SOLUBLE GLOBOTRIAOSYLKERAMIDE AS A POTENTIAL SYSTEMIC AND LOCAL INHIBITOR OF HIV INFECTION

By

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ABSTRACT

Previously we have identified the glycosphingolipid globotriaosylceramide (Gb₃/Pk) as an inhibitor and resistance factor against HIV-1 infection in vitro. Here we show that a novel, soluble, completely synthetic Gb₃ analogue, FSLGb₃, inhibits infection of X4 strains of HIV-1 in the Jurkat T-cell line and both R5 and X4 strains in PBMCs. FSLGb₃ absorbs into cellular plasma membranes and membrane adsorbed FSLGb₃ was able to inhibit subsequent HIV-1 infection.

We have also developed a mouse model to test in vivo the efficacy of soluble Gb₃ analogs in the prevention of mucosal viral infection. Soluble Gb₃ was incorporated into gel or alone and applied directly to the vaginal and rectal mucosal tissue of mice. We have not yet shown a statistically significant reduction in infection, although a trend towards inhibition is evident. Our studies show synthetic Gb₃ to be an inhibitor of HIV-1 infection and further exploration of therapeutic strategies are warranted.
ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr. Donald Branch and Dr. Clifford Lingwood, for the opportunity to conduct HIV research. I am very grateful that I was able to attend many conferences and meetings the past two years, especially the International AIDS conference in Sydney, Australia. Thank you for your advice, guidance and support.

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Finally, thank you James for always believing in me and for your unconditional love and support. Thanks for keeping me company at the lab on the many weekends and late nights of work!
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<th>Description</th>
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<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AdamantylGb₃</td>
<td>Adamantyl globotriaosylceramide</td>
</tr>
<tr>
<td>APOBEC</td>
<td>Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G</td>
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<tr>
<td>ARV</td>
<td>Anti-retroviral</td>
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<td>AZT</td>
<td>Azidothymidine</td>
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<td>Capsid</td>
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<td>CCR5</td>
<td>CC-chemokine receptor 5</td>
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<td>Cer</td>
<td>Ceramide</td>
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<tr>
<td>CONRAD</td>
<td>Contraceptive research and development program</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic lymphocyte</td>
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<tr>
<td>CXCR4</td>
<td>CXC-chemokine receptor 4</td>
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<td>DCs</td>
<td>Dendritic cells</td>
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<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin</td>
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<tr>
<td>DGJ</td>
<td>1-Deoxygalactonojirimycin</td>
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<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
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<td>Envelope</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
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<td>Full Form</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FDA</td>
<td>US Food and drug administration</td>
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<tr>
<td>FIs</td>
<td>Fusion inhibitors</td>
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<tr>
<td>FITC</td>
<td>Flourescein isothiocyanate</td>
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<tr>
<td>FSLGb&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Functional Head-Spacer-Lipid Tail Globotriaosylceramide</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>Gag</td>
<td>Group-specific antigen</td>
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<td>GalCer</td>
<td>Galactosylceramide</td>
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<tr>
<td>Gb&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Globotriaosylceramide</td>
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<td>Globotetraaosylceramide</td>
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<td>Glycoprotein</td>
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<td>Glycosphingolipid</td>
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<tr>
<td>GT</td>
<td>Trisialoganglioside</td>
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<tr>
<td>HAART</td>
<td>Highly active anti-retroviral therapy</td>
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<td>HEPS</td>
<td>Highly exposed persistently seronegative</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HR-2</td>
<td>Heptad repeat-2</td>
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<tr>
<td>HSCs</td>
<td>Haematopoietic stem cells</td>
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<td>Abbreviation</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRs</td>
<td>Integrase inhibitor</td>
</tr>
<tr>
<td>LTNP</td>
<td>Long term nonprogressor</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosal associated lymphoid tissue</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC-1</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage chemotactic protein</td>
</tr>
<tr>
<td>m.o.i.</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MRC</td>
<td>Maraviroc</td>
</tr>
<tr>
<td>MSM</td>
<td>Men who have sex with men</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>N9</td>
<td>Nonoxynol-9</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative effector</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor and activator of transcription</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-integration complex</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T-cell expressed and secreted</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative luciferase units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SGC</td>
<td>3’ Sulphogalactosylceramide</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually transmitted infection</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal factor</td>
</tr>
<tr>
<td>VPAC</td>
<td>Vasoactive intestinal peptide and pituitary adenylate cyclise activating polypeptide receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>Vpr</td>
<td>Viral protein r</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein u</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular stomatitis virus envelope G protein</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>VSV/HIV</td>
<td>Pseudotyped vesicular stomatitis and HIV virus</td>
</tr>
<tr>
<td>VT</td>
<td>Verotoxin</td>
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</table>
CHAPTER 1

INTRODUCTION
1.1 HIV and AIDS

1.1.1 Introduction to HIV/AIDS

The acquired immunodeficiency syndrome (AIDS) was first described in 1981 in a small group of homosexual men in California exhibiting rare *Pneumocystis carinii* pneumonia. It was noted that the underlying immunosuppression typified by these patients was most likely the result of CD4+ T helper cell depletion (Gottlieb, Schroff, et al, 1981). Within two years of this first clinical description, three different groups described a new virus isolated from patients exhibiting the symptoms of AIDS, coined the lymphadenopathy-associated virus (LAV) (Barre-Sinoussi, Chermann, et al, 1983), the human T-lymphotropic virus type III (HTLV-III) (Gallo, Sarin, et al, 1983) and AIDS-associated retrovirus (ARV) (Levy, Hoffman, et al, 1984). The first two descriptions were published in the same issue of *Science*. Decades of argument ensued over which group was the official first discoverer of HIV. A political agreement was eventually reached between the French and American governments with American Robert Gallo of the National Institutes of Health, and French scientist Luc Montagnier of the Pasteur Institute receiving equal credit and the virus was subsequently renamed the human immunodeficiency virus (HIV) (Coffin, Haase, et al, 1986). However, the Nobel Prize in Physiology or Medicine for 2008 was recently co-awarded to both Francoise Barre-Sinoussi and Luc Montagnier of France for discovering HIV (Lever, and Berkhout, 2008) without reference to Gallo.

Since 1983, immense worldwide research interest in HIV/AIDS has resulted in a tremendous amount of scientific data, however our understanding of the virus that causes AIDS and its pathogenesis is still limited and we are yet to discover a cure or vaccine to
combat the pandemic.

1.1.2 Natural history

While the earliest cases of HIV and AIDS were first noted in the United States, it was soon realized that the virus was rooted in Africa (Piot, Quinn, et al, 1984, Van de Perre, Rouvroy, et al, 1984). Current evidence shows that HIV is a zoonotic infection acquired through nonhuman primates (Keele, Van Heuverswyn, et al, 2006). HIV-1 is most closely related to the simian immunodeficiency virus type cpz (SIVcpz) isolated from chimpanzees, specifically the species *P. t. troglodytes* of southern Cameroon (Keele, Van Heuverswyn, et al, 2006). HIV-2 is most closely related to SIVsm from Sooty Mangabeys of Western Africa (Keele, Van Heuverswyn, et al, 2006). In both cases, the regions where the most genetically diverse HIV-1 or HIV-2 are found, overlaps with the natural habitat of the species from which the virus originates (Keele, Van Heuverswyn, et al, 2006). HIV-2 shares about 40-50% homology with HIV-1 but is more similar to SIVsm, sharing 75% homology (Markovitz, 1993). Recent evident shows that HIV-2 originated in the human population at least as early as the 1940’s (Lemey, Pybus, et al, 2003). Both HIV-1 and HIV-2 likely entered the human population over multiple transmissions from simian hosts (Lemey, Pybus, et al, 2003). HIV-2 is less pathogenic than HIV-1, and progression to AIDS is more prolonged compared to HIV-1 infection (Leligdowicz, and Rowland-Jones, 2008).

Additionally, it has been shown that HIV/AIDS circulated in the United States for approximately 12 years prior to its recognition in 1981, and that the oldest epidemic outside of Africa is found in Haiti (Gilbert, Rambaut, et al, 2007).

1.1.3 Epidemiology

The current global status of the virus is devastating, particularly in the developing world and countries of low socioeconomic status. The pandemic has formed two broad patterns globally comprising epidemics that are sustained in the general population of much of Sub-Saharan Africa, and epidemics in the rest of the world that are found in discreet populations of groups that are of highest risk (UNAIDS/WHO, 2007). The most recent epidemiology estimates 33.2 million people are infected with HIV-1 globally, with 2.5 million of these being new infections (UNAIDS/WHO, 2007). 2.1 million people died of AIDS and AIDS related illnesses in 2007; the majority came from developing nations. These recent statistics however represent a sizeable decrease from 2006 estimates, when 39.5 million were approximated infected worldwide. This decline is largely due to substantial changes in the methodology at the ground level used to estimate the epidemic. However, the decreases seen in Kenya, Uganda and Zimbabwe among other countries, are attributed to an actual decline in new infections (UNAIDS/WHO, 2007). Figure 1.1 maps the global prevalences of the HIV pandemic.
Figure 1.1: A global view of regional HIV prevalence
The greatest burden of disease remains in the developing world. Of the 2.1 million deaths due to AIDS in 2007, 76% occurred in sub-Saharan Africa, where AIDS remains the primary cause of death. Additionally, 68% of new infections occurred in this region as well. Women are increasingly shouldering the burden of the disease in sub-Saharan Africa, where almost 61% of adults living with HIV-1 in 2007 were women. However, it must be noted that these most recent statistics represent an overall decline in new infections for the region since 2001. This trend was also seen in South and South-East Asia and Eastern Europe, where declines in new infections were also noted compared to 2001 statistics (UNAIDS/WHO, 2007). Despite these positive trends, it must be made clear that these regional figures mask the fact that the overall number of individuals infected with the virus has increased substantially. For example, the total number of people living with the disease in Eastern Europe increased 150% from 2001 (UNAIDS/WHO, 2007).

The latest data for Canada shows that vulnerable populations, such as injection drug users, aboriginal peoples and men who have sex with men (MSM), continue to be disproportionately infected compared to the general population (UNAIDS/WHO, 2008). 58,000 people are currently infected with the virus, a 16% increase between 2002 and 2005 (UNAIDS/WHO, 2008). Of the new infections, 45% can be attributed to unprotected sex between MSM (UNAIDS/WHO, 2008). Women are also a vulnerable population, particularly women of aboriginal ancestry and those originating from endemic countries (UNAIDS/WHO, 2008). This data highlights the dire need for increased access to, and knowledge about prevention, including new prevention modalities.
Overall, the global burden of disease from infection with HIV-1 remains overwhelming and stresses the need for a greater understanding of viral pathology, new treatment modalities and novel ways to prevent new infections.

1.1.4 Transmission

The transmission of HIV occurs under conditions that facilitate the exchange of bodily fluids and blood. The three main modes of transmission are through sexual contact, parenteral injection, and from mother-to-child via delivery and breastfeeding (UNAIDS/WHO, 2007). The virus exists in bodily fluids such as semen both as free virus and within infected lymphocytes (Coombs, Reichelderfer, and Landay, 2003). During sexual intercourse, HIV will enter the host’s body through the mucosal tissue of either the vagina or rectum, or through the infection of specific target cells including CD4+ T lymphocytes, dendritic cells (DCs), Langerhans cells (LCs) and macrophages (Wu, 2008). Transfer of the virus across mucosal tissue is aided by disruption of the barrier through sexual intercourse or by co-infections with other sexually transmitted infections (STIs) such as genital herpes that promote inflammation (Chan, 2006). DCs, like macrophages, act as reservoirs for the virus in lymphatic tissue. These cells express the lectin-like receptor DC-SIGN (DC-specific intercellular adhesion molecule 3-grabbing nonintegrin) that can bind HIV virions and can display HIV antigen to T cells, thereby promoting their infection (Steinman, Granelli-Piperno, et al, 2003, Geijtenbeek, Kwon, et al, 2000). Epithelial cells of the mucosa can also become transiently infected as HIV-1 can enter these cells by transcytosis or endocytosis mediated by the glycosphingolipid galactosylceramide (GalCer) (Fotopoulos, Harari, et al, 2002). It is debated whether these cells are sites of productive infection; their role in the HIV
lifecycle may be involved in sequestering the virus to other target cells (Morrow, Vachot, et al, 2007).

After initial entry into the host’s system, the virus disseminates through the lymphatic system and colonizes lymphatic tissues of the spleen, tonsils, gut mucosa and lymph nodes, which become reservoirs for the virus, and the depletion of GI associated T cells is a hallmark of initial infection (Veazey, DeMaria, et al, 1998, Mehandru, Poles, et al, 2004, Haase, 2005).

In the early stages of the epidemic, infection was identified mainly in homosexual men and it was at first thought the disease was limited to this particular demographic group, however this was due to a bias in screening in Western countries. Heterosexual transmission is the predominant mode of infection globally (UNAIDS/WHO, 2007).

Another important mode of transmission is through inoculation and the sharing of blood products. Intravenous drug abusers often share needles and with this, direct blood to blood contact and the transmission of the virus can occur between individuals (UNAIDS/WHO, 2007). This is an important mode of infection, particularly due to the fact that sexual transmission can then occur in other groups of adults. Additionally, in the early stages of the epidemic, haemophiliacs and other patients consistently using blood products acquired the virus through contaminated blood (van den Berg, ten Cate, et al, 1986). Public health measures that ensure all donated blood is now tested for HIV before use have nearly eliminated this mode of transmission in the Western Hemisphere (Greenwald, Burstein, et al, 2006).

The predominant mode of transmission to children is through their infected mothers. Infants can acquire the virus through the placental blood vessels, through the
birth canal during delivery, and after delivery through infected colostrum and milk (Mofenson, and McIntyre, 2000, Newell, 1998). The most important mode of infection is during delivery through the ingestion of blood and vaginal fluids by the infant (Gaillard, Verhofstede, et al, 2000). Antiretroviral treatment that greatly reduces the mother’s viral load has nearly eliminated transmission to infants in the Western Hemisphere, along with precautionary planned caesarean sections and bottle feeding (Mofenson, and McIntyre, 2000, Townsend, Cortina-Borja, et al, 2008). In the developing world however, limited access to antiretrovirals and maternal care means infants continue to become unnecessarily infected with the virus (Mofenson, and McIntyre, 2000). In situations where these treatments are available, access to clean water to prepare infant formula is often an issue, which forces many mothers to breastfeed even if infected with the virus.

1.1.5 Pathophysiology of HIV infection

The pathophysiology of an HIV infection can be broken down into three main phases: (1) acute retroviral syndrome (ARS), (2) a chronic phase and (3) full-blown AIDS (Kumar, V., Abbas, A.K. and Fausto, N., 2005, Weiss, 2008). ARS represents the initial phase of HIV infection, typically the first few weeks post infection, and is characterized by widespread viremia and seeding of lymphatic tissues. Clinical symptoms resembling mononucleosis such as fever, rash, sore throat, weight loss and fatigue may occur (Simon, Ho, and Abdool Karim, 2006). HIV targets mainly CD4 positive (CD4+ ) cells of the immune system such as T helper cells and cells of the monocyte/macrophage lineage, and thus a decrease in the number of CD4+ cells results (Haase, 2005). The most active site of HIV replication in early infection is the mucosal associated lymphoid tissue (MALT) (Stebbing, Gazzard, and Douek, 2004). Studies
using macaques have shown replication to occur in infected macrophages from cervical
and vaginal tissue samples (Haase, 2005). From this main initial source of infection,
infected dendritic cells migrate to the lymph nodes and present the virus to T helper cells
(Weiss, 2008).

This stage is followed by a vigorous host immune response, in which a decrease
in viremia or viral load occurs. This fall in viral load has been attributed to the
appearance of cytotoxic T cells (CD8+) and CD4 helper cells specific to the virus (Simon,
Ho, and Abdool Karim, 2006, Weiss, 2008). Other theories suggest that MALT infection
is so severe that HIV target cells are depleted to the point where viral replication
significantly halts due to a lack of cells to become infected (Brenchley, Schacker, et al,
2004, Brenchley, Price, and Douek, 2006). At this time, the chronic phase is reached, also
tерmed clinical latency, where the patient may be asymptomatic for a period lasting up to
a decade or more. CD4+ cells continue to be depleted during the latency phase, however
they are regenerated (Weiss, 2008). As more CD4+ helper cells become infected and die,
this balance shifts and CD4+ cells decrease (Weiss, 2008). When the level of CD4+ cells
fall below 200 cells/µL the host becomes increasingly susceptible to opportunistic
infections. A low CD4 count and/or opportunistic infections are the diagnostic criteria
for AIDS (Pantaleo, Graziosi, and Faucci, 1993). AIDS-associated opportunistic infections
such as *P. carinii, Mycobacterium tuberculosis* (TB), and cancer, specifically Kaposi’s
sarcoma, overwhelm the host (Safai, Johnson, et al, 1985). Other clinical features of
AIDS, such as dementia due to neurological dysfunction, dislipidemia and also wasting
syndrome may occur (Ances, and Ellis, 2007). The host will eventually succumb to the
virus without intervention. Figure 1.2 depicts the typical course of an HIV infection.
Figure 1.2: Graphical representation of the course of HIV infection

Redrawn from (Kumar, V., Abbas, A.K. and Fausto, N., 2005)
1.2 HIV Biology

1.2.1 Classification of the virus

HIV is a virus of the Retroviridae family and Lentivirus subfamily. Infection with the virus leads to acquired immunodeficiency syndrome (AIDS) in the majority of human hosts without treatment (Simon, Ho, and Abdool Karim, 2006). There are two genetically different forms of HIV, HIV-1 and HIV-2. HIV-1 is the most common form and is the virus associated with the current pandemic of HIV/AIDS (Butler, Pandrea, et al, 2007). HIV-2 causes a similar disease, however individuals infected with this strain are mainly concentrated in Western Africa and the pathology is much less severe than HIV-1 in the majority of infected individuals (Butler, Pandrea, et al, 2007). HIV-1 isolates are extremely variable, with most of this variability concentrated in the genes encoding the envelope glycoproteins, as envelope proteins are essential to viral entry and are the targets of host immune responses. This variability has resulted in a classification system for HIV-1 viruses based on genetic differences. The three groups are denoted $M$ (Major), $O$ (Outlier) and $N$ (Neither) with the $M$ group the most common globally. Within the $M$ group there are at least nine different genetic HIV-1 subtypes or clades (Butler, Pandrea, et al, 2007) (Table 1.1).
### Table 1.1: HIV strains

<table>
<thead>
<tr>
<th>HIV-1</th>
<th>Sub-classification</th>
<th>Description</th>
<th>Geographic region</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group M Subtype</td>
<td>A</td>
<td>Subtypes A, A1, A2</td>
<td>E, W, S Africa</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>America, W Europe, Australia</td>
<td></td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>S and E Africa, India</td>
<td></td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>E and W Africa</td>
<td></td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>Subtypes F1 and F2</td>
<td>W, Central Africa</td>
<td>Very low</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td></td>
<td>W, Central Africa</td>
<td>Very low</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td></td>
<td>W, Central Africa</td>
<td>Very low</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td></td>
<td>W, Central Africa</td>
<td>Very low</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td></td>
<td>W, Central Africa</td>
<td>Very low</td>
</tr>
<tr>
<td>Group O Subtype</td>
<td></td>
<td></td>
<td>Cameroon, Senegal</td>
<td>Very low</td>
</tr>
<tr>
<td>Group N Subtype</td>
<td></td>
<td></td>
<td>Cameroon</td>
<td>Very low</td>
</tr>
</tbody>
</table>
Figure 1.3: Schematic diagram of HIV virion structure
The HIV-1 virion is spherical and comprised mostly of a protein core and RNA viral genome surrounded by a lipid bilayer derived from host cells (Figure 1.3). The core consists of the two copies of the RNA genome, the p24 capsid protein, p7 nucleocapsid protein and the three viral enzymes, protease (PR), integrase (I) and reverse transcriptase (RT). Accessory proteins found associated with the enzymes include Vpr, Vif and Vpu (Clements, and Zink, 1996). The viral genomic RNA includes the genes typically found in retroviruses: gag, pol and env as well as the genes that encode the accessory and regulatory proteins (vif, vpr, vpu, tat, rev and nef) (Clements, and Zink, 1996). Glycoproteins project from the viral membrane and are integral to host cell binding, fusion and entry (Center, Leapman, et al, 2002). These include the gp120 surface glycoprotein and the gp41 transmembrane glycoprotein (Butler, Pandrea, et al, 2007). Please refer to table 1.2 for a more detailed overview of the components of the HIV-1 virus. The HIV genome is depicted in Figure 1.4.
Figure 1.4: Illustration of the HIV genome and open reading frame for HIV genes
### Table 1.2: HIV-elicited proteins, enzymes and auxiliary proteins

<table>
<thead>
<tr>
<th>HIV Proteins</th>
<th>Abbr.</th>
<th>Encoding Gene Segment</th>
<th>Gene Product</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface proteins</td>
<td>SU gp120</td>
<td>Env</td>
<td>Envelope</td>
<td>Attachment of HIV to specific target cells</td>
</tr>
<tr>
<td>Transmembrane Protein</td>
<td>TM gp41</td>
<td>env</td>
<td>Envelope</td>
<td>Fusion of viral and host cell membranes, facilitating entry</td>
</tr>
<tr>
<td>Matrix</td>
<td>MA</td>
<td>gag</td>
<td>Structural</td>
<td>Required for mature virion structure, transport of proviral DNA across nuclear envelope for integration</td>
</tr>
<tr>
<td>Capsid</td>
<td>CA p24</td>
<td>gag</td>
<td>Structural</td>
<td>Required for mature virion structure, Potential role in early stage of replication</td>
</tr>
<tr>
<td>Nucleocapsid</td>
<td>NC</td>
<td>gag</td>
<td>Structural</td>
<td>Required for mature virion structure</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>RT</td>
<td>pol</td>
<td>Enzyme</td>
<td>Synthesis of proviral DNA into host-genome</td>
</tr>
<tr>
<td>Integrase</td>
<td>IN</td>
<td>pol</td>
<td>Enzyme</td>
<td>Integration of proviral DNA into host genome</td>
</tr>
<tr>
<td>Protease</td>
<td>PR</td>
<td>pol</td>
<td>Enzyme</td>
<td>Cleavage of immature virion structure post-budding</td>
</tr>
<tr>
<td>Transcriptional transactivator</td>
<td>Tat</td>
<td>tat</td>
<td>Regulatory</td>
<td>Enhancement of transcription of HIV genes from proviral DNA</td>
</tr>
<tr>
<td>Regulator of virion gene expression</td>
<td>Rev</td>
<td>rev</td>
<td>Regulatory</td>
<td>Regulates splicing and efficient transport of unspliced and singly spliced viral RNAs from the nucleus</td>
</tr>
<tr>
<td>Negative regulatory factor</td>
<td>Nef</td>
<td>nef</td>
<td>Regulatory</td>
<td>Enhancer of viral infectivity by several mechanisms; facilitates packing, down-regulated CD4 and MHC-1,etc</td>
</tr>
<tr>
<td>Viral infectivity factor</td>
<td>Vif</td>
<td>vif</td>
<td>Regulatory</td>
<td>Enhancer of viral infectivity, specifically overcomes host-restriction activity ie APOBEC3G</td>
</tr>
<tr>
<td>Viral protein r</td>
<td>Vpr</td>
<td>vpr</td>
<td>Regulatory</td>
<td>Associated functions include G2-M cell cycle arrest, apoptotic cell death, transcription activity</td>
</tr>
<tr>
<td>Viral protein U</td>
<td>Vpu</td>
<td>vpu</td>
<td>Regulatory</td>
<td>Facilitates virion packaging and down regulates CD4, ion transport</td>
</tr>
</tbody>
</table>
1.2.2 HIV Attachment, fusion and entry

HIV productively infects mainly CD4$^+$ lymphoid cells such as T lymphocytes, monocytes, macrophages and dendritic cells (Dalgleish, Beverley, et al, 1984). HIV can also infect resting non-replicating CD4$^+$ cells of these lineages which may become latent reservoirs for the virus that are difficult to eradicate, particularly in the gut mucosa, brain and lungs and kidney (Lackner, and Veazey, 2007, Kinter, Moorthy, et al, 2003). Specificity for these cells is mediated by the HIV envelope glycoprotein’s affinity for the CD4 molecule on these cells (Dalgleish, Beverley, et al, 1984). The interaction is initially mediated by positive charges on the viral envelope which interact with negative electrostatic charges found at the cell surface which allows the virus to come into close proximity to its CD4 primary receptor (Ugolini, Mondor, and Sattentau, 1999). Binding to CD4 is then mediated by the viral envelope glycoprotein gp120, specifically the C2 loop region (Jones, Korte, and Blumenthal, 1998, Kwong, Wyatt, et al, 1998). Binding of gp120 to CD4 leads to a conformational change in gp120, which exposes the V3 loop and a binding motif for a chemokine co-receptor (Berger, Murphy, and Farber, 1999) (Kwong, Wyatt, et al, 1998, Rizzuto, Wyatt, et al, 1998). These motifs allow for interaction between gp120 and the chemokine co-receptor found in lipid rafts, which is required for efficient infection (Berger, Murphy, and Farber, 1999). The chemokine co-receptors utilized by HIV belong to two groups: the $\alpha$-chemokine receptors of which CXCR4 is most important and the $\beta$-chemokine receptors of which CCR5 is of principal importance (Berger, Murphy, and Farber, 1999, Littman, 1998). X4 viruses utilize the CXCR4 receptor to infect T-cells, while R5 viruses utilize CCR5 to infect monocytes, macrophages, and DCs (Doms, and Trono, 2000, Dragic, Litwin, et al, 1996, Alkhatib,
Figure 1.5: Tropism of HIV strains based on co-receptor preference
Transmission of HIV is mediated chiefly by R5 viruses utilizing CCR5 while X4 strains mostly accumulate in the host in late stage infection (Connor, Sheridan, et al, 1997).

Binding to the chemokine co-receptor results in a conformational change to the transmembrane glycoprotein gp41 whereby the coiled protein opens to project three fusion domains through the lipid bilayer of the host cell (Chan, and Kim, 1998). A subsequent hairpin formation promotes virion and target cell membrane fusion, which leads to the deposition of the core virus into the host cell (Chan, and Kim, 1998).

The paradigm described above for HIV binding, fusion and entry has been controversial and the role of chemokine coreceptors was met with initial scepticism. Indeed, there are many instances where the supposed CD4$^+$ plus CXCR4/CCR5 model does not fit, such as the case in which CD4$^+$ CXCR4$^+$ monocyte/macrophage U937 clones show variable infection levels (Moriuchi, Moriuchi, et al, 1997). Additionally, cells that do not express CD4 can become infected (Furuta, Eriksson, et al, 1994) (Harouse, Laughlin, et al, 1991, Tateno, Gonzalez-Scarano, and Levy, 1989). These discrepancies could be explained by alternative receptors utilized by the virus. In fact, one such example is VPAC1, the receptor for the vasoactive intestinal peptide (VIP) which is expressed on most CD4$^-$ cells and has a sequence similar to the CD4 binding region of gp120 (Pert, Ruff, and Hill, 1988), recognizing a sequence within the CD4 binding C2 loop of HIV gp120, and thus could serve as an alternative receptor to CD4 (Branch, Valenta, et al, 2002, Veljkovic, Metlas, et al, 1992). As well, as mentioned above (Section 1.1.4), DC-SIGN mediates the non-productive infection of DCs which carry HIV and transfer infectious virus to CD4$^+$ target cells (Miller, and Shattock, 2003).

The identification of another receptor for HIV-1, the C-type lectin receptor
langerin found on Langerhan’s cells (LCs) located in the mucosal epithelium, has caused recent controversy as these cells have been associated with both the prevention and transmission of HIV infection depending on host immune status (de Jong, and Geijtenbeek, 2009). This receptor provides protection against HIV-1 infection whereby langerin captures HIV-1, leading to viral degradation intracellularly (de Witte, Nabatov, et al, 2007). However, when co-infections with other STIs and inflammation occurs, LCs become activated, which results in HIV-1 replication in these cells, which then migrate to lymph nodes and transmit the virus to CD4$^+$ cells (de Jong, de Witte, et al, 2008; de Jong, and Geijtenbeek, 2009).

1.2.3 Viral life cycle

Once the virus has entered the host cell, the viral RNA genome is reverse transcribed by the viral enzyme RT resulting in a linear double stranded cDNA flanked by long terminal repeats (LTR) (Nisole, and Saib, 2004, Rausch, and Le Grice, 2004). Reverse transcriptase does not have 3’ to 5’ exonuclease activity to repair errors of transcription, and 1 in 1000 base pairs are mismatched (Keulen, Nijhuis, et al, 1997). This high mutation rate allows for the immense genetic diversity exhibited by the virus, and especially when coupled with selection pressures and a high turnover rate, allows for different viruses to exist within a single host (Lal, Chakrabarti, and Yang, 2005, Saag, Hahn, et al, 1988). The final product of reverse transcription is the HIV preintegration complex (PIC) consisting of viral cDNA, IN, matrix protein, Vpr, RT and the DNA binding protein HMGI(Y) (Miller, Farnet, and Bushman, 1997). The PIC translocates to the nucleus via nuclear pores (Miller, Farnet, and Bushman, 1997). Upon nuclear entry the proviral DNA may form 2-LTR circles, the function of which is debated (Delelis,
Petit, et al, 2005). Viral IN integrates the viral genome into the host genome, particularly into regions where active genes are located (Miller, Farnet, and Bushman, 1997, Schroder, Shinn, et al, 2002) and integrated viral DNA or provirus can remain dormant until the host cell becomes activated (Han, Wind-Rotolo, et al, 2007). An overview of the HIV lifecycle is depicted in Figure 1.6.
Figure 1.6: Overview of the HIV lifecycle
The LTR sequences of the provirus contain regions that bind nuclear factor κB (NF-κB), nuclear factor of activated T cells (NFAT), and Ets family members (Jones, and Peterlin, 1994). NF-κB is activated after antigen and cytokine mediated immune events when cytoplasmic kinases phosphorylate its inhibitor I-κB, releasing NF-κB to translocate to the nucleus where it binds to κB sequences in the promoter of different genes, including those for proinflammatory cytokines. Since these similar sequences exist within the LTR region of the HIV genome, an immune response activating CD4+ T cells will also trigger the transcription of the HIV provirus (Greene, and Peterlin, 2002). More than twelve HIV specific transcripts are generated from the transcription of the viral genome. Multiple splicing of HIV transcripts that are processed cotranscriptionally generate the mRNA sequences that encode the early viral proteins Nef, Tat and Rev, and these are transported to the cytoplasm (Greene, and Peterlin, 2002). Single splicing or HIV transcripts that are not spliced yield the structural accessory or late proteins such as Gag-Pol glycoprotein precursor molecule and the viral RNA genome required to produce progeny virions (Greene, and Peterlin, 2002).

Progeny virions are assembled and budding occurs at the plasma membrane of the host cell. Viral RNA is incorporated into capsids that bud from the cell surface and take up the viral envelope precursor protein, whose maturation occurs within the budding virion aided by viral protease. The viral membrane is derived from the host cell during budding (Greene, and Peterlin, 2002). It is interesting to note that the viral protein Nef is essential in productive HIV infection. CD4 is down-regulated by the protein which is necessary in preventing the interaction of precursor gp-120 molecules with CD4 which would hinder viral budding (Whetter, Ojukwu, et al, 1999). The viral protein Vif also
plays a critical role in preventing the host anti-viral proteins such as APOBEC3G from functioning (Goila-Gaur, and Strebel, 2008, Lecossier, Bouchonnet, et al, 2003, Sheehy, Gaddis, et al, 2002). (Figure 1.6)

The main mechanism of T cell immunodeficiency seen in HIV infection is via T cell lysis, which occurs when the cells are productively infected and progeny virions are produced (Hazenberg, Hamann, et al, 2000). Additionally most T cells can become depleted by other means such as induced apoptosis through the chronic activation of uninfected cells, loss of CD4\(^+\) cells through the formation of syncytia, and via the CD8\(^+\) cytotoxic T cell induced death of uninfected CD4\(^+\) T cells that are coated with gp120 from infected cells (Hazenberg, Hamann, et al, 2000).

1.2.4 Resistance factors

Natural resistance to HIV infection was first identified when CD4\(^+\) cells from individuals exposed to the virus without seroconversion were shown to resist HIV-1 infection with R5 (macrophage) tropic viruses (Paxton, Martin, et al, 1996). It was later determined that these individuals possessed a mutation in the gene encoding the CCR5 chemokine receptor which renders the receptor nonfunction and prevents entry of R5 HIV viruses (Huang, Paxton, et al, 1996). The Nairobi cohort of resistant sex workers in Kenya is another example of a population remaining uninfected with multiple known exposures (Fowke, Nagelkerke, et al, 1996). These individuals, termed “highly exposed persistently seronegative” (HEPS) remain uninfected despite numerous exposures (Fowke, Kaul, et al, 2000, Kulkarni, Butera, and Duerr, 2003). Other individuals become infected but control the infection well, maintaining a low viral load, have slower
progression to AIDS and are termed long term nonprogressors (LTNP) (Kulkarni, Butera, and Duerr, 2003). Mechanisms of resistance that have thus far been identified include genetic factors such as the CCR5-Δ32 mutation mentioned above, and human leukocyte antigen (HLA) alleles that may result in HIV antigen presentation that elicits an immune response (MacDonald, Matukas, et al, 2001). Other factors may include HIV specific IgA antibodies, and acquired immune system mediated responses including humoral immune responses, helper T cell function, and cytotoxic lymphocyte responses (Devito, Broliden, et al, 2000, Broliden, Hinkula, et al, 2001, Kulkarni, Butera, and Duerr, 2003).
1.3 The treatment and prevention of HIV/AIDS

Currently there are approved agents for the treatment of HIV/AIDS across six drug classes, based on mechanism. These classes include: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors (FIs), CCR5 antagonists and integrase inhibitors (IRs) (NIH, January 29, 2008). A summary of key drugs is provided in Table 1.3.1. Drugs initially targeted one or more of three HIV-1 proteins: reverse transcriptase, protease and the envelope glycoprotein gp41 (Gulick, Lalezari, et al, 2008). These strategies have been successful, however toxicity, patient compliance, and the development of HIV strains resistant to the drugs are areas of key concerns (Mocroft, Devereux, et al, 2000, Phillips, Dunn, et al, 2005). Additionally, current treatments are extremely expensive and not available to everyone, particularly those living in the developing world. For these reasons, the continued development of new treatment modalities is imperative, particularly those that prevent new infections. This section provides an overview of currently approved drugs and future prospects for the treatment and prevention of HIV/AIDS.

1.3.1 Drugs for the treatment of HIV/AIDS

There are currently 13 approved NRTIs for the treatment of HIV/AIDS (FDA, 2008). The first approved drug to treat HIV was in fact the NRTI zidovudine commonly referred to as AZT (Yarchoan, and Broder, 1987). These drugs target RT, and are structurally 2’,3’-dideoxynucleosides with a modified 3’hydroxyl group that cannot form phosphodiester bonds between nucleotides.
Table 1.3: FDA approved drugs for the treatment of HIV/AIDS

## Multi-class Combination Products

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Manufacturer Name</th>
<th>Year Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>efavirenz, emtricitabine and tenofovir disoproxil fumarate</td>
<td>Bristol-Myers Squibb and Gilead Sciences</td>
<td>2006</td>
</tr>
</tbody>
</table>

## Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

<table>
<thead>
<tr>
<th>Generic Name(s)</th>
<th>Manufacturer Name</th>
<th>Year Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>lamivudine and zidovudine</td>
<td>GlaxoSmithKline</td>
<td>1997</td>
</tr>
<tr>
<td>emtricitabine, FTC</td>
<td>Gilead Sciences</td>
<td>2003</td>
</tr>
<tr>
<td>lamivudine, 3TC</td>
<td>GlaxoSmithKline</td>
<td>1995</td>
</tr>
<tr>
<td>abacavir and lamivudine</td>
<td>GlaxoSmithKline</td>
<td>2004</td>
</tr>
<tr>
<td>zalcitabine, dideoxycytidine, ddC</td>
<td>Hoffmann-La Roche</td>
<td>1992</td>
</tr>
<tr>
<td>zidovudine, azidothymidine, AZT, ZDV</td>
<td>GlaxoSmithKline</td>
<td>1987</td>
</tr>
<tr>
<td>abacavir, zidovudine, and lamivudine</td>
<td>GlaxoSmithKline</td>
<td>2000</td>
</tr>
<tr>
<td>tenofovir disoproxil fumarate and emtricitabine</td>
<td>Gilead Sciences, Inc.</td>
<td>2004</td>
</tr>
<tr>
<td>enteric coated didanosine, ddI EC</td>
<td>Bristol Myers-Squibb</td>
<td>2000</td>
</tr>
<tr>
<td>didanosine, dideoxyinosine, ddI</td>
<td>Bristol Myers-Squibb</td>
<td>1991</td>
</tr>
<tr>
<td>tenofovir disoproxil fumarate, TDF</td>
<td>Gilead</td>
<td>2001</td>
</tr>
<tr>
<td>stavudine, d4T</td>
<td>Bristol Myers-Squibb</td>
<td>1994</td>
</tr>
<tr>
<td>abacavir sulfate, ABC</td>
<td>GlaxoSmithKline</td>
<td>1998</td>
</tr>
</tbody>
</table>

## Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

<table>
<thead>
<tr>
<th>Generic Name(s)</th>
<th>Manufacturer Name</th>
<th>Year Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>etravirine</td>
<td>Tibotec Therapeutics</td>
<td>2008</td>
</tr>
<tr>
<td>delavirdine, DLV</td>
<td>Pfizer</td>
<td>1997</td>
</tr>
<tr>
<td>efavirenz, EFV</td>
<td>Bristol Myers-Squibb</td>
<td>1998</td>
</tr>
<tr>
<td>nevirapine, NVP</td>
<td>Boehringer Ingelheim</td>
<td>1996</td>
</tr>
</tbody>
</table>

## Protease Inhibitors (PIs)

<table>
<thead>
<tr>
<th>Generic Name(s)</th>
<th>Manufacturer Name</th>
<th>Year Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>amprenavir, APV</td>
<td>GlaxoSmithKline</td>
<td>1999</td>
</tr>
<tr>
<td>tipranavir, TPV</td>
<td>Boehringer Ingelheim</td>
<td>2005</td>
</tr>
<tr>
<td>indinavir, IDV,</td>
<td>Merck</td>
<td>1996</td>
</tr>
<tr>
<td>saquinavir (no longer marketed)</td>
<td>Hoffmann-La Roche</td>
<td>1997</td>
</tr>
<tr>
<td>saquinavir mesylate, SQV</td>
<td>Hoffmann-La Roche</td>
<td>1995</td>
</tr>
<tr>
<td>lopinavir and ritonavir, LPV/RTV</td>
<td>Abbott Laboratories</td>
<td>2000</td>
</tr>
<tr>
<td>Fosamprenavir Calcium, FOS-APV</td>
<td>GlaxoSmithKline</td>
<td>2003</td>
</tr>
<tr>
<td>ritonavir, RTV</td>
<td>Abbott Laboratories</td>
<td>1996</td>
</tr>
<tr>
<td>darunavir</td>
<td>Tibotec, Inc.</td>
<td>2006</td>
</tr>
<tr>
<td>atazanavir sulfate, ATV</td>
<td>Bristol-Myers Squibb</td>
<td>2003</td>
</tr>
<tr>
<td>nelfinavir mesylate, NFV</td>
<td>Agouron Pharmaceuticals</td>
<td>1997</td>
</tr>
</tbody>
</table>
### Fusion Inhibitors

<table>
<thead>
<tr>
<th>Generic Name(s)</th>
<th>Manufacturer Name</th>
<th>Year Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>enfuvirtide, T-20</td>
<td>Hoffmann-La Roche &amp; Trimeris</td>
<td>2003</td>
</tr>
</tbody>
</table>

### Entry Inhibitors (CCR5 Co-Receptor Antagonist)

<table>
<thead>
<tr>
<th>Generic Name(s)</th>
<th>Manufacturer Name</th>
<th>Year Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>maraviroc</td>
<td>Pfizer</td>
<td>2007</td>
</tr>
</tbody>
</table>

### HIV Integrase Strand Transfer Inhibitors

<table>
<thead>
<tr>
<th>Generic Name(s)</th>
<th>Manufacturer Name</th>
<th>Year Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>raltegravir</td>
<td>Merck &amp; Co., Inc.</td>
<td>2007</td>
</tr>
</tbody>
</table>

(FDA, 2008)
They therefore function by terminating the nucleotide chain of viral DNA during reverse transcription. The NNRTIs are also RT inhibitors, however their mechanism of action is different in that NNRTIs block RT function by binding to allosteric sites on the enzyme and inhibit the movement of protein domains of RT that are required to carry out DNA synthesis. The first approved drug in this class was nevirapine (Merluzzi, Hargrave, et al, 1990) and there are currently four approved NNRTIs (FDA, 2008). Another drug class targets the HIV-1 enzyme protease. PIs bind to the active site of protease and inhibit the post-translational processing of proteins making up the viral core, as well as the other viral enzymes. Its inhibition leads to the production of immature non-infectious virions (Vella, 1994). The first drug approved in this class was Saquinavir mesylate in 1995, and there are currently 11 approved PIs (FDA, 2008).

Novel drug classes provide patients undergoing treatment failure due to drug resistant viruses alternative options (Soulie, Malet, et al, 2008). These classes of drugs often constitute what is referred to as ‘salvage therapy’ against viruses resistant to the other drug classes and include the fusion inhibitors (FI), entry inhibitors (EI) and integrase inhibitors (IR).

The first FI, enfuvirtide (ENF) or T20 was approved in 2003 (FDA, 2008). ENF is a 36 amino-acid peptide that mimics the amino acids in heptad repeat-2 (HR-2) of the HIV-1 gp41 envelope protein. ENF binds to residues in HR-1 and blocks the conformational change in gp41 required for fusion therefore preventing viral entry (Chen, Kilby, and Saag, 2002, Matthews, Salgo, et al, 2004). ENF must be administered via subcutaneous injection and the cost associated with 1 year of treatment is approximately $25,000 U.S (Hardy, and Skolnik, 2004). It is used as a salvage therapy when other
ARVs fail (FDA, 2008). Raltegravir, the first and thus far only IR, was approved by the FDA in 2007 (FDA, 2008). IN catalyzes the insertion of HIV-1 DNA into the host genome. IRs work by inhibiting strand transfer, where the 3’ end of viral DNA are covalently linked to the host DNA (Hazuda, Felock, et al, 2000). The first entry inhibitor Maraviroc (MRC) was approved in 2007 and is a CCR5 antagonist (FDA, 2008). By binding to CCR5, MRC prevents gp120 binding the coreceptor, and subsequent conformational changes allowing fusion to occur (Briz, Poveda, and Soriano, 2006). MRC, like the other new classes of drugs is indicated for treatment experienced patients undergoing ARV failure. Of particular concern with MRC use is the accumulation of X4 strains which are associated with late stage infection and the onset of AIDS (Soriano, Geretti, et al, 2008). Additionally, MRC use has already been shown to drive mutations in the V3 loop of gp120 (Soulie, Malet, et al, 2008). It remains to be seen what the long-term effects of CCR5 inhibitors will have since little is known about the normal function of this receptor, however individuals with the CCR5-Δ32 mutation show few negative consequences. Other CCR5 inhibitors (eg vicriviroc) not currently approved for use have been associated with hepatotoxicity and lymphoma (Emmelkamp, and Rockstroh, 2007). A recent study also showed that CCR5 deficiency was associated with extreme risk of West Nile virus infection (Glass, McDermott, et al, 2006). CXCR4 antagonists are also in various stages of development (Rusconi, Scozzafava, et al, 2007) however there have been reports of toxicity due to the relative importance of the CXCR4 receptor in haematopoiesis (Hendrix, Collier, et al, 2004).
1.3.2 HAART

Modern treatment is termed highly active antiretroviral therapy (HAART) and uses at least 3 drugs in combination in order to prevent resistant viral strains from evolving for as long as possible (Hammer, Eron, et al, 2008). The most common regimes for treatment naive patients utilize one NNRTI with two NRTIs or one PI with two NRTIs (NIH, January 29, 2008). The key goal of HIV/AIDS therapy is to decrease viral load to limits below the detection threshold (<50copies/mL) while increasing CD4 count and therefore immune function. Therapy is usually initiated under the following conditions: CD4 count less than 350cells/mm\(^3\), presentation of an AIDS defining illness, pregnancy, HIV nephropathy or Hepatitis B or TB coinfection (NIH, January 29, 2008). Adverse events have been reported for all ARVs and is the main reason for the discontinuation of treatment (O’Brien, Clark, et al, 2003). These can include but are not limited to hepatitis, hyperlipidemia, and cardiovascular disease (FDA, 2008). However, the complications that arise from untreated HIV/AIDS are worse than potential side effects of treatment.

Once treatment is initiated it must be continued for the duration of the patients’ lifetime as cessation of treatment or brief noncompliance leads to a swift return of detectable virus and a decreased CD4 count (Yeni, Hammer, et al, 2004, Weiss, 2008).

1.3.3 Topical strategies to prevent infection

The ultimate strategy to prevent HIV infection would be the development of a vaccine. Decades of research has thus far failed to produce a suitable candidate, and, in fact, a recent high profile clinical trial of a potential adenoviral vaccine actually increased infection and did not suppress viral load of those subsequently infected (Robb, 2008,
Anonymous, 2007, Watkins, Burton, et al, 2008). Condoms are an excellent way to prevent infection, however in many countries where the HIV incidence is high, men are reluctant to use them (Lederman, Offord, and Hartley, 2006). There has thus been renewed interest in new prevention strategies, and in particular, in the development of topical agents or “microbicides” for vaginal and/or rectal mucosal use for the purpose of preventing HIV transmission. These prevention methods are largely targeted to women, and would allow women to protect themselves in the event of condom refusal. These agents have a variety of mechanisms of action, and could be formulated as a gel or cream, cervical cap or ring. The ideal candidate however would be safe, and must not cause irritation or abrasion of the mucosal surface, as this would likely serve to increase HIV infection by the recruitment of target cells or viral entry into the bloodstream. There are currently 18 ongoing microbicide trials in various stages of testing (Alliance for Microbicide Development, 2008).

The issue of product safety is paramount and not as simple as it may seem. Many phase I and II studies have shown potential microbicide compounds to be safe, however in larger trials this has not been the case (Moscicki, 2008). Nonoxynol-9 (N9) a common spermicide detergent found in gel and foam preparations and as an ingredient in condoms, was deemed to be safe initially as a microbicide agent. However, in a large trial involving 700 women (Van Damme, Ramjee, et al, 2002), the relative risk of acquiring HIV infection was higher for those in the N9 group compared to control. For women reporting at least 3.5 applications of the gel daily, the risk of acquiring HIV was nearly double that seen in the placebo group (Van Damme, Ramjee, et al, 2002). Trials since then have had disappointing results. Two recent studies using a cellulose sulfate...
gel, an anionic polymer that blocks gp120 binding sites, were halted. First, the
CONRAD trial was stopped due to safety concerns similar to N9, as more HIV infections
were seen in the cellulose sulfate group compared to placebo (Van Damme, Govinden, et al, 2008). Another similar trial led by Family Health International was stopped due to lack of evidence supporting any efficacy (Moscicki, 2008). The trial involving C31G, a surfactant, was halted because there were too few seroconversions (HIV positive from negative status) to conduct any useful statistics on the data (Moscicki, 2008). Lastly, the trial involving the Population Council’s Carraguard, a seaweed based product that binds to the envelope of HIV-1 and inhibits cell trafficking of macrophages, showed no efficacy in reducing HIV infections (Skoler-Karpoff, Ramjee, et al, 2008).

Current trials largely target HIV machinery to either inhibit replication or viral fusion and entry. Accelerated trials involving ARVs already used as therapeutics include the Tenofovir gel trials, UC-781 and TMC120 (Moscicki, 2008, Alliance for Microbicide Development, 2008). A new product, SPL7013, (VivaGel) binds electrostatically to the viral envelope and prevents entry (Moscicki, 2008). For a listing of current microbicides in clinical trials see Table 1.4.
## Table 1.4: Microbicide candidates in ongoing clinical trials

<table>
<thead>
<tr>
<th>Phase</th>
<th>Candidate Name</th>
<th>Title of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>PRO 2000/5 gel</td>
<td>Efficacy and safety of 0.5% PRO 2000/5 gel for the prevention of vaginally acquired HIV infection</td>
</tr>
<tr>
<td>2B</td>
<td>Tenofovir gel</td>
<td>Safety and effectiveness of the vaginal microbicide 1% tenofovir gel to prevent HIV infection in women in South Africa (CAPRISA 004)</td>
</tr>
<tr>
<td>2/2B</td>
<td>PRO 2000/5 gel and Buffergel</td>
<td>Safety and effectiveness study of the vaginal microbicides BufferGel and 0.5% PRO2000/5 gel for the prevention of HIV infection in women</td>
</tr>
<tr>
<td>2</td>
<td>Tenofovir gel</td>
<td>Adherence and pharmacokinetics study of the oral and vaginal preparations of tenofovir</td>
</tr>
<tr>
<td>1/2</td>
<td>VivaGel</td>
<td>Assessment of local retention and duration of activity of SPL7013 following vaginal application of 3% SPL7013 gel in healthy volunteers</td>
</tr>
<tr>
<td>1</td>
<td>Dapivirine gel</td>
<td>Safety and pharmacokinetics of two intravaginal dapivirine gel formulations in healthy HIV negative women</td>
</tr>
<tr>
<td>1</td>
<td>Ethanol in Emollient gel</td>
<td>Safety and acceptance of 62% ethanol in emollient gel as a topical male microbicide</td>
</tr>
<tr>
<td>1</td>
<td>HEC/CS/N9</td>
<td>Assessment of markers of inflammation after vaginal use</td>
</tr>
<tr>
<td>1</td>
<td>PRO 2000</td>
<td>Postcoital anti-viral activity of cervicovaginal secretions following intravaginal application of 0.5% PRO 2000/5gel</td>
</tr>
<tr>
<td>1</td>
<td>Tenofovir gel</td>
<td>Pharmacokinetic study of the vaginal microbicide agent 1% tenofovir gel</td>
</tr>
<tr>
<td>1</td>
<td>Tenofovir gel</td>
<td>Maternal pharmacokinetics and placental perfusion of tenofovir/PMPA gel</td>
</tr>
<tr>
<td>1</td>
<td>Tenofovir gel</td>
<td>Effect of repeated applications of tenofovir gel on mucosal mediators of immunity and intrinsic antimicrobial activity of cervicovaginal excretions</td>
</tr>
<tr>
<td>1</td>
<td>UC-781 gel</td>
<td>Safety and persistence of 0.1% UC-781 vaginal gel in HIV-1 seronegative women</td>
</tr>
<tr>
<td>1</td>
<td>UC-781 gel</td>
<td>Safety and acceptability study of the UC-781 vaginal microbicide gel formulation applied rectally in HIV-1 seronegative adults</td>
</tr>
<tr>
<td>1</td>
<td>UC-781 gel</td>
<td>Safety and acceptability of 0.1% and 0.25% UC-781 topical vaginal microbicide and acceptability in their male partners</td>
</tr>
<tr>
<td>1</td>
<td>UC-781 gel</td>
<td>Male tolerance study</td>
</tr>
<tr>
<td>1</td>
<td>UC-781 gel</td>
<td>Safety and acceptability of UC-781 topical vaginal microbicide in heterosexual women and male partners</td>
</tr>
<tr>
<td>1</td>
<td>VivaGel</td>
<td>Safety and acceptability of 3% w/w SPL7013 Gel applied vaginally in sexually active young women</td>
</tr>
</tbody>
</table>

(Alliance for Microbicide Development, 2008)
Currently there is no standard in safety testing of these products *in vivo* and this problem is enhanced by a lack of understanding of the inflammatory process at the mucosal level of vaginal and rectal tissue. There are studies however that address this concern, and the recent failures, particularly the N9 trial, have led to a better understanding of the markers of inflammation that could potentially act to increase HIV infection. Exposure to N9 has been shown to increase the inflammatory cytokines MCP-1, RANTES, MIP-2, IL-1α and β, IL-6 and TNF and cause epithelial sloughing, therefore resulting in the recruitment HIV target cells (Galen, Martin, et al, 2007). Studies such as these will be useful in the safety screening of microbicide candidates *in vivo*.

### 1.3.4 Male circumcision

Epidemiological data has shown that male circumcision is associated with decreased risk of infection (Weiss, Quigley, and Hayes, 2000). The foreskin of the penis is rich in HIV target cells (Patterson, Landay, et al, 2002). It is also thought that a lack of circumcision can result in prolonged inflammation due to other sexually transmitted infections (STIs), which may act to increase susceptibility by the further recruitment of target cells (Weiss, 2007). The first randomized trials comparing circumcised to uncircumcised men, confirmed these observations, as male circumcision prevented female to male transmission of the virus by 60% (Gray, Kigozi, et al, 2007)(Auvert, Taljaard, et al, 2005). Currently there are widespread clinical programs in key endemic areas such as Africa to raise awareness, and many men have been willing to undergo circumcision to protect themselves from the virus.
1.4 Glycosphingolipids

1.4.1 Introduction to glycosphingolipids: Classification, biochemistry and synthesis

Glycosphingolipids (GSLs) are an important component of cellular membranes. These carbohydrate-lipid conjugates consist of a hydrophobic ceramide backbone that contains a conserved sphingolipid base joined to a variable fatty acid chain. This backbone embeds in the lipid bilayer of mammalian cells. The hydrophilic head group is the carbohydrate portion of the GSL and faces the extracellular space (Stults, Sweeley, and Macher, 1989, Degroote, Wolthoorn, and van Meer, 2004). Greater than 60 sphingolipid bases and 300 distinct oligosaccharide chains have been characterized so far (Degroote, Wolthoorn, and van Meer, 2004). GSLs are classified as either neutral, acidic or basic (Hakomori, 2003). The acidic group consists of GSLs containing sialic acid or a sulphate group. There are four major groups of neutral GSLs and they are characterized by their core structure: globo-(Galα1-4Gal), lacto-(Galβ1-3GlcNAc), neolacto-(Galβ1-4GlcNAc) and ganglio-(Galβ1-3 GalNAc).

The first step in GSL synthesis is the condensation reaction of serine and palmitoyl-CoA to 3-ketosphinganine, which is the precursor molecule for all sphingolipid bases (Degroote, Wolthoorn, and van Meer, 2004). Dihydroceramide is produced by the acylation of this molecule which is then oxidized to form ceramide. This step occurs in the cytosolic leaflet of the rough ER. Ceramide then follows one of two biosynthetic pathways as follows: (1) It can be converted in the ER lumen to gala-series glycolipids via the addition of galactose by β-glycosidic link producing galactosylceramide (GalCer) (Sprong, Kruithof, et al, 1998). The addition of a sulphate group to the 3-position of galactose on GalCer produces Sulphatide (SGC). (2) Ceramide transported to the Golgi is
converted to glucosylceramide (GlcCer) by the addition of glucose via a $\beta$-glycosidic link. The enzyme responsible is glucosyltransferase, and GlcCer is the precursor molecule of most GSLs (Futerman, and Pagano, 1991). This reaction occurs at the cytosolic surface of the Golgi. GlcCer is translocated by P-glycoprotein (a flippase) to the lumen of the Golgi (De Rosa, 2003) where the synthesis of all other GSLs occurs by means of specific glycosyltransferases (Lannert, Gorgas, et al, 1998). GlcCer is first modified to produce lactosyl ceramide (Gal $\alpha$1-4Glc-cer, LacCer) which can then be sialated to produce siaolosyl derivatives including the (a) monosialogangliosides GM3 (NeuNAc $\alpha$2-3 Gal$\beta$1-4 Glc-cer), GM2 and GM1; (b) disialogangliosides GD3, GD1a and GD1b and (c) trisialoganglioside GT1b. LacCer can also be galactosylated to form globotriaosylceramide (Gb$_3$; Gal$\alpha$ 1-3 Gal$\beta$1-4Glc-cer). Gb$_3$ can then convert to globotetraosylceramide (Gb$_4$; GalNAc $\beta$1-3 Gal $\alpha$1-4 Gal $\beta$1-4Glc-cer). Synthesized GSLs are integrated into the outer layer of the plasma membrane.

1.4.2 Cellular Functions

GLSs play a crucial role in embryonic development. This is apparent when evaluating the resultant phenotypes of knockout mice. When the major synthetic pathway of GSLs is stopped by targeted disruption of the gene encoding glucosylceramide synthase in mice, the resultant embryos are non-viable as tissue development is arrested (Yamashita, Wada, et al, 1999). This suggests an essential role for GSLs in the process of development, particularly in tissue differentiation. Other models show GSLs are important in nervous system development, spermatogenesis, and the maintenance of skin integrity (Sheikh, Sun, et al, 1999, Takamiya, Yamamoto, et al, 1998, Inoue, Fujii, et al, 2002). It is not surprising, given that GSLs are critical to
development, that abnormal GSL expression has been associated with neoplasia. Several 
GSLs have been identified as tumour-associated antigens (Hakomori, and Kannagi, 1983)
and may aid tumor progression through adhesion functions or by modulating tumour 

GSLs also act as cellular markers or antigens including blood group antigens of 
the Lewis, ABH, I/i and P/P₁/Pᴾ blood groups. The P/P₁/Pᴾ blood group will be discussed 
further in a later section. Gb₃, also known as CD77, is a marker of B cell differentiation 

1.4.3 Lipid Rafts

GSLs are a key component of ordered membrane domains called lipid rafts. 
These domains consist primarily of GSLs, sphingomyelin (SM), phospholipids and 
cholesterol with associated proteins such as glycosylphosphatidylinositol (GPI) anchored 
proteins (Simons, and Ikonen, 1997). Caveolae are tiny indentations in the cell 
membrane associated with caveolin scaffolding and are a morphological characteristic of 
rafts (Kurzchalia, and Parton, 1999). Compared to the non-raft membrane, components of 
lipid rafts are tightly associated due to the saturated hydrocarbon chains of the GSLs and 
phospholipids which enable close interaction with cholesterol (Rajendran, and Simons, 
2005, Simons, and Vaz, 2004). Additionally, these structures are mobile within the 
membrane and may combine to form larger lipid raft domains within the membrane 
(Rajendran, and Simons, 2005).

Many studies have shown lipid rafts to play an important role in cell signalling. 
The carbohydrate portion of GSLs as mentioned above, can play a role in cell recognition 
and corresponds to some blood group and development associated antigens.
Additionally, ligand binding with GSL components has been shown to induce signaling cascades (Hakomori, Yamamura, and Handa, 1998, Iwabuchi, Zhang, et al, 2000) and rafts may also be involved in the intracellular trafficking of raft-associated lipids and proteins (Ikonen, 2001, Mukherjee, and Maxfield, 2000).

### 1.4.4 GLSs as receptors for pathogens

Many GSLs have been shown to play an important role in the interactions between pathogens and host cells, including viruses, bacteria and bacterial toxins, and can initiate pathogenic events including entry of the pathogen into the host cell, adhesion, changes in growth, differentiation, migration and apoptosis (Degroote, Wolthoorn, and van Meer, 2004). Several GSLs have been identified as receptors for bacteria and bacterial toxins (Lingwood, 1998). *Helicobacter pylori* which has been identified as causative agent associated with peptic ulcers and gastritis and has been shown to bind to SGC, LacCer and other gangliosides (Tang, Seino, et al, 2001). Additionally, the globo-series of GSLs expressed on the urogenital epithelium are sites of infection for *Escherichia coli* (Stapleton, Nudelman, et al, 1992, Stapleton, Stroud, et al, 1998). Soluble bacterial toxins bind to GSL receptors. Gb\(_3\) is the receptor for the *E. coli* elicited verotoxin (VT) and the *Shigella* spp. elicited Shiga toxin, while GM1 is used by the cholera toxin from *Vibrio cholerae* (Lingwood, 2003, Lingwood, 1999, Lencer, Hirst, and Holmes, 1999). Additionally, GSLs are involved in the attachment and fusion of viruses to host cells (Haywood, 1994).
1.5 Globotriaosylceramide (Gb\textsubscript{3})

Studies have particularly implicated the GSL globotriaosylceramide (Gb\textsubscript{3}) as important in HIV infection. This section will provide a brief overview of Gb\textsubscript{3}. A review of the role of Gb\textsubscript{3} in HIV infection will follow in the subsequent section.

1.5.1 Characteristics and cellular functions

Gb\textsubscript{3} (also referred to as CD77/P\textsuperscript{k}) is a neutral sphingolipid containing the trisaccharide core unit Gal\textalpha\textsubscript{1}-4Gal\textbeta\textsubscript{1}-4Glc linked to a ceramide backbone (Figure 1.7). Like other GSLs, the length and degree of saturation and/or hydroxylation of the fatty acid chain can vary between different isoforms. Gb\textsubscript{3} is expressed mainly in the kidney cortex and medulla, in addition to the heart, spleen, placenta and some cells of the hematopoetic system (Boyd, and Lingwood, 1989, Mangeney, Richard, et al, 1991, Kojima, Fukumoto, et al, 2000). In B cells, Gb\textsubscript{3} (CD77) is expressed at a specific stage of tonsillar B lymphocyte differentiation in the germinal centre (Mangeney, Richard, et al, 1991, Wiels, Mangeney, et al, 1991). Additionally, Gb\textsubscript{3} (P\textsuperscript{k}) is a blood group antigen (section 1.5.2).

Gb\textsubscript{3} is particularly well known as the receptor for \textit{E. Coli} elicited verotoxin (VT) (Lingwood, 1999) and is thus critical to the induction of VT derived pathologies such as haemolytic uremic syndrome (HUS) and hemorrhagic colitis (Karmali, 1989, Lingwood, Law, et al, 1987, Lingwood, 1999).
1.5.2 The P/P<sub>1</sub>/P<sup>k</sup> blood group system

A short overview of the P blood group system is necessary, as Gb<sub>3</sub> belongs to the class of P/P<sub>1</sub>/P<sup>k</sup> blood group antigens. P (also known as globoside or Gb<sub>4</sub>) and P<sup>k</sup> (Gb<sub>3</sub>) antigens are found on nearly all individuals. As well, 80% of Caucasians also exhibit P<sub>1</sub> antigens (Steffensen, Carlier, et al, 2000). When all three antigens are expressed, the individual is of the P<sub>1</sub> phenotype. When P<sub>1</sub> is not expressed the phenotype is denoted P<sub>2</sub>.
P₁ and P₂ are the most common phenotypes found in this blood group (Spitalnik, and Spitalnik, 1995).

Genetic anomalies result in other, rare phenotypes. Individuals with a mutation in the B3GALNT1 gene are deficient in the P antigen, which is caused by a non functioning Gb₄ synthase (βGalNAc transferase). This mutation results in increased expression of the precursor molecule, Pᵏ (Gb₃). These individuals may or may not express the P₁ antigen. Those expressing the P₁ antigen are of the P₁ᵏ phenotype, while those not expressing P₁ are of the P₂ᵏ phenotype (Spitalnik, and Spitalnik, 1995). Individuals with a mutation in the A4GALT gene do not express any P/P₁/Pᵏ antigens due to a non-functional Gb₃ synthase (α4Gal transferase) and exhibit the rare p phenotype (Furukawa, Iwamura, et al, 2000) completely lacking Gb₃ expression.

### 1.5.3 Fabry Disease

Defects in the enzymes responsible for the degradation of GSLs in lysosomes can lead to their accumulation in these organelles and subsequent serious pathologies result in the organism. Fabry disease for example, is a rare X-linked lysosomal storage disorder caused by a genetic deficiency of the enzyme α-galactosidase A (Brady, Gal, et al, 1967) (Ries, Clarke, et al, 2007). Lipids containing α-D-galactosyl moieties such as globotriaosylceramide (Gb₃), fail to catabolize and accumulate in the lysosomes of affected tissue (Brady, Gal, et al, 1967, Ries, Clarke, et al, 2007). Their accumulation can lead to kidney failure, cardiac abnormalities, peripheral nerve defects and an overall shortened and decreased quality of life (Ries, Clarke, et al, 2007). Enzyme replacement therapy is now available, however it is very costly (Rohrbach, and Clarke, 2007).
1.6 Role of Glycosphingolipids in HIV-1 infection

1.6.1 GSLs and HIV infection


GSLs are also involved in other events of the HIV viral life. Virion production and budding is dependant on GSL containing lipid rafts (Brown, 2002). Aberrant GSL expression has been reported in HIV infected individuals and antibodies to the GSLs Gb₃, GM3 and GD3 have been described as well (Fantini, Tamalet, et al, 1998, Misasi, Sorice, et al, 2000). Additionally, GSLs have been implicated in the infection of CD4⁺ cells, chiefly GalCer through transcytosis (Meng, Wei, et al, 2002).

1.6.2 Gb₃ and HIV infection

The GSL globo tria osylceramide (Gb₃) has been particularly implicated in HIV infection, namely in the mechanism of virus and host cell fusion (Hug, Lin, et al, 2000, Puri, Hug, et al, 1999, Puri, Hug, et al, 1998). In Fantini’s model (Fantini, Hammache, et al, 2000) gp120 binds to Gb₃ in lipid rafts and this binding facilitates the migration of the CD4-gp120 complex to the chemokine coreceptor, as mentioned earlier. Specifically, initial binding of gp120 to CD4 causes a conformation change, exposing the V3 loop and binding motifs for the chemokine co-receptor as well as a Gb₃ binding motif (Delezay, Hammache, et al, 1996, Xiao, Owen, et al, 1998). Interaction with the GSL and the chemokine receptor would thus lead to membrane fusion and implies that Gb₃ is an important facilitator of viral fusion (Cormier, and Dragic, 2002, Delezay, Hammache, et al, 1996, Mahfoud, Mylvaganam, et al, 2002, Sakaida, Hori, et al, 1998). However, Gb₃ is not expressed on CD4⁺ human T cells; thus, this model has severe limitations.

1.6.3 Gb₃: A novel resistance factor in HIV infection

In contrast to the above, studies conducted by our group support a role for Gb₃ in resisting HIV. We have developed a Gb₃ analogue, adamantylGb₃ that is water soluble and maintains its receptor function, as confirmed through VT/Gb₃ binding assays that

We have shown that adamantylGb3 markedly reduces HIV infection of Jurkat T-cells and peripheral blood mononuclear cells (PBMCs) regardless of viral tropism or resistance to antivirals, by preventing viral fusion (Lund, Branch, et al, 2006). PBMCs from individuals with Fabry disease, the lysosomal storage disease discussed earlier, where a defective α-galactosidase A enzyme leads to an accumulation of Gb3 in cells, show resistance to HIV infection (Lund, Branch, et al, 2005). These studies were further supported by data showing differences in susceptibility to HIV infection of PBMCs expressing varying amounts of Gb3. PBMCs from the blood group p phenotype which lack Gb3 are highly susceptible to HIV infection compared to controls. In contrast, the P1k phenotype, which highly expresses Gb3, are resistant to infection (Lund, Olsson, et al, Submitted). Furthermore, we have shown that the chemical induction of Gb3 accumulation in HIV-1 infectable cell lines results in resistance to infection, while pharmacological blocking of the Gb3 synthetic pathways increases cellular susceptibility to HIV-1 infection (Ramkumar, Sakac, et al, 2008).

These results propose an overall protective function for Gb3 and suggest that Gb3 could be a novel resistance factor in HIV infection. It is imperative that new strategies be developed to combat the virus and these findings support the further study of Gb3 and HIV infection, in particularly to elucidate a possible function for Gb3 as a therapeutic.
1.6.4 FSLGb₃, a novel Gb₃ analogue

We have obtained a new soluble Gb₃ mimic termed FSLGb₃ (Functional head Spacer Lipid tail-Gb₃) from KODE Biotech New Zealand (See Figure 1.8). The carbohydrate moiety of Gb₃ is maintained and coupled to phosphatidyl ethanolamine through a phosphate linker. The 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. Animal studies have shown no toxicity at mM quantities systemically.

![Figure 1.8: Structure of the new soluble Gb₃ analogue, FSLGb₃.](image)

1.6.5 Potential for a mouse model of to test GSL microbicide candidates using Pseudotyped HIV

All clinical trials testing potential microbicides against HIV infection in humans have thus far failed. It is therefore imperative that new methods to prevent infection continue to be explored. The most relevant currently available model to test potential
microbicide compounds is the nonhuman primate model using macaques and a SHIV virus (Kish-Catalone, Pal, et al, 2007). These model systems are extremely expensive, therefore it is prudent to be completely confident in the efficacy of new products before translating basic in vitro data to a nonhuman primate model. To further complicate in vivo testing of HIV therapeutics, a mouse model, which is the gold standard in many other model systems of human disease is not relevant here, since HIV does not infect normal mouse cells. To address this however, mouse models have been developed using severe combined immunodeficient mice (SCID) which have been reconstituted with cells of the human hematopoietic system, in particular HIV target cells. These mice can be infected with HIV-1 viruses, however low infection rates have held back their use (D'Cruz, and Uckun, 2007). Additionally, not all research centres, including the University of Toronto, have access to Level 3 biosafety animal facilities that would enable testing in these mice with intact HIV-1. HIV viruses pseudotyped with amphotropic envelopes of viruses such as the vesicular stomatitis virus (VSV) could potentially be used in the development of a mouse model system to test microbicide candidates. We acknowledge that a significant weakness to this model system would be the lack of gp120 envelope glycoproteins that are involved in HIV infection. We have however determined that the VSV envelope protein VSV-G binds to GSLs in a similar manner as HIV gp120, and that VSV-G binds to Gb3 (Mahfoud, and Manis, 2008). Additionally, VSV-G contains a sequence that is similar to the GSL binding motif found on gp120. The HIV genome used, (NL43-luc) is replication deficient in that it is env negative and the function of nef has been abolished due to a luciferase (luc) insertion (Figure 1.9). The virus therefore has only one infection cycle and viral entry can be
monitored by PCR for HIV cDNA or through the luciferase reporter. Therefore, we believe that a pseudoenvelope-typed HIV virus (VSV/HIV) could provide the basis for a new model system to test microbicide candidates \textit{in vivo}, and in particular, to test the efficacy of Gb\textsubscript{3} in the prevention of mucosal infection.
Figure 1.9: Genome structure of NL4-3luc HIV-1 used to produce replication deficient virions
1.7 Hypothesis and Specific Aims

1.7.1 Hypotheses

1) Through a collaboration with KODE Biotech™ of New Zealand we have obtained a new soluble construct of Gb₃, FSLGb₃. We hypothesize that FSLGb₃ will inhibit HIV-1 infection similarly to adamantylGb₃, with the added advantages that mass production in standardized conditions is possible, and that minimal cellular toxicity has been observed. If effective, FSLGb₃ would thus be a good candidate to explore as an HIV therapeutic.

2) New strategies to combat HIV-1 are necessary to decrease the global devastation of the pandemic, particularly in the developing world. Previously, we have shown that the soluble GSL analogue of Gb₃, adamantylGb₃, markedly reduces both X4 and R5 tropic HIV-1 strains in vitro.

   We hypothesize that when added to a vaginal cream or gel, adamantylGb₃ would prevent or decrease transmission of the virus at the level of the vaginal and/or rectal mucosa by preventing viral fusion as consistent with our previous studies, and could therefore be utilized as a microbicide to prevent new infections. Here, our goal is to obtain in vivo data that could provide rationale to move to a macaque nonhuman primate model investigating soluble Gb₃ as a microbicide. We propose to develop a mouse model using VSV/HIV that will potentially infect mice. Pilot studies conducted by our group have shown adamantylGb₃ to inhibit both VSV/HIV and HIV-1 infection in vitro. If FSLGb₃ proves to be an effective inhibitor of HIV-1, we would test the compound against our mouse model of mucosal infection as well.
1.7.2 Specific Aims

1) Determine the efficacy of FSLGb3 as an inhibitor of HIV-1 infection *in vitro* in both cell lines and human PBMCs.

2) Develop a mouse model for the study of mucosal transmission of HIV-1 for use in a Level 2 biosafety environment using a modified HIV-1 pseudovirus (VSV/HIV).

3) Use this mouse model to test the efficacy of soluble Gb3 as a microbicide against HIV-1 infection.

4) Determine if application of soluble Gb3 to rectal and vaginal mucosa is safe by evaluating proinflammatory cytokine profiles of mouse lavage fluid over time after application of the compounds.
CHAPTER 2: MATERIALS AND METHODOLOGY
2.1 Compounds

2.1.1 AdamantylGb₃

AdamantylGb₃ was produced in the laboratory of Dr. Clifford Lingwood at the Hospital for Sick Children’s Research Institute by Ronghua Yuan and Myls Mylvaganam. Gb₃ was first purified from human kidney, which was obtained from an autopsy as described (Strasberg, Grey, et al, 1989). Briefly, human kidney from the Pathology Department at Toronto General Hospital (stored at -20°C) was minced into ~0.5cm pieces, mixed with an equivalent volume of PBS and blended to homogeneity. The homogenate was added to 20 volumes of CHCl₃:MeOH (2:1, vol/vol) and stirred vigorously for 18 hours. The extract was filtered and water was added to it to obtain a Folch partition (CHCl₃:MeOH:H₂O, 2:1:0.6, vol/vol/vol) which was shaken vigorously and allowed to separate overnight. The lower (organic) phase was collected and dried using a rotating evaporator. The sample was dissolved in a minimal volume of CHCl₃:MeOH (98:2, vol/vol) and run through a batch elution silica column to isolate the neutral glycolipids (neutral glycolipids elute with acetone:methanol (9:1, vol/vol)). The elutant was saponified (2-5mg lipid/mL 1M NaOH in MeOH and pH adjusted to ~8 with HCl) where lipid contaminants (base-labile phospholipids and other glycerolipids) were removed as they partition into the upper phase of a Folch partition. The lower phase was isolated and run through a DEAE-Sephadex A-25 column to remove sulfated glycolipids and finally the sample was loaded onto a second silica column to fractionate remaining GSLs and isolate Gb₃. Purity was determined when a single band (corresponding to a known standard) appeared on a TLC sprayed with orcinol when an excess (~10µg) of the sample was loaded.
AdamantylGb3 was prepared as described previously (Mylvaganam, and Lingwood, 1999). Dimethylformamide was added to a solution of oxalyl chloride in dichloromethane. Adamantane acetic acid was then slowly added over 30 minutes. After stirring at room temperature for 2 hours, oxalyl chloride in excess and solvent were removed under a stream of N2, and residual adamantane acetic acid was dissolved in dichloromethane. LysoGb3, prepared by hydrolysis of Gb3, was suspended in dichloromethane and pyridine, and then 2 aliquots of the adamantane acetic acid solution were added at 30-minute intervals. After the reaction, the mixture was dried under N2 (TLC; chloroform:methanol:water, 80:20:2 (v/v/v)) and the product purified on a mini silica column. Stock solutions were obtained by dissolving the powdered compound in PBS.

### 2.1.2 FSLGb3

FSLGb3 (Functional head Spacer Lipid tail) was obtained from KODE™ Biotech (Auckland, New Zealand). FSLGb3 was synthesized by Lectinity Inc. (Moscow, Russia) by conjugating spacer-armed Gb3 trisaccharides to dioleoyl-phosphatidyl-ethanolamine with the help of an adipate linker as previously described (Frame, Carroll, et al, 2007). Stock solutions were obtained by dissolving the powdered compound in PBS. Purity was verified by mass spectroscopy by KODE.

### 2.2 Cell lines

Jurkat C were from Dr. Branch and 293T cells were purchased from American Type Culture Collection (ATCC, Rockville, Maryland, USA). NIH3T3 cells were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, Maryland, USA). The HEC1A human endocervical cell line and the Vk2 human vaginal...
keratinocyte cell line were a kind gift from Dr. Charu Kaushic (McMaster University, Hamilton, ON). The Jurkat C non-adherent cell line was maintained in complete RPMI (RPMI-1640, 10% FBS, 10μM gentamicin antibiotics (Gibco-Invitrogen, Burlington, ON)). Both adherent 293T cells and NIH3T3 cells were maintained in complete DMEM (DMEM, 10% FBS, 10μM gentamicin (Gibco)). These adherent cells were cultured on 10cm plates and split twice weekly by first washing with PBS and then applying 1mL of trypsin-EDTA (Gibco) for 5 minutes. Cells were then washed with PBS and centrifuged to remove the trypsin. The Hek1A cell line was maintained in 90% McCoy’s 5a medium (Gibco) supplemented with 10% FBS. Vk2 cells were maintained in keratinocyte-serum free media (Gibco) supplemented with the following: 0.1 ng/mL human recombinant EGF, 0.05 mg/mL bovine pituitary extract and 44.1 mg/mL calcium chloride (Gibco). Both of these adherent cell lines were maintained on 10cm plates as described above. All cell lines were maintained at 37°C and at 5% CO₂.

2.3 Human peripheral blood mononuclear cells (PBMCs)

Whole blood was obtained from healthy volunteers after informed consent. Blood was mixed with an equal volume of phosphate buffered saline (PBS) without calcium and magnesium (Gibco) and heated to 37°C in a water bath. In 50mL tubes, 35mL of blood was overlayed onto 15mL of Ficol-Paque separation medium (GE Healthcare, Baie d’Urfé, Quebec) and peripheral blood mononuclear cells were isolated using density gradient centrifugation at 1800 rpm for 30 minutes without the break on. PBMCs were then washed with PBS and centrifuged at 1200 rpm in 15mL falcon tubes three times until the PBS was clear. PBMCs were resuspended in complete RPMI as described
above, and activated for 3 days with 5µg/mL phytohaemagglutinin (PHA, Sigma-Aldrich Chemical, Oakville, ON) and interleukin-2 (IL-2; Invitrogen, 100U/mL) After 3 days the PBMCs were washed and centrifuged at 1200rpm and resuspended in RPMI media containing just IL-2 prior to infection by HIV-1.

2.4 HIV-1 viruses and in vitro infections

2.4.1 Viruses

X4 HIV-1\text{IIIB} and R5 HIV-1\text{Ba-L} were obtained from the NIH AIDS Research and Reference Reagent Program (NIH/AIDS). Viral stocks were grown in Jurkat C for HIV-1\text{IIIB} or PBMCs (Ba-L) and multiplicity of infection was calculated using MT-4 cells for HIV-1\text{IIIB} or calculated based on total p24\text{gag} and infectivity for HIV-1\text{Ba-L}, measured by enzyme-linked immunosorbant assay (ELISA; ZeptoMetrix, Buffalo, NY). Infections were carried out in the Level 3 containment facility at the University of Toronto.

2.4.2 Viral pre-incubation experiments

Briefly, for pre-incubation experiments, HIV-1\text{IIIB} or HIV-1\text{Ba-L} was incubated for 1 hour at 37°C with adamantylGb\text{3} or FSLGb\text{3} over the concentration range 50 - 1000µM prior to HIV-1 infection. 1x10\text{6} Jurkat C or PBMCs were infected for 1 hour at 37°C. For infections using PBMCs, cells were activated with PHA/IL-2 for 3 days prior to infection. After infection, cells were washed extensively (4X) with PBS without MgCl\text{2}/CaCl\text{2} and cultured in complete RPMI or complete RPMI supplemented with IL-2 (PBMCs). Culture supernatants were taken 3 days after infection and every other day thereafter, up to day 12 for measurement of p24\text{gag} antigen production by ELISA.

2.4.3 Cell pre-incubation experiments

For cell pre-incubation experiments with FSLGb\text{3}, 1x10\text{6} Jurkat C cells were pre-
incubated for 1 hour at 37°C with varying concentrations of FSLGb$_3$ or RPMI media as control. Cells were then washed extensively with PBS without MgCl$_2$/CaCl$_2$. These cells were then transported to the Level 3 facility as described previously, and infected with HIV-1$_{\text{IIIB}}$ (m.o.i.; 0.1) for 1 hour at 37°C. Cells were then washed extensively with PBS and cultured as described above.

### 2.4.4 Infection of adherent cervical and vaginal cell lines

For infection of Hec1A and Vk2 cells, 5x10$^5$ cells were plated 24 hours before infection in 6 well plates and maintained in 2mL of their respective media as described above. HIV-1$_{\text{IIIB}}$ was pre-treated with DNase I as per kit directions (Promega). Next, 400µM FSLGb$_3$ was incubated with the HIV-1 DNAse I treated virus for 1 hour (final volume 100µL). 100µL of the respective media for these cells was then added to the viral mixture. Media was removed from the cell monolayers and the 200µL virus mixture was added to the cells which were then incubated at 37°C and 5% CO$_2$ for 2 hours. The infection solution was removed and cells washed 4 times with PBS lacking MgCl$_2$/CaCl$_2$. The cell monolayers were then incubated for 24 hours. Cells were then trypsinized and lysed for removal from the BSL-3 Facility using protocols approved by the University of Toronto for testing of viral cDNA by PCR.

### 2.5 FACS analysis

To determine the effects of pre-incubation of FSLGb$_3$ on cell surface Gb$_3$ expression and/or cell surface receptor expression, 5x10$^5$ Jurkat cells were incubated with varying concentrations of FSLGb$_3$ for 1 hour at 37°C. Cells were then washed with PBS and pelleted in eppendorf tubes for 5 minutes by centrifugation at 4000 rpm. Cells were
resuspended in 100µL 10% human serum plus FACS buffer (PBS, 2% FBS, 0.1% sodium azide, 5mM EDTA) and incubated on ice for 20 minutes, then centrifuged again and resuspended in 100µL FACS buffer. For Gb3 staining, 1.5µL of mouse anti-Gb3 monoclonal antibody (clone BGR23, SeikagakuCorp., Tokyo, Japan) was added per tube, then incubated on ice for 30 minutes, washed and resuspended in 100µL FACS buffer. Then 10µL of the secondary antibody APC-labelled goat anti-mouse (10% solution; Invitrogen Molecular Probes) was added, followed by a 30 minute incubation on ice and washing as above. If another conjugated antibody was used after this, the cells were blocked with 20µL 10% mouse serum in FACS buffer for 20 minutes on ice. After washing and resuspending in 100µL FACS buffer, 5µL of the following conjugated antibodies were added: CXCR4-PE (Seotec Ltd., Oxford, UK) CD4-FITC (BD Biosciences) and incubated on ice for 30 minutes. The cells were then washed, resuspended in 500µL FACS buffer and transferred to FACS tubes through filter tops. Data was collected on a Becton Dickson FACSCalibur cell cytometer and analyses were conducted using CellQuest software.

2.6 Verotoxin1 TLC overlay

At least 2x10^6 Jurkat cells per treatment group were centrifuged and washed in PBS lacking MgCl2/CaCl2 and stored at -20°C to disrupt the cell pellet. GSLs were then extracted from Jurkat Cells by vigorously shaking cells overnight in chloroform/methanol (C:M, 2:1). The mixture was filtered through glass wool and the lipids collected were dried under nitrogen gas. Extracts were saponified to isolate the glycolipid fraction by resuspending in 1 M NaOH in methanol for 1 hour at 37°C. The mixture was then neutralized with 1N acetic acid to achieve a slightly basic pH. Volumes were adjusted to
form a Folch partition, C:M:W, 8:4:3 and centrifuged to separate the upper and lower phases. The upper phase was removed and discarded and then replaced with an equal volume of C:M:W, 1:47:48. After another round of centrifugation, the upper phase was discarded and the lower phase was dried as described. The dried extract was re-suspended in C:M, 2:1 to approximately 105 cells/20µL and the aliquots were applied to the TLC plate and then pre-cleared by C:M, 98:2. GSLs were then separated by TLC (C:M:W, 65:25:4) and the GSL species were detected by verotoxin-1 (VT1) binding using TLC overlay (Nutikka, Binnington, and Lingwood, 2003).

For the VT1 overlay, the TLC plate was run in the appropriate solvent (C:M:W, 65:25:4) and then dried in the fume hood. The plate was blocked with 1% bovine gelatin and after incubation at 37°C, the plate was washed with 50mM TBS at pH 7.4. The plate was incubated for 45 minutes at room temperature with purified VT1, diluted to a concentration of 1µg/10mM in TBS. After washing, the plate was incubated for 45 minutes with a monoclonal anti-verotoxin B subunit (Boyd, Magnusson, et al, 1994; Nutikka, Binnington, and Lingwood, 2003) diluted 1/2000 and then with HRP-conjugated goat anti-mouse IgG (diluted 1/2000; Bio-Rad). The plate was developed for 10 minutes with 3mg/mL solution of 4-chloro-1-naphthol (4-CN) in methanol freshly mixed with 5 volumes of TBS and 1/2000 dilution of 30% H2O2.

2.7 VSV-G binding assays

The VSV-G protein and the anti-VSV-G polyclonal rabbit antibody were obtained from the Gaudin laboratory (Molecular and Structural Virology, Gif sur Yvette, France). The VSV-G protein was used at a concentration of 3µg/mL in the discontinuous sucrose gradient and the antibody was used at a dilution of 1:1000 in TBS during
immunodetection. gp120 samples used were R5 strains obtained from the NIH AIDS Research Reference and Reagents Program. Catalog #4961, used predominantly throughout the experiments, is a recombinant HIV-1_{BaL} strain.

In order to study the binding of gp120 to GSLs in a raft-like environment, detergent-resistant membrane (DRM) constructs consisting of glycolipid and cholesterol were made in a detergent-containing non-ionic buffer (1% Triton in MES, w/vol). A 2:1 (50µg:25µg or 0.69mol:1mol) ratio of glycolipid to cholesterol were placed in a glass tube and dried under nitrogen flow, resuspended in excess ethanol and re-dried under nitrogen in order to remove residual water. The samples were suspended in 750µL of a 1% Triton containing MES buffer (pH 7), vortexed for 1 minute, sonicated in a water for 1 minute and placed in a 55°C water bath for 5 minutes. 750µl of 70% sucrose in MES buffer (w/vol) is then added to the sample (giving a 35% sucrose solution) and the samples are let to sit for 1 hour at RT.

The vesicles, now in 5mL Beckman ultra-clear ultracentrifuge tubes, were then overlaid with a discontinuous sucrose gradient (1.5mL 35%, 2mL 30%, 1.5mL 5%) containing 3µg/mL of gp120 (or 1µg/mL VT-1/CT, or 3µg/mL VSV-G, or 1µg/mL anti-Gb3, or 1µg/mL anti-GM1) in the first 1ml 30% fraction. The gradient includes the sucrose density corresponding to DRMs, which is found at the 5%/30% sucrose interface. The vesicles are separated on the sucrose gradient by ultracentrifugation (72h, 20°C, 34,000rpm, SW55Ti swinging bucket rotor) and 10 0.5ml fractions were collected from the top of the gradient and analyzed for gp120 content (indicating DRM binding) by immunodetection. Although cell DRMs are often isolated at 4°C, we found that separation was not altered at RT.
Aliquots of the gradient fractions were loaded onto nitrocellulose using a slotblot apparatus (Shliecher & Schuell Minifold I microsample filtration manifold). The samples were washed with 50mM TBS, 150mM NaCl containing 1% skim milk blocking solution and left at 37°C for 1 hour. The membrane was rinsed with TBS and incubated with anti-VSV-G polyclonal rabbit antibody (1:1000 in TBS) for 2 hrs at room temp. After washing, bound antibody was detected using an appropriate HRP conjugated anti-species antibody. Visualization was achieved either by addition of a 4-chloronaphthol solution (20mL TBS: 4 mL 4-CN: 10µL H₂O₂) (2-10 minutes at RT) which reacts with HRP to give a purple color or by a 1 minute incubation with a 1:1 (vol/vol) ratio of the ECL reagents followed by exposure of a film.

For the Langmuir trough monolayer surface pressure assay, the surface pressure was measured with a fully automated microtensiometer (µTROUGH S, Kibron Inc. Helsinki, Finland). The apparatus allowed the recording of the kinetics of interaction of a ligand with the monomolecular film using a set of specially designed Teflon troughs. Experiments were carried out in a controlled atmosphere at 23°C ± 0.5°C. Monomolecular films of Gb₃ or Gb₃ analogues (1-2µg) ± cholesterol were spread on pure water subphases (400µL) from hexane/chloroform/ethanol solution. After spreading the film, 5 min was allowed for solvent evaporation. To measure the interaction of gp120 and VSV-G with Gb₃ monolayers, the ligand was injected in the subphase with a 10µL Hamilton syringe, and pressure increases produced were recorded for the indicated time. The data were analyzed with the Filmware version 3.57 program (Kibron Inc. Finland). The accuracy of the system under our experimental conditions was ± 0.25mN.m⁻¹ for surface pressure. This work was conducted by A. Manis and R. Mahfoud.
2.8 Pseudoenvelope-typed HIV-1 viruses

To generate replication deficient, vesicular stomatitis virus-G (VSV-G) enveloped NL4-3\(luc\)HIV-1 luciferase containing recombinant virions, 293T cells were co-transfected with plasmids containing the VSV-G envelope (a kind gift from Dr. Michel Tremblay, Quebec City, PQ) and the NL43-\(luc\) HIV-1 genome lacking \(env\) and with the luciferase gene inserted into the \( nef\) gene (NIH AIDS Reagent Program). 2.5\(\times\)10\(^6\) 293T cells were plated 24 hours previously on 10cm plates in 10mL DMEM supplemented with 10% FBS. 10\(\mu\)g of plasmid containing the amphotropic VSV-G envelope and 15\(\mu\)g of plasmid containing the HIV-1 \(env\) lacking NL4-\(luc\) genome were mixed and the cells were transfected using the Clontech CalPhos\textsuperscript{TM} Mammalian Transfection Kit (Clontech, USA). 12 hours post-transfection cells were washed with PBS and then incubated for 72 hours at 37\(^\circ\) C in fresh media. Viral supernatant was then collected, centrifuged for 10 minutes, and filtered through a 0.45\(\mu\)M filter. Virions (VSV/HIV) were pelleted by ultracentrifugation in 8mL aliquots over 400\(\mu\)L 20% glucose for 1 hour at 19 000 RPM. Pelleted VSV-G/NL4-3\(luc\) virions were resuspended in 800\(\mu\)L TNE buffer (20mM Tris, pH 7.5, 1mM EDTA, 100mM NaCl), aliquoted and stored at -80\(^\circ\)C until further use. Virion content was determined by p24 ELISA (ZeptoMetrix) however infectious dose was determined by luciferase assay.

2.9 Infectivity of pseudoenvelope-typed viruses

To determine the infectivity level of produced VSV-G/NL4-3\(luc\) pseudoenvelope-typed virus, 2\(\times\)10\(^5\) Jurkat C cells were plated in a 96 well tray in triplicates in 100\(\mu\)L RPMI media supplemented with 10% FBS and lacking phenol red. 20\(\mu\)L volumes of virus and media up to a total volume of 200\(\mu\)L per well was then added. Cells were
incubated for 48 hours, then lysed using cell culture lysis reagent (CCLR, Promega Corporation, Madison, WI) and frozen at -80 °C overnight. 100μL of luciferase assay substrate (Promega Corporation) was added to 20μL of the cell lysate and luciferase activity was measured using a luminometer (Luminoskan Ascent, Thermo Electron Corporation, Vantaa, Finland).

2.10 Infection of NIH3T3 cell line by pseudoenvelope-typed virus

To determine if the recombinant virus would infect a mouse epithelial cell line, 1x10⁶ NIH3T3 cells were infected with 25μL and 75μL aliquots of VSV-G/NL4-3luc virus and media up to a total volume of 200μL and incubated in DMEM for 2 hours. DNA was then isolated using the Qiagen DNEasy DNA isolation kit and subject to PCR to detect reverse transcribed HIV-1 cDNA as described below. Infection was also measured using the luciferase assay as described above with slight modifications. 2x10⁵ NIH3T3 cells were plated in triplicate in 96 well plates 24 hours before infection. 20μL of virus was added or media control and cells were incubated at 37ºC and 5% CO₂ for a 48 hour period. The cells were then washed and lysed and luciferase activity was measured as described above.

2.11 PCR

Detection of viral entry and intracellular intact HIV-1 cDNA or pseudotyped NL4-3luc of infected cells was assessed by DNA-PCR using primers specific for the LTR region of the HIV-1 genome as described previously (Bokaei, Ma, et al, 2007). HIV-1IIIb or VSV/HIV was treated before infection of cells or mouse vaginal and/or rectal mucosa with DNAse I (Promega) to degrade any possible DNA that could contaminate the
samples. DNA was isolated using the Qiagen DNeasy Tissue and Blood kit (Qiagen).

The following primers were used for DNA amplification: forward, LTR 5’-
GGGACTGGAAGGGCTAATTC-3’ and reverse, L1 5’-
AGGCAAGCTTTATTGAGGCTTAAGC-3’. For nested PCR forward, L2 5’-
CTGTGGATCTACCACACACA AGGCTAC-3’ and reverse, LTR U3 5’-CTCCCT
GGAAAGTCCCCAGC-3’. Cell lysates were also amplified using primers specific for human β-globin or mouse β-actin as DNA comparison controls. The primers for the β-globin gene were: forward, 5’-CAACTTCATCCACGTTCACC- 3’ and reverse, 5’-
GAAGAGCCAAGGACAGGTAC-3’ and for the β-actin gene forward: 5’:
ATCTGGCACCACACCTTCTA-3’ and reverse 5’- GAGGCATACAGGGACAGCAC-
3’. 50ng total genomic DNA was used for amplification. The amplification cycle used was: denaturation at 95°C for 4min, then amplified by 35 cycles at 94°C for 1min, 58°C for 1 min, 72°C for 1 min and a final extension step of 7min at 72°C. Amplified products were run on a 1.5% agarose gel and visualized by ethidium bromide.

### 2.12 Quantitative PCR

To detect the level of pseudotyped NL4-3\textit{luc} HIV-1 cDNA in mouse tissue samples (as described later) and HIV-1\textsubscript{IIB} cDNA from infected cervical cell lines, the LTR region of the genome was measured by quantitative real time PCR (qPCR). DNA was isolated from cervical cell lines following infection with HIV-1 and from mouse vagina and rectum, described below. Reaction components were obtained from the LightCycler® FastStart DNA Master\textsuperscript{PLUS} SYBR Green I kit (Roche, Germany). The following reaction conditions were used: pre-incubation at 95°C for 10 minutes, followed
by 45 amplification cycles of denaturation at 95°C for 10 seconds, annealing at 65°C, extension at 72°C for 10 seconds, melting curve analysis at 65°C for 15 seconds and a continuous acquisition mode of 95°C with a temperature transition rate of 0.1°C/s. The real time PCR was performed in a final volume of 20µL containing 1X Master SYBR Green Plus buffer, 25µM forward and reverse primers and 2µL template DNA from mouse samples (50ng/µL) or NL4-3luc HIV-1 plasmids (from 2.7x10^{10} to 2.7 copies for standard curve generation). The primers used for amplification were: Forward 5’-TTAGACCAGATCTGAGCCTGGGAG-3’ and reverse 5’-GGGTCTGAGGGATCTCTAGTTACC-3’. Primers were purified with high performance liquid chromatography (HPLC) to ensure purity. All H2O used in reaction components was PCR grade. Analysis was performed using LightCycler 2.0 software, which allows for the determination of the threshold cycle (Ct). This represents the cycle number where fluorescence intensity of the PCR product is significantly higher than background fluorescence (Gibellini, Vitone, et al, 2004). This Ct value is directly proportional to the log_{10} copy number of the inputted NL4-3luc plasmid templates that were diluted in serial in order to generate a standard curve for comparison with unknown samples. This enabled a copy number value to quantify the actual number of NL4-3luc or HIV-1 genomes in unknown samples.

2.13 Mice and husbandry

Animal Utilization Protocols (AUPs) were first approved by the University Health Network Animal Care Committee. Male and female outbred immunocompetent CD1 mice were obtained from Charles River Laboratories, Montreal, Quebec at 6-8 weeks of
age. Mice were housed in groups in plastic cages with environmental enrichment at the University Health Network Animal Facility, 67 College Street, Toronto. The housing facility was on a 12 hours light and 12 hours dark photo-cycle and food and water were offered *ad libitum*. All animal housing and maintenance practices were in compliance with the Canadian Council for Animal Care (CCAC) guidelines. Prior to the commencement of experiments, mice were rested for at least 1 week.

### 2.14 Progesterone treatment of female mice

Female CD1 mice were acclimatized for 1 week and then injected with 2.5 mg of Depo-Provera (medroxyprogesterone acetate) subcutaneously (Sigma). Progesterone treatment produces a diestrous-like state whereby the vaginal stratified squamous epithelial layer of dead and dying cells is eliminated that helps protect the vagina from mechanical abrasion and pathogens. The epithelium becomes uniformly columnar and decreases variability between female mice (Cone, Hoen, et al, 2006). Female mice were used in toxicity and direct application of FSLGb$_3$ experiments one week after inoculation.

### 2.15 Mouse infection model using pseudoenvelope HIV-1

To determine if the pseudoenvelope typed VSV/HIV virus infects mouse mucosal tissue, male and female CD1 mice were first euthanized and then the vaginal and rectal cavities were challenged with 25µL of VSV/HIV virus (enough virus to produce consistent RLU via *in vitro* studies) administered via pipette for 2 hours and incubated at 37°C. Rectal and vaginal tissue was then surgically removed. Tissue was quick frozen in liquid nitrogen until DNA isolation. Thawed tissue was homogenized for 1 min with a
grinder and DNA was isolated via the Qiagen DNEasy Kit for DNA isolation from tissue. For studies evaluating infection through a cream, an FDA approved vaginal cream (Wellspring Pharmaceuticals, Oakville, Canada) was mixed with varying ratios of virus and 15µL of each concentration was used to infect the vaginal and/or rectal mucosa of mice for 2 hours. For gel studies, a Carbopol base gel was obtained from Wellspring Pharmaceuticals. Gel was created by mixing the powdered gel component with dH₂O in a 1:5 ratio. While vortexing the dH₂O at 1200rpm, the gel powder component was slowly added to the water phase. This was continued for 5-10 minutes until a smooth polymer product was created. 1-5µL 1MNaOH was then added while vortexing to solidify the product into a gel. 15µL of gel containing FSLGb₃, adamantylGb₃ or placebo was applied to the vaginal and/or rectal cavity via specialized pipette for viscous materials for 30 minutes and then the mice were challenged with virus as above for 1.5 hours. For FSLGb₃ direct application studies, 15µL of the compound in PBS or placebo was added directly to the mucosal surface and then mice were challenged with VSV/HIV similarly to the gel studies.

2.16 Cytokine assay

The Bio-Plex system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure mouse inflammatory cytokines in both vaginal and rectal lavaged samples after administration of FSLGb₃ and this study was modeled after a toxicity protocol for microbicide candidates in a murine model (Cone, Hoen, et al, 2006). A 2% solution of Nonoxynol-9 (N9) (Sigma-Aldrich, Oakville, ON, Canada) was used as a positive control and PBS as negative control. Female CD1 mice were progesterone primed 1 week prior
to the commencement of experiments. Groups of 4 mice were used for each sample type and both vaginal and rectal samples were taken. Baseline lavage samples were taken prior to delivery of the test agents. 25µL of the test agent (1mM FSLGB₃, PBS or 2% N9) was delivered to the vaginal or rectal cavity of CD1 mice via pipette. The vagina and rectum of the mice in each group were then lavaged with 50µL PBS (about 20 times in and out via pipette) at 4, 12, 24 and 48 hours after test agent delivery. The lavage fluid was then centrifuged and frozen at -80°C until analysis. Samples were diluted 1:3 with PBS prior to analysis. Concentration of cytokines in the samples was determined using the Bio-Rad cytokine analysis kit and the Bio-Plex assay system at the Ontario Cancer Biomarker Network. The inflammatory cytokines chosen to test were those determined to be informative in previous microbicide studies (Cone, Hoen, et al, 2006) and also those available for analysis with the Bio-Plex machine. Those tested were: IL-1α, IL-1β, IL-6, IL-10, IL-13, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α.

2.17 Statistics

Data is represented as mean ± SEM, where the Student’s t test was used to determine statistical significance. Data was considered statistically significant if p< 0.05.
CHAPTER 3

SOLUBLE SYNTHETIC Pk MIMIC INHIBITS HIV-1 INFECTION
3.1 Chapter Overview

The purpose of this study was to extend research by our group on the semi-synthetic analogue of the glycosphingolipid, globotriaosylceramide (Gb₃), (adamantylGb₃) and its role in the inhibition of HIV-1 infection by the prevention of viral fusion. Here I have evaluated a totally synthetic form of Gb₃, coined FSLGb₃, (Functional head-Spacer-Lipid tail) that is completely water dispersible and has low toxicity, for its potential to inhibit HIV-1 infection *in vitro*. To assess this, I started by confirming the previous results obtained with adamantylGb₃ against HIV-1<sub>IIIB</sub> infection. By viral pre-incubation of HIV-1 with FSLGb₃ over the concentration range of 50-1000µM, HIV-1<sub>IIIB</sub> (X4 virus) infection in Jurkat and phytohaemagglutinin (PHA)/interleukin-2 (IL-2) activated peripheral blood mononuclear cells (PBMCs), and HIV-1<sub>Ba-L</sub> (R5 virus) infection of PHA/IL-2 activated PBMCs was monitored by p24<sub>Gag</sub> ELISA *in vitro*. Viral pre-incubation with FSLGb₃ showed near complete inhibition of both X4 and R5 viral infection in PBMCs with a dose-dependent manner with an IC<sub>50</sub> of approximately 200µM. Next, using FACS I determined that FSLGb₃ would absorb onto pre-incubated Jurkat cells with no significant effect on the cell surface receptors CD4 and CXCR4. In addition, I found that pre-incubation of Jurkat cells with FSLGb₃ for 1 hour before infection with HIV-1<sub>IIIB</sub> maintained a similar inhibitory effect as viral pre-incubation experiments. Through FACS analysis of Jurkat cells treated with FSLGb₃ over time and lipid extraction of these cells, I determined that FSLGb₃ is cell-associated after treatment but this association is lost within 24 hours. This led me to explore the effect of daily vs. once only treatment of Jurkat cells with FSLGb₃ on HIV-1<sub>IIIB</sub> infection, where I determined that a once daily treatment was significantly more effective at
inhibiting infection than a single initial dose. In conclusion, these results suggest that FSLGb₃ may be a novel therapeutic candidate for the inhibition of systemic HIV infection and could potentially offer a new treatment and/or prevention approach for HIV/AIDS.
3.2 AdamantylGb$_3$ inhibits HIV-1 infection of Jurkat T-cells

I commenced the study by first evaluating a new batch of adamantylGb$_3$ for inhibitory action against HIV-1$_{IIIb}$ infection in Jurkat *in vitro* in order to ensure batch efficacy and accuracy of technique. An m.o.i of 0.1 was used to permit reliable evaluation of p24 expression 3-5 days after infection. In accordance with previously published work (Lund, Branch, et al, 2006) I found that a 1 hour viral pre-incubation with increasing concentrations of adamantylGb$_3$ resulted in increasing inhibition of Jurkat cell infection, (LD$_{50}$ =100µM) reaching 100% inhibition at 400µM (Fig. 3.1), however some toxicity to cells was noted by visual observations when using 400µM adamantylGb$_3$. 
Figure 3.1: AdamantylGb$_3$ inhibition of HIV-1$_{111B}$ Jurkat T-cell infection by viral pre-incubation. HIV-1$_{111B}$ (m.o.i., 0.1) was pre-incubated with adamantylGb$_3$ for 1 hr at 37°C prior to infection of Jurkat C. The p24$^{gag}$ antigen expression level was monitored 5 days after infection. Jurkat C infection in quadruplicate [**$P<0.001$ adamantylGb$_3$ in comparison to positive control].
3.3 FSLGb3 inhibits HIV-1 infection of Jurkat T-cells

FSLGb3, a totally synthetic and water soluble analogue of Gb3 obtained from KODE Biotech New Zealand, was tested first for efficacy against HIV-1_{IIIB} infection of Jurkat C T-cells. Using an m.o.i of 0.1 to permit reliable evaluation of infection by p24 expression, I found that a 1 hour pre-incubation of HIV-1_{IIIB} with an increasing dose range of FSLGb3 resulted in complete inhibition of HIV-1 infection by 400-600µM with an IC\textsubscript{50} of approximately 200µM and with no noticeable toxicity to cells even at the highest concentration of 1000µM (Fig. 3.2 A). Next, using sufficient HIV-1_{Ba-L} to permit reliable evaluation of infection via p24\textsuperscript{gag} expression by day 6 post infection, I found that using a 1 hour viral pre-incubation, FSLGb3 did not show inhibition of HIV-1_{Ba-L} infection, which I found puzzling and will be contrasted to data in PBMCs in the next section (Fig. 3.2 B).
FIGURE 3.2

A)

**P<0.001

B)
Figure 3.2 FSLGb3 inhibition of HIV-1\textit{IIIb} Jurkat T-cell infection by viral pre-incubation. HIV-1\textit{IIIb} (m.o.i., 0.1) or sufficient HIV-1\textit{Ba-L} was pre-incubated with FSLGb3 for 1 hr at 37°C prior to infection of Jurkat C. The p24\textsuperscript{gag} antigen expression level was monitored 5-8 days after infection. (A) HIV-1\textit{IIIb} infection of Jurkat C in quadruplicate, representative of three similar experiments [\(*\ast P<0.001\) FSLGb3 in comparison to positive control]. (B) HIV-1\textit{Ba-L} infection of Jurkat C in quadruplicate, representative of two similar experiments.
3.4 FSLGb₃ inhibits HIV-1 infection of PBMCs

HIV-1 infection of primary PBMCs in vitro represents a more physiological approximation of infection in vivo, therefore I also examined the effect of FSLGb₃ on HIV-1 infection of these cells. For both X4 and R5 HIV-1 infection, PBMCs were activated for 3 days by treatment with PHA/IL-2. FSLGb₃ was slightly better at inhibiting X4 HIV-1_{IIIB} (m.o.i., 0.3) infection of PBMCs than Jurkat C, with increasing inhibition over a similar concentration range but reaching complete inhibition by 200-600µM with an IC₅₀ of approximately 100µM (Fig.3.3A). In contrast to results using Jurkat T cells, using PBMCs, treatment of R5 HIV-1_{Ba-L} with FSLGb₃ resulted in increasing inhibition of infection over the concentration range with near complete inhibition at 400µM with an IC₅₀ of approximately 300µM (Fig.3.3B). PBMCs were maintained in culture up to 7 days post-infection where inhibition at this time-point was still maintained as shown by p24₇₀₉₉ ELISA.
**FIGURE 3.3**

A) 

![Graph showing HIV-1 IIB Infection (p24 pg/mL) across different treatments from Day 7.](image)

*P<0.05; 200-1000µM

B) 

![Graph showing HIV-1 Ba-L Infection (p24 pg/mL) across different treatments from Day 7.](image)
Figure 3.3 FSLGb3 inhibition of X4 HIV-1IIIB and R5 HIV-1Ba-L infection of activated human peripheral blood mononuclear cells by viral pre-incubation. PBMCs were isolated from the blood of healthy donors after informed consent and isolated via Ficoll-Paque density gradients. PBMCs were activated for 3 days prior to infection by (A) HIV-1IIIB (m.o.i., 0.3) or (B) HIV-1Ba-L with PHA and IL-2 and were maintained in media containing IL-2 after infection. Virus was pre-incubated for 1 hr at 37°C with increasing doses of FSLGb3 as compared to media control. p24gag ELISA was used to measure infection between days 5-7 post infection. (A and B) One of three similar experiments were completed in triplicate or quadruplicate [*P<0.05 in comparison to media control].
3.5 Direct application of FSLGb₃ to Jurkat T-cells results in 100% Gb₃ expression on cell surface and inhibition of HIV-1_{IIIb} infection.

To further explore the inhibitory effect of FSLGb₃ and because of the specific chemistry of FSLGb₃ which should allow this molecule to insert into the plasma membrane of cells I investigated whether Jurkat cells pre-incubated with 1mM or 3mM FSLGb₃ showed increased Gb₃ expression via FACS analysis, since cell incubation would most closely mimic the scenario in treatment of HIV. Untreated Jurkat cells revealed no Gb₃ expression (Fig. 3.4A) which after 1 hour exposure to FSLGb₃ increased to 100% expression with 1mM FSLGb₃ (Fig. 3.4B). MFI increased by nearly half log intensity from 1 to 3mM (Fig. 3.4C) treatment with FSLGb₃. I was then interested in determining whether these Gb₃ expressing Jurkat cells would show decreased infectability to HIV-1. I incubated the Gb₃-expressing Jurkat cells with HIV-1_{IIIb} and HIV-1_{Ba-L} for 1 hour and measured infection by p24 expression 3 to 6 days post-infection. I found that cells pre-treated with FSLGb₃ inhibited HIV-1_{IIIb} infection (Fig. 3.4D) which showed a dose response over the concentration range tested and was significant at 3mM treatment [*P = 0.016 in comparison to untreated control]. Infection levels of HIV-1_{Ba-L} also revealed an increased initial infection at 1mM which was not significant [P=0.09] and an overall slight decrease in infection at 3mM, but this decrease was not statistically significant [P=0.33] (Fig. 3.4E). These initial experiments should be replicated.
FIGURE 3.4

A) March 11, 2008.002

Untreated Control

B) March 11, 2008.006

1mM FSLGb3

C) March 11, 2008.004

3mM FSLGb3

D) HIV-1 IIIB Infection (p24 pg/mL)

CTRL 1000µM 3000µM

E) HIV-1 Ba-L Infection (p24 pg/mL)

CTRL 1000µM 3000µM
Figure 3.4: Jurkat T-cell treatment with FSLGb3 results in 100% Gb3 expressing cells and a subsequent inhibition of HIV-1 infection

Jurkat C cells were incubated for 1 hr with FSLGb3 and then FACS analysis was performed to detect surface Gb3 expression using anti-Gb3 antibodies. (A) Percentage of untreated Jurkat C cells expressing Gb3. (B) Percentage of 1mM treated Jurkat C expressing Gb3. (C) Percentage of 3mM treated Jurkat C expressing Gb3. Treated and untreated cells were then infected with HIV-1IIIB (m.o.i., 0.1) or sufficient HIV-1Ba-L and monitored for p24 expression 3 to 6 days following infection. (D) HIV-1IIIB infection in triplicate of Jurkat cells following cell pre-incubation with FSLGb3 [*P = 0.016 in comparison to untreated control]. (E) HIV-1Ba-L infection in triplicate of Jurkat cells following cell pre-incubation with FSLGb3.
3.6 Kinetics of absorption and retention of Gb₃ following Jurkat treatment with FSLGb₃

To determine the kinetics of retention of different amounts of FSLGb₃ adsorbed onto HIV target cells, Jurkat C cells were treated for 1 hour with FSLGb₃ over the concentration range 100-1000µM and Gb₃ expression was followed for a period of up to 2 days by FACS and VT1 overlay. By FACS analysis, at 100µM there was no difference between control and treated groups for MFI (Fig. 3.5B) however percent Gb₃ expressing cells increased from 0 to 18% (Fig. 3.5A). By 200µM, MFI increased significantly to 1765 and nearly 100% of cells were expressing Gb₃ (Fig. 3.5B), and by 1000µM, MFI had reached 3028 (Fig. 3.5B). Retention of Gb₃ however was reduced to background levels by both MFI and Percent Expression 24 hours after treatment, for all concentrations except 1000µM which retained 92% Gb₃ expressing cells, but MFI was near background levels (Fig. 3.5 A and B). Additionally, total cellular GSLs were extracted from Jurkat cells treated with 400µM FSLGb₃ before and 1 hour after treatment, and at 24 and 48 hours post treatment. I found Gb₃ to be cell-associated after initial 1 hour incubation with FSLGb₃ but 24 hours later was no longer detected in the cell population (Fig. 3.5 C).
FIGURE 3.5

A) B)

C)
Figure 3.5: Gb$_3$ expression over time following FSLGb$_3$ treatment of Jurkat T-cells

Jurkat T-cells were incubated for 1 hour at 37°C with 100-1000µM FSLGb$_3$. Aliquots of cells were taken before treatment, after treatment and at 24 and 48 hours for GSL extraction and Verotoxin/TLC overlay (400µM for GSL extraction) and FACS analysis and washed and pelleted prior to analysis. FACS analysis of Gb$_3$ expression using anti-Gb$_3$ antibody was conducted 1hr, 24 and 48 hours following treatment. (A) Percent expression of Jurkat C Gb$_3$ expression over time. (B) MFI of Jurkat C expressing Gb$_3$ following treatment over time. (C) Verotoxin1 TLC overlay, Lane 1, Gb$_3$ standard; lane 2, FSLGb$_3$ standard; lane 3 GSL from Jurkat C before treatment; lane 4, GSL from Jurkat C after 1 hour incubation with FSLGb$_3$; lane 5, GSL from Jurkat C 24 hours after treatment; lane 6, GSL after 48 hour treatment.
3.7 FSLGb\textsubscript{3} effects on cell surface receptor expression of Jurkat T-cells.

Jurkat cells exposed to FSLGb\textsubscript{3} for 1 hr showed no difference in cell surface CD4 expression over time by MFI or percent expression (Fig. 3.6 A and B). CXCR4 expression increased at 24 hours post FSLGb\textsubscript{3} treatment for the 100µM group by MFI, however this was not detected at 48 hours (Fig. 3.6C). No other significant differences in CXCR4 expression were noted (Fig. 3.6 C and D). CCR5 expression was not detectable in this cell line both prior to and after FSLGb\textsubscript{3} treatment (not shown).
FIGURE 3.6

CD4:

A)  

B)  

CXCR4:

C)  

D)
Figure 3.6: FSLGb₃ effects on cell surface receptor expression on Jurkat T-cells.

Jurkat C cells were treated with 100-1000μM FSLGb₃ and FACS analysis of CD4 and CXCR4 expression was performed 1hr, 24 and 48 hours following treatment. Cells were stained with anti-CD4 or anti-CXCR4 antibodies. CCR5 was not detectable on these cells (not shown). (A) Percentage of Jurkat C expressing CD4 before and following 1hr FSLGb₃ treatment over time. (B) MFI of Jurkat C expressing CD4 before and following 1hr FSLGb₃ treatment over time. (C) Percentage CXCR4 expression before and following 1hr FSLGb₃ treatment over time. (D) MFI of Jurkat C expression of the population in (C).
3.8 Daily administration of FSLGb\textsubscript{3} is effective at maintaining inhibition of HIV-1\textsubscript{IIIb} infection

In order to assess the most effective administration schedule for FSLGb\textsubscript{3}, I compared infection levels of Jurkat cells treated once before infection to those treated initially and then once-daily for a 5 day infection period with HIV-1\textsubscript{IIIb}. I chose a treatment concentration of 200µM, since this was the minimum dose required to absorb FSLGb\textsubscript{3} resulting in 100% Gb\textsubscript{3} expressing cells by FACS analysis. Additionally this was the lowest concentration to produce significant inhibition of HIV-1\textsubscript{IIIb} infection by viral pre-incubation (Fig 3.2A). Cells were incubated with FSLGb\textsubscript{3} for 1 hour at 37°C and then infected with HIV-1\textsubscript{IIIb} (m.o.i., 0.1). For cells in the daily treatment group, 200µM FSLGb\textsubscript{3} was added daily based on a total culture volume of 1mL. Infection was monitored 5 days post infection by p24 ELISA. I found that compared to cells treated once only with FSLGb\textsubscript{3} (Fig. 3.7A), those receiving a daily treatment, showed significant inhibition of HIV-1\textsubscript{IIIb} infection (Fig 3.7B).
FIGURE 3.7

A)

B)
Figure 3.7: Daily culture treatment with FSLGb$_3$ is more effective in inhibiting long-term HIV-1$_{IIIb}$ infection when compared to one cellular treatment of Jurkat C Jurkat C were pre-incubated for 1 hr at 37°C with 200µM FSLGb$_3$ prior to infection with HIV-1$_{IIIb}$ (m.o.i., 0.1). Infections were carried out in triplicate. HIV-1 infection was determined by p24$_{gag}$ ELISA 5 days post-infection (A) Jurkat C treated once with FSLGb$_3$ compared to untreated control. (B) Jurkat C treated once initially as in (A) and then a daily dose of 200µM FSLGb$_3$ based on total media volume was added to the cell culture every day sequentially up to 5 days post-infection, compared to untreated control [*P = 0.04].
CHAPTER 4

SOLUBLE Gb₃ AS A POTENTIAL MICROBICIDE FOR THE PREVENTION OF HIV-1 INFECTION
4.1 Chapter Overview

We have shown that both adamantylGb\textsubscript{3} and the new KODE construct FSLGb\textsubscript{3}, inhibit X4 HIV-1 in Jurkat cell lines and both X4 and R5 HIV-1 viruses in primary cells. Here, I have also evaluated FSLGb\textsubscript{3} as an inhibitor of HIV-1\textsubscript{IIIb} infection in two mucosal cell lines (vaginal and cervical) by quantitative PCR and determined that in these cell lines, FSLGb\textsubscript{3} inhibited infection.

Novel methodologies are imperative to curb new HIV infections globally. Microbicides, which are cream or gel preparations for vaginal and/or rectal use that contain compounds that inhibit HIV infection, are one such possible method. In this Chapter I show my work to develop a novel mouse model using a pseudoenvelope-typed replication-deficient VSV-G/NL4-3\textit{luc} HIV-1 virus (VSV/HIV), which is approved for Level 2 biocontainment in order to test the efficacy of soluble Gb\textsubscript{3} as a potential microbicide compound. I show that the modified virus infects mouse fibroblasts \textit{in vitro}. I demonstrate by PCR after 2 hour infection that the VSV/HIV virus infects the rectum and vagina of CD1 mice alone and through FDA approved cream formats for topical vaginal use. Next, a gel was prepared containing soluble Gb\textsubscript{3} and male and female CD1 mice were challenged rectally and/or vaginally after topical application of the gel with or without soluble Gb\textsubscript{3}. DNA from vaginal and rectal tissue was isolated after 2 hours. PCR and quantitative PCR (qPCR) was conducted to detect the reverse transcribed HIV-1 cDNA. Soluble Gb\textsubscript{3} incorporated into gel and applied directly to mucosa has not shown significant inhibitory activity to subsequent viral challenge although there appears to be a trend for this and additional animals tested could prove significance. Finally, I began preliminary examination for the potential for toxicity by measuring inflammatory and
other cytokines of vaginal and rectal lavage fluid after challenge with soluble Gb₃.

In conclusion, this mouse model of mucosal infection of VSV/HIV is an effective surrogate for HIV sexual transmission but using soluble Gb₃ in gel format has not as yet shown efficacy, however significant in vitro data warrants further study in this model and other animal model systems.
4.2 The search for a small animal model system to test soluble Gb3 microbicide candidates

Since HIV-1 does not infect mouse cells, the immediate problem was first in establishing a model system in which to test our soluble Gb3 analogues as microbicides against mucosal infection. There are established models using transplanted SCID mice engraftment with human tissues, haematopoietic stem cells (HSCs) or peripheral blood mononuclear cells (PBMCs) (Di Fabio, Giannini, et al, 2001; Shultz, Ishikawa, and Greiner, 2007) and more recent model systems using Rag2\(^{-/-}\)γc\(^{-/-}\) (RAG-hu) mice transplanted with HSCs of the CD34\(^+\) lineage (Berges, Akkina, et al, 2008) that express human HIV-1 target cells and are infectable by HIV-1. However, currently at the University of Toronto, there are no Level 3 biosafety facilities for animal studies using intact HIV virions. As an alternative, I turned to a pseudoenvelope-typed HIV-1 virus. The NL43-luc HIV-1 genome used in the current study is envelope negative, and has a luciferase reporter gene inserted into the nef gene region of the HIV-1 genome, rendering the virus replication deficient. To create virions that would infect mice, this plasmid was co-transfected with the plasmid containing the gene for the vesicular stomatitis virus envelope (VSV-G) which infects rodent cells to create the pseudoenvelope typed-HIV-1 virus. Viral entry and thus infection would be detectable by PCR using primers specific for HIV-1 reverse-transcribed cDNA. In addition, it was a requirement that this modified virus also be inhibited by the compounds of interest, adamantylGb3 and FSLGb3, as this would correlate to the results obtained using intact HIV-1 (Lund, Branch, et al, 2006; Chapter 3 Results). Prior to the commencement of this study it was determined in pilot experiments that adamantylGb3 was an excellent inhibitor of VSV/HIV infection in vitro, which enabled this current study to proceed.
4.3 Reactivity of VSV-G and gp120 with Gb₃

Both VSV and gp120 bind Gb₃ with similar kinetics (Fig. 4.1 A and B) and adamantylGb₃ and FSLGb₃ inhibit both VSV/HIV (next section) and HIV-1. Thus, VSV/HIV is a reasonable surrogate virus model to determine adamantylGb₃ or FSLGb₃ efficacy for mucosal inhibition and should be transferable to HIV-1 infection.
FIGURE 4.1

This figure was contributed by A. Manis and R. Mahfoud
Figure 4.1: VSV-G binding to Gb₃.

A) VSV-G protein (kindly supplied by Dr Y. Gaudin CNRS, Paris) was found to bind to Gb₃/cholesterol vesicles immobilized on nitrocellulose, as seen for gp120. Binding was detected using anti VSV-G. Unlike gp120, VSV-G binding to GalCer was less than to Gb₃ and VSV-G also bound Gb₄ vesicles which are not bound by gp120. Thus, VSV-G target cell binding should also be affected by the host cell membrane Gb₃ content and Gb₃ mimics. No bound VSV-G was detected in samples lacking VSVG or replaced with gp120. B) In surface pressure monolayer studies (Langmuir trough), gp120 was shown to bind and insert into Gb₃ water/air interface monolayers. Specificity is established by increasing initial surface pressure to prevent VSV-G insertion.
4.4 Viral production and *in vitro* testing of infectivity in Jurkat T cells and mouse fibroblasts

Batches of virus were produced by co-transfecting the HIV-1 NL4-3luc plasmid with a plasmid containing the gene for the VSV-G envelope in 293T cells until a highly infectious candidate was produced, and which was subsequently used for the duration of the experiments in this study. I tested *in vitro* infection of the virus by both luciferase activity and DNA-PCR in Jurkat T cells and NIH3T3 mouse fibroblasts. I first tested the VSV/HIV in Jurkat T-cells to confirm infectivity via viral entry by luciferase (Fig 4.2A) and DNA-PCR (Fig. 4.2B). To further validate produced virus I tested for p24<sup>gag</sup> using ELISA (not shown). I found p24<sup>gag</sup> concentration to not be a good indication of viral potency, therefore I used viral volume known to provide significant luciferase activity as our main marker of concentration of infectious virions. This volume was determined to be 20μL per reaction. I next needed to verify that VSV/HIV would infect mouse cells. The NIH3T3 mouse fibroblast line was determined to be infectable via both luciferase activity and DNA-PCR (Fig. 4.2 C and D).
FIGURE 4.2

A)       B)

Control VSV/HIV Infected

RLU

Treatment

H₂O  NL43-luc CTRL 25µL 75µL

HIV cDNA

β-Globin

C)       D)

H₂O  NL43-luc CTRL 25µL 75µL

HIV cDNA

β-Actin
Figure 4.2: VSV/HIV infects both mouse fibroblasts and Jurkat T-cells in vitro

Jurkat C and mouse fibroblasts (NIH3T3 cell line) were incubated with VSV/HIV for 1 hour at 37°C and then washed. For the measurement of infection by luciferase activity, cells were cultured for 48 hours, lysed and a luminometer was used to determine relative luciferase activity (RLU). Results are in RLU and represent experiments conducted in triplicate. For detection of infection by DNA-PCR, cells were infected for 1 hr at 37°C, and then DNA was isolated. 50ng of DNA was used for each reaction. (A) Jurkat infection by detection of luciferase activity. Results of infected verses uninfected control are shown and represent a 20µL infection volume. (B) Jurkat infection by DNA-PCR. NL4-3luc plasmid was used as positive control, and water and uninfected cells as negative controls. 25 and 75µL viral infections and expression of HIV-1 proviral cDNA are shown. (C) NIH3T3 infection by detection of luciferase activity. Results of infected cells using two viral concentrations are shown (20 and 50µL) compared to uninfected control. (D) NIH3T3 infection by DNA-PCR. NL4-3luc plasmid was used as positive control, and water and uninfected cells as negative controls. 25 and 75µL viral infections and expression of HIV-1 proviral cDNA are shown.
4.5 VSV/HIV is inhibited by both adamantylGb3 and FSLGb3

We next assessed the ability of adamantylGb3 and FSLGb3 to inhibit VSV/HIV infection in vitro in Jurkat T cells. I found that pre-incubating the virus with increasing concentrations of adamantylGb3 and FSLGb3 inhibited Jurkat cell infection. For adamantylGb3, 50% inhibition was reached by 200µM (Fig. 4.3A). A higher dose of FSLGb3 was required to inhibit VSV/HIV infection. 75% inhibition was reached at 3mM concentration (Fig. 4.3B).
FIGURE 4.3

A)

![Graph A showing RLU values for different treatments.]

B)

![Graph B showing RLU values for different treatments.]

uninfected  AZT  Infected  50µM  100µM  200µM  400µM

CTRL (neg)  AZT  CTRL (pos)  100µM  500µM  1000µM  3000µM

Treatment

RLU

CTRL (neg)  AZT  CTRL (pos)  100µM  500µM  1000µM  3000µM

Treatment

RLU
Figure 4.3: Both adamantylGb\textsubscript{3} and FSLGb\textsubscript{3} inhibit VSV/HIV infection of Jurkat C by viral pre-incubation. VSV-G/NL4-3\textsubscript{luc} pseudoenvelope-typed HIV-1 virus (20µL/reaction) was incubated at 37°C with adamantylGb\textsubscript{3} and FSLGb\textsubscript{3} for 1 hr and then Jurkat cells were infected for 1 hr at 37°C. Cells were then washed and cultured. 48 hours after infection cells were lysed and luciferase activity was measured with a luminometer. (A) Effect of adamantylGb\textsubscript{3} on VSV/HIV infection of Jurkat C compared to untreated control. Results are in relative luciferase units (RLU) and represent experiments conducted in triplicate. (B) Effect of FSLGb\textsubscript{3} on VSV/HIV infection of Jurkat C compared to untreated control. RLU results represent experiments conducted in triplicate.
4.6 FSLGb₃ inhibits HIV-1ᵢᵢᵢB infection of human endocervical and vaginal cell lines by quantitative PCR

The vaginal mucosa is a major site of infection by HIV-1. The vaginal and cervical epithelium has been shown to be transciently infectable by HIV-1 and most likely plays a role in transporting the virus to target cells, resulting in productive systemic viral infection (Campbell, Crowe, and Mak, 2001). This is also the surface targeted for novel microbicide therapeutics. Since FSLGb₃ was an effective inhibitor of X4 HIV-1 infection in Jurkat cells and both X4 and R5 HIV-1 in PBMCs, I assessed the capacity of FSLGb₃ to inhibit infection of the Vk2 vaginal keratinocyte cell line and the Hek1A endocervical cell line which are infectable by HIV-1 (Kaushic, C., Personal communication; Berlier, Bourlet, et al, 2005). The Vk2 cell line expresses Gb₃ while the Hek1A does not, confirmed by VT binding assay (Lingwood A., Personal communication). We found the HIV-1ᵢᵢᵢB (m.o.i., 0.1) was pre-incubated with 400µM FSLGb₃ which was the minimum concentration to show complete inhibition of HIV-1ᵢᵢᵢB infection in Jurkat and PBMCs by viral pre-incubation. Cells were plated in 6 well plates 24 hours prior to infection, and then infected with the treated HIV-1ᵢᵢᵢB for 1 hour at 37°C. Cells were washed and cultured for 24 hours and then lysed and removed from the BSL-3 facility for DNA extraction and quantitative PCR for proviral DNA. I found that FSLGb₃ treatment reduced the proviral copy number of HIV-1 cDNA from 42 to 37 copies/20µL reaction (Fig. 4.4A) in the Hek1A cell line, which was an 11% reduction in infection compared to untreated controls. In the Vk2 cell line, FSLGb₃ treatment reduced the proviral copy number from 71 to 32 copies/reaction (Fig. 4.4B), a 55% reduction in infection levels compared to control.
FIGURE 4.4

Hek1A Cell Line

A)

Vk2 Cell Line

B)
Figure 4.4: FSLGb₃ treatment inhibits HIV-1 infection of human endocervical and vaginal cell lines. HIV-1ⅢB (m.o.i., 0.1) was pre-incubated with 400µM FSLGB₃ for 1 hour prior to infection of both Hec1A and Vk2 cells which were plated 24 hours previously. Quantitative real time PCR of total genomic cellular DNA (50ng/reaction) was used to detect proviral HIV-1 cDNA and genome copy number was determined based on comparison to standards. Results are from pooled triplicates. (A) Hek1A human endocervical cell line infection and inhibition by FSLGb₃. (B) Vk2 human vaginal keratinocyte cell line infection and inhibition by FSLGb₃.
4.7 VSV/HIV infects vaginal and rectal mucosa of CD1 mice

After confirming that the Gb$_3$ compounds inhibited VSV/HIV infection, I continued by optimizing the mouse model I would use to test the compounds \textit{in vivo}. CD1 mice were used in all experiments. I first began by infecting mice vaginally and rectally via pipette with 25µL of VSV/HIV and allowing infection to proceed in live mice for 2 hours. I then euthanized the mice and determined infectivity by DNA-PCR of the DNA samples from homogenized tissue. I found infection to be variable. I then anesthetized the mice with a ketamine and xylazine combination for up to 2 hours during infection to immobilize the mice and ensure the virus remained in the vagina or rectal cavity. Infectivity continued to be variable. I next found that by first euthanizing the mice and then challenging the vaginal or rectal cavity with virus for 2 hours that I could get reproducible and consistent infection by DNA-PCR. This protocol was approved by the Animal Use Committee of the University Health Network and this organ culture system was used in all subsequent experiments. Infection of both rectal and vaginal mouse mucosal tissue was confirmed (Fig. 4.5 A and B).
FIGURE 4.5

A)

B)
Figure 4.5: VSV/HIV infects both vaginal and rectal mucosa of CD1 mice.

CD1 outbred mice were first euthanized and then 25µL of VSV/HIV was administered via pipette to the vaginal and/or rectal cavity and incubated at 37°C for 2 hours. The vagina or rectum was then removed and frozen in liquid nitrogen. Thawed samples were homogenized and then total DNA was isolated from the tissue samples. DNA-PCR (50ng total DNA) was conducted to detect proviral DNA. (A) Expression of HIV-1 cDNA in rectal samples of 2 CD1 mice. NL4-3luc plasmid was used as a positive control and uninfected mice were used as a negative control. (B) Expression of HIV-1 cDNA in vaginal samples of 2 CD1 mice. NL4-3luc plasmid was used as a positive control and an uninfected mouse was used as a negative control.
4.8 Mouse infection model using a vaginal cream

I proceeded to obtain a water soluble vaginal cream from Wellspring Pharmaceuticals as a possible microbicide delivery system. In order to determine if soluble Gb₃ would inhibit VSV/HIV infection \textit{in vivo} and thus show potential as a microbicide, it was first necessary to determine if infection through the delivery agent was possible and at which virus to cream ratio. This was essential in order to demonstrate that any inhibition was due to the compound and not an inhibitory effect of the cream. I mixed the cream with the virus in various ratios of cream to virus and then infected the mice as above. I found that infection was possible at 1:2 and 1:3 ratios of virus to cream in both vaginal and rectal samples (Fig. 4.6A).
Figure 4.6: VSV/HIV infects vaginal and rectal mucosa through cream

CD1 mice were euthanized and then infected with 25μL of virus and vaginal cream mixture in one of three ratios of cream to virus: 1:1; 1:2; or 1:3 both vaginally and rectally. The vagina or rectum was then removed and frozen in liquid nitrogen. Thawed samples were homogenized and then total DNA was isolated from the tissue samples. DNA-PCR (50ng total DNA) was conducted to detect proviral DNA. (A) Expression of HIV-1 cDNA in rectal and vaginal samples. Vaginal lane 1; 1:1 ratio, lane 2; 1:2 ratio, lane 3; 1:3 ratio, Rectal lane 1; 1:1 ratio, lane 2; 1:2 ratio, lane 3; 1:3 ratio. NL4-3Luc plasmid was used as a positive control, and water and uninfected mice as negative controls.
4.9 Mouse infection model using gel and quantitative real time PCR

Mixing the virus with cream as explained above compromised the integrity of the cream and created a diluted cream/virus mixture that would not be consistent with a delivery system for potential use in humans. It was necessary to amend the model to a more real world scenario. This was accomplished in two ways. First, I obtained an aqueous carbopol based gel that I could insert accurate concentrations of the Gb₃ compounds into without changing the consistency of the gel. This gel was similar to gels currently used in clinical trials of the microbicide candidates (Carballo-Dieguez, Balan, et al, 2007; Morrow, Rosen, et al, 2003; Rosen, Morrow, et al, 2008; Williams, Newman, et al, 2007). In addition, in subsequent experiments, I first applied the gel to the mucosa and then challenged with virus, which is what would occur in the real world use of HIV prophylactics. To resolve the problem of quantifying infection levels, I used quantitative real time PCR to detect viral copy number and thus enabled the quantification of infection level. This technique was first optimized and a standard curve was created using concentrations of known HIV-1 DNA to compare unknown samples to. Standards were kindly supplied by Dr. Chen Liu of Montreal.
4.10 Effect of AdamantylGb$_3$ and FSLGb$_3$ in gel on VSV/HIV infection

To determine if adamantylGb$_3$ or FSLGb$_3$ would inhibit VSV/HIV infection by a gel containing the compounds, I created a 1mM adamantylGb$_3$, 1mM FSLGb$_3$ gel and PBS control gel. This was applied to the rectal and vaginal mucosa for 30 minutes followed by 1.5hr infection duration. I found no significant difference in either vaginal and rectal samples between the control group with placebo gel and the compound-containing gel groups (Fig. 4.7 A and B). There was a slight increase in infection in mice given adamantylGb$_3$ containing gel, however this was not significant and most likely due to sample variations (Fig. 4.7 A and B). When outliers were removed there is an indication for a trend towards inhibition, particularly in the FSLGb$_3$ group (Fig. 4.7 C and D).
FIGURE 4.7

A) VAGINAL

B) RECTAL

C) VAGINAL

D) RECTAL
Figure 4.7: Effect of application of AdamantylGb₃ and FSLGb₃ containing gel to vaginal and rectal mouse mucosa on VSV/HIV infection. CD1 mice were euthanized and then 15µL of gel containing 3mM FSLGb₃, 1mM AdamantylGb₃ or gel without compounds as control was applied to the rectal or vaginal mucosa for 30 minutes and incubated at 37°C. VSV/HIV was then administered to the mucosal surface and infected for a period of 1.5 hours at 37°C. The tissue was removed and frozen in liquid nitrogen. The tissue was then homogenized and DNA was isolated. Quantitative Real Time PCR was used to detect proviral HIV-1 cDNA. Results are shown in mean copy number of HIV-1 cDNA and represent n=10 in the control and adamantylGb₃ groups, and n=5 in the FSLGb₃ group. 

(A) Vaginal mucosal infection of VSV/HIV in CD1 mice after administration of control gel, or gel containing 3mM FSLGb₃ or 1mM adamantylGb₃. 

(B) Rectal mucosal infection of VSV/HIV in CD1 mice after administration of control gel, or gel containing 3mM FSLGb₃ or 1mM adamantylGb₃. 

(C) With outliers removed n=8 CTRL; n=8 adaGb₃; n=4 FSLGb₃ (vaginal). 

(D) With outliers removed n=8 CTRL; n=8 adaGb₃; n=4 FSLGb₃ (rectal).
4.11 Effect of direct application of FSLGb$_3$ on VSV/HIV infection

Next, I sought to determine if 3mM FSLGb$_3$ could inhibit VSV/HIV infection when applied directly in PBS to the vaginal and rectal mucosa before viral challenge. I found no significant difference between the PBS control and FSLGb$_3$ group (Fig. 4.8 A and B). However, some of the values were apparent outliers and if removed and the effect recalculated, a trend towards inhibition was evident in both the vaginal and rectal experiments (Fig. 4.8C and D). Additional animals may confirm this effect.
FIGURE 4.8

A) RECTAL

B) VAGINAL

C) RECTAL

D) VAGINAL
Figure 4.8: Effect of direct application of FSLGb<sub>3</sub> to mouse vaginal and rectal mucosa on VSV/HIV infection. CD1 mice were euthanized and then 15µL of either PBS or PBS containing FSLGb<sub>3</sub> was applied to the rectal or vaginal mucosa for 30 minutes and incubated at 37°C. VSV/HIV was then administered to the mucosal surface and infected for a period of 1.5 hours at 37°C. The tissue was removed and frozen in liquid nitrogen. The tissue was then homogenized and DNA was isolated. Real time PCR was used to detect proviral HIV-1 cDNA. Results are shown in mean copy number of HIV-1 cDNA and represent n=5 in each group. (A) Rectal mucosal infection of VSV/HIV in CD1 mice after administration of PBS control, or PBS containing 3mM FSLGb<sub>3</sub>. (B) Vaginal mucosal infection of VSV/HIV in CD1 mice after administration of PBS control, or PBS containing 3mM FSLGb<sub>3</sub>. (C) With outliers removed n=4 (rectal). (D) With outliers removed n=4 (vaginal).
4.12 Effect of FSLGb$_3$ on mouse vaginal and rectal mucosal cytokine profiles

The Bio-Plex results indicated that exposure of high dose of FSLGb$_3$ to vaginal and rectal mucosa had varying effects on cytokines compared to N9 control. Baseline controls were conducted as well, however at the time of submission I was unable to run these samples, and it will be prudent to complete this work as part of future experiments resulting from this study. All results at this point are in comparison to a 2% N9 positive control and n=8 baseline control before treatment.

In vaginal samples compared to N9 control I observed that IL-1$\alpha$ concentration initially decreased but then increased in the FSLGb$_3$ group compared to N9 at 24 and 48hrs, however these concentrations were lower than the initial baseline sample (Fig 4.9A). For IL-1$\beta$, at 4hrs FSLGb$_3$ increased in comparison to N9 (Fig. 4.9B). For IL-6, in both N9 and FSLGb$_3$ groups the cytokine concentration increased at 4hrs and then went back to baseline (Fig. 4.9C). There was a significant ($P<0.05$) increase in IL-10 secretion after 24hrs in the FSLGb$_3$ group compared to N9 (Fig. 4.9D). This effect was maintained at 48hrs ($P=0.055$), and was also significant in comparison to baseline levels of IL-10 ($P=0.04$). IL-13, GM-CSF, MCP-1, and TNF-$\alpha$ showed no significant change over time in comparison to baseline samples for each group (Figs 4.9 E,F,I,M). IFN-$\gamma$ increased in the N9 group at 12 hours (Fig 4.9G). However, baseline levels of this cytokine were already increased in the FSLGb$_3$ group in initial samples. Murine KC (human IL-8 equivalent) increased in both N9 and FSLGb$_3$ groups at 4hrs (Fig. 4.9H). For MIP-1$\alpha$, concentration was consistent over time in the FSLGb$_3$ group, however expression was decreased in the N9 group in comparison to FSLGb$_3$ at 24 and 48 hrs and this was a significant different ($P<0.05$) (Fig. 4.9J). This trend was also observed
similarly for MIP-1β (Fig. 4.9K) For RANTES, concentration decreased in the N9 group for 24 and 48 hr samples (Fig 4.9L).

For rectal samples, IL-6 was undetectable for all samples (Fig. 4.10C). For IL-1α the FLSGb3 group showed a decreased concentration at time 4hrs which returned to baseline by 48hrs (Fig. 4.10A). For IL-1β, N9 caused a spike in concentration after 4hrs, which was not mirrored in the FLSGb3 group (Fig. 4.10B). IL-10 and IFN-γ were nearly undetectable, whereas IL-13 concentration increased over time in the FLSGb3 group over time (Figs. 4.10 D,G,E). KC (human IL-8) increased in the N9 group after 4hrs but not the FSLGb3 group (Fig. 4.10H). Results for GM-CSF were variable; expression was not seen at all time points (Fig. 4.10F). MCP-1 was expressed only at 4hrs for both groups and then again at 48hrs in the FSLGb3 group (Fig. 4.10I). MIP-1α was low or undetectable, with highest expression at 12hrs in the FSLGb3 group (Fig. 4.10J). For MIP-1β, N9 induced higher expression at 4hrs which was not seen in the FSLGb3 group (Fig. 4.10K). RANTES expression was nearly undetectable, but increased at 12hrs in the N9 group (Fig. 4.10L). Lastly, for TNF-α, the FSLGb3 group saw the highest level of expression, particularly at 4 and 48hrs post treatment (Fig. 4.10M).
FIGURE 4.9

A. IL-1α

B. IL-1β

C. IL-6

D. IL-10

E. IL-13

F. GM-CSF

G. IFN-γ

H. KC

I. MCP-1

J. MIP-1α

K. MIP-1β

L. RANTES

M. TNF-α
Figure 4.9: Cytokine levels in cervicovaginal lavage samples after application of N9 and FSLGb₃. Cervicovaginal lavage samples were collected initially, and at 4, 12, 24 and 48 hours after 25μL application of 2% N9 or 1mM FSLGb₃. Samples were frozen at -80°C until analysis with the Bio-Plex machine. Each value represents the mean +/- SEM for a sample size of n=4 in each treatment group. Baseline values represent n=8 before treatment. (A-M) correspond to the following cytokines in order: IL-1α, IL-1β, IL-6, IL-10, IL-13, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α.
FIGURE 4.10
Figure 4.10: Cytokine levels in rectal lavage samples after application of N9 and FSLGb3. Rectal lavage samples were collected initially, and at 4, 12, 24 and 48 hours after 25 µL application of 2% N9 or 1mM FSLGb3. Samples were frozen at -80°C until analysis with the Bio-Plex machine. Each value represents the mean +/- SEM for a sample size of n=4 in each treatment group. Baseline values represent n=8 before treatment. (A-M) correspond to the following cytokines in order: IL-1α, IL-1β, IL-6, IL-10, IL-13, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α.
CHAPTER 5:

DISCUSSION AND FUTURE DIRECTIONS
5.1 Impact

The HIV/AIDS pandemic continues to have a devastating global effect. In the developing world, where treatment options are limited, infection is synonymous with a severely shortened lifespan as death from AIDS occurs about a decade after initial infection (Rees, 2008). In the developed world, where treatment is more accessible, HIV/AIDS has become a chronic condition, although current regimens are in no way ideal due to side effects, resistance, compliance issues and cost. Knowledge of the pathogenesis of HIV is clearly incomplete and only through continued research will our understanding improve, and new methodologies to prevent and treat infection be identified. Previous findings (Lund, Olsson, et al, Submitted; Lund, Branch, et al, 2005; Lund, Branch, et al, 2006; Ramkumar, Sakac, et al, 2008) have suggested that the pathogenesis of HIV infection is inversely proportional to the expression of the glycosphingolipid, Gb$_3$. The research described in this Thesis has further contributed to the understanding of HIV pathogenesis and identified a novel, completely synthetic and water soluble Gb$_3$ analogue as a potent inhibitor of HIV infection. Additionally, I have established a mouse model to test Gb$_3$-based microbicide candidates using a modified HIV virus that is approved for Biosafety Level 2. Overall, my findings may contribute to the development of potential novel therapeutics for the prevention and/or treatment of HIV/AIDS.

5.2 Summary of key findings

5.2.1 Rationale

Previous studies by our group using different experimental approaches have implicated Gb$_3$ as a resistance factor in HIV infection. We have shown that a soluble Gb$_3$
mimic, adamantylGb₃, which binds gp120, (Mahfoud, Mylvaganam, et al, 2002) is a fusion inhibitor of both X4 and R5 HIV-1 and HIV-2 infection of both HIV target cell lines and primary cells by the prevention of membrane fusion (Lund, Branch, et al, 2006). PBMCs from patients with Fabry disease, who accumulate Gb₃ cellularly due to a defective α-galatosidase A required for Gb₃ degradation are resistant to R5 HIV-1 infection (Lund, Branch, et al, 2005). Gb₃ is also a blood group antigen denoted Pᵏ (Spitalnik, and Spitalnik, 1995). This blood group comprises Pᵏ (Gb₃), P (Gb₄) and P₁ (a pentaosyl ceramide). Individuals with the p phenotype express no Gb₃ due to a defective α galactosyl transferase. We have shown that PBMCs from p individuals, who do not express Gb₃ are highly susceptible to both X4 and R5 HIV infection, while P₁ᵏ variants, who accumulate Gb₃, resisted infection (Lund, Olsson, et al, Submitted). Also, Gb₃ synthase gene silencing using siRNAs results in increased HIV infection while introduction of Gb₃ synthase into cells causes resistance to HIV infection (Lund, Olsson, et al, Submitted). Additionally, the chemical induction of Gb₃ accumulation in HIV-1 infectable cell lines results in resistance to infection, while pharmacological blocking of the Gb₃ synthetic pathways increases cellular susceptibility to HIV-1 infection (Ramkumar, Sakac, et al, 2008). These findings taken together strongly argue that Gb₃ can play a major inhibitory role in HIV infection and warrants further exploration into the relationship between Gb₃ and HIV pathogenesis.

5.2.2 FSLGb₃: A novel synthetic Gb₃ analogue that inhibits HIV infection

My first aim was to evaluate a new soluble Gb₃ analogue obtained from KODE Biotech™ that I hypothesized would inhibit HIV infection similarly to adamantylGb₃ (Lund, Branch, et al, 2006). FSLGb₃ is structured so that the carbohydrate moiety of Gb₃
is coupled to phosphatidyl ethanolamine via a phosphate linkage. I have found that pre-
incubation of HIV with FSLGb$_3$ inhibits HIV-1$_{\text{IIIB}}$ infection of both Jurkat T cells and
PBMCS and HIV-1$_{\text{Ba-L}}$ infection of PBMCs similarly to previous data with
adamantylGb$_3$. The apparent lack of ability of FSLGb$_3$ to inhibit HIV-1$_{\text{Ba-L}}$ infection of
Jurkat T cells is puzzling as FSLGb$_3$ was clearly shown to effectively inhibit HIV-1$_{\text{Ba-L}}$
infection of PBMCs. Although Jurkat cells become productively infected with
HIV-1$_{\text{Ba-L}}$, I have determined using FACS that CCR5 co-receptor is not detectable on the
Jurkat cells. It is not uncommon for the co-receptor expression to be below FACS
detectable limits as detection is dependent upon the particular anti-CCR5 and the level of
cell surface expression. I did not use RT-PCR to confirm CCR5 mRNA in Jurkat cells
but this should be done. It is unlikely that the particular Ba-L strain that I was using has
become contaminated with the IIIB strain as FSL-Gb$_3$ inhibits HIV-1$_{\text{IIIB}}$ infection of
Jurkat very well. It is possible that our Ba-L strain recognizes additional entry
components that it can utilize that are present on the Jurkat cells whereby soluble Gb$_3$ is
unable to inhibit its entry.

_**In vivo**, in mice FSL compounds have been shown to have no maximum tolerated
dose up to 100 mg/kg (Dr. Stephen Henry, personal communication). Indeed, when used
in millimolar concentrations FSLGb$_3$ had no observable toxicity to cells _in vitro_. Low
toxicity would be critical if the compound were to be further explored for human use as a
systemic and/or local potential treatment. Our _in vitro_ observation could be qualified
further by evaluation for any increase in apoptotic and necrotic cells after treatment with
FSLGb$_3$ using FACS and Annexin V-FITC/PI staining. Also, more extensive _in vivo_
toxicity testing is warranted.
I have found that pre-incubation of the Jurkat T cell line with FSLGb3 render these Gb3-negative cells 100% Gb3-positive by FACS analysis. Coating of the cells with FSLGb3 caused them to resist HIV infection, at least at 3 days post infection. With HIV-1IB2, I show a significant inhibition of infection, and while infection with Ba-L was not significant, a trend towards overall inhibition was noted. This is an important finding, since this was not observed with adamantylGb3 in previous experiments (Lund, Branch, et al, 2006). AdamantylGb3 is a more bioactive molecule than FSLGb3, where adamantylGb3 can enter cells and act as an alternative substrate in GSL biosynthesis, although this property has not been directly tested with FSLGb3. The finding that FSLGb3 can convert Gb3-negative cells into cell surface-expressing Gb3-positive cells offers potential ways to utilize the compound as a therapeutic, both through systemic inhibition and through the coating of mucosal target cells in a microbicide formulation.

Cellular GSL analysis via VT1 TLC overlay revealed that FSLGb3 was cell-associated initially after treatment but was lost from the cell within 24 hours. These results were also consistent with our findings via FACS analysis. Additionally, treatment with FSLGb3 had no effect on the HIV receptors CXCR4 and CD4. Consistent with our results that FSLGb3 was lost from the cell membrane within 24 hours, I found that when Jurkat cells were treated initially and then once daily for a 5 day period, that addition of FSLGb3 to the culture medium was required to maintain a significant inhibition of long-term HIV infection in culture compared to cells treated once only. At this time, I am
unsure of the mechanism of FSLGb₃ loss from membranes, but hypothesize that the molecule is lost into the culture medium or is internalized and rapidly degraded through cellular catabolic pathways. Although the finding that FSLGb₃ is lost within a 24 hour period is disappointing, this is not at all unusual for pharmaceutical agents where daily dosing or even multidoses taken daily are required for therapeutic benefit. However, further studies exploring the kinetics of FSLGb₃ loss from cells and the mechanism involved would be valuable in determining its suitability as a therapeutic.

5.2.3 Mouse in vivo model for testing Gb₃ based microbicides

The next aims of the study were to establish an in vivo mouse model of mucosal infection for the testing of Gb₃ based microbicides candidates and to use the model to determine efficacy of soluble Gb₃ analogues in VSV/HIV infection. The finding that VSV-G binds to GSLs similarly as to gp120 and that a similar binding sequence to the GSL binding site in gp120 was found in VSV-G by our group validates use of a mouse model using a VSV-G pseudoenvelope-typed HIV as an appropriate alternative to assess the effect of soluble Gb₃ analogues on VSV/HIV infection in vivo. This finding not only establishes the validity of our model, but may also provide greater understanding of Rhabdoviruses in general, as although their promiscuity for nearly all cell types is widely known, the receptor responsible for viral attachment is not (Coil, and Miller, 2004; Roche, Albertini, et al, 2008).

We found that the consistent infection of mice required for our purposes was met by utilizing a protocol whereby the mouse was first euthanized and then, within 1-2 hours, the vagina or rectum/colon was infected with VSV/HIV. Infection through cream was possible, however a significant dilution of the base product was required to permit
infection. Reliable baseline infection levels were necessary to determine potential
efficacy of soluble Gb$_3$ in inhibiting infection in future studies. For this reason, I
obtained a gel similar to that used in current microbicides trials for use in our model and
opted to pursue a qPCR system to determine copy number of HIV cDNA, allowing a
quantitative analysis of infection. Our findings currently show no significant difference
between control gel or PBS and FSLGb$_3$/adamantlyGb$_3$ containing gel or direct
application of FSLGb$_3$ in PBS to mucosa. While this is disappointing, there was
significant variability between the level of infection of some individual mice, which has
worked to skew the results. When these “outliers” are removed, a trend toward inhibition
is seen. A larger sample size and/or using an inbred strain of mice, as opposed to the
outbred CD1 mice, would be required to determine if this trend is significant.

A higher concentration of the Gb$_3$ analogues was required to inhibit VSV/HIV
infection compared to intact HIV-1. Even a slight decrease in VSV/HIV infection in this
model could correlate to a greater inhibition of intact HIV. The VSV/HIV virus is highly
promiscuous in terms of its cellular targets and this property could make it challenging to
reduce infection of cells in vivo compared to the more selective HIV-1. Indeed I found
in another model using cervicovaginal epithelial cells, that when challenged with HIV-1
pre-treated with FSLGb$_3$, I saw a reduction in HIV proviral copy number by qPCR.
Cumulatively, our findings support further study of the Gb$_3$ analogues in a different
model such a tissue explant system, Rg$^{\phi}$ SCID/hu HIV-1 mouse model, or the use of
nonhuman primates.
5.2.3 Cytokine analysis for toxicity

Our last aim was to attempt to evaluate safety of FSLGb$_3$ and adamantylGb$_3$ through the evaluation of cytokine profiles after administration to mucosal tissue in a mouse lavage model. Due to a lack of sufficient adamantylGb$_3$ at the time, these experiments were conducted using only FSLGb$_3$. I used N9 as a positive control as this compound has been documented to increase early inflammatory cytokines and cause an increase in HIV infection (Cone, Hoen, et al, 2006; Fichorova, Tucker, and Anderson, 2001; Hillier, Moench, et al, 2005; Van Damme, Ramjee, et al, 2002). At this time, our data is not complete and due to variability between samples it is imperative that I increase our animal numbers to obtain validity of results, as well as conduct baseline PBS negative controls. However, given these caveats, I nevertheless obtained some interesting preliminary results.

There are significant differences between the mucosal tissues of the vaginal and rectum. The vagina consists of a stratified squamous epithelium that is able to resist abrasion and mechanical forces that are typical of intercourse (McGowan, Elliott, et al, 2007). In contrast, the rectal epithelium consists of a single layer of columnar epithelial cells that has little ability to withstand trauma. Beneath the epithelial barriers of the mucosa lies a lamina propria layer that contains immune cells that are targets for HIV infection. Since the rectal epithelium is easily breached, it is highly sensitive to HIV infection (McGowan, Elliott, et al, 2007). From this it would be expected that immunological reactions and inflammation due to trauma would be more severe in rectal mucosa, however, thus far there have been few trials to characterize rectal safety of HIV microbicides (McGowan, Elliott, et al, 2007).
Of the proinflammatory cytokines, IL-1α and β, IL-6, IL-8 (KC in mice), and IFN-γ have significant association with increased risk of HIV infection through NF-κB mediated LTR activation and through the recruitment of HIV-1 susceptible cells which could become infected by the virus (Aranha, Gupta, and Reddy, 2008; Fichorova, 2004). IL-10 is a significant immunosuppressive that inhibits the production of various inflammatory cytokines (Conti, Kempuraj, et al, 2003). The CCR5 ligands MIP-1α, MIP-1β and RANTES are also associated with anti-HIV suppression (Cocchi, DeVico, et al, 1995; Gonzalez, Kulkarni, et al, 2005).

Interestingly, our preliminary results in rectal samples suggest that FSLGb₃ is not associated with inflammation, whereas in the vaginal samples, there is some indication of inflammation as the inflammatory cytokines IL-1α, IL-1β, IL-6 and IL-8 were all elevated compared to baseline values. However, the anti-inflammatory cytokine IL-10 was elevated in the FSLGb₃ group. As well, compared to N9, which showed a down-regulation of the cytokines involved in suppressing HIV activity (MIP-1α, MIP-1β, RANTES) this observation was not seen after application of FSLGb₃. Finally, proinflammatory TNF-α was elevated in the rectal FSLGb₃ group at three time points. Inflammation could have been mediated by trauma to the vaginal/rectal canal and it would be necessary to compare the results found here to PBS negative controls to normalize inflammation due to technique.

Overall, these preliminary studies show that cytokine levels in the vaginal and rectal mucosa differ greatly, both in their initial baseline values and after application of the same compounds. This work shows that in the development of any potential
microbicide that it is necessary to conduct studies evaluating toxicity in both mucosal surfaces.

5.3 Globotriaosylceramide and HIV-1 infection

As I have discussed previously, GSL containing lipid rafts are a central part of the HIV lifecycle. CD4 must interact with the co-receptor within lipid rafts in order for virus-host cell fusion to occur (Fantini, Hammache, et al, 2000; Liao, Cimakasky, et al, 2001). CXCR4 is not initially associated with rafts, but becomes recruited to GM3 containing rafts after CD4 binding to gp120 (Nguyen, Giri, et al, 2005; Sorice, Garofalo, et al, 2001). It has been shown that CD4 and CCR5 may interact with lipid rafts that contain the GSLs GM3 and Gb₃ (Hammache, Piéroni, et al, 1998; Manes, Lacalle, et al, 2001; Sorice, Parolini, et al, 1997). The model proposed by Fantini et al suggests that a cooperation between GSLs, CD4 and chemokine receptors facilitates the fusion process (Fantini, Hammache, et al, 2000; Hammache, Yahi, et al, 1999). Specifically, in that gp120 binds to GSLs in lipids rafts which then facilitates the migration of the CD4-gp120 complex to the chemokine receptor (Fantini, Hammache, et al, 2000; Hammache, Yahi, et al, 1999). Recently I have determined that gp120 binding to Gb₃ may be dependent on the fatty acid moiety of Gb₃, as the molecule can exist in different isoforms based on levels of saturation and chain length of the fatty acid component (Mahfoud, and Manis, 2008).

The work conducted here supports previous studies by our group implicating Gb₃ as a resistance factor in HIV infection (Lund, Olsson, et al, Submitted; Lund, Branch, et al, 2005; Lund, Branch, et al, 2006; Ramkumar, Sakac, et al, 2008) despite previous reports that suggest Gb₃ was as a facilitator of membrane fusion (Fantini, Hammache, et
al, 2000). However, as Gb₃ is either lacking or expressed at very low levels on resting or activated peripheral blood T-cells, a role for Gb₃ as facilitating the HIV infection through Gb₃ is unlikely. Our model proposes the following (Fig 5.1). Initially, the gp-120-C2 loop that binds CD4 causes the V3 loop of gp120 to undergo a conformational change which exposes both the GSL (Gb₃) and chemokine co-receptor binding motifs. The binding motif XXXGPGGRAFXXX (Delezay, Hammache, et al, 1996) within the V3 loop which binds Gb₃ overlaps with the chemokine consensus binding sequence S/GXXXGPGXXXXXXXE/D (Xiao, Owen, et al, 1998) also in the V3 loop. Fantini suggests that following this, the V3 loop interacts with GSLs such as Gb₃, and a subsequent interaction with the chemokine receptor follows, leading to the virus-host cell fusion reaction. I suggest here that Gb₃ binds to its motif within the V3 loop and thus prevents subsequent binding of the V3 loop with a chemokine co-receptor, which then prevents the conformational change necessary for gp41-mediated viral fusion (Figure 5.1). Thus, if soluble Gb₃ can be introduced into the blood stream where it can interact with circulating HIV-1 directly or be adsorbed onto the Gb₃-negative T-cell targets of HIV-1, effective inhibition or prevention of HIV-1 infection may be attained.
Indeed, since Gb₃ binds gp120 (Mahfoud, Garmy, et al, 2002; Mylvaganam, and Lingwood, 1999) it is not difficult to picture the situation where Gb₃ is over-expressed, or what could occur if external Gb₃ is added to the process. Increased Gb₃ could lead to more Gb₃-gp120 interactions, which could sequester the virus and prevent infection, since Gb₃ could compete for chemokine co-receptor binding to the V3 loop. Additionally, soluble Gb₃ adsorbed onto cellular membranes could restrict the lateral movement of the HIV-1gp120-CD4 complex within the membrane by steric hindrance, and thus prevent co-receptor interaction. Indeed, reduction in lateral CD4 movement has been shown to inhibit infection (Rawat, Zimmerman, et al, 2008).
5.7 Conclusions

Understanding the role of GSLs such as Gb$_3$ in HIV/AIDS has the potential to lead to novel therapeutic strategies to both topically prevent infection, and treat patients systemically. Overall, this study has contributed to understanding the role of Gb$_3$ in HIV pathogenesis. Specifically, I have shown that a novel, synthetic, completely water soluble Gb$_3$ compound having low toxicity inhibits infection \textit{in vitro} through multiple mechanisms; the coating of both the virus and target cells. These properties make FSLGb3 an ideal candidate for further examination as both a topical preventative and systemic treatment for HIV/AIDS. Current results and future data stemming from these results will support further studies using non-human primate models for the future development of Gb$_3$-based systemic and local therapeutics.
5.8 Future directions

While this study has made a significant contribution to understanding the potential inhibitory role of Gb₃ in HIV infection, there are areas of research that should be explored further, and several questions remain.

1. Additional toxicity studies examining the safety of both adamantylGb₃ and FSLGb₃ by the measurement of inflammatory cytokines in mouse mucosa and evaluating the potential for direct mucosal toxicity. While I have begun these studies, it is necessary to increase subject numbers and establish baseline controls. It would also be necessary to conduct additional systemic toxicity studies that would include determining maximum tolerated dose (MTD), histological studies, and other pre-clinical trial analyses of toxic effects using mice, rabbits and other animals prior to moving to a non-human primate model.

2. Studies of Gb₃ levels in vaginal and rectal mucosal cells and the pharmacological manipulation of these cells using DGJ and cellular absorption of Gb₃. These studies would be used for the development of a potential Gb₃ based microbicide.

3. Additional studies testing the direct application of Gb₃ containing gel or cream for the prevention of mucosal HIV infection. This would be done by (a) upscaling our current model system by using greater subject numbers and (b) using human cervical and vaginal cell lines and an in vitro model of mucosal transcytosis.

4. Moving the Level 2 studies to Level 3 mouse model using Rg⁻/⁻ SCID/human mouse model and whole HIV-1 virions. The mechanism of resistance to HIV-1 infection by cell surface expressed Gb₃ has not yet been elucidated. We hypothesize that inhibition is the result of Gb₃ binding to the Gb₃ binding motif of
the V3 loop of gp120, preventing chemokine receptor binding, gp41 activation and thus viral fusion/entry. To test this we will (a) use transmission electron microscopy with a primary antibody to gp120 followed by a secondary antibody coupled to colloidal gold. We would expect to see free virions and virions bound to cell membranes but no fusion to cell entry. (b) Pharmacological manipulation of Gb3 expressing cell line models using DGJ to increase Gb3 expression and siRNAs to silence Gb3 synthase mRNA and then to monitor viral entry by quantitative PCR and/or RT detection.

5. It is necessary to determine the kinetics of absorption in vivo and the potential of soluble Gb3 as an inhibitor of systemic HIV infection. Here an in vivo mouse model could be used to determine the optimal route and schedule of drug administration (oral or intravenous, and once or twice daily, etc.) through the monitoring of Gb3 expression of mouse hematopoietic cells, CD4+ and CD8+ cells, and monocytes.
CHAPTER 6:

BIBLIOGRAPHY


