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Glycosphingolipids in HIV/AIDS: The Potential Therapeutic Application

Clifford A. Lingwood^{2,3,6} and Donald R. Branch^{1,2,4,5}

*Departments of¹Medicine, ²Laboratory Medicine and Pathobiology
and ³Biochemistry, University of Toronto,*

⁴Division of Cell and Molecular Biology, Toronto General Research Institute,

⁵Research and Development, Canadian Blood Services, Toronto, Ontario,

*⁶Research Institute, Hospital for Sick Children, Toronto, Ontario
Canada*

1. Introduction

It has been 30 years since the original description of acquired immunodeficiency syndrome (AIDS) was first reported (Gottlieb et al., 1981). Since this initial discovery, human immunodeficiency virus (HIV) and HIV pathogenesis ranks near the top as one of the most studied human diseases in the history of medical science. Unfortunately, we are no closer now than we were back in the early 1980s at finding a cure. Although there has been significant progress in treatment, there continues to be an increase in the numbers of infected people and those dying from AIDS throughout the world.

Current dogma says that HIV type 1 (HIV-1) is the most common HIV virus and that it infects immune cells called helper T-cells. HIV can also infect other cells involved in the immune response such as monocytes, macrophages and dendritic cells. The virus has an envelope that mediates its tropism for immune cells. The viral envelope protein gp120 first recognizes and binds to the CD4 molecule located on the cell-surface of CD4⁺ helper T-cells (Dalglish et al., 1984). Although researchers insisted for over a decade that CD4 was the only cellular receptor required for HIV to infect cells, some in the research community were sceptical and aware from clinical and laboratory findings that something else must be required for HIV infection. Indeed, in 1996 it was discovered that CD4 does not act alone. Another family of cell-surface receptors, the chemokine receptors, were shown to be required, in conjunction with CD4, for successful infection with HIV-1 (Feng et al., 1996, Alkhatib et al., 1996). These receptors are important for the tropism of the virus. Thus, the CXCR4 chemokine receptor, directs infection of T cells by T cell-tropic HIV-1, whereas the CCR5 and CCR3 chemokine receptors are responsible for infection of monocytes with monocyte-tropic HIV-1 virions. However, as more studies were undertaken, it became clear that many other chemokine receptors could support HIV-1 infection and that the tropism was directed more to the chemokine co-receptor than to the cell type. Thus, HIV viruses that require CXCR4 are now known as X4 HIV-1 while virus that recognizes CCR5, or other members of this family of chemokine receptor, are now known as R5 HIV-1. (Dragic et al., 1996, Littman, 1998). Thus, the current paradigm for

HIV infection is that X4 or R5 HIV-1 first binds via its envelope gp120 to CD4 on T-cells expressing either CXCR4 or CCR5. The binding to CD4 results in a conformational change in the structure of a part of the virus envelope gp120 known as the variable V3 loop. This change in conformation of the gp120 exposes a binding site for either chemokine co-receptor. Following binding of the virus to the chemokine co-receptor, another conformational change occurs in the gp120 that exposes another viral membrane protein called gp41. It is the gp41 that then is able to cause the fusion of the virus envelope to the host cell membrane so that the virus can release its contents into the host target cell and begin the infectious process.

Perhaps not that surprising given the history of HIV/AIDS, the HIV-1 paradigm for productive infection continues to change as more studies are undertaken. A role for a family of cell-surface-expressed neuropeptide receptors has been proposed to be important for productive HIV infection (Branch et al., 2002) and cell-surface-expressed glycosphingolipids (GSLs) have been proposed to act as HIV-1 fusion receptors (Fantini et al., 1997; Nehete et al., 2002). Thus, despite 30 years of intense research, we continue to find new and surprising aspects of HIV pathogenesis that have eluded us over the years. One of these more recent findings is the possible therapeutic potential of GSLs in HIV/AIDS.

2. Glycosphingolipids

2.1 Biochemistry, biosynthesis and degradation

GSLs are carbohydrate-lipid conjugates almost exclusively restricted to the outer leaflet of the plasma membrane bilayer of mammalian cells. The hydrophobic backbone, ceramide, consists of a fatty acid chain linked to a sphingosine base, and is common to all GSLs. The alkyl chains of the lipid moiety (ceramide) are embedded in the bilayer and vary in chain length, saturation and hydroxylation (Huwiler et al., 2000; Hakomori, 1993) (Figure 1).

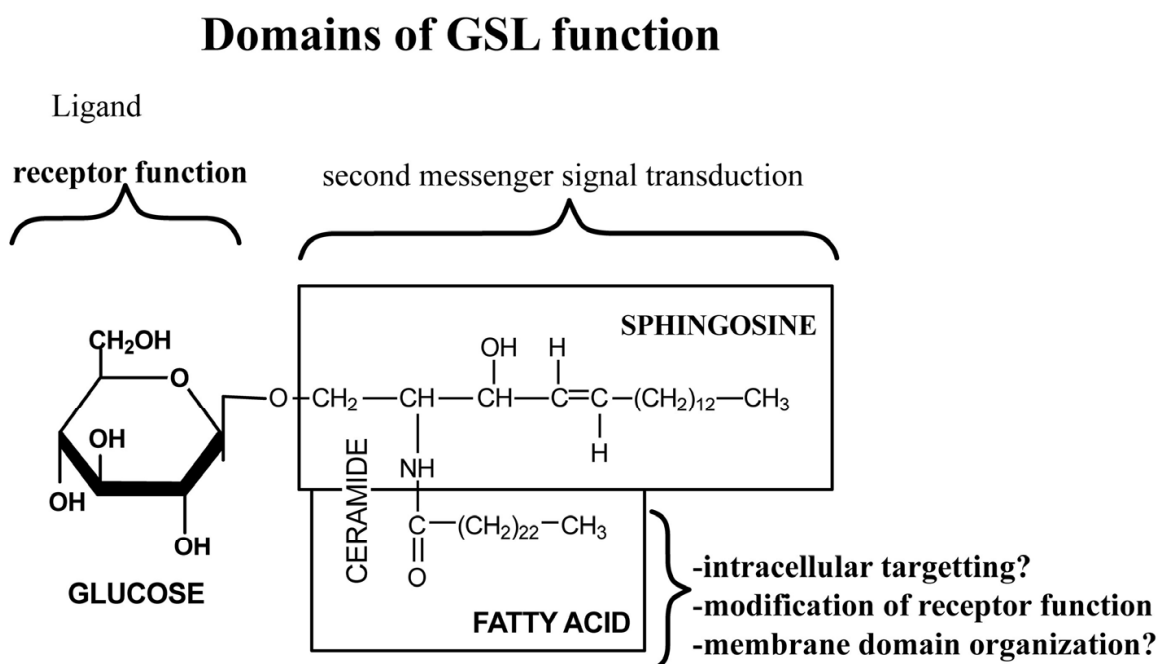


Fig. 1. Different functional domains of GSLs.

The hydrophilic core sugar sequence defines the carbohydrate moieties, and these protrude into the extracellular space (Stults et al., 1989). The different moieties comprising GSLs have different roles in these multifunctional membrane lipids. GSLs are classified as neutral, acidic (anionic) and basic (cationic) glycolipids (Hakomori, 1986). Acidic GSLs usually contain either a sialic acid, which largely encompass the gangliosides, or a sulphate group. Basic glycolipids are very rare, but include plasmalopsychosine or glyceroplasmalopsychosine (Hikita et al., 2002). For the most part, GSLs are comprised of four groups, characterised by their basic core structure: globo- (defined by Gal α 1-4 Gal), lacto- (Gal β 1-3GlcNAc), neolacto- (Gal β 1-4GlcNAc) and ganglio- (Gal β 1-3GalNAc) series.

The ceramide backbone of GSLs is synthesized on the cytosolic leaflet of the rough ER, through condensation of L-serine and fatty-acyl co-enzymeA and subsequent enzymatic modification (Huwiler et al., 1998). Ceramide may be converted in the lumen of the ER into gala-series glycolipids by addition of galactose, via β -glycosidic linkage, producing the first in the series, galactosylceramide (GalCer) (Sprong et al., 1998). The addition of a sulphate group to the 3-position of the sugar residue on GalCer will give rise to sulphatide (SGC). Ceramide may alternatively be transported to the Golgi apparatus where the first sugar added is glucose, via β -glycosidic link, producing glucosylceramide (GlcCer). The precursor for most GSL structures is GlcCer, which is synthesised by glucosyltransferase located in the cytosol (Futerman & Pagano, 1991). GlcCer can be then translocated by the flippase function of the drug resistance pump, P-glycoprotein (P-gp), to the Golgi lumen (De Rosa et al., 2004, Lala et al., 2000). Here subsequent synthesis of all other GSLs takes place through highly specific glycosyltransferases (Lannert et al., 1998). The first product that is formed from GlcCer is lactosyl ceramide (Gal β 1-4Glc cer, LC), which can then be sialylated, to give monosialoganglioside (sialic acid α 2-3 Gal β 1-4Glc cer, GM3). Alternatively, LC is galactosylated to form globotriaosyl ceramide (Gal α 1-4 Gal β 1-4Glc cer, Gb₃), which can be further converted to globotetraosyl ceramide (GalNAc β 1-3 Gal α 1-4 Gal β 1-4Glc cer, Gb₄) (Figure 2). The major GSLs contain ~5 sugars or less although GSLs containing over 60 sugar residues have been described (Miller-Podraza et al., 1993), and more than 400 GSL species have been reported (Hakomori, 2008). Newly synthesised GSLs follow anterograde vesicular traffic through the Golgi compartments and are directed to the plasma membrane, where they are integrated into the outer leaflet. GSLs follow a process of recycling between intracellular compartments and the plasma membrane, before final endocytosis and transportation through endosomal compartments to the lysosomes (Huwiler et al., 1998). Here, highly specific glycosylhydrolases remove the terminal sugar sequentially from the GSLs, to release the ceramide backbone, which is subsequently catabolised or recycled. It is important to note here, that deficiencies in specific glycosylhydrolases manifest specifically as lysosomal storage diseases, where there is an accumulation of GSL in the lysosome (Kolter & Sandhoff, 1998). These include: Tay-Sachs disease, which accumulates GM2; Gaucher's disease, which accumulates glucocerebroside; and Fabry's disease, which accumulates Gb₃ (Kanfer & Hakomori, 1983).

2.2 Cellular functions

The biological functions of GSLs are many and varied, and may particularly relate to the distribution pattern within the membrane. One such functional role is attributed to the maintenance of membrane structural rigidity, and the ordering of the membrane structure in lipid rafts (discussed below).

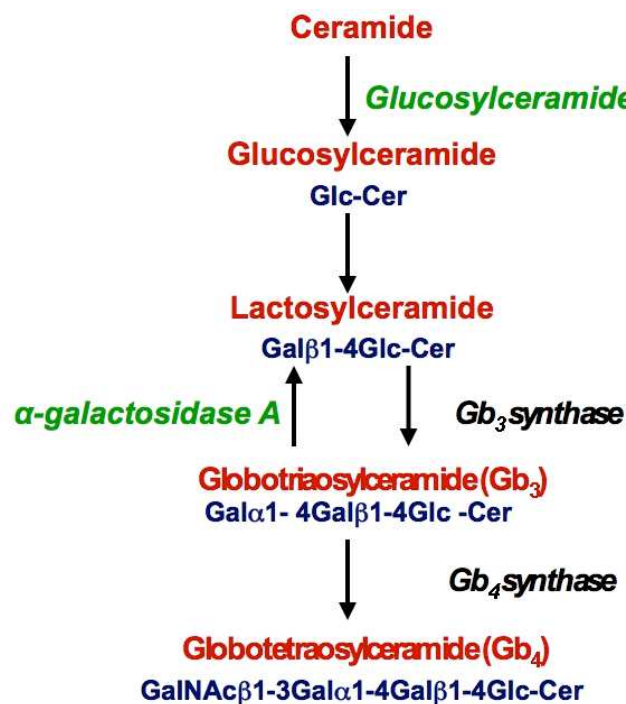


Fig. 2. Biosynthesis of Globotriaosylceramide (Gb₃).

Several GSLs act as cellular antigens or cell-type specific markers, although the functional significance of this is not well understood. Perhaps the most well-known cell-type specific antigens are the GSLs that comprise the histo-blood group antigens, which include members of the Lewis (Le), ABH, I/i and P/P₁/P^k blood groups. Differential expression of GSLs is also particularly depicted during development. The GSLs Le^x (stage-specific embryonic antigen 1, SSEA-1), which is also a Lewis blood-group antigen, Gb₅ (SSEA-3) and monosialyl-Gb₅ (SSEA-4) are variably expressed at specific stages of embryonic development (Kannagi et al., 1983a, Kannagi et al., 1982, Kannagi et al., 1983b, Solter & Knowles, 1978). This differential expression profile has been shown to be important for cell adhesion and cell-cell contact, and such (carbohydrate-carbohydrate) interactions may be essential in developmental processes (Eggens et al., 1989). Indeed, SSEA-4 is a human multipotent stem cell marker.

Interestingly, cell-specific expression of GSLs is also common during differentiation and this is well demonstrated in the haematopoietic cell system. Myeloid cells are characterised by Le^x, Neutrophils specifically express the GSL marker LC (CDW17) and the major T-cell (and monocyte) GSL is ganglioside GM3 (Schwartz-Albeiz et al., 1991, Sorice et al., 2004). Furthermore Gb₃, which has been defined as CD77, is a marker of germinal centre B cells in humans, and thus a marker of differentiation (Mangeney et al., 1991, Wiels et al., 1991).

Given the role of GSLs in development and differentiation, it is not surprising that GSL expression may be aberrant in tumour development, and several have been identified as tumour-associated antigens (Hakomori, 1985). Developmentally regulated GSLs may be re-expressed, or the GSL profile modified, to specifically aid in tumour progression through adhesion functions or tumour growth modulation (Hakomori, 1996, Hakomori, 2002).

It is interesting to note that certain GSLs have been documented to act as cell adhesion molecules, and even as functional receptors, on the cell surface. The GSL sialyl 6-sulfo Le^x acts as an adhesion ligand for selectins on leukocytes and activated endothelial cells, facilitating the process termed "rolling", a critical step in migration of cells from the blood

stream during an immune response (Sperandio, 2006) Furthermore, cell-cell interactions can take place between GSLs, and these interactions have been documented between GM3-Gg3, GM3-LacCer, and SGC-GalCer (Kojima & Hakomori, 1991, Koshy et al., 1999) and more recently, between Gb3-GalCer and Gb3-GlcCer (Mahfoud et al., 2010) and between Gg4-GM1, Gg4-Gg3 and Gg4-LacCer (Emam et al., 2010). GSLs acting as functional receptors include sulphated galactolipids (SGC, SGG), which are receptors for hsp70's (Boulanger et al., 1995, Mamelak et al., 2001b); GM1, a co-receptor for FGF2 (Rusnati et al., 2002); and nerve cell gangliosides GD1a and GT1b that bind myelin-associated glycoprotein (MAG), and inhibit nerve regeneration (Vyas et al., 2002).

GSLs, particularly gangliosides, may also have an impact on cell growth and motility (Hakomori & Igarashi, 1993). In terms of cell growth, several GSLs have been shown to interact with growth factor receptors, such as FGFR and EGFR, and modulate growth (Bremer, 1994, Weis & Davis, 1990). GM3 interaction with the insulin receptor is important in type 2 diabetes (Tagami et al., 2002, van Eijk et al., 2009). While the mechanisms of inhibitory or stimulatory effects are not well understood, in many cases receptor-associated tyrosine kinases are inhibited. Cell motility is controlled by integrin function, and is specifically affected by gangliosides. The GSL, GM3 is able to inhibit motility by interaction within a complex of N-glycosylated alpha3 integrin and tetraspanin CD9 (Ono et al., 2001).

2.3 Lipid rafts

Biological membrane lipids are not homogeneously distributed but can be organized into heterogeneous microdomains or lipid rafts of increased membrane order. Lipid rafts within the plasma membrane of eukaryotic cells present different physical assemblies of proteins and lipids. Specifically, rafts are comprised of increased concentrations of GSLs, certain phospholipids, and cholesterol, as well as scaffold and/or functional membrane proteins (Hooper, 1999, Simons & Ehehalt, 2002, Simons & Ikonen, 1997). Several membrane proteins preferentially associate with lipid rafts, and these include glycosylphosphatidylinositol (GPI)-anchored cell surface proteins within the outer leaflet, and cytosolic palmitoylated and myristoylated proteins, and cholesterol- or phospholipid-binding proteins (Rajendran & Simons, 2005). The proteins and lipids cooperate to form dynamic membrane assemblies to facilitate transmembrane information flow (Lingwood & Simons, 2010) One morphologically identifiable raft structure is caveolae, which are flask-shaped invaginations of the membrane associated with caveolin scaffolding protein (Kurzchalia & Parton, 1999).

Lipid rafts are small, highly dynamic and detergent-insoluble, and while these assemblies are fluid, they represent a more ordered region within the membrane. This "liquid-ordered" domain is more tightly packed than the surrounding bilayer, and this is largely due to the saturated hydrocarbon chains of raft-associated GSL and phospholipids (Simons & Vaz, 2004). Thus, the degree of saturation and hydroxylation of GSLs may greatly affect the "liquid-ordered" state of the membrane, as well as the degree of clustering or association with membrane proteins (Brown & London, 1997, Hakomori et al., 1998b). Because raft formation is dependant on lipid structure, lipids of the appropriate structure are capable of forming microdomains in model membranes (Dietrich et al., 2001, Radhakrishnan et al., 2000). However such model membrane systems do not fully reflect plasma membrane microdomains (Kaiser et al., 2009).

Two fundamental properties of lipid rafts associated with their physical attributes, are their capacity to selectively incorporate proteins, and their ability to coalesce to form larger

domains. It is not surprising therefore, that lipid rafts play a role in protein sorting, membrane trafficking and signal transduction (Brown & London, 2000, Lajoie & Nabi, 2010). Because of the thermodynamic formation of lipid rafts, the cell utilizes them as centres or “hot spots” for transmembrane signal transduction for a variety of membrane receptors (Hakomori & Igarashi, 1995, Simons & Toomre, 2000). Ligand-induced receptor dimerization and successive cytosolic phosphorylation cascades occur in microdomains, and as such membrane receptors often partition into such domains upon ligation, and may subsequently be internalised and traffic through said domains (Dykstra et al., 2001). This can result in direct ligand interaction with GSLs (Hakomori et al., 1998a, Iwabuchi et al., 2000) without necessarily, the involvement of a transmembrane protein (Katagiri et al., 1999, Mori et al., 2000). This implies a mechanism of communication between the cell surface and the cytosolic lipid bilayer leaflets. Cytosolic signal transduction proteins, such as *src*-family tyrosine kinases and small G-proteins, are often associated with the cytosolic surface of such domains in a transient and surface ligand-regulated manner (Dykstra et al., 2001, Hakomori, 2000, Katagiri et al., 1999).

Lipid rafts are also involved in internalisation and intracellular trafficking of proteins and lipids (Lajoie & Nabi, 2010, Mukherjee & Maxfield, 2000) and likely, their attendant signalling. An endocytic role has been established for caveolae and lipid rafts, which may translocate and endocytose GPI-anchored proteins in particular (Parton & Richards, 2003). Other raft-mediated routes of internalisation have been identified where the GPI-anchor acts as a targeting signal in the traffic to an endosomal organelle called the GPI-anchored protein enriched early endosomal compartment (Sabharanjak et al., 2002). It is clear therefore that several distinct raft-mediated trafficking pathways exist. It is important to note however, that lipid rafts are not distributed randomly in the endosomal pathway, but are excluded from the degradative compartments, although this is not well understood (Nichols et al., 2001, Simons & Gruenberg, 2000).

2.3.1 Defining a new assay for lipid raft formation

Lipid rafts are isolated from cells from the Triton insoluble fraction separated on a discontinuous sucrose ultracentrifuge gradient. Due to their atypical density, the rafts separate as a band above the 30% sucrose layer. The majority of proteins sediment to the bottom, while components found in this fraction are deemed lipid raft associated. This has not been studied for purified (glyco)lipids. We have developed this procedure as a new method for examining the ‘raft’ forming capability of glycolipids. Soluble adamantylGb₃ (adaGb₃), natural Gb₃ or Gb₃+cholesterol were mixed with Triton and placed at the bottom of the sucrose gradient, below the 30% layer, the lower half of which now contains FITC-labeled VT1 B subunit. The gradients are centrifuged at 66K rpm for 3 days. Any raft structures formed will float up through the FITC-VT1 B layer and the raft band should thus be fluorescently labeled. When this was performed with Gb₃ alone, no fluorescent band was formed. In contrast, a distinct fluorescent band was formed for Gb₃+cholesterol. However adaGb₃ formed the strongest labeled ‘raft’ band (Mahfoud et al., 2002b). While the characteristics of the structures formed by adaGb₃ in this band remain to be fully characterized, this supports the “raft-like” character of adaGb₃. Moreover, this is an excellent method for determining the properties and components required for optimal raft formation (Nutikka & Lingwood, 2004). We have shown that cholesterol is one requirement. A fifty fold molar excess of the SPC3 peptide from the glycolipid binding V3 loop of gp120 of HIV, which strongly binds adaGb₃ (Mahfoud et al., 2002b) is able to eliminate FITC-VT1 B

labeling of the adaGb₃ 'raft' band. The raft band is still formed –seen under visible light- in the presence of SPC3. Thus both the SPC3 peptide (and presumably, gp120 and the intact HIV virus) and VT1 B selectively bind the same Gb₃ containing raft structures. This would correlate with the raft requirement for HIV infectivity and VT cytotoxicity (Falguieres et al., 2001).

2.4 Pathogens and GSL receptors

GSLs have been shown to play a role in many pathogen interactions with host cells. As previously described, several GSLs represent histo-blood group molecules, and there is a longstanding association between pathogens and these particular blood groups, which are not necessarily limited to expression on erythrocytes. Such interactions have been defined both in protective qualities conferred by a specific blood type, and in pathogen interactions with blood group antigens (Moulds & Moulds, 2000, Rios & Bianco, 2000)

Several GSLs, including those categorised as blood group antigens, have been identified as adherence receptors for bacteria, or bacterial toxins (Lingwood, 1998). The globo-series of GSLs expressed on urogenital epithelia, particularly monosialyl-Gb₅, are infection sites for *Escherichia coli* (Stapleton et al., 1992). The Le^b antigen is required for surface adherence of *Helicobacter pylori*, known to cause gastritis and peptic ulcers, to gastric mucosa, and Group O Le (b+) secretors are thus likely most susceptible to this pathogen (Borén et al., 1993). The minimal structure of sialyl-lactosylceramide (GM3) is crucial for colonization and adherence to epithelium via fimbria-dependant binding of *Haemophilus influenzae*, which causes a variety of diseases from meningitis to upper respiratory infection (van Alphen et al., 1991). Ganglioseries GSLs such as asialoGM1(Gg4), are binding targets for the pili of certain *Pseudomonas aeruginosa* strains, which are opportunistic pathogens that target and colonize epithelial cells of the lung (Comolli et al., 1999). These GSLs are not receptors for these organisms (Emam et al., 2006) but can assist host cell invasion (Emam et al., 2010). Finally, bacterial toxins, which are soluble proteins, often bind to GSLs to elicit their effects. Glycolipid receptors include ganglioside GM1, bound by cholera toxin (De Haan & Hirst, 2004) from *Vibrio cholerae*, and Gb₃, which is utilized by *Escherichia coli* elicited verotoxins (VT) (Petruzzello et al., 2009), susceptibility to cholera toxin (and *E. Coli* LT) is blood group O related.

The involvement of GSLs in the host cell attachment of viruses, and also fusion in terms of enveloped viruses, has long been recognised (Haywood, 1994). The initial step of viral attachment to the susceptible cell is crucial in the process of establishing an infection. The sialic acid motif, which is widely presented on acidic GSLs, is perhaps the most broadly recognised adhesion component utilised by viruses, from small non-enveloped DNA polyomaviruses to larger enveloped RNA influenza viruses (Gilbert & Benjamin, 2004, Miller-Podraza et al., 2000, Tsai et al., 2003). The GSL neolactotetraosylceramide (nLc₄Cer) is a key receptor for the enveloped Dengue virus, an infectious agent transmitted by mosquitoes (Aoki et al., 2006). The ganglioside GD1a has been identified as a critical component for viral binding of Sendai virus, and fusion of this enveloped virus with its target is abolished if GD1a is not present for initial contact (Epand et al., 1995).

2.5 Pathogens and lipid rafts

GSLs and lipid rafts themselves are important for many microbial pathogens and often form preferential sites for pathogen interactions (Lafont et al., 2002, Samuel et al., 2001, van der Goot & Harder, 2001, Vieira et al., 2010). Pathogenic interactions may be vast and varied. For example, lipid rafts serve as key platforms for entry of parasitic agents, such as *Plasmodium*

falciparum, which causes malaria. Following attachment of the *P. falciparum* merozoite to erythrocytes, the membrane invaginates taking up the parasite within a parasitophorous vacuolar membrane (PVM) (Haldar et al., 2002). Lipid rafts are critical for the formation of the PVM, as are the raft-associated proteins internalized with the vacuole (Lauer et al., 2000). Indeed, even 'non-classic' infectious agents require lipid rafts, as demonstrated by the requirement for the prion proteins which partition into rafts during the conversion of PrP^c to infectious PrP^{sc} (Simons & Eehalt, 2002).

Bacteria often favour lipid rafts during host-cell interactions (Heung et al., 2006). Raft association may provide a platform for colonisation, through signalling, cytoskeleton rearrangements and membrane ruffling (Manes & Martinez, 2004). Intracellular bacteria rely on lipid rafts to enter host-cells, which provides protection from degradation and immune detection. This is demonstrated by *Mycobacterium* spp., which exploits rafts to generate phagosomes in which to survive within the cell, allowing the bacterium to evade antigen processing (Gatfield & Pieters, 2000). Toxins produced by non-intracellular bacteria, are particularly dependant on rafts for host-cell interaction, which mediates oligomerization, internalization and intracellular trafficking (Fivaz et al., 1999). Cholera toxin binding ganglioside GM1 is the current "gold standard" for identification of such rafts (Lencer, 2001). Lipid rafts are integral in the retrograde transport of cholera toxin to the Golgi (Lencer & Saslowsky, 2005). VT is also dependant on the organization of its receptor, Gb₃, into rafts for intracellular routing (Falguieres et al., 2001) and cytopathology (Khan et al., 2009).

Lipid rafts are also fundamental in viral infection, predominantly in the process of viral entry, both for enveloped and non-enveloped viruses (Manes et al., 2003). In the case of non-enveloped viruses, lipid rafts are important in the process of viral attachment and subsequent internalization and trafficking to the appropriate sub-cellular niche. Caveolae lipid rafts are required for non-enveloped simian virus 40 (SV40) interactions with MHC-I and viral entry, a process that can be inhibited with cholesterol chelators (Norkin, 1999). Lipid rafts facilitate the traffic of SV40 to the ER through the Golgi (Parton, 1994). Interestingly, rafts appear to be involved in a sorting process in viral trafficking, as other viruses, such as the echovirus, enter by caveolae but do not traffic to the ER (Marjomaki et al., 2002). Enveloped viruses are particularly dependant on lipid raft domains for the process of viral/cell membrane fusion. Cholesterol and sphingolipids, which define these domains, have been identified as critical in the process of fusion for alphaviruses, such as the Semliki-forest virus (Ahn et al., 2002). Interestingly, disrupting raft formation by replacing cholesterol with androstenol did not hinder envelope glycoprotein insertion, but replacing sphingolipids with dipalmitoylphosphatidylcholine was inhibitory, emphasizing the importance of GSLs in the process of fusion (Waarts et al., 2002). Finally, lipid rafts are critical in the process of enveloped virus assembly and budding for many viruses, including influenza, measles, filoviruses and HIV (Bavari et al., 2002, Luo et al., 2008, Manie et al., 2000, Scheiffele et al., 1999). Membrane rafts are an efficient system of concentrating viral proteins in a specific region, may provide a specific lipid composition for the virus, and also exclude/include host-proteins from the viral envelope (Manes et al., 2003).

3. Globotriaosylceramide

3.1 Characteristics and expression

The neutral glycolipid, globotriaosylceramide (Gb₃), is defined by the trisaccharide core unit (Gal α 1-4Gal β 1-4Glc) linked to a ceramide backbone, and as such belongs to the globo-series

of GSLs. Gb₃ shares the amphipathic characteristics of all GSLs, and their fatty acid chain length, saturation and hydroxylation, may vary yielding various Gb₃ isoforms. Gb₃ may also partition into lipid-rafts and interact with raft-associated proteins. Gb₃ is widely expressed in a variety of tissues, but is a major GSL of human renal cortex (Boyd & Lingwood, 1989), heart, spleen and placenta; (Kojima et al., 2000). Moreover, it has been described in a number of epithelial and endothelial cell lines. Gb₃, or CD77, is expressed as a differentiation antigen on a subset of tonsillar B lymphocytes in the germinal center, where expression is very specific, and only occurs at a restricted stage (Mangeney et al., 1991, Wiels et al., 1991). It is interesting to note that human Burkitt lymphoma cells, which are characteristically derived from B cells, also express Gb₃ (Wiels et al., 1981; Kim et al., 2011). Finally, human erythrocytes are characterized by two major globo-series GSLs, Gb₃ or P^k, being one, the other being Gb₄, or P. Gb₃ is also upregulated in many human tumours (Devenica et al., 2010)

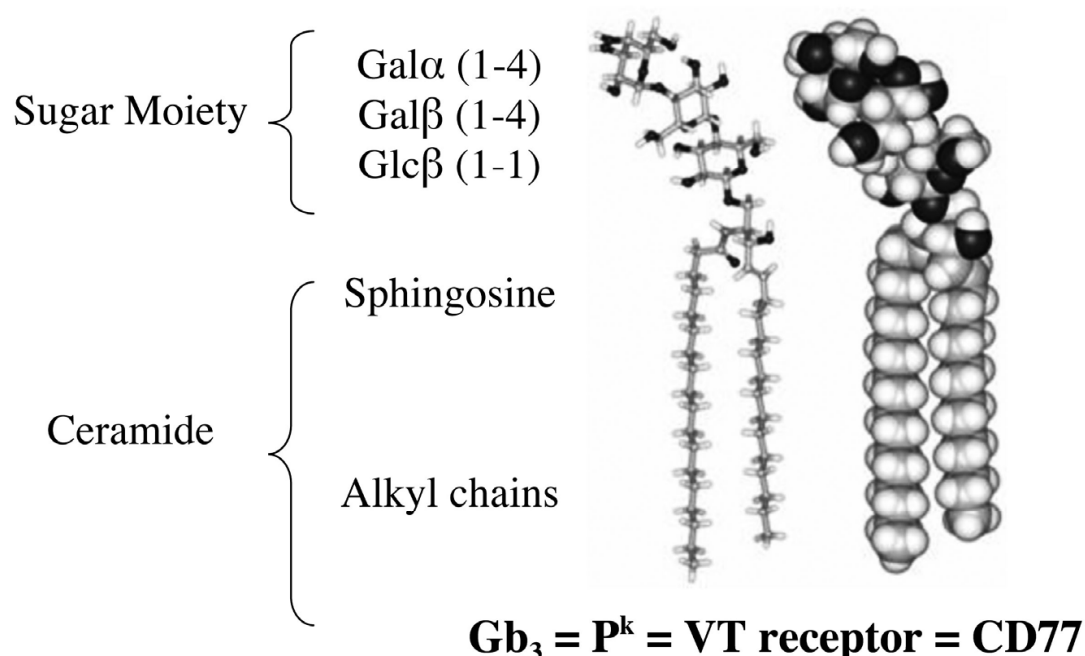


Fig. 3. Chemical structure of globotriaosylceramide.

3.2 Function

Gb₃ expressed on B-lymphocytes, has specifically been implicated in signal transduction resulting from CD19 engagement (Maloney & Lingwood, 1994). Indeed, the extracellular domain of CD19 presents a Gb₃ binding site, (with sequence similarity to the Gb₃-binding VT1B subunit of *Escherichia coli*). Gb₃ is crucial for CD19 induced homotypic adhesion of B cells and this suggests a potential role for Gb₃ in adhesion during B cell development (Maloney & Lingwood, 1994). Gb₃ has further been shown to mediate CD19 directed apoptosis of B cells, which may be important during B cell selection (Khine et al., 1998). This occurs following CD19 ligation, where Gb₃ mediates targeting and intracellular traffic of CD19 to the ER and nuclear envelope (Khine et al., 1998).

Gb₃ expression has also been shown to affect the binding capacity of IFN- α for its receptor, $\alpha 2$ interferon receptor IFNAR1, on B lymphoid cells (Ghislain et al., 1992). The amino

terminus of IFNAR1 is able to bind to Gb₃, sharing sequence similarity with the VT1B subunit (Lingwood & Yiu, 1992) and binding to Gb₃ likely affects the subsequent signaling. Gb₃ has thus been shown to be critical for IFNAR1-dependant α 2IFN induced growth inhibition, mediated by short chain fatty acid Gb₃ isoforms (Khine & Lingwood, 2000). Furthermore, Gb₃ is important in α 2IFN IFNAR1 signaling to induce antiviral activity, which is mediated by long fatty acid isoforms of Gb₃ (Khine & Lingwood, 2000). Gb₃ has been implicated in angiogenesis and is found in tumour neovasculature (Heath-Engel & Lingwood, 2003), and can promote tumour metastases (Kovbasnjuk et al., 2005)

3.3 Blood group antigens

Gb₃ belongs to the P1PK and GLOB blood group system, that have red cell phenotypes termed P/P₁/P^k (Table 1) and whose expression profile is not limited to erythrocytes. The structure galabiose (Gal α 1-4Gal) is the terminal structure of P^k, also known as Gb₃, and P₁ blood group antigens, whilst it is the precursor for P antigen, also known as globoside or Gb₄, which terminates with β 1-3GalNAc (Spitalnik & Spitalnik, 1995). P₁ and P₂ are the two common P/P₁/P^k-related blood group phenotypes. P₁ individuals (~80% of Caucasians but only ~20% of Asians) (Daniels, 2002) express P and P₁ but only expose low amounts of P^k antigens on their cell surfaces. P₂ individuals (~20% of Caucasians and ~80% of Asians) (Daniels, 2002) express only P and low amounts of P^k antigens. There are also rare phenotypes defined by a deficiency in one or more of the P/P₁/P^k blood group antigens. Individuals deficient in P antigen have mutations in the *B3GALNT1* gene causing lack of functional Gb₄ synthase (β 3GalNAc transferase) (Hellberg et al., 2002, Hellberg et al., 2004), and consequently express high amounts of unmodified precursor, P^k. These individuals may express P₁ antigen (P₁^k phenotype) or not (P₂^k). Although uncertain for many years (Hellberg et al., 2005; Iwamura et al., 2003), the molecular basis for P₁/P₂ has recently been elucidated (Thuresson et al., 2011). Individuals who do not express any P/P₁/P^k antigens have mutations in the *A4GALT* gene, causing lack of functional Gb₃ synthase (α 4Gal transferase), and have the rare p blood group phenotype (Furukawa et al., 2000, Hellberg et al., 2002, Hellberg et al., 2003, Steffensen et al., 2000). Similar to the ABO blood group system, naturally occurring antibodies are formed against the P/P₁/P^k antigens when missing (Spitalnik & Spitalnik, 1995). Recent studies show anti-P^k is present in all normal sera (Pochechueva et al., 2010)

Phenotype	Frequency	Red Blood Cell Antigens	Serum Antibodies
P ₁	75%	P ₁ , P, P ^k	None
P ₂	25%	P, P ^k	Anti-P ₁
P ₁ ^k	Rare	P ₁ , P ^k	Anti-P
P ₂ ^k	Rare	P ^k	Anti-P ₁ , anti-P
P	Rare	None	Anti-P ₁ , anti-P, anti-P ^k

Table 1. Red Blood Cell Phenotypes in the P1Pk and GLOB Blood Group System (Spitalnik & Spitalnik, 1995; Branch, 2010)

4. Relationship to disease

The P1PK and GLOB blood group system antigens are of particular interest, with many defined pathogen interactions. Both P^k (or Gb₃) and P (or Gb₄) are receptors for P pili of uropathogenic *E. coli* (Leffler & Svanborg-Eden, 1981). P^k has been shown to act as a receptor for the porcine bacteria *Streptococcus suis* (Haataja et al., 1994). Indeed, P^k is also known to act as a receptor for bacterial toxins, Shigella or Verotoxins specifically, produced by *Shigella dysenteriae* and Enterohemorrhagic *E. coli* (Bitzan et al., 1994, Pellizzari et al., 1992) but no association with P blood group status has been found (Jelacic et al., 2002). Viruses have also been shown to have interactions with blood group antigens. In terms of the P blood group system, Parvovirus B19 utilizes the P antigen as its receptor to infect cells, and individuals with the p phenotype lacking P are resistant to the virus (Brown et al., 1994). More recently, the P1PK and GLOB blood group system antigens, specifically, P^k, has been implicated as having a role in HIV infection (Branch, 2010)(see below).

4.1 Verotoxin-induced disease

Enterohemorrhagic *Escherichia coli* induce disease, characteristically haemolytic uremic syndrome (HUS), by the production of verotoxins (VT). VTs are capable of binding to Gb₃, thus Gb₃ contributes to the pathology of VT-induced disease (Lingwood, 2000, Lingwood et al., 1987) VT is comprised of a single toxic 'A' subunit and non-covalently associated pentameric 'B' subunits responsible for receptor (Gb₃) binding. Only cells with Gb₃ surface expression are sensitive to VT toxicity (Okuda et al., 2006, Waddell et al., 1990). VT interaction with the sugar moiety of Gb₃ is dependant on the lipid moiety in its membrane environment (Arab & Lingwood, 1996, Kiarash et al., 1994, Pellizzari et al., 1992), which is crucial in internalization and subcellular targeting of VT (Arab & Lingwood, 1998 , Smith et al., 2006). The intracellular routing of VT thus is also dependant on the organization of Gb₃ into lipid rafts (Falguieres et al., 2001). Indeed, VT binding to cell surface Gb₃ within lipid microdomains has been shown to activate cytosolic raft-associated src kinase ((Katagiri et al., 1999, Mori et al., 2000)) indicating Gb₃ can mediate transmembrane signals. Furthermore, Gb₃ containing cells where Gb₃ is not present in rafts are insensitive to VT cytotoxicity (Falguieres et al., 2001, Ramegowda & Tesh, 1996). In cells sensitive to VT cytotoxicity, the toxin is internalized via both clathrin independent/dependant pathways(Lauvrak et al., 2004) and undergoes retrograde transport via the reverse of the secretory system to the Golgi and ER/nucleus (Arab & Lingwood, 1998, Khine & Lingwood, 1994, Sandvig et al., 1994) Highly VT sensitive cells contain higher levels of short fatty acid containing Gb₃ isoforms and retrograde transport the VT/Gb₃ complex to the ER/nucleus. Less VT sensitive cells have longer fatty acid containing Gb₃ isoforms and retrograde transport VT to the Golgi only (Arab & Lingwood, 1998). Interestingly, Gb₃ is maintained in lipid rafts during retrograde transport (Smith et al., 2006).

4.2 Fabry disease

Fabry disease is an X-linked lysosomal storage disorder, as a result of a genetic defect in the lysosomal enzyme α -galactosidase A, which results in reduced enzyme activity (Brady, 1967). This enzyme is normally responsible for the removal of the terminal Gb₃ galactose residue, through hydrolysis of the α 1-4 glycosidic linkage. Thus, Gb₃, and potentially other α -galactose terminal lipids accumulate in the lysosomes to abrogate their normal function and the function of these organelles. Clinical manifestations of the disorder are related to the

cell-type-specific expression of Gb₃ (Huwiler et al., 2000). Thus patients with Fabry disease typically experience renal dysfunction, myocardial and skin lesions, and joint pain, which relate to the major tissue distribution of Gb₃ (Hakomori, 1986).

5. HIV and GSLs

5.1 GSL receptors

Initial contact of HIV with the host cell surface must occur before the virus can initiate infection. HIV envelope glycoprotein gp120 targets CD4 and CCR5 or CXCR4 chemokine co-receptors on monocytes and T-cells respectively, as the major HIV-host cell interaction (Alkhatib et al., 1996, Dalglish et al., 1984, Feng et al., 1996). GSLs have been implicated in HIV infection since the original description of the binding of GalCer and sulfatide (3' sulfogalactosyl ceramide, SGC) by the HIV adhesin gp120 (Bhat et al., 1993, Bhat et al., 1991) and indeed gp120 binding to these species is considered the primary mechanism by which non-CD4 expressing cells are 'infected' by HIV (Dorosko & Connor, 2010, Harouse et al., 1995, Magerus-Chatinet et al., 2007, Ullrich et al., 1998). GSLs bound by gp120 include GalCer, SGC, Gb₃ and the ganglioside GM3 (Delezay et al., 1996, Hammache et al., 1998a). It has been suggested that GM3 is bound only by gp120 from R5 strains whereas Gb₃ is bound by both X4 and R5 strains (Hammache et al., 1999). GSL analogues have been shown to inhibit HIV infection (Fantini et al., 1997, Faroux-Corlay et al., 2001, Garg et al., 2008, Lund et al., 2006, Weber et al., 2000) and the efficacy of such analogues depends on the nature of both the carbohydrate and lipid moieties. In addition, GalCer binds to gp120 associated gp41 (Alfsen & Bomsel, 2002), the fusion heptad repeat C-terminal peptide of which, mediates viral/host membrane fusion (Shnaper et al., 2004). Nevertheless, the exact role of the GSLs in HIV infection remains unclear. Early suggestions were that GSL binding within lipid rafts facilitated a simultaneous recognition of CD4 and chemokine receptor by gp120 (Fantini, 2003). However, the fact that the GSL-binding site (Delezay et al., 1996), defined as 2 alpha helices with a central aromatic amino acid sequence (Mahfoud et al., 2002a), responsible for gp120-GSL binding, is contained within the same V3 loop as amino acids crucial for chemokine receptor binding (Xiao et al., 1998), suggest that the binding of GSLs within the V3 loop would more likely provide an inhibitory, rather than stimulatory effect on chemokine receptor binding. To address this potential dichotomy the unusual membrane properties of GSLs must be considered.

5.1.1 GSL conformation and lipid heterogeneity

A single glycosphingolipid (i.e. a single carbohydrate species with a heterogeneous ceramide moiety) can differentially recognize two or more ligands, specific for the carbohydrate sequence. This can be based on differential recognition of the hydroxyl groups within the sugar sequence as has been shown for Verotoxin variants and monoclonal anti-Gb₃ (Chark et al., 2004). Differential binding of anti-GM1 and cholera toxin to GM1 lipid isoforms has also been reported (Iglesias-Bartolome et al., 2009). This is consistent with differential ligand recognition of GSL lipid isoforms by ligands which bind the same carbohydrate sequence. The oligosaccharide moiety of glycolipids shows considerable flexibility in conformation and nine potential energy minima have been defined by molecular modeling (Nyholm & Pascher, 1993). This potential for differential carbohydrate conformation which can be regulated by the relative plane of the plasma membrane may therefore reflect the lipid composition and its membrane microenvironment. Indeed,

cholesterol can induce a fatty acid-dependent GSL conformational change (Lingwood et al., 2011). The identification of a family of fatty acyl co-A selective ceramide synthases (Stiban et al., 2010, Teufel et al., 2009) provides the metabolic means to regulate the differential synthesis of such GSL fatty acid isoforms.

As indicated, cell membrane GSLs can be organized into cholesterol enriched microdomains. Such microdomains are typically correlated with resistance to detergent extraction *in vitro* (Lingwood & Simons, 2007). While this procedure can induce domain pooling and the relationship between detergent resistance and natural cell membrane GSL domains has yet to be established (Westerlund & Slotte, 2009), detergent resistance indicates stronger lateral membrane interactions. Detergent resistant, cholesterol enriched plasma membrane domains have been shown to be important for HIV infection by most studies (Del Real et al., 2002, Gummuluru et al., 2003, Liao et al., 2001, Manes et al., 2000, Nguyen & Hildreth, 2000, Popik et al., 2002, Raulin, 2002) but not all (Percherancier et al., 2003).

5.1.2 GSLs and HIV infection

The role of GSLs in HIV infection must be considered both in terms of GSL species generally distributed in the membrane or restricted to lipid microdomains. Several studies have shown that the binding of the gp120 HIV adhesin to GSL is dependent not only on the carbohydrate, but also the lipid moiety of the GSL (Mahfoud et al., 2009, Mahfoud et al., 2002b, Villard et al., 2002).

The interaction of GSLs with cholesterol is modulated by the fatty acid chain length and the binding of HIV gp120 to Gb₃/cholesterol vesicles has been shown to be a function of the fatty acid composition in that C16 fatty acid Gb₃ was bound but C17, C18 and C20 Gb₃ were not. C22 and C24 fatty acid containing Gb₃s were bound (Mahfoud et al., 2009). The Gb₃ fatty acid isoforms not recognized by gp120 in this context, have fatty acid chain lengths which are of the order of the dimensions of the cholesterol molecule, suggesting that these fatty acid isoforms have the minimum 'hydrophobic mismatch' (Niemela et al., 2009) and therefore interact more effectively with cholesterol. The interaction of GSLs with cholesterol has been shown in modeling studies to induce a conformation change in the headgroup to become parallel to the plane of the cholesterol containing membrane rather than perpendicular, as seen in the absence of cholesterol (Hall et al., 2010, Lingwood et al., 2011, Yahi et al., 2010). In such a membrane parallel carbohydrate format, the accessibility of the carbohydrate to carbohydrate binding ligands, such as gp120, will be restricted (but lateral interaction with the membrane may be enhanced). In GSL/cholesterol model detergent resistant membranes separated by sucrose gradient centrifugation, the major GSL fraction was not recognized by gp120 (GSLs -sulfatide, galactosyl ceramide and Gb₃) (Mahfoud et al., 2010). Only a minor fraction of smaller vesicles were bound. Such smaller vesicles may display the GSL in a more disperse format, even in the presence of cholesterol, and thereby defray the effect of this potential cholesterol-induced conformational change. Moreover, the fatty acid isoforms of Gb₃ negative for gp120 binding were dominant negative in mixtures of saturated Gb₃ fatty acid isoforms, whereas addition of the unsaturated C24:1 Gb₃ was dominant positive, suggesting that membrane fluidity in these vesicles could be a key factor in determining availability of the GSL carbohydrate for gp120 binding (Mahfoud et al., 2009). Thus the interaction of gp120 with membrane GSLs is extremely complex depending also, on the membrane bilayer organization and perhaps curvature and fluidity. In addition, host cell GSLs taken up into the viral membrane at the time of plasma membrane budding may also play a direct role in HIV dendritic cell targeting (Hatch et al., 2009) and T cell infection.

This differential availability of cell membrane Gb₃ for example, is dramatically highlighted by the differential binding of various monoclonal antibodies to Gb₃ and verotoxin B subunit to lymphoid cells which synthesize Gb₃ (Kim et al., 2011), despite the fact that these antibodies show similar efficacy to detect the Gb₃ once extracted from the cells and separated by TLC. Gb₃ positive cells which do not bind any Gb₃ ligands have been reported (Sekino et al., 2004).

The masking of membrane GSL is also dependent on the relative cholesterol concentration. In model GSL membranes, the thickness of the carbohydrate layer was an inverse function of the cholesterol concentration, suggesting that the sugar can adopt intermediate conformations between the membrane perpendicular (thickest) and parallel (thinnest) conformation according to membrane cholesterol content (Lingwood et al., 2011), extending the potential for conformational regulation of GSL receptor function.

The differential expression of membrane GSLs within and without cholesterol enriched lipid rafts may provide the explanation for the differential function ascribed to GSLs in HIV infection; first as promoters of fusion/infection (Puri et al., 1998, Puri et al., 2004) and then as inhibitors of these functions (Lund et al., 2009, Ramkumar et al., 2009). It is conceivable that different GSL carbohydrate conformers can play different roles at different (or the same) times in infection.

Amino acids within the gp120 V3 loop defined by mutational analysis as crucial for chemokine receptor binding (Xiao et al., 1998) coincide with 3 amino acids of the consensus GSL binding site at the V3 loop apex (Delezay et al., 1996), together with 2 distinct amino acids, one in the base of each alpha helix comprising the V3 loop (Xiao et al., 1998). The GSL hexapeptide binding domain has been synthesized as a separate peptide and shown to bind the same GSLs *in vitro* as observed for the intact gp120 (Delezay et al., 1996). The V3 loop must open following CD4-gp120 binding to allow the chemokine receptor to bind (Wang et al., 1999) whereas gp120-GSL binding is observed in the absence of CD4 (Mahfoud et al., 2009). Thus GSL binding to the apex of the V3 loop could well alter the alpha helix conformation at the base of the V3 loop to modulate chemokine receptor binding. It is possible that binding of different GSLs, or different lipid isoforms of the same GSL, could differentially alter the conformation of the V3 loop to enhance or inhibit CCR5 binding.

This concept is consistent with NMR studies which indicate that the N terminus of CCR5 binds within the base of the V3 loop (Huang et al., 2007). In combination, soluble CD4 and CCR5 reduced proteolytic susceptibility of the V3 loop of gp120, consistent with binding. A model was proposed (Huang et al., 2007) by which the CCR5 N terminus bound to the base of the V3 loop (via tyrosine sulfate) and then the second extracellular loop of CCR5 associated with the V3 loop apex. Alternatively, the extracellular CCR5 loop associated with the V3 loop apex first, followed by CCR5 N terminus binding to the V3 loop base. The binding of the CCR5 N terminus to the V3 loop base was found to cause a conformational change to rigidify the V3 loop. Such a conformational change might be impeded or promoted by GSL binding within the GSL binding site at the V3 loop apex (Figure 4).

If the first GSL sugar is primarily responsible for binding, it is possible that the effect on V3 conformation could be dependent on the number and character of additional sugar residues. In the absence of the gp120 conformational change induced by CD4 binding, V3 loop binding to GSLs via this apical binding site could mediate a less effective mechanism for HIV internalization. Cell membrane GSLs undergo a natural process of internalization and recycling and GSLs function in receptor mediated endocytosis of appropriate GSL binding ligands. This could thereby provide a basis for the observed association of galactosyl

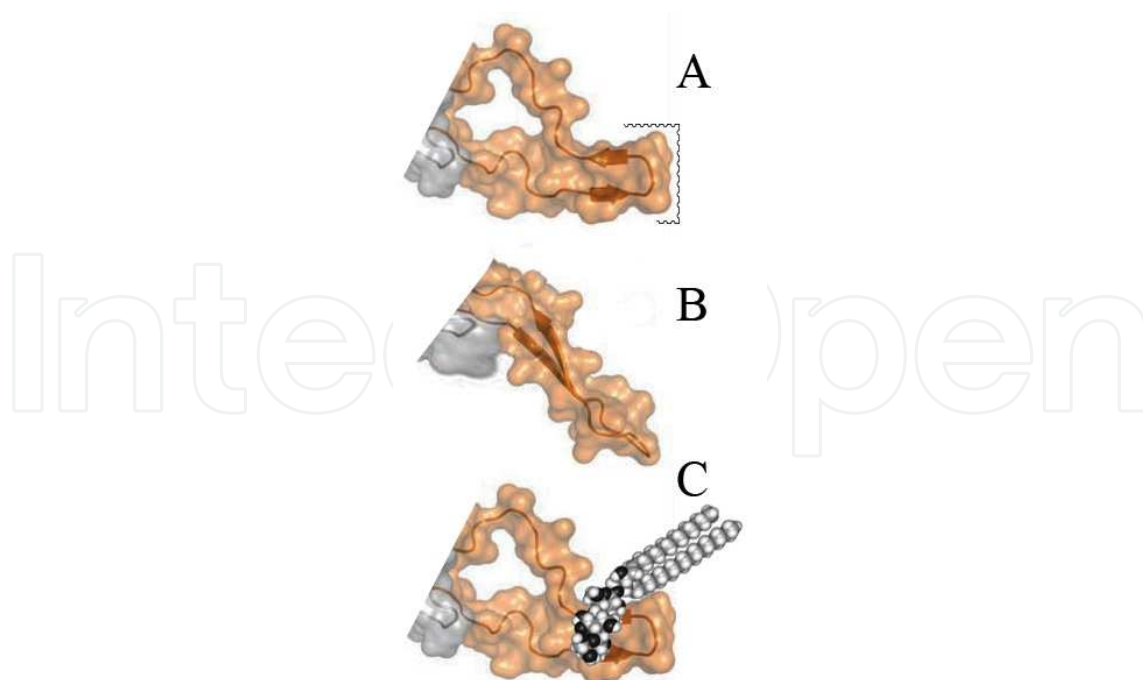


Fig. 4. Conformational change in gp120 induced by CCR5 binding and relation to GSL binding. The NMR structure of the V3 loop alone (A) or with bound CCR5 N terminus (B) is shown according to Huang et al., 2007. The amino acids of the GSL binding site at the loop apex are boxed in (A). The V3 loop initially disorganized, becomes more rigid on binding of the CCR5 N terminus to the V3 loop base. Gb₃ has been arbitrarily placed and oriented with its glucose moiety stacked over the phenylalanine of the CCR5 unbound loop (C) to illustrate the potential of GSL binding to affect this V3 loop conformational change.

ceramide in gastroepithelial/neuronal/renal cells targeted by HIV (Harouse et al., 1995). Infectious HIV utilizes GSLs and lipid rafts to traverse the host mucosa and access underlying susceptible target cells during transmission. This process is called transcytosis, whereby “receptors” mediate the transcellular traffic of the virus across the tight epithelial cell barrier, rather than productive infection (Bomsel and Alfsen 2003). Thus uptake of the virus occurs at one pole of the cells and infectious virus is released at the opposite pole, gaining access to the submucosa. GalCer has been shown to bind to both HIV-gp120 and gp41 (Alfsen and Bomsel 2001). However, HIV binding to GalCer in epithelial cells does not result in HIV/host cell fusion necessary for productive infection but rather mediates HIV transcytosis (Bomsel, 1997), Bomsel and David 2002). This has been demonstrated in epithelial cell lines, where HIV ‘hijacks’ the vesicular pathway in order to cross the cell. The process of transcytosis via GalCer has also been shown to occur in primary intestinal epithelial cells, specifically for R5 HIV-1 strains, and is particularly dependant on lipid rafts, as disruption of rafts substantially reduces uptake (Meng Wei 2002). It has further been suggested that transcytosis may occur across specialized M cells, which provide an epithelial barrier, to lymphoid Peyer’s patches in the gastrointestinal tract (Fotopoulos 2002). Mucosal dendritic cells express GalCer which can mediate HIV uptake and transfer to T cells (Magerus-Chatinet et al., 2007). To date, the vast majority of evidence suggests transcytosis is a process that facilitates HIV transmission across the gastrointestinal mucosa, and limited data has been shown for vaginal mucosal transmission

5.2 Membrane fusion

For productive infection, HIV enters cells directly via plasma membrane penetration, which requires fusion of the viral envelope with the host cell membrane (Marsh & Helenius, 1989). Membrane fusion is particularly dependant on lipid rafts, which have a central role in HIV infection. Depletion of cholesterol, a key component of lipid rafts, renders cells resistant to infection and membrane fusion, a phenotype rescued upon re-introduction of cholesterol (Manes, del Real 2000, Liao, Cimasky et al 2001). GSLs, important components of lipid rafts, have also been shown to play a role in membrane fusion. Complete lack of GSLs protects CD4 positive cells from HIV infection (Hug et al., 2000, Puri et al., 2004, Rawat et al., 2003). Interestingly, reconstitution of GSL deficient cells with Gb₃, and to a lesser extent GM3, was able to restore membrane fusion in these model systems (Hug et al., 2000, Puri et al., 1998, Puri et al., 1999). However, no other GSLs were able to rescue this phenotype. This impediment could also be overcome by the over-expression of CD4 and CXCR4, suggesting the role for GSLs is facilitative (Hammache et al., 1999, Puri et al., 1998, Puri et al., 1999, Rawat et al., 2003). These findings have been supported by reports that both Gb₃ and GM3, when introduced into the cell membrane of CD4⁺ T lymphocytes, have the potential to enhance HIV-1 fusion and entry of a broad range of isolates (Hug et al., 2000). It has also been shown that non-human cells expressing CD4, ordinarily not permissive to HIV-1 infection, become permissive to membrane fusion upon introduction of Gb₃ (Puri et al., 1998). Our studies using a different glucosyl ceramide synthase inhibitor are consistent with an inhibitory role for GSLs(Gb₃)(Ramkumar et al., 2009) and HIV resistance is also conferred by high GM3 levels (Rawat et al., 2004).

For membrane fusion to proceed, HIV-gp120 binding to CD4 and chemokine co-receptor, must initiate conformational change in gp120 and the associated transmembrane gp41 (Freed et al., 1992, Jones et al., 1998). At physiological levels, CD4 and the co-receptors are not physically associated in the membrane in the absence of HIV-1 (Jones, Korte et al 1998). However, CD4 and CCR5 are both present in lipid rafts, albeit separate rafts, and their associations with rafts have been shown to be required for infection. Indeed, both CD4 and CCR5 may interact with lipid rafts containing GM3 and Gb₃ (Hammache et al., 1999, Hammache et al., 1998b, Manes et al., 2001, Millan et al., 1999, Sorice et al., 1997). Interestingly, CXCR4 is not normally associated with rafts, and is separated from CD4, which is ordinarily associated with GM3 rafts. Upon HIV-gp120 interactions with CD4 however, CXCR4 is physically recruited into these rafts for membrane fusion (Sorice et al., 2000, Sorice et al., 2001).

CD4 has been shown to insert into Gb₃ or GM3 monolayers, as has HIV-gp120 (Hammache et al., 1999). Since both CD4 and chemokine receptors are found in, or are recruited to, lipid rafts for HIV infection, it was proposed that CD4 binds GSLs in rafts to promote gp120/GSL interactions (Fantini et al., 2000). GSL within rafts may then function to promote the migration of the CD4-gp120 complex to an appropriate, initially distal, coreceptor (Hammache et al., 1999). This would in turn promote clustering and thus co-operative interactions between the CD4-GSL-chemokine coreceptors (Rawat et al., 2006). HIV-gp120 binding interactions within the GSL-containing domain could then induce the conformational changes necessary to effect membrane fusion. Indeed, the fusion complex has specifically been shown to assemble in lipid rafts (Manes, et al., 2000).

5.3 Infectivity and viral egress

It is interesting to note that there appears to be an overall role for GSL containing lipid rafts in HIV infection (Manes et al., 2000, Popik et al., 2002). More specifically, HIV not only

requires lipid rafts in the process of entry, but in immune evasion, suppressing host-cell signalling during replication, and egress of the virus from the host (Manes et al., 2000, Nguyen & Hildreth, 2000, Peterlin & Trono, 2003)

HIV Nef is a myristoylated protein that is associated with rafts, and this association is necessary for its function. Nef is involved in down-regulation of CD4 and MHC-I molecules, crucial for viral infectivity and immune evasion. These functions are dependant on the Nef targeting and trafficking function, through clathrin coated pits and early endosome associations, and are thus dependant on lipid raft (Bresnahan et al., 1998, Piguët & Trono, 1999). Interestingly, Nef has been shown to inhibit Gb₃ retrograde transport (Johannes et al., 2003).

GSL-enriched lipid rafts are required for viral egress in addition to entry. Assembly and incorporation of envelope glycoproteins in the virion envelope is regulated by an interaction between the gp41 cytoplasmic tail and the MA domain of the Gag precursor peptide (Hourieux, Brand 2000). During post-transcriptional modification, the MA domain of Gag is myristoylated, and the envelope precursor gp160 is palmitoylated. These modifications target these proteins to lipid rafts, which promote the assembly of budding virions (Ono & Freed, 2001). Furthermore, because HIV selectively buds from lipid rafts, the viral envelope is enriched in lipid raft components, including cholesterol and GlcCer (Brugger et al., 2006, Nguyen & Hildreth, 2000). The viral membrane GSL content can affect infectious potential (Hatch et al., 2009)

5.4 Clinical links to GSL

Increased Gb₃ and GM3 synthesis can be detected at an early stage in HIV-1 infected individuals. In addition, antibodies to these GSLs have been detected in HIV patients (Fantini et al., 1998b). These GSLs have important functions within the immune system, with regards to cell growth, signalling and motility. They are of particular importance as markers in lymphocyte differentiation, where Gb₃ is a marker of B cell development (Wiels et al., 1991) and GM3 of monocytes and T-cells. In addition, GM3 containing microdomains are functional in T cell motility (Gomez-Mouton et al., 2001) and signalling (Sorice et al., 2000). Thus, perturbations in GSL expression, and antibodies produced to GSL in HIV-1 infection may be immunosuppressive.

It is of interest to note that HIV infected patients are more prone to haemolytic uremic syndrome (HUS) (Turner et al., 1997). HUS is characterised by thrombotic microangiopathy of the renal glomeruli mediated by verotoxin/Gb₃ binding (Muthing et al., 2009). It is thus interesting that transgenic mice, in which the HIV genome has been incorporated into the germ line, show renal Gb₃ synthesis is selectively upregulated to induce renal disease (Liu et al., 1999)

5.5 Inhibiting HIV at the membrane level

In the quest for new drug targets, such as the entry inhibitors, and subsequent potential microbicide candidates, attention has been turned to HIV interactions with lipid rafts and GSLs. Several studies have investigated cholesterol-depletion as a means of disrupting lipid rafts to prevent HIV-1 fusion and entry (Liao et al., 2001; Liao et al., 2003) It has also been proposed that increasing ceramide levels in CD4⁺ lymphocytes and monocyte-derived macrophages may block HIV infection, perhaps inhibiting HIV fusion by disrupting normal lipid raft organization and function (Finnegan & Blumenthal, 2006). These studies have used several mechanisms to increase ceramide, including pharmacological agents, such as N-(4-

hydroxyphenyl) retinamide and fenretinide, treatment with sphingomyelinase or addition of long-chain ceramide. Lipid-raft altering compounds may have dual efficacy in treatment of HIV/AIDS. Microorganisms causing opportunistic infections in AIDS patients often rely on lipid-raft mediated mechanisms to elicit their effect, thus HIV treatments altering lipid-rafts may be protective.

As GSLs are critical in the process of HIV infection and pathogenesis, targeting of these molecules may give rise to the development of novel therapeutics. Not only are GSLs key components of lipid rafts, but they also play several roles during HIV binding and host cell fusion. The efficacy of inhibiting HIV infection by targeting GSLs has already been demonstrated *in vitro*. Peptide analogues of the V3 loop of gp120, including those that define the GSL binding site, are effective as inhibitors of HIV-membrane fusion (Delezay et al., 1996, Savarino et al., 2003). Furthermore, analogues of galactosyl ceramide have been found to be protective against T cell infection *in vitro*, where the hydrophobic aglycone moiety of GalCer played an important role (Fantini, 2000, Fantini et al., 1997, Faroux-Corlay et al., 2001)

6. Generation of GSL mimics

Given the importance of GSLs in HIV infection, and the demonstrated anti-HIV potential of GalCer analogues, it is particularly advantageous to develop soluble GSL analogues. GSL binding and receptor function is significantly regulated by lipid modulation (Lingwood, 1996). Despite the fact that the carbohydrate moiety of the GSL defines the specificity of binding interactions, the lipid-free sugar shows minimal binding activity (Boyd et al., 1994, Mamelak et al., 2001a). Thus, gp120 binding to the GSL receptors is abrogated if the lipid moiety, that is the anchor to the cell membrane, is removed (Faroux-Corlay et al., 2001, Mylvaganam & Lingwood, 1999b, Villard et al., 2002). In the membrane bilayer, GSLs comprise three domains - the external aqueous sugar domain, the internal liquid crystalline domain and the "interface" between them. The "interface" region modulates the receptor function of the carbohydrate in response to the liquid crystalline domain, and likely plays a role in lipid raft organization.

In order to generate GSL analogues and maintain the interface character, an adamantane frame was used to replace the fatty acid (Mylvaganam & Lingwood, 2003). This rigid, globular, cage-like hydrophobic structure close to the interface region perturbs the lateral packing of the glycolipid, and thus bilayer structure formation, thereby promoting solubility. The Gb₃ analogue, adamantylGb₃ (adaGb₃), was shown to preferentially partition into water in an organic/aqueous solvent system (Mylvaganam & Lingwood, 1999a). This compound, unlike the lipid-free Gb₃ sugar, maintained its receptor function and was able to inhibit VT/ Gb₃ binding, protecting cells against this toxin (Mylvaganam & Lingwood, 1999a).

We utilized the same strategy to develop a soluble analogue of SGC and GalCer. We substituted the fatty acid of SGC with an adamantane or with a norbornane (smaller) frame and, as with the Gb₃ case, the conjugates partitioned into water (although adamantylGalCer was significantly less soluble), rather than the organic phase (Whetstone & Lingwood, 2003). AdamantylSGC retained its receptor activity (Mamelak et al., 2001a, Whetstone & Lingwood, 2003)

6.1 AdaGb₃ as a mimic of lipid rafts: a 'superligand' for HIV gp120

We have found that adaGb₃ has a variety of additional unusual physical properties which indicate that adamantyl-glycolipids may have unusual biological effects, particularly in

modulating host/microbial interactions. In collaboration with Fantini's group, we showed that gp120 can insert into a Gb₃ monolayer at the water/air interphase in a Langmuir trough (Mahfoud et al., 2002b). However, there was a 2 hour lag-phase prior to binding/insertion which then proceeded at a sigmoidal rate. AdaGb₃, although water-soluble, also can form a monolayer at a water/air surface interface. Binding and insertion of gp120 into such a monolayer was exponential and immediate. Thus adaGb₃ is by far (>1000x), a superior ligand for gp120 than the native Gb₃. These results were duplicated using the SPC3 peptide from the V3 loop of gp120 which contains the GSL binding domain (Delezay et al., 1996). In the studies with the peptide, the lag-phase prior to Gb₃ binding/insertion was even more exaggerated, being three hours as compared to immediate insertion into adaGb₃. The lag phase observed for gp120 insertion into Gb₃ monolayers was removed if the monolayer is formed with 20% cholesterol. This suggests that the gp120 may be interacting with Gb₃ containing lipid rafts or microdomains (of which cholesterol is a key component). The lag phase seen in the absence of cholesterol, may be a function of the ability of Gb₃ to organize into suitable microdomains for gp120 binding and the sigmoidal curve suggests a cooperative effect, once a few domains have been formed. The immediate binding and insertion into adaGb₃ monolayers suggests that this organization required for gp120 insertion is already present in the adaGb₃ monolayer. Interestingly, although gp120 binds SGC, we found no evidence for gp120 insertion into SGC monolayers, even in the presence of cholesterol. Similarly, no gp120 insertion into adamantylSGC monolayers was seen (Mahfoud et al., 2002b). Our recent work showing that 50% cholesterol can mask membrane Gb₃ from gp120 (Mahfoud et al., 2010) indicates a bimodal concentration dependent cholesterol effect.

SGC was shown to inhibit HIV infection of CD4 negative HT29 cells (Fantini et al., 1998a) without inhibition of HIV cell binding. In these studies, SGC was incorporated into the host cell membrane thereby increasing HIV binding, since gp120 binds SGC (Bhat et al., 1993), but fusion with the host cell membrane was inhibited (Fantini et al., 1998a). Although these studies also implicated GalCer in these cells as mediating HIV infection, HT29 cells are Gb₃ positive. Thus, this is consistent with a role for Gb₃ rather than SGC, in HIV-cell fusion. It is possible that in addition to forming microdomains poorly itself, SGC could interfere with rafts containing other GSLs. AdamantylSGC (Whetstone & Lingwood, 2003) is a soluble inhibitor of gp120-SGC binding and may prove more effective than the poorly soluble SGC.

6.2 AdaGb₃ inhibits HIV infection

Comparison of the "compressibility" of Gb₃ and adaGb₃ monolayers shows that the adaGb₃ structure is more rigid and able to withstand greater increases in surface pressure without collapsing (Mahfoud et al., 2002b). This is consistent with a microdomain format for the adaGb₃ monolayer. If adaGb₃ is a "superligand" for gp120 as our studies indicate, adaGb₃ might be able to interfere with the process of HIV infection even for (T) cells which do not express Gb₃ (Akashi et al., 1988). We therefore tested whether adaGb₃ was able to modify HIV infectivity *in vitro*. 200 μM adaGb₃ was able to reduce HIV infectivity in Jurkat T cells using a multiplicity of infectivity ratio of 0.6 (60x higher than standard practice) by ~70% over a 4 day infection period (figure 9) as monitored by ELISA of host cell production of viral nucleoprotein p24^{gag}. Amino adamantane itself showed no inhibition. *Thus, this approach does represent a novel basis for the control of HIV infectivity.* Moreover, in our studies to use adaGb₃ to protect mice against VT, we have shown that adaGb₃ itself (4mg/kg) shows no side effects *in vivo*.

In collaboration with Blumenthal's group at NIH we have found that adaGb₃ is also able to inhibit gp120/CD4/chemokine coreceptor dependent host cell fusion irrespective of gp120 type (R5 or X4 tropic, HIV-1 or HIV-2) as monitored in an indicator system in which gp120 is transfected into one indicator cell and the chemokine receptor into another (Lund et al., 2006).

6.3 FSL-Gb₃

Recently, additional GSL analogues have been shown to act to inhibit HIV-1 infection *in vitro*. A completely synthetic water soluble analogue of Gb₃ termed Functional head Spacer Lipid tail-Gb₃ (FSL-Gb₃) was shown to inhibit X4 and R5 HIV-1 infection with a similar 50% inhibitory activity (IC₅₀) as adaGb₃ (Harrison et al., 2010). This Gb₃ analogue was unique in that the lipid tails were replaced with phosphatidylethanolamine and a spacer region containing multiple ionic residues allowed for complete solubility in aqueous media. A novel synthetic process maintains the carbohydrate moiety of Gb₃ coupled to phosphatidylethanolamine through a phosphate linker. This molecule gains its solubility through the insertion of charged nitrogen and phosphate containing groups that are located between the glycone and aglycone moieties. The molecule is completely synthetic, completely soluble in aqueous solutions, and available in large quantities for testing. In addition, animal studies have shown no toxicity at millimolar quantities systemically. The unusual tail also allows for this analogue to insert itself into cell membranes and convert an HIV-permissive Gb₃-negative T-cell into a Gb₃-positive T-cell that resists HIV infection (Harrison et al., 2010).

Harrison et al. (2010) have used the FSL-Gb₃ to show that it can inhibit HIV infection by two different mechanisms. First, as with adaGb₃, mixing the FSL-Gb₃ with either X4 or R5 HIV-1 results in inhibition of HIV infection with approximately the same IC₅₀ as with adaGb₃ (Harrison et al., 2010). This was shown both for laboratory strains of HIV-1 as well as for clinical isolates of R5 HIV-1 viruses. In addition, FSL-Gb₃, apparently due to its particular hydrophobic tail structure, was shown to insert itself into cell membranes, retaining proper cell-surface conformation of the carbohydrate moieties. Cellular insertion of FSL-Gb₃ was able to result in conversion of a human CD4⁺ T-cell that completely lacked Gb₃ expression into a T-cell that highly expresses Gb₃. This property of FSL-Gb₃ to convert a permissive HIV target cell into a less permissive cell for HIV infection is a major finding; thus, providing acquired resistance to HIV-1 infection as a possible therapeutic approach. The following: "In addition, preliminary work has shown potential for soluble Gb₃ analogues, including FSL-Gb₃, to act as microbicides to inhibit mucosal HIV transmission (Harrison et al., 2011). FSL-Gb₃ shows great promise as a possible therapeutic, *in vivo*, as it would be potentially capable of inhibiting HIV infection both systemically to reduce viremia but also by its insertion into CD4⁺ T cells resulting in inhibition of HIV infection by blocking viral entry into its normal primary host targets.

6.4 Multimeric GSLs

The finding that a soluble GSL analogue was capable of inhibiting HIV infection soon led to other studies where the investigators used a series of C-glycoside synthetic analogues of GalCer (Garg et al., 2008). These investigators showed that two of six analogues were able to bind gp120 and inhibit X4 and R5 strains and dual-tropic HIV-mediated fusion and entry in the absence of any significant cytotoxicity. In addition to HIV, these investigators also showed that soluble GSLs may inhibit additional enveloped viruses such as vesicular stomatitis virus (VSV) (Garg et al., 2008).

Rosa Borges et al. (2010) found that if the synthesized analogues utilized multimeric Gb₃ sites, increased efficacy for inhibition of HIV-1 could be achieved. These investigators covalently attached multiple head groups of either Gb₃ or GM3 to a dendrimer core to produce multivalent dendrimeric compounds that were water soluble and showed a much lower IC₅₀ for inhibition of HIV-1 infection, compared to either FSL-Gb₃ or adaGb₃ (Rosa Borges et al., 2010). Thus, soluble Gb₃ analogues have important therapeutic potential to block HIV from interacting with CD4⁺ target cells (Figure 5D).

7. Pharmacologic modulation of Gb₃ expression

1-Deoxygalactonojirimycin (DGJ) is an alkylated imino sugar with a galactose head and a one-carbon side chain. It was found to be highly tolerable *in vivo* and established as a potent competitive inhibitor of *α-galactosidase A* (α -Gal A) (Hamanaka et al., 2008). DGJ has been proposed as a specific chemical chaperone for treatment of diseases including Fabry (Fan et al., 1999). Studies have indicated that oral administration of DGJ to transgenic mice expressing a human mutant α -Gal A substantially elevated enzyme activity in major organs (Fan et al., 1999). Because Gb₃ has now been suggested by the studies of Lund et al. (Lund et al., 2006) as an important component for prevention of the HIV entry mechanism, the use of DGJ to pharmacologically increase Gb₃ expression may be useful for HIV prevention strategies.

Recent studies (Ramkumar et al., 2009) used DGJ to increase the cell-surface expression of Gb₃ on the monocyte cell line, THP-1, which is infectable with X4 HIV-1. DGJ used at 100 μ M was able to increase the cell-surface expression of Gb₃ of THP-1 cells by approximately 20-fold. Subsequent X4 HIV-1 infection was decreased significantly.

To inhibit Gb₃ expression in these cells, these investigators used the compound, D-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4) which inhibits GlcCer synthase, the enzyme responsible for producing GlcCer (Inokuchi & Radin, 1987). This is a key enzyme in the biosynthesis of GSLs because most are glucosylceramide-based. P4 is the most potent inhibitor of this glycosyltransferase (Lee et al., 1999). Using 2 μ M P4, Ramkumar et al. (Ramkumar et al., 2009) were able to completely inhibit Gb₃ expression with subsequent X4 HIV-1 infection increased up to 20-fold. DGJ had little effect on the infection of a Gb₃-negative subclone of THP-1 cells and FACS analysis indicated that after DGJ treatment, CD4 and HIV co-receptor levels were similar in the Gb₃ expressing and non-expressing THP-1 cell lines by these two compounds. Also, as DGJ was found to be non-toxic in the THP-1 cells up to concentrations of 300 μ M, the inhibitory effect was not a result of cytotoxicity of DGJ treatment. The authors concluded that their results indicated that the difference in HIV infection was due solely to the modulation of the expression of the levels of Gb₃.

To examine the effects of DGJ and P4 treatment on subsequent infection with R5 HIV-1 virus, the glioblastoma cell line, U87, that had been transfected to express CD4 and the chemokine co-receptor, CCR5, was used. Ramkumar et al. (Ramkumar et al., 2009) again found that treatment of these cells with DGJ resulted in a significant inhibition of R5 HIV-1 infection while treatment with P4 caused a doubling in the infection. They concluded that pharmacologically increasing Gb₃ expression using DGJ treatment or inhibition of Gb₃ expression using P4 demonstrates a linear relationship between Gb₃ expression and infection with either X4 or R5 HIV-1. In addition, their studies suggest that pharmacologically increasing Gb₃ is an effective and novel means to prevent HIV-1 infection *in vitro* and that this approach should be explored for *in vivo* treatment of HIV infection.

8. HIV infection of CD4 negative cells

The current paradigm indicates that infection with HIV-1 depends entirely on the recognition of its primary and co-receptors for viral fusion and entry into a target cell. Unfortunately, this paradigm is insufficient to completely explain the pathogenesis of HIV-1. This is because there are many instances of HIV-1 infection where either the primary and/or co-receptors are missing from the infected cell. Indeed, HIV-1-infected CD4 negative cells have been identified *in vivo*, including various brain cells (Pumarola-Sune et al., 1987, Ward et al., 1987, Wiley et al., 1986) epithelial cells (Nelson et al., 1988), cardiomyocytes (Barbaro et al., 1998), CD4 negative lymphocytes (Livingstone et al., 1996, Saha et al., 2001a), renal tubular epithelial cells (Marras et al., 2002, Wyatt & Klotman, 2007), hepatocytes (Fromentin et al., 2011) and thymocytes (Kitchen et al., 1997). HIV-1 has also been shown to infect CD4-negative neural and epithelial cells *in vitro*, although not productively (Clapham et al., 1989, Tateno et al., 1989). However, it has been shown that HIV-1 can productively infect CD4-CD8⁺ T lymphocytes *in vitro* (Saha et al., 2001b).

Our own work supports the idea of HIV infection of CD4-negative cells. Using kidney-derived cell lines such as ACHN and 293 as well as a colon-derived cancer cell line called Caco-2, we have been able to show transient infection with an X4 virus (Figure 5). We have also shown that soluble Gb₃ can inhibit the infection of CD4-negative Caco-2 epithelial cell lines (Figure 6) as well as human CD4-negative cell lines derived from the cervix or endometrium (Harrison et al., 2011). Although, the infection of these cell lines is not robust

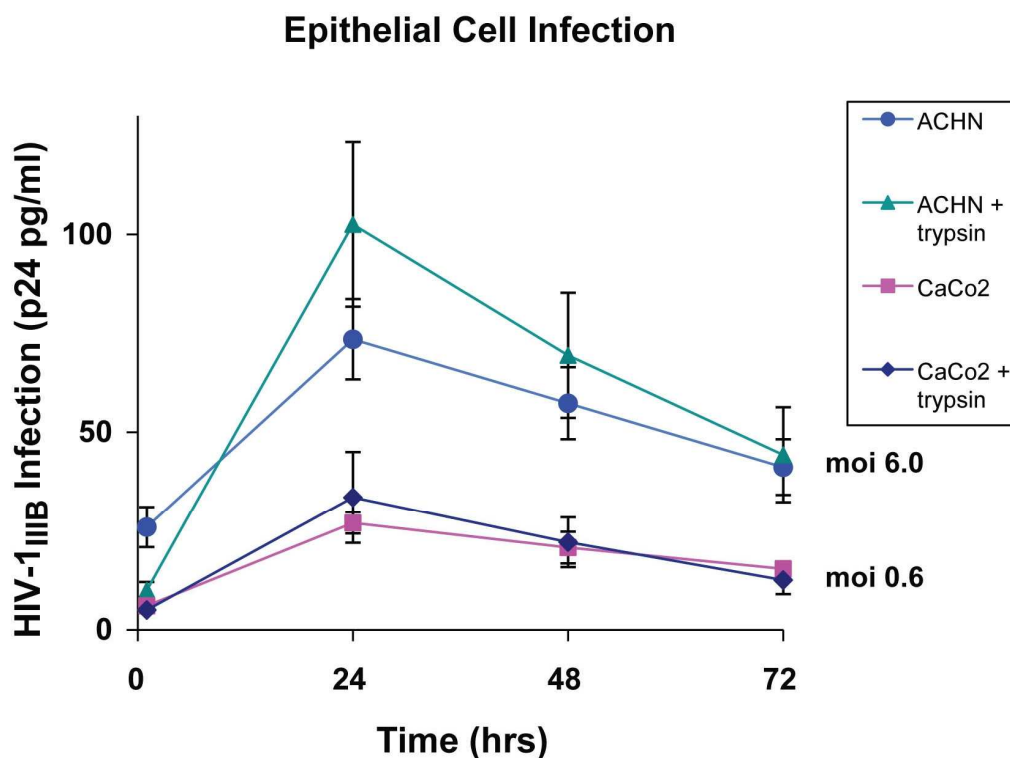


Fig. 5. **HIV can infect CD4-negative epithelial cells.** HIV infection of ACHN kidney-derived cell line and Caco-2 colon cancer derived epithelial cell line. Trypsin is used to insure that the p24 antigen being used as a measure of productive HIV infection is not derived from external virions sticking to the cell membranes but from budding virions indicating a round of replication of the virus.

and appears transient, these infected cells could serve as reservoirs of latent HIV provirus and may become activated under certain conditions to produce a round of progeny virions which would have the potential to infect other cells such as CD4⁺ T-cells and maintain or re-establish an active infection.

Several hypotheses have arisen to explain the infection of CD4-negative cells within the current paradigm of only CD4 and chemokine co-receptors playing a role. A popular theory is that the availability of CXCR4 in CD4-negative cells is sufficient for viral fusion and entry. However, the evidence addressing this idea is contradictory. In support, human CD4-CCR5-CXCR4⁺ pre-T cell lines can be infected with HIV-1 (Borsetti et al., 2000). Furthermore, CD4⁺CXCR4⁻ human megakaryocytic cells are fully resistant to HIV-1 infection until they are transfected to over-express CXCR4 (Baiocchi et al., 1997). In contradiction, the CD4-negative human B-cell line Raji is not permissive to HIV-1 infection, even though it expresses functional CXCR4 (Speck et al., 1999). Therefore, the absolute dependence of HIV-1 on CXCR4, even in the absence of CD4, does not completely account for the ability of HIV-1 to infect CD4⁻ cells.

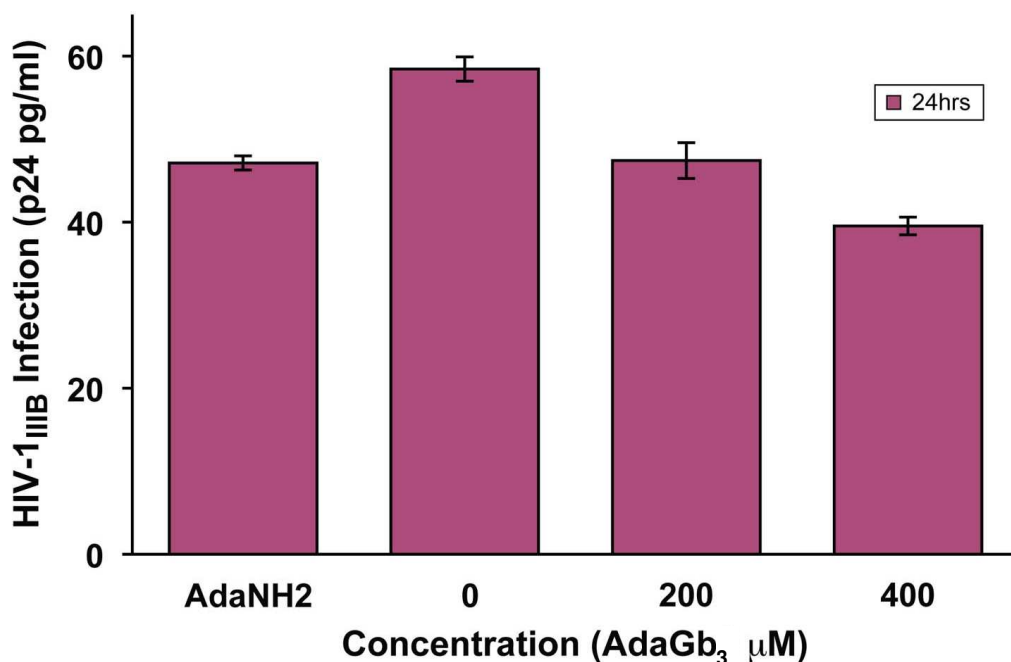


Fig. 6. **Inhibition of HIV infection of epithelial cells by soluble Gb₃.** Caco-2 CD4-negative epithelial cells are infected by HIV but the infection can be inhibited using soluble adaGb₃. adaNH2 is a control soluble GSL for adaGb₃

Taken together, the current paradigm that requires the availability of both a primary CD4 receptor plus a co-receptor, either CXCR4 or CCR5, in order for HIV to infect a target cell is not sufficient to explain other cell infections where either the primary receptor and/or the co-receptor are not present, or where there is a lack of infection when both receptors are present. Indeed, there is ample evidence that these receptors are not always sufficient for viral infection. Further examples include human CD4-negative astrocytes that express functional CCR5 and CXCR4 and are resistant to infection by HIV-1 strains (Boutet et al., 2001) and CD4⁺CXCR4⁺ cells, also resistant to infection with HIV-1 (Moriuchi et al., 1997). This was shown by infecting U937 monocyte-derived cell lines that were shown to be either permissive or nonpermissive for infection by HIV-1. All but one of these cell lines expressed

both functional CXCR4 and CD4. One of these cell lines that was nonpermissive lacked CXCR4, but when this receptor was transfected back into this cell line, it remained nonpermissive to infection 1 (Moriuchi et al., 1997).

9. Summary

Studies have indicated that human PBMCs with an intracellular or cell-surface accumulation of Gb₃ were less susceptible to HIV infection (Lund et al., 2005, Lund et al., 2009). These PBMCs were derived from patients with Fabry disease and from healthy P₁^k blood group phenotype individuals having a pathologic or natural, respectively, elevation of Gb₃. AdaGb₃, FSL-Gb₃, and multivalent dendrimeric-Gb₃, all soluble Gb₃ analogues, have been shown to be effective inhibitors of HIV regardless of strain or tropism, and also to inhibit drug resistant HIV strains and prevent HIV infection of CD4-negative epithelial cells. Therefore, Gb₃ may be a natural host resistance factor and increasing its expression *in vitro* using soluble analogues, such as FSL-Gb₃, that can insert into T-cells that do not naturally express Gb₃, and/or the use of a pharmacologic agent, such as DGJ, to increase Gb₃ expression, may decrease HIV-1 susceptibility. Importantly, the further development of soluble Gb₃ analogues, especially multivalent analogues expressing multiple Gb₃ sugar moieties having increased affinity and avidity for the V3 loop of HIV gp120, may provide for novel and highly effective HIV therapeutics to prevent or treat HIV/AIDS (Figure 7).

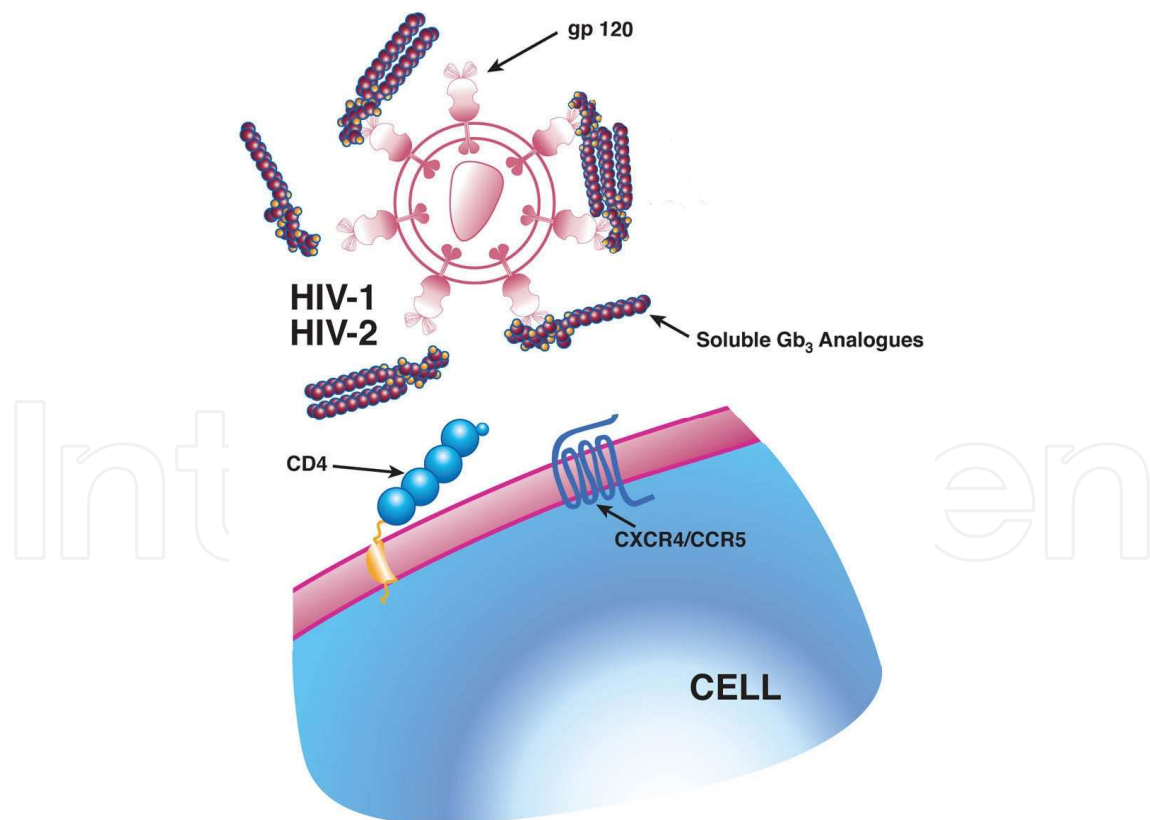


Fig. 7. **Potential novel HIV therapeutic.** Soluble Gb₃ analogues may be able to bind to HIV gp120 protruding from the HIV envelop and prevent HIV from interacting with the primary and/or co-receptors for HIV; thus, preventing HIV infection.

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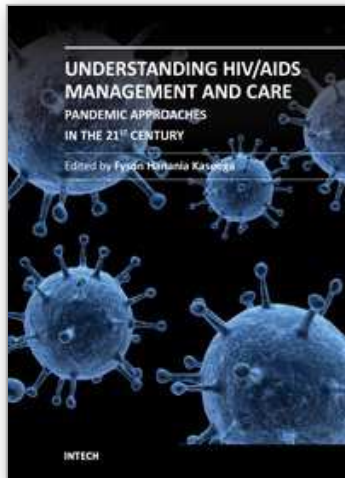
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Like any other book on the subject of HIV/AIDS, this book is not a substitute or exhausting the subject in question. It aims at complementing what is already in circulation and adds value to clarification of certain concepts to create more room for reasoning and being part of the solution to this global pandemic. It is further expected to complement a wide range of studies done on this subject, and provide a platform for the more updated information on this subject. It is the hope of the authors that the book will provide the readers with more knowledge and skills to do more to reduce HIV transmission and improve the quality of life of those that are infected or affected by HIV/AIDS.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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