of SCA was possible up to 10 $\mu$g/mL without an appreciable increase in the APTT (activated partial thromboplastin time), as compared to a 7-fold increase in the APTT when cells were treated with

the same concentration of both HS and DS.[52] Therefore, due to the better therapeutic profile of SCA compared to HS and DS, it represents a more attractive compound to pursue for further anti-HIV drug development.

## 3.3. Polysaccharides Isolated from Marine Organisms

### 3.3.1. Marine Plants

A number of polysaccharides have been isolated from marine algae that, in the sulfated form, have anti-HIV properties. Algae are classified into six groups based primarily on their colors, such as red, green, or brown.[60] Algae can be harvested from both fresh water and seawater sources. In the ocean, multicellular alga are classified as seaweeds and are distributed in all manners from tidal to deep water, anchored and not.[60] Polysaccharides from marine algal sources are readily extractable using hot water, under either acid or basic conditions.[61] All marine algal species produce at least one sulfated polysaccharide, the function of which is not well understood but may be involved in maintaining the structural integrity of the plant, preventing the plants from drying out under low tide conditions, and in the absorption of potassium and calcium selectively from the seawater.[61] Other naturally unsulfated polysaccharides from algae become antiviral upon chemical sulfation. The antiviral properties of these natural products have been known since the 1950s, with anti-HIV attributes being discovered in 1987 by Nakashima and co-workers.[61,62] Many research groups have studied the anti-HIV properties of these polysaccharides to determine the specific mechanism of action and optimum structural features to maximize activity. A few of these studies are summarized below; however, for a more complete review of anti-HIV alga, see the cited reviews.[60,61]

**3.3.1.1. Naturally Sulfated Polysaccharides.** Calcium spirulan (Ca-SP) is a complex polysaccharide found in African and Central and South American lakes with high salt concentrations. This polysaccharide is composed of the sugars rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, glucuronic acid, and galacturonic acid, and has approximately 3% sulfur content.[63] This polysaccharide was isolated from the blue-green algae *Spirulina platensis* and evaluated by Hayashi and co-workers for anti-HIV activity using syncytium formation, p24, and cytopathicity assays.[64] These researchers also assessed the anticoagulant properties and structural requirements for anti-HIV activity of Ca-SP, including sulfation and calcium chelation. The results of their studies indicate that Ca-SP has a low toxicity profile for CD4+ cells, and therapeutic concentrations were achieved without significantly increasing the APTT. The therapeutic levels required to inhibit HIV-1 replication were found to be similar to those for DS, 9.3 $\mu$g/mL in p24 antigen assays. They also confirmed that, like HS and DS, the sulfate groups were required for anti-HIV activity, and even in the presence of the sulfate esters, if calcium was absent, antiviral activity was lost, suggesting that Ca chelation induces a specific polysaccharide conformation essential for biological activity.

Another naturally occurring sulfated polysaccharide, isolated from the Mediterranean red alga *Asparagopsis armata*, was found to have anti-HIV activity in the range of 8−10 $\mu$g/mL, as measured in syncytium forming and reverse transcriptase activity assays.[65] The complex galactan polysaccharide isolated from this organism is derived from the cell wall and takes on different forms depending on the reproductive stage of the algae; gametic, carposporic, and tetrasporic. Interestingly, only the gametic and tetrasporic stage polysaccharides had anti-HIV activity. The active compounds have molar ratios of galactose:anhydrogalactose:sulfates of 1:0.01:1.23 and 1:0.01:1.13 for the gametic and tetrasporic stages, respectively, and 1:0.04:0.47 for the inactive carposporic stage. The authors concluded that the sulfate content was critical for activity, and they confirmed this by removing the sulfate groups from the active gametic polysaccharide, which resulted in the loss of anti-HIV activity. The gametic polysaccharide was nontoxic to 500 $\mu$g/mL, a concentration well above the therapeutic level *in vitro*.

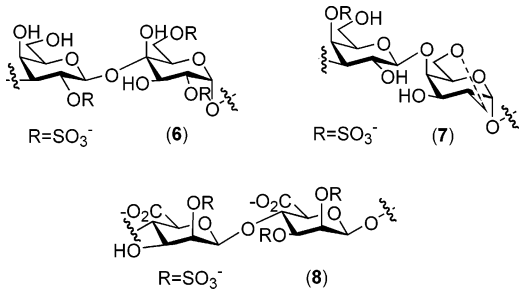



**Figure 9.** Structures of algal-derived sulfated polysaccharides.

**3.3.1.2. Chemically Modified/Sulfated Polysaccharides.** In addition to naturally sulfated algal polysaccharides, chemically modified polysaccharides from natural sources have been exploited as potential anti-HIV agents. In one study by Yamada and co-workers, low molecular weight *O*-acylated derivatives of $\lambda$- and $\kappa$-carrageenans were prepared from native polysaccharides to increase the anti-HIV activities of the polysaccharides, while minimizing the native anti-coagulant properties.[66] $\lambda$-Carrageenan is isolated from two species of algae that grow together in the North Atlantic coastal region from Norway to North Africa, *Gigartina aciculaire* and *Gigartina pistillata*, and has a repeating (1→3)-linked disaccharide of 2-sulfate $\beta$-D-galactopyranosyl (1→4)-α-D-galactose sulfated at the 2 and 6 positions (**6**) (Figure 9).[61] $\kappa$-Carrageenan is isolated from *Eucheuma cottonii* and is characterized by a repeating unit of 4-sulfate-$\beta$-D-galactopyranosyl (1→4)-3,6-anhydro-α-D-galactose linked (1→3) (**7**) (Figure 9).[61] Yamada and co-workers determined that the butanoylated derivatives with 1.1−1.4 mol of butanoyl per disaccharide unit and 3 mol of sulfate per disaccharide unit for $\lambda$-carrageenan gave anti-HIV $IC_{50}$ values of 3.9 $\mu$g/mL as compared to that for DS at 7.8 $\mu$g/mL.

Another example of an anti-HIV chemically modified polysaccharide is from a brown algae (species not given).[67] The active molecule is composed of the monosaccharide sulfated $\beta$-D-mannuronic acid linked (1→4) (**8**), has an average molecular weight of 8 kDa, and is known as sulfated polymannuroguluronate (SPMG) (Figure 9). In their studies, Meiyu and co-workers used a combination of SPR and flow cytometry assays to measure binding and inhibition of binding, respectively, of SPMG to gp120 and the inhibition of CD4/gp120 and CD4+ T-cells−gp120 binding mediated by SPMG. Their studies revealed that SPMG bound to the V3 loop peptide with high affinity (1.38 nM) and multivalently, where one SPMG interacted simultaneously with three or four V3 loop peptides. Computer docking experiments confirmed that an octasaccharide of SPMG covered the V3 loop. It was further determined by SPR that a

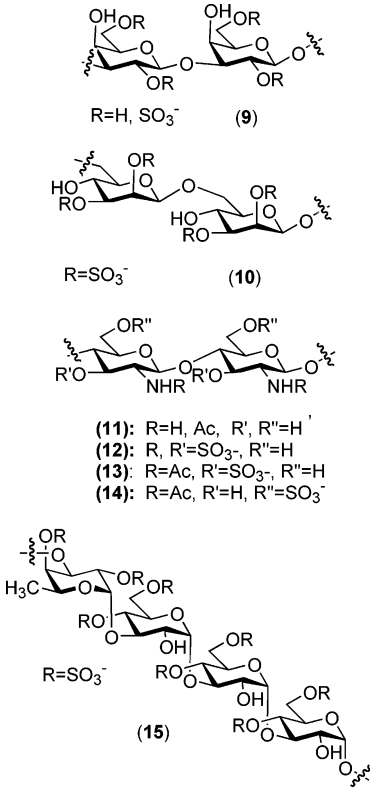



**Figure 10.** Sulfated polysaccharides isolated from marine invertebrate animals.

hexasaccharide was the minimum chain length capable of binding gp120, with 15−16-mers having binding similar to that of the full size polymer. The larger oligomers were also shown to interact with gp120 at multiple locations and, additionally, with multiple copies of gp120.[68] These observations again highlight the importance of both polysaccharide length and conformation, as noted in the previous studies (*vide supra*). This compound (Figure 9, compound **8**) has now entered into phase II clinical trials in China.[69]

### *3.3.2. Marine Invertebrate Animals*

In addition to numerous reports of isolated marine algae sulfated polysaccharides being found to have anti-HIV properties, there have also been reports of marine animals such as clams, tunicates, and sponges having sulfated polysaccharides with inherent anti-HIV activities. These reports highlight the diversity of sulfated polysaccharides produced in a variety of marine invertebrates. A species of marine clam, *Meretrix petechialis*, was found to contain a homopolymer D-galactan sulfated polysaccharide with $\beta$-(1→3)-glycosidic linkages and partial sulfation at the C-2 and C-6 positions, as determined by methylation analysis/degradation and NMR (**9**) (Figure 10).[70] Preliminary anti-HIV studies were conducted using syncytium forming assays. It was found that the clam polysaccharide was a moderate inhibitor of syncytium formation by 33 and 56% at 100 and 200 $\mu$g/mL concentrations, respectively. DS was used as a control and, at the same concentrations, led to inhibition of syncytium formation by 65 and 95%.

Another homopolysaccharide was isolated from the mucus secretions of the pacific tunicate *Didemnum molle*, known as kakelokelose, derived from the Hawaiian word meaning slimy.[71] The tunicate was collected in Pohnpei, Micronesia, and also in Manado, Indonesia. The structure of this

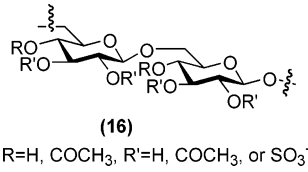



**Figure 11.** Structure of an anti-HIV glucan isolated from a lichen.

polysaccharide was determined by NMR to be a repeating mannose Man-$\beta$-(1→6)-Man, with sulfate groups at C-2 and C-3, but not at C-4 (**10**) (Figure 10). The sulfur content was determined to be 38.3% of the dry weight, with a ratio of sulfate/Man estimated to be 1.8:1. The anti-HIV activity of kekelokelose was determined to have 100% effectiveness in the prevention of infection of CEM cells by HIV strain RF, at 0.3 $\mu$g/mL, with no cytotoxicity at concentrations up to 15 $\mu$g/mL in the same cell line.

Sulfated chitosan has also been found to possess anti-HIV activity. Chitin, the parent polymer, is a naturally occurring amino-polysaccharide consisting of $\beta$-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranose or 2-amino-2-deoxy-D-glucopyranose and can be isolated from crustacean shells and insect cuticle (**11**) (Figure 10).[72] Nishimura and co-workers prepared three derivatives of chitosan, one with the C-2 amino groups and C-3 hydroxyls sulfated (23S, **12**), the second with the C-2 amino groups acetylated and the C-3 hydroxyls sulfated (3S, **13**), and the third with the C-2 amino group acetylated and the C-6 hydroxyl sulfated (6S, **14**) (Figure 10).[73] The researchers found that compound 23S had greater anti-HIV activity than did 3S or 6S, with the $EC_{50}$ values 0.28, 9.6 and 57 $\mu$g/mL, respectively. The authors concluded that specific sulfation sites were much more important in interacting with gp120 than the total degree of sulfation on the sugars.

Finally, a more complex linear polysaccharide, known as rosacelose, was isolated from an aqueous extract of the sea sponge *Mixylla rosacea* collected in the fiords of Galicia (Santiago de Compostela, Spain).[74] Sulfated polysaccharides in sponges play an important role in sponge/cell aggregation and may play a similar role in maintaining the structure of the sponges, similar to proteoglycans in the connective tissues of vertebrates.[75] The structure of the polysaccharide was shown to be composed of 4,6-disulfated 3-*O*-glycosylated α-D-glucopyranosyl and 2,4-disulfated 3-*O*-glycosylated α-L-fucopyranosyl residues in a 3:1 ratio by chemical degradation and NMR spectroscopy (**15**) (Figure 10). The anti-HIV activity ($IC_{50}$ 5 $\mu$g/mL) of rosacelose was determined in a syncytium forming assay with MT4 cells.

## 3.4. Polysaccharides Isolated from Land Plants

In addition to the wealth of unique anti-HIV sulfated polysaccharides that have been isolated from saline lakes and oceans, terrestrial organisms have been found to have polysaccharides that, when chemically sulfated, yield polysaccharides with potent anti-HIV activities.

An early study reported that the lichen *Umbilicaria esculenta* possesses a partially acetylated $\beta$-(1→6) glucan, known as GE-3, that, when chemically sulfated to give GE-3-S (**16**, Figure 11), has the ability to inhibit syncytium formation in HIV-infected Molt-4 cells at a concentration of 62.5 $\mu$g/mL, to suppress viral antigen expression at 31 $\mu$g/mL, and to inhibit HIV plaque formation by 50% at a concentration of 19.5 $\mu$g/mL;[76] however, weak toxicity in animals was noted. From the sap of a Chinese lacquer tree

(*Rhus vernicifera*), a polysaccharide consisting of a 1,3-$\beta$-galactopyranosidic main chain with complex branched 4-*O*-methyl glucuronic acid terminating chains was discovered.[77] The monosaccharide composition was reported to be D-galactose, 4-*O*-methyl-D-glucuronic acid, L-arabinose, and L-rhamnose. When chemically sulfated, the lacquer polysaccharide had mild anti-coagulant properties, and it was found that the high molecular weight polysaccharides (10.6−42.7 kDa) had the most potent anti-HIV activities in an MTT assay, with reported $EC_{50}$ values ranging from 0.4 to 0.8 $\mu$g/mL. The cytotoxicities of these same compounds were reported as $CC_{50}$ (cytotoxic concentration needed for 50% cell death) and ranged from 457 to above 1000 $\mu$g/mL, well above the effective anti-HIV concentrations. Therefore, even though the land organism-based polysaccharides are not naturally sulfated, chemical sulfation of these compounds yielded polyanionic polysaccharides with potent anti-HIV activities that rival the activities observed for the naturally sulfated polysaccharides already discussed.

## 3.5. Synthetic/Semisynthetic Polysaccharides

In addition to harvesting anti-HIV activities of naturally occurring polysaccharides, much work has been reported on synthetic manipulations of polysaccharides, generation of novel synthetic oligosaccharides from monosaccharides, and the effects of the modifications on HIV activities. The structural features of these oligo-/polysaccharides as they relate to activity have also been a major focus of the studies to further elucidate the basis of their anti-HIV properties.

### 3.5.1. Curdlan Sulfate

Curdlan sulfate (CRDS) is the sulfated derivative of curdlan, a naturally occurring polysaccharide isolated from two types of bacteria, *Agrobacterium biobar* and *Agrobacterium radiobactor*. This polysaccharide is a 1,3-$\beta$-D-glucan (**3**) with sulfate ester incorporation possible at C-2, C-4, or C-6, an average molecular weight of 79 kDa and sulfur content of approximately 15% (Figure 6). The anti-HIV properties of CRDS were first reported in 1990 when Yoshida and co-workers determined that CRDS polysaccharides with high sulfur content (12.1−14.7%) had the ability to protect MT-4 cells against HIV infection better than higher molecular weight CRDS with low sulfur content (5.6−8.9%).[78] The minimum inhibitory concentration of CRDS was determined to be 3.3 $\mu$g/mL. Antibody binding studies of CRDS to regions of gp120 indicated the binding region for CRDS is within the V3 loop and the CD4 binding site.[79] A phase I/II clinical trial of CRDS was carried out in 1994 where it was determined that CRDS had low toxicity (up to 5000 $\mu$g/mL in cell culture) and only affected blood clotting time at high doses (200−300 mg). The authors also noted a dose-related increase in CD4-positive cells in HIV-positive patients.[80] Finally, Jeon et al. performed NMR studies whereby the highly basic α-6 helical region of gp120 (amino acids 506−518, Thr-Lys-Ala-Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg) was mimicked by using poly-Lys to study the ionic interactions between CRDS and gp120-α-6.[81] The authors noted that these interactions may be important in induction of conformational changes in gp120, and modulation of the viral adhesion process.

### 3.5.2. Anhydrosugar Oligosaccharides

Anhydrosugars have been used to create unique non-natural polysaccharides that are then sulfated to create new potential anti-HIV agents. In an early report, Hatanaka and co-workers used a ring-opening polymerization on a benzyl-protected 1,6-anhydro-$\beta$-D-pyranose derivative to yield a stereoregular (1→6)-α-D-mannopyranan polymer with an average molecular weight of 75 kDa, upon deprotection (**17**) (Figure 12).[82] Upon sulfation with piperidine-*N*-sulfonic acid, sulfate esters were found incorporated at C-2, C-3, and C-4, with a maximum degree of sulfation of 2.12. The compounds with the highest anti-HIV activities (reported as $EC_{50}$ values) had degrees of sulfation of 1.19−1.83 and activities of 3.3 $\mu$g/mL.

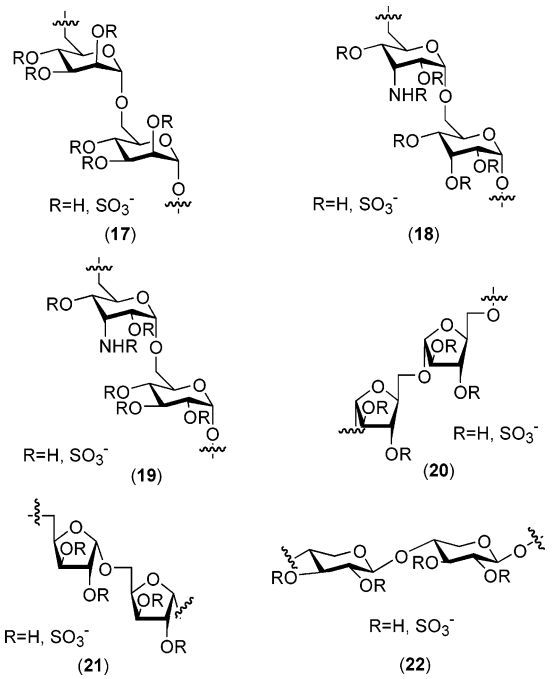
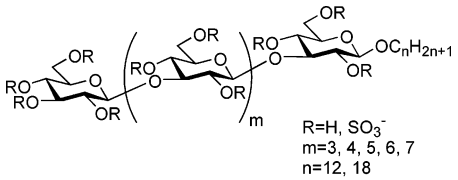


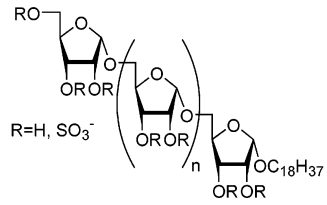

**Figure 12.** Synthetic sulfated polysaccharides derived from anhydrosugars.

Reports of other non-natural sulfated polysaccharides, created by ring-opening polymerizations, as anti-HIV agents have been published. In one example, a series of sulfonated 3-amino-3-deoxy-(1→6)-α-D-allopyranan copolymers was created by reacting various ratios of 1,6-anhydro-3-azido-2,4-di-*O*-benzyl-3-deoxy-$\beta$-D-allopyranose with either 1,6-anhydro-2,3,4,-tri-*O*-benzyl-$\beta$-D-allopyranose or 1,6-anhydro-2,3,4,-tri-*O*-benzyl-$\beta$-D-glucopyranose and a $PF_5$ catalyst.[83] Upon reduction of the azido group, deprotection of the benzyl groups, and subsequent sulfation of the amines and hydroxyls, the anti-HIV properties of the unnatural polymers were evaluated (**18, 19**) (Figure 12). The derivatives with the highest activity were the sulfamido allose/glucose co-polysaccharides (**19**) with $EC_{50}$ values ranging from 0.2 to 0.5 $\mu$g/mL. The sulfamido allose/allose co-polysaccharides (**18**) had lower activities with $EC_{50}$ values ranging from 0.8 to 0.9 $\mu$g/mL. Interestingly, the $CC_{50}$ values were higher for the allose/glucose copolymer (>1000 $\mu$g/mL) and lower (more toxicity) for the allose/allose copolymer (740−797 $\mu$g/mL). The amino allose unit also contributed to blood anticoagulant activity.

A final example of unnatural anhydrosugar-derived sulfated anti-HIV polysaccharides was reported by Yoshida et al. in 2001.[84] In this study, the authors used two anhydropentose monomers with different configurations of substituents at C-2 and C-3 than D-ribose, to study the effects of structure/anti-HIV activities. They used 1,4-anhydro-2,3-di-*O*-*tert*-butyldimethylsilyl-α-L-arabinofuranose and -D-xylo-




(**23**)




(**24**)

**Figure 13.** Alkylated sulfooligosaccharides.

furanose as monomers and catalyzed the ring-opening polymerization with $PF_5$ to give (1→5)-α-L-arabinofuranan and (1→5)-α-D-xylofuranan derivatives, which, upon deprotection and sulfation, gave the target anti-HIV pentosan compounds (**20, 21**) (Figure 12). A commercially available xylan, (1→4)-β-D-xylopyranan, was also sulfated for comparison (**22**) (Figure 12). The degree of sulfation was found to be important for activity, with higher sulfate content resulting in higher anti-HIV activities. All of the compounds tested had significant anti-HIV activity ($EC_{50}$'s: 0.1−3.3 μg/mL) except for the xylofuranan derivative with a degree of sulfation less than one. The toxicity profiles of all of the derivatives except one xylan derivative exceeded 1000 μg/mL; however, the anticoagulant activities for the arabinofuranan and the xylofuranan derivatives were higher than that of either CRDS or DS. The authors attributed this to a more flexible polysaccharide backbone, resulting from the five-membered ring structure.

### 3.5.3. Alkyl Oligosaccharides

Sulfated alkyl oligosaccharides have been known to have potent anti-HIV activities since the early 1990s. The general strategy involved utilizing oligosaccharides derived from polysaccharides such as curdlan (laminaraoligosaccharides) and cyclodextrins (maltooligosaccharides), and appending a variety of linear, branched, or cyclic aliphatics to the reducing end. The lipid chain was attached to create surface-active agents whereby the alkyl chains coalesced.[85]

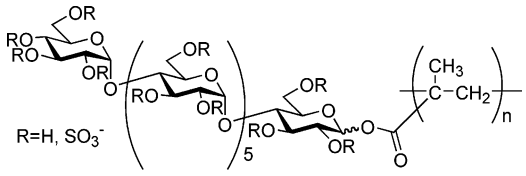
In an early report by Katsuraya et al., laminaraoligosaccharides ranging from pentaose through nonaose were prepared by acid hydrolysis of curdlan.[85] These oligosaccharides were then reacted with either dodecyl alcohol or octadecyl alcohol, followed by sulfation in sulfur trioxide pyridine, to give the sulfated alkyloligosaccharides (**23**) (Figure 13). The sulfur content ranged from 14 to 17.5% by elemental analysis, with degrees of sulfation ranging from 2.3 to 3.2 sulfates/glucose residue. The anti-HIV activities of the sulfated alkyl oligosaccharides were determined using an MTT assay, and $EC_{50}$ values were determined. It was found that the sulfated dodecyl oligosaccharides had $EC_{50}$ values ranging from 0.1 to 0.18 μg/mL, nearly equivalent to that reported for highly active CRDS (0.18 μg/mL). The cytotoxicities for the dodecyl derivatives were also low, with $CC_{50}$ values of above 1000 μg/mL. The sulfated octadecyl oligosaccharides also possessed good anti-HIV activity with $EC_{50}$ values ranging from 0.2 to 0.63 μg/mL; however, the cytotoxicity profiles were not as good as for the dodecyl series, with $CC_{50}$ values ranging from 180 to 240 μg/mL. In a subsequent study by the same research group, maltooligosaccharides ranging from pentaose through heptaose and laminaraoligosaccharides in chain lengths of pentaose and undecaose sugar residues were modified with alkyl chains of 12, 14, and 18 carbons in length.[86] The sulfated octadecyl maltoheptaoside, sulfated dodecyl laminarapentaoside, and sulfated dodecyl laminaraundecaoside had the highest anti-HIV activities with $EC_{50}$ values of 0.5, 0.2, and 0.1 μg/mL, respectively, and $CC_{50}$ values ranging from 810 to 2000 mg/mL.

In a later report by the same research group, the structure/activity relationships between oligosaccharide length, degree of sulfation, and type/length of alkyl aglycone were investigated. It had been noted in previous studies that all of the sulfated alkyl oligosaccharides evaluated had good anti-HIV activity but that the length of the alkyl chain affected cytotoxicity, with longer chains giving lower $CC_{50}$ values.[87] In this report, laminaraoligosaccharides ranging from four to six glucose residues were alkylated with a variety of groups, sulfated, and evaluated for anti-HIV activity and cytotoxicity. These studies showed that the sulfated tetrasaccharide had only one thirtieth the anti-HIV activity of the sulfated pentasaccharide, both of which had a butyl alkyl chain ($EC_{50}$ values of 43 vs 3.4 μg/mL, respectively). Maximal activities were found with degrees of sulfation greater than two sulfate groups/sugar residue. Interestingly, for the derivatives with the lowest degrees of sulfation (less than one), anti-HIV activity was not apparent, and the $CC_{50}$ values were lower, meaning the compounds were more toxic. Finally, the authors investigated the effect of different alkyl substituents on both anti-HIV activities and toxicities. They noted that more hydrophilic alkyloligoethyleneoxy chains typically gave low anti-HIV activities (18−110 μg/mL), while long, branched, or cyclic chains gave high anti-HIV activities (0.24−0.97 μg/mL). The most active compound in this series was a laminarapentaoside with a cholesterol aglycone.

Finally, an unnatural ribo-oligosaccharide with an octadecyl aglycone was synthesized by Choi and co-workers by ring-opening polymerization of a 1,4-anhydro-2,3-di-*O*-benzyl-α-D-ribopyranose with boron trifluoride etherate as a catalyst.[88] The ultimate product, after derivatization with the octadecyl group and sulfate esters was a (1→5)-α-D-ribofuranan compound with sulfate esters at C-2 and/or C-3 (**24**) (Figure 13). The sulfur content of the oligosaccharides ranged from 13 to 16.2%. It was determined that, for a derivative with a small molecular weight of 6000 Da, the $EC_{50}$ value was 0.6 μg/mL, similar to that of the reference compound CRDS (0.43 μg/mL), but for a derivative with only 21% alkyl incorporation and 7000 Da, the $EC_{50}$ value was only 13 μg/mL. This clearly illustrates the importance of the alkyl chain for anti-HIV activity, especially in light of the fact that both sulfur contents were ∼15.6%. Interestingly, a larger nonalkylated ribofuranan derivative with a higher molecular weight of 9000 Da and 14.7% sulfur also gave a value for the $EC_{50}$ on par with the small alkylated derivative of 0.6 μg/mL, indicating that the alkyl chain increases the anti-HIV activity of smaller molecular weight sulfated oligosaccharides.

### 3.5.4. Polymer-Anchored Oligosaccharides

The finding that sulfated oligosaccharides with attached alkyl chains yielded potent anti-HIV activity and low




(**25**)

**Figure 14.** Polymer-anchored sulfated maltoheptaose.

toxicities inspired studies using organic polymers as scaffolds for oligo-/polysaccharides presentation. In one report, a linear polymethacrylate polymer was created with sulfated maltoheptaose side chains (**25**) (Figure 14).[89] Yoshida and coworkers determined that having the sulfated maltoheptaose in every repeating unit of the backbone polymethacrylate resulted in low anti-HIV activity ($EC_{50}$: 15−62 $\mu$g/mL), while having a sulfated maltoheptaose side chain composition of 22 mol % resulted in high anti-HIV activity ($EC_{50}$: 0.3 $\mu$g/mL). These copolymers also exhibited low cytotoxicities, with reported $CC_{50}$ values of greater than 1000 $\mu$g/mL, and lower anticoagulant values as compared to DS. They concluded that the biological activity was influenced by the spatial distance of the side chains as well as the conformation.

## 3.6. Summary of Important Structural Features of Polysaccharide-Based Inhibitors

From the myriad of polysaccharide structures studied with good anti-HIV activity, several common structural motifs emerge that are important for activity. These structural features might be useful in the design of the next generation of more potent synthetic carbohydrate-based inhibitors. With regard to functionality, it is absolutely critical that the polysaccharide be polyanionic. The type of anionic group is also significant. *N*-/*O*-sulfates are required for activity, whereas carboxyl groups do not promote antiviral activity. Amino groups lend to the antiviral activity when the amine is either sulfated or acetylated, but they decrease activity as the free amine. The positioning of the *O*-sulfates on specific sugars appears to be important, but there is no general pattern as to where sulfates give the most anti-HIV activity. Rather, the density of sulfate groups on sugars appears to be more critical with between one and two sulfate groups per sugar giving the best activity. The size of the polysaccharide that gives antiviral activity also varies, but in general, no activity is seen, even for highly sulfated molecules, when the chain is less than a hexasaccharide in length, and the activity for these short oligosaccharides is not as good as that for polysaccharides until the chain length reaches about 15 residues. This is possibly due to the formation of higher order structures, such as helices, that may recognize complementary regions on the protein. Finally, in modifying the polysaccharides with groups other than acetate or sulfates, it has been noted that hydrophobic alkyl chains lend to the cytotoxic profile of the compounds but that hydrophilic side chains do not (*vide supra*). Additionally, in anchoring polysaccharides to polymers, the density of the sugar groups is important. If they are too densely populated on the polymer backbone, antiviral activity is compromised.

# *4. HIV Surface Protein gp120 and its Role in the Viral Entry of CD4-Negative Cells*

In addition to HIV-1 having the ability to infect CD4-positive cells, it is also known to infect cells that do not bear the CD4 surface marker, such as epithelial cells. The mechanism by which the HIV gains entry to these cells is through uptake. Virtually all modes of sexual transmission of HIV-1 involve initial contact of the virus with epithelial cells lining the genital and gastrointestinal tracts. GalCer (galactosylceramide, **26**, Figure 15) is the primary receptor for env on epithelial cells, which in combination with R5 mediates uptake in acute infections.[90] In striking contrast to CD4, env interactions with GalCer do not result in unmasking of the fusion domain. Instead, the virus is taken up and shuttled to cells that support viral amplification.[91]

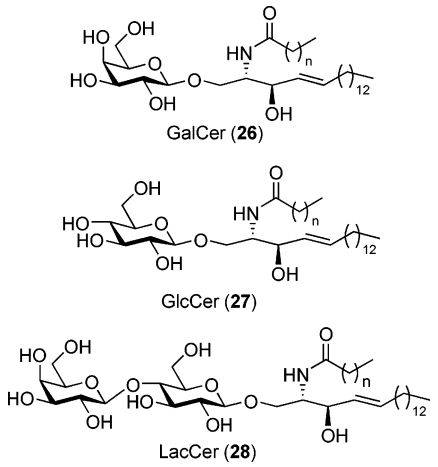



**Figure 15.** Naturally occurring cell-surface glycosphingolipids.

The uptake mechanism is not well understood, but some important interactions between env and GalCer have been elucidated. For example, both subunits of env (gp41 and gp120) interact with GalCer. Gp41 has a lectin-binding site corresponding to amino acids 650−685 that specifically bind GalCer (i.e., glucosyl ceramide (GlcCer, **27**) does not bind).[92] Thermodynamic studies of GalCer interactions with peptide fragments containing the gp41 lectin domain show that the association constant ($K_a \sim 10^8$ $M^{-1}$) is on the same order as that for antigen/antibody association. It is also notable that the gp41 lectin domain is exposed in native virus, implicating its involvement in viral adhesion and transport.

## 4.1. Biotin–Neutravidin Adhesion Assay Screening of Biotinylated GalCer Analogues

It is now well-established that GalCer is an alternate receptor for gp120, but in early studies the specificity of these interactions was not clear. For example, it had been reported that GlcCer was not a receptor for gp120 in enzyme-linked immunosorbent assay (ELISA),[93] yet it did show activity in liposome flotation assays.[94] Similarly, lactosyl ceramide (LacCer, **28,** Figure 15) was reported to be only a weak receptor in high performance thin-layer chromatography (HPTLC) studies,[95] but it showed comparable activity to GalCer in liposome flotation experiments. The ELISA had been performed on polystyrene plates, and the glycosphingolipids (GSLs) were adhered to the plate via hydrophobic interactions. At the same time, the hydrophilic portions of the GSLs differed, making it possible that some molecules (particularly the disaccharide LacCer) would be more susceptible to rinsing off the plate during the assay. In contrast, the HPTLC experiments were performed on silica gel with the GSLs interacting with the plate via the carbohydrate portion. Considering these factors, it seemed plausible that the binding data reflected differences in

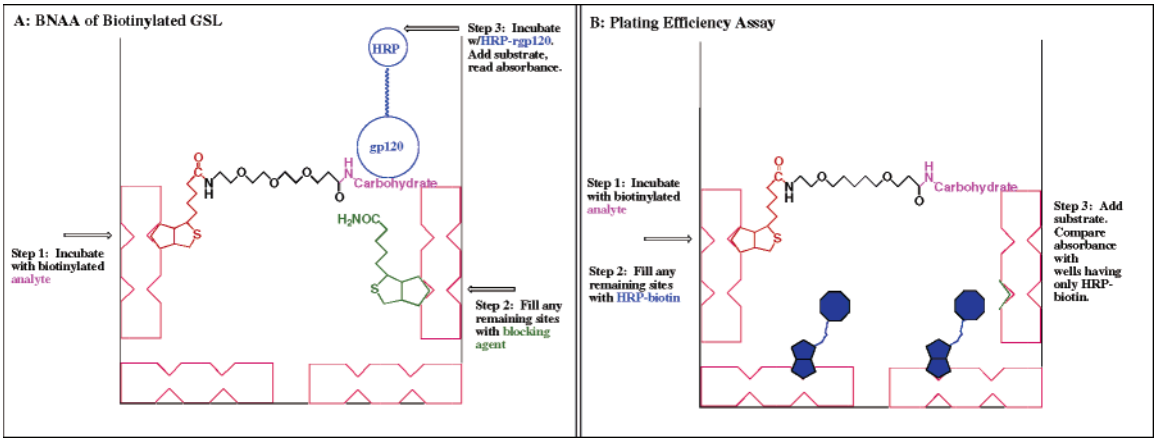



**Figure 16.** (A) Biotin−NeutrAvidin adhesion assay (BNAA) of biotinylated GSLs to HRP-rgp120. (B) Plating efficiency was determined by independently monitoring the number of vacant sites after the first incubation.

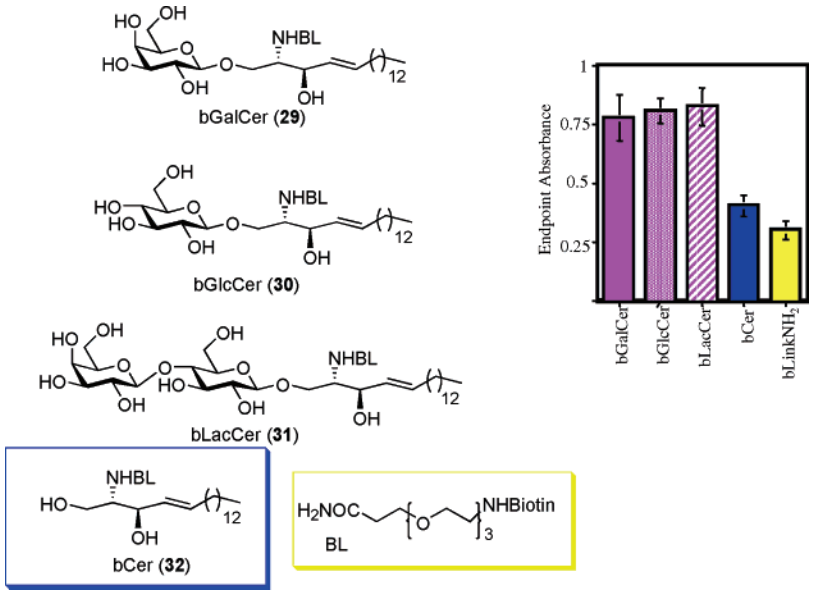



**Figure 17.** Biotinylated GSLs evaluated in BNAA. BL = biotinylated linker.

receptor presentation geometries, rather than gp120 recognition.

To ensure that all ligands were similarly displayed, a modified ELISA was developed that relied upon the strong association of biotin and NeutrAvidin to adhere receptors to the microtiter wells (Figure 16A).[96] Biotinylated analogues of GalCer (bGalCer, **29**), GlcCer (bGlcCer, **30**), and LacCer (bLacCer, **31**) were synthesized and adhered to NeutrAvidin-coated plates (Figure 17). This so-called biotin−NeutrAvidin adhesion assay (BNAA) allowed rapid evaluation of GSLs as receptors for gp120. Horseradish peroxidase (HRP)−rgp120 from baculovirus expression of HIV-IIIb was used in each assay, and appropriate control experiments were implemented to ensure that the putative receptors did not bind to HRP. Similarly, a biotinylated ceramide analogue (**32**, Figure 17) was prepared to ensure that HRP−rgp120 association was specific for the carbohydrate and not a result of hydrophobic interactions with the lipid.

Perhaps the most important feature of the BNAA was the ability to independently determine relative amounts of receptor bound to the plate (Figure 16B). For example, after the NeutrAvidin-coated wells were incubated with the biotinylated receptors, HRP-biotin could be introduced to measure the relative number of unoccupied biotin sites. Assuming that the wells had approximately equal amounts of NeutrAvidin to begin with, differences in absorbance could be related to plating efficiency. The biotinylated glycosphingolipids (bGSLs) plated with high efficiency, making it possible to directly correlate differences in BNAA results to the ability of the receptors to bind HRP−rgp120.

The utility of the BNAA was quickly realized in the screening of several natural and unnatural bGSLs.[97,] The results showed that all of the naturally occurring biotinylated GSLs, were receptors for HRP−rgp120 (Figure 17).

## 4.2. Total Internal Reflectance Fluorimetry Assay of GalCer Analogues

While the BNAA was a useful screening tool, the results could only be interpreted qualitatively, since the assay was not performed under equilibrium conditions. In order to obtain quantitative information about the recognition processes, total internal reflectance fluorimetry (TIRF) was employed.[96,97a] In the TIRF experiments (Figure 18), GSLs were reconstituted at 5 mol % in supported planar lipid bilayers composed of 95% 1-palmitoyl-2-oleoylphosphatidylcholine (POPC). Fluorescein isothiocyanate (FITC)-labeled rgp120 was introduced, and the fluorescence intensity as a function of protein concentration was monitored. The

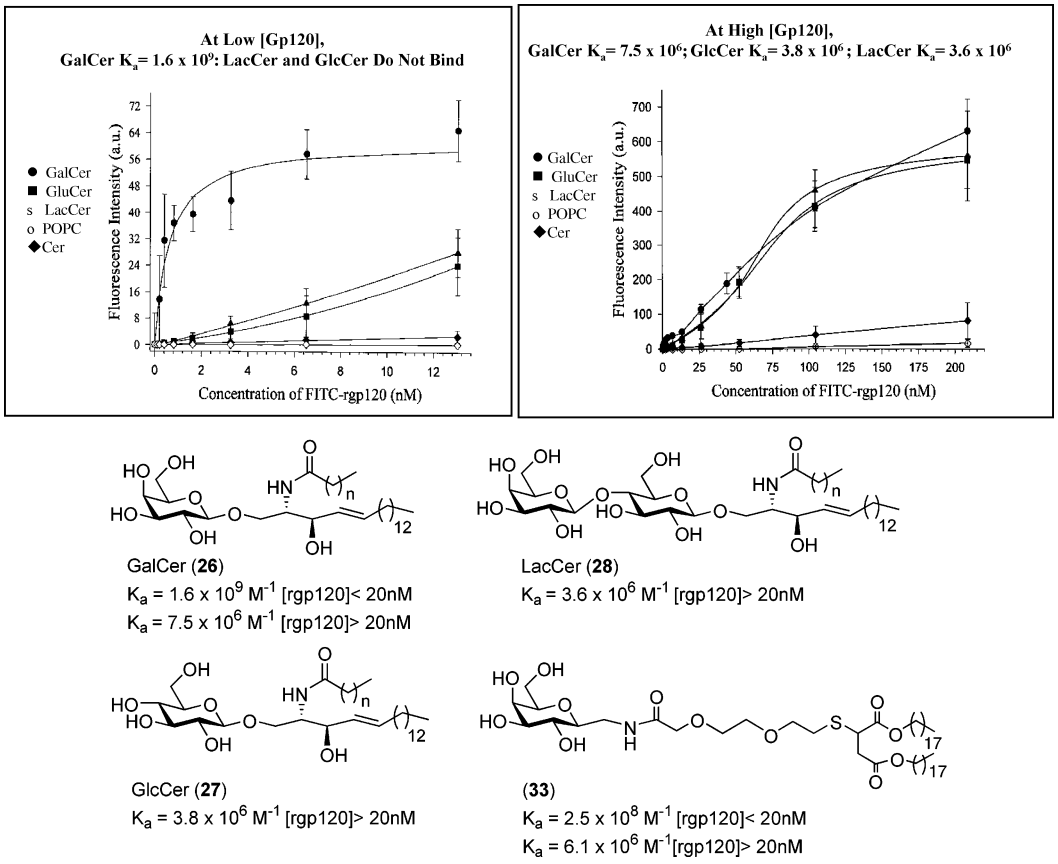



**Figure 18.** Glycolipids evaluated in TIRF experiments.

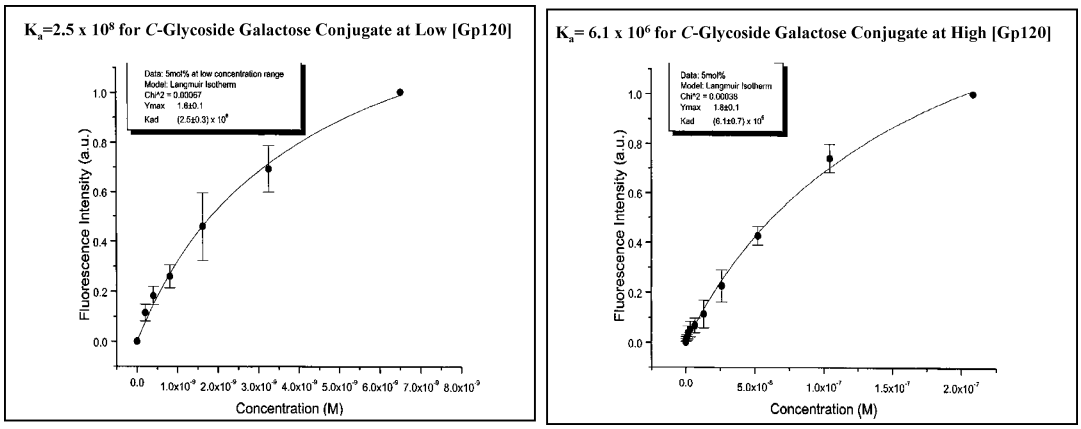



**Figure 19.** TIRF isotherm data for FITC-labeled rgp120 interactions with **33**.

TIRF isotherms for GalCer (**26**), GlcCer (**27**), and LacCer (**28**) were similar at protein concentrations ranging from 25 to 220 nM, and the association constant ($K_a$) was calculated to be $\sim 10^6$ $M^{-1}$. In contrast, at lower concentrations of protein, only GalCer absorbed protein with a $K_a \sim 10^9$ $M^{-1}$. The isotherm data were consistent with a cooperative binding model, and the GalCer recognition event observed at low rgp120 concentrations involved only a small fraction of the available receptor sites.

Further studies demonstrated that the Gp120 concentration dependency on GalCer association is independent of the lipid component. Methodologies for synthesizing *C*-glycoside amine analogues of the carbohydrates corresponding to both natural and unnatural GSLs were developed in order to independently probe interactions between rgp120 and the carbohydrate head group. TIRF (Figure 19) indicated that the *C*-amidomethylgalactoside (**33**)[98] showed similar trends to GalCer. At low concentrations of rgp120, the $K_a$ was measured to be $2.5 \times 10^8$ $M^{-1}$ whereas at higher concentrations of protein the association constant decreased ($K_a = 6.1 \times 10^6$ $M^{-1}$). More, recent studies involving the synthesis and biological evaluation of gold nanoparticles functionalized with both glucose and galactose indicate that the lipid component is not required for efficient binding of gp120 if the carbohydrates are presented as multivalent constructs (section 4.5).[99]

It is possible that the structure of the protein changes with concentration. At low rgp120 concentrations, a specific interaction with galactose is evident from the TIRF studies. At higher concentrations, protein aggregation may hinder Gal-specific recognition. It is also possible that carbohydrate/carbohydrate interactions are prevalent at higher concentrations of the protein. The studies conducted thus far have relied upon the use of commercially available monomeric

rgp120 expressed in baculovirus. This particular form of rgp120 has 22 Asn sites, and each one is glycosylated with high mannose structures.[100] A critical next step in these investigations will surely compare binding data with trimeric gp120 expressed in mammalian cells to determine if alternate carbohydrate components mediate the recognition process.

## 4.3. Polyvalent gp120-Binding GalCer Analogues in CD4-Negative Cells

In addition to evaluating the binding affinities of monovalent GalCer constructs to rgp120, there have also been recent reports of the synthesis and biological investigations of polyvalent GalCer analogues. Multivalent inhibitors of gp120 binding rely upon molecular mimicry of GalCer rafts that present the carbohydrate in clusters on the cell surface in combination with CD4 and CCR5 and that may be the site at which HIV entry occurs.[101] Additionally, polyvalent GalCer glycoconjugates can serve to further strengthen interactions between the viral proteins and the polyvalent inhibitors through what is known as the multivalent effect; where multiple copies of receptors, in this case gp120, bind multiple copies of GalCer glycoconjugates, leading to enhanced inhibition of binding to the normal cell surface receptors such as CD4 and the coreceptors.

There are currently two types of multivalent GalCer analogues that have been reported as potential anti-HIV agents. One utilized a dendrimeric scaffolding system, and the other gold nanoparticles.

## 4.4. Dendrimeric GalCer Analogues

Dendrimers are synthetic branched macromolecules of defined size. Dendrimers are synthesized in generations, where the number of reactive end groups is generally doubled in each generation. Glycodendrimers are dendrimers incorporating carbohydrates into their structures, either as the core, as the entire dendrimer, or as pendent surface groups. The first report of a glycodendrimer with anti-HIV properties was by Schengrund and co-workers in 2004, in which synthetic sulfated and nonsulfated GalCer analogues were prepared and subsequently anchored onto the surface of a commercially available dendrimer, polypropyleneimine (DAB-Am) (**34**, Figure 20), generations 1−5.[102] The evaluated GalCer analogues were a truncated nonsulfated GalCer (**35**), a thioglycoside GalCer analogue (**36**), a 3-*O*-sulfate thioglycoside GalCer analogue (**37**), and a randomly sulfated thioglycoside GalCer analogue (**38**) (Figure 21). These glycoconjugates were then appended via an amide linkage to the amino-terminated Dab-Am dendrimers, generations 1−5. The glycodendrimers were evaluated for binding to rgp120 using surface plasmon resonance (SPR). All of the GalCer analogues, when appended to Dab-Am generations 3−5 (with 16, 32, and 64 amino end groups, respectively), gave equilibrium dissociation constants ($K_D$) on the order of $10^{-9}$ M. The binding affinities for the GalCer glycodendrimers were roughly 2 orders of magnitude lower than that observed for the known standard, DS ($2.29 \times 10^{-11}$ M). SPR also indicated that the binding of both the GalCer glycodendrimers and DS was 1:1 with respect to rgp120.

In addition to evaluating the binding affinities of the GalCer glycodendrimers, Schengrund and associates also tested the ability of the glycodendrimers to inhibit HIV infection of U373-MAGI-CCR5 cells by HIV-1 Ba-L, again utilizing DS as a standard.[102] These cells express CD4 and

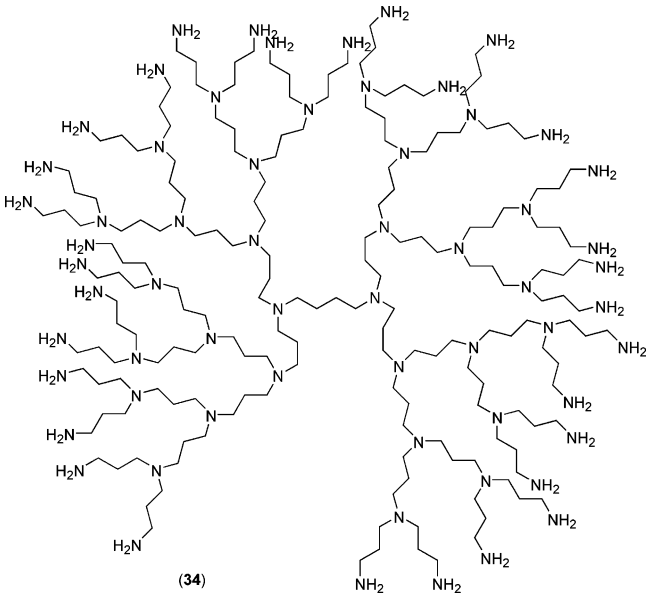



**Figure 20.** Commercially available generation 4.0 Dab-Am dendrimer (**34**).

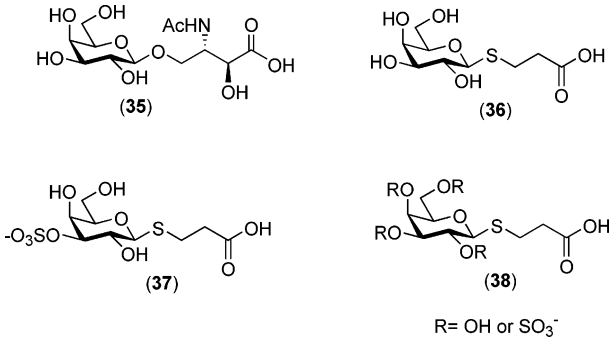



**Figure 21.** Synthetic GalCer derivatives.

included either cloned CCR5 or CXCR4 coreceptor genes.[103] It was determined that none of the nonsulfated GalCer glycodendrimers were able to inhibit HIV infection *in vitro*. However, one of the sulfated derivatives (**38**), when attached to the higher order dendrimers (generations 3−5), gave $EC_{50}$ values of approximately 90, 70, and 20 $\mu$M, respectively. Dextran sulfate was found to be a superior inhibitor with a measured $EC_{50}$ value of less than 1 $\mu$M. The authors concluded that DS was a better inhibitor than any of the GalCer glycodendrimers tested due to the number of sulfate groups per sugar residue (2.3) on the DS, compounded by the size of the DS polysaccharide (50kDa), to yield approximately 387 sulfate groups per DS molecule, while the largest sulfated GalCer glycodendrimer tested only had 34 sulfate groups per molecule.

The same group of investigators created a generation 5 Dab-Am glycodendrimer bearing a GalCer thioglycoside group with an average of two sulfate groups per sugar and evaluated the infectivity inhibition *in vitro* with three different HIV-1 strains: HIV-1 IIIB, HIV-1 NL4-3, and HIV-1 89.6.[104] This time, the $EC_{50}$ value obtained for the GalCer generation 5 Dab-Am glycodendrimer (64 terminal GalCer groups) was nearly equivalent to that of DS. The $EC_{50}$ values were reported as follows. For HIV-1 IIIB: DS, ∼10 nM; glycodendrimer, ∼100 nM. For HIV-1 NL4-3: DS, ∼4.0 nM; glycodendrimer, 2.0 nM. For HIV-1 89.6: DS, ∼2.0 nM; glycodendrimer, ∼0.6 nM. Toxicity studies using the same cell line as that used for the infectivity studies

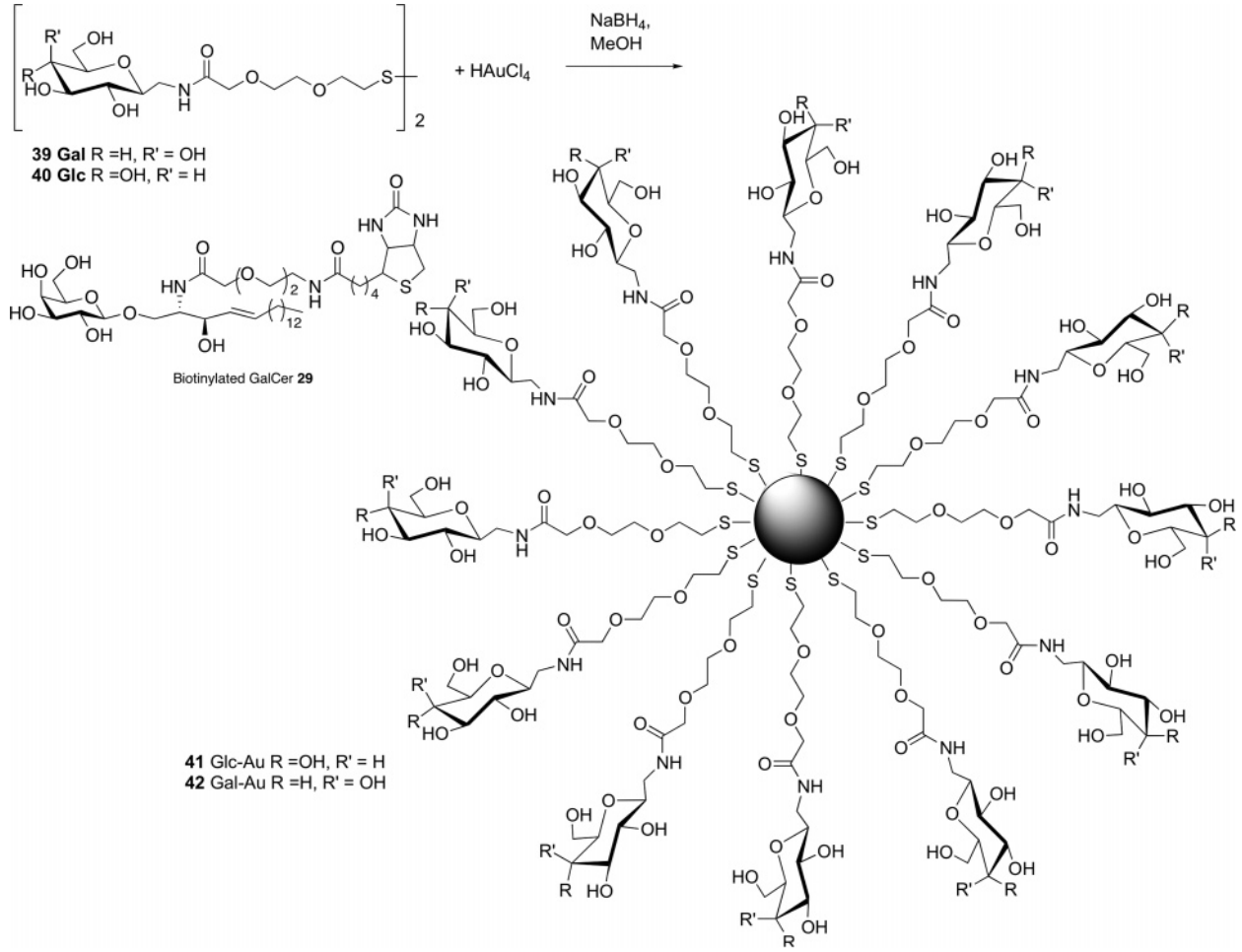



**Figure 22.** Synthesis of glyconanoparticles.

showed no significant toxicity for either the glycodendrimer or the DS, when evaluated up to 3 mg/mL. The authors therefore concluded that this new, more highly sulfated GalCer glycodendrimer could serve as a lead compound for the development of further HIV-1 antagonists. Finally, it should be noted that it is still unknown whether these GalCer glycodendrimers are capable of inhibiting HIV-1 infection in CD4-negative cells.

## 4.5. Gold Glyconanoparticles

An excellent review on the synthesis of nanoparticles and their uses in glycoscience has recently been published by de la Fuente and Penadés,[105] so the discussion in this section will be limited to the application of glyconanoparticles as HIV chemotherapeutics. As of this writing, there are no published articles describing nanoparticles with antiviral activity; however, an abstract from the 13th European Carbohydrate Symposium reported that mannose-functionalized nanoparticles inhibited binding of HIV-1 to DC-SIGN-positive cells.[106]

As part of ongoing efforts to develop HIV-entry inhibitors, the Gervay-Hague group reported investigations of multivalent interactions of Au glyconanoparticles containing galactosyl and glucosyl headgroups with recombinant gp120 (Figure 22). These particles were prepared from disulfides containing *C*-glycosides linked to triethylene glycol via an amide bond using a modification of the Brust method.[107] Results from high-resolution transmission electron microscopy (HR-TEM), atomic force microscopy (AFM), UV/Vis absorption spectroscopy, HR-TEM, and elemental analysis data indicated that the nanoparticles averaged 2 nm in diameter and contained approximately 120 carbohydrate head groups per particle. The BNAA was used to evaluate the ability of the Au glyconanopraticles to displace rgp120 from plate-bound GalCer (Figure 23). The results showed divalent disulfides were <10% as active as biotinylated GalCer; however, when these same carbohydrates were presented in a polyvalent display, they were greater than 350× times more active than the disulfides and at least 20× more active than biotinylated GalCer. These results collectively demonstrate the potential utility of polyvalent display of ligand arrays on nanoplatforms.

In follow-up experiments, these same researchers patterned thiolated GalCer ligands on mica and studied surface interactions with rgp120.[108] Self-assembled monolayers of GalCer in combination with octanethiol or decanethiol were prepared, and the ligands were spacially and geometrically arranged using AFM-based nanografting. Recombinant gp120 was introduced onto these platforms, and binding interactions were monitored *in situ* using AFM. These studies demonstrated that protein adhesion occurred only where GalCer was patterned on the surface and that interactions were enhanced in regions where dimers or trimers of rgp120 could overlap with the glycosurface.

These combined studies are consistent with glycodendrimer studies, which show enhanced activity with multivalent constructs. Demonstration of antiviral activity is a critical next step in these investigations.

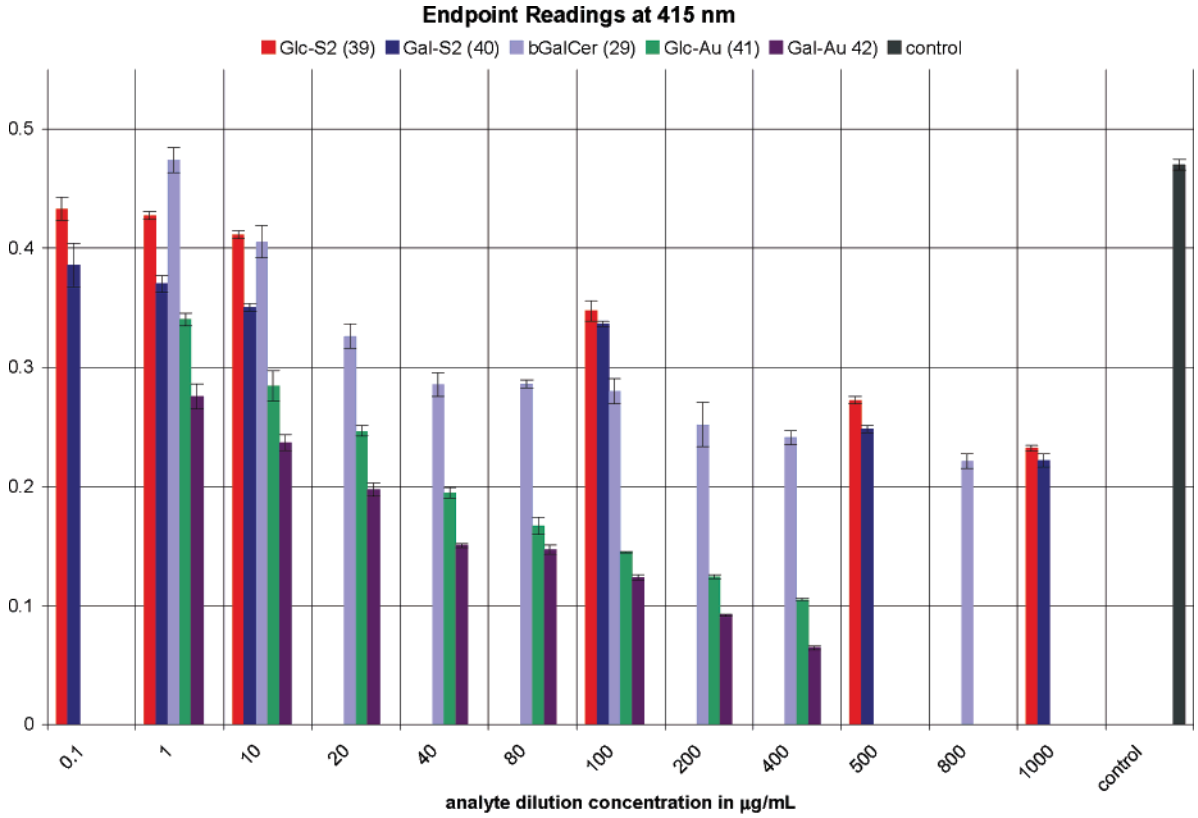



**Figure 23.** BNAA competition assay data for disulfides **39** and **40**, Au nanoparticles **41** and **42**, and biotinylated GalCer **29** shown as end-point readings at 415 nm after 20 min. As a control, the data for bGalCer correspond to maximal absorbance (no exposure to ligands in the competition step).

## 5. Perspectives

HIV-viral adhesion, transport, and entry into host cells are complex processes mediated by proteins, glycans, and glycosphingolipids. Understanding the molecular details of these interactions has been the focus of numerous studies over the past two decades. It is clear that viral infection of T-cells and macrophages involves specific interactions between HIV-env and cell-surface receptors such as CD4, CCR5, and CXCR4. Electrostatic interactions between gp120 and HSPGs also play a critical role in initial contacts. Recent findings suggest that these interactions specifically involve the 6-*O*-sulfate groups of HSPGs and Arg 298 found in the V3 loop of gp120. A similar electrostatic interaction occurs between the V3 loop of gp120 and a sulfated tyrosine residue found in CCR5. HSPG recognition processes appear to facilitate HIV entry by allowing the virus to attach to cell surfaces with low levels of CCR5 expression. Several anionic carbohydrates that inhibit these electrostatic contacts have been identified from numerous sources; however, many of these compounds cross-react with cytokines, chemokines, and growth factors. Understanding the specificity of polyanionic carbohydrate/receptor interactions is a critical next step in the drug discovery process.

Early studies probing gp120 interactions with neutral host-associated glycolipids indicated that GalCer is an alternate receptor, particularly in CD4-negative cells. Most of these studies used recombinant gp120 expressed in baculovirus to probe inhibition. Native viral gp120 is expressed as trimeric spikes on the virus surface, whereas rgp120 is typically monomeric. These important differences may explain why antivirals based upon GalCer interactions have proven difficult to develop. Lipid raft structure may also play a key role in viral adhesion, and understanding these interactions at a molecular level will likely advance the design of polyvalent inhibitors of HIV replication and or transport. In some circles, GalCer has become a less attractive target, replaced by enthusiasm for C-type lectin interactions that involve HIV-associated high mannose structures. The latter topic, which has recently been reviewed,[109] represents a promising new direction that will complement ongoing studies with HSPGs and glycosphingolipids. Further elucidation of the roles of carbohydrates in HIV infection and transport is likely to lead to new advances in chemotherapeutic interventions.

## 6. Acknowledgments

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## 7. Note Added after ASAP Publication

After this paper was published ASAP (on April 17, 2007), we became aware of a review that includes important references covering anti-HIV GalCer analogues and dendrimeric GalCer derivatives that would be of interest to readers of this review: Rico-Lattes, I.; Blanzat, M.; Perez, E.; Soussan, E.; Stefaniu, C. *Biophys. Rev. Lett.* **2006**, *1* (4), 423 and references therein. The version with this reference added was published ASAP on April 26, 2007.

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