Molecular Engineering of a Banana Lectin that Inhibits HIV-1 Replication

by

Michael Dennis Swanson

A dissertation submitted in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
(Immunology)
in The University of Michigan
2010

Doctoral Committee:

Professor David M. Markovitz, Chair
Associate Professor Kathleen L. Collins
Associate Professor David Miller
Assistant Professor Gary D. Luker
Assistant Professor Akira Ono

Dedication:

To my wife Christina, the best discovery I made as a graduate student.

Acknowledgements

I would like to thank my thesis mentor, Dr. David Markovitz, for his support and guidance during my time as a graduate student. In addition, I greatly appreciate his help with my oral and written communication skills.

I wish to thank the past and current members of David's laboratory. They have been great people to work with and have provided me with technical assistance. I would like to thank Dr. Nirit Mor-Vaknin for her help with the isolation of lymphocytes from peripheral blood and Drs. Ferdinand Kappes and Michael Khodadoust for their technical advice. I would like to thank Marta Gonzalez-Hernandez for her help and Rafael Contreras for his help with real-time PCR. I would also like to thank Maureen Legendre for her help with protein purification and obtaining reagents.

Numerous individuals have been kind enough to share their technical expertise with me by providing advice or by performing experiments. I would like to thank Dr. Irwin Goldstein for introducing me to BanLec along with his helpful input and advice. In addition, I would like to thank Dr. Harry Winter for providing BanLec and performing the agglutination assays and the micro-calorimetric assays for the evaluation of lectin:carbohydrate interactions. I would also like to thank Drs. Jeanne Stuckey and

Jennifer Meagher for determining the crystal structure of our BanLec mutant protein in an unbelievably short amount of time. Drs. Kathy Collins, David Miller, Gary Luker, and Akira Ono have been excellent committee members, and I would like to thank them for their constructive criticism and their helpful suggestions. I would also like to thank Dr. Blake Roessler for his mentorship and encouragement over these past years and Dr. Mark Kaplan for his support and input on my project. I would also like to thank Mark for directing me to the International Partnership for Microbicides, which provided the data comparing the mitogenic activity of BanLec and Cynaovirin-N.

Numerous individuals at University of Michigan helped me with learning how to work with HIV-1. I am grateful for Dr. Steve King for providing me with BSL-3 training and advice on various HIV-1 related assays. I am also grateful for the hard work he puts into maintaining the BSL-3 facility. I wish to thank Drs. Jason Norman and Christoph Carter of Kathy Collin's laboratory for their assistance and advice on working with HIV-1.
In addition to the people at the University of Michigan, I am extremely grateful for the NIH AIDS Reference and Reagent Program since nearly every HIV-1-related experiment I performed utilized one or more of their reagents.

I would like to my parents, Dennis and Mary Swanson, and my brothers, Matt and Ryan Swanson, for their support and encouragement, and the rest of my family for their support. In addition, I am grateful for my in-laws, Jeff and Alicen Rogers, for accepting me into their family and tolerating the studying and writing I performed at their home during the holidays. Finally, I would like to thank my wife Christina. She has been very

supportive emotionally and intellectually. Without her, I would have not obtained my Ph.D

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

HIV-1 Biology

Human immunodeficiency virus type 1 (HIV-1) is a lentivirus, which is a genus of the retroviradae family. HIV-1 is an enveloped virus with two copies of single-stranded RNAcomprising its genome. This genome is approximately 9 kb long and encodes for 9 genes and 15 proteins. The *gag* gene encodes the structural proteins, which include the matrix (p17), capsid (p24), and nucleocapsid (p6 and p7) proteins. The HIV-1 enzymes integrase, reverse-transcriptase, and protease are encoded by the *pol* gene. The *env* gene encodes for the envelope proteins: the surface protein gp120 and the transmembrane protein gp41. The *tat* and *rev* genes encode regulatory proteins of the same name that are essential for viral replication. Finally, the accessory proteins, which are dispensable for HIV-1 replication *in vitro*, are encoded by genes of the same name: *vif*, *vpr*, *vpu*, and *nef* (Figure 1-1).

HIV-1 was first isolated in 1983 and later found to be the cause of acquired immunodeficiency syndrome (AIDS). Evidence shows that HIV-1 originated from cross-species transmission of a chimpanzee simian immunodeficiency virus (SIVcpz) to humans [1]. It is estimated that the jump from chimpanzee to humans occurred between 1890 and 1940 and the earliest human sample found to be HIV-1 positive can be traced back to 1959 [2, 3]. Today there are over 30 million individuals infected with HIV-1 [4].

Although the rate of new infections world-wide appears to have peaked in the year 1996, it is estimated there were 2.7 million new infections in 2008 [4].

The persistence of HIV-1 can be attributed to its genetic diversity, which arises from multiple factors that include an error-prone reverse transcriptase that introduces numerous mutations in its genome, frequent recombination events, rapid viral turn-over, and selective pressures stemming from the immune response and transmission bottlenecks [5-7]. This variability of the virus makes the design of vaccines extremely difficult and creates resistance to anti-retroviral drugs. To put this in perspective, more viral genetic diversity exists within one HIV-1 positive individual than can be found in a world-wide influenza epidemic [8]. As a result of this genetic diversity, the virus population that comprises the world-wide HIV pandemic is extremely diverse and can be further categorized into several groups and subgroups based on genetic identity.

HIV-1 is divided into three major phylogenetic groups for HIV-1: group M (main), group O (outlier), and group N (neither belonging to M or O) [9]. The vast majority of HIV-1 infections are caused by group M viruses and these viruses are further divided into subtypes, also referred to as clades, and are assigned a letter [9]. Subtypes are grouped so they are approximately genetically equidistant from each other. However, the genetic variability of genes within each subtype is not identical, e.g. there is 15% genetic variability for the *gag* gene while the variability for the *env* gene is 25% [10].

The most prevalent HIV-1 subtypes are A, C, and D, with nearly half of the HIV-1 infections worldwide being from subtype C. Subtype A viruses are concentrated in central and eastern Africa and in eastern European countries.. Subtype B is the dominant form found in western and central Europe, the Americas, and Australia and is associated with homosexual transmission [10]. The association of homosexually or heterosexually transmitted HIV-1 to a particular subtype is likely due to founder effects and not due to a genetic preference for transmission [10]. Subtype C is dominant in countries with greater than >80% of all global HIV-1 infections, including southern Africa and India [10].

Viral attachment, Fusion, and Entry

The stages of the HIV-1 replicative cycle are as follows: attachment, fusion/entry, reverse-transcription and uncoating, integration, transcription and RNA processing, nuclear export, assembly, budding, and maturation (Figure 1-2). Attachment and fusion are the most pertinent to the work described below, and will be emphasized here.

The trimeric envelope protein complex of gp120 and gp41 drives virus attachment and fusion to the target membrane (Figure 1-3). The primary cellular receptor for HIV-1 is CD4, and binding to this receptor initiates the entry process. Other cellular membrane proteins, such as the integrin α4β7, DC-SIGN, and heparan sulfate proteoglycans (HSPG), have also been shown to bind to HIV-1 [11-13]. However, these proteins are unable to initiate viral entry. Binding of gp120 to CD4 induces a conformational change of the envelope protein. This results in the formation and exposure of the co-receptor

binding site, also known as the bridging sheet [14, 15]. The co-receptor is typically the chemokine receptor CCR5, but it can also be CXCR4. Viruses that utilize CCR5 are known as R5 tropic viruses, and those that utilize CXCR4 are known as X4 tropic viruses. These viruses were once designated as macrophage-tropic and T-cell-tropic, respectively, since these were the cell types that allowed efficient replication of the virus. However, this designation is no longer used since many early R5 tropic viruses prefer to replicate in T-cells over macrophages and there are X4-tropic viruses that can efficiently replicate in macrophages [16-18]. Some viruses are able to utilize either CCR5 or CXCR4 for entry, and these viruses are known as dual-tropic viruses, also designated as R5/X4. In addition, other receptors can be used for entry, but they are believed to be of less significance.

It is believed that CCR5 is of greater importance to infection than other receptors since individuals that are homozygous for a 32 bp deletion in the CCR5 gene (CCR5Δ32) are resistant to HIV infection [19, 20]. This has been further corroborated by what is known as the "Berlin Patient". Briefly, an HIV-1-infected individual received a bone marrow transplant from a CCR5Δ32 homozygous individual and has not had detectable HIV-1 in the two years since the transplant [21]. In addition, the majority of HIV-1 isolates that cause sexual transmission are R5 tropic.

Upon co-receptor binding, further conformational changes allow the hydrophobic fusion peptide gp41 to insert into the target cell membrane. Two trimeric coil-coil structures of

gp41 undergo extensive rearrangement to form a structure that provides the energy for fusion of the target cell and the viral membranes [22].

Pathogenesis

The majority of HIV-1 infections are acquired through heterosexual transmission, but the exact mechanism behind sexual transmission remains to be elucidated. The current working hypothesis of HIV-1 pathogenesis is based on experiments with simian immunodeficiency virus (SIV) infection models, along with clinical findings from infected individuals. The following paragraph describes the current model of male to female sexual transmission of HIV-1 and its progression to AIDS.

A few hours after exposure, the virus is able to cross the vaginal epithelial barrier, where a very small number of viruses are able to establish infection in a resident CD4+ T cell population present in the mucosa. The virus found in this initial population is known as the founder virus, and it has been shown that infections can be traced back to one virus that was able to productively infect a cell [18]. A period of virus amplification occurs locally for approximately one week. The virus and infected cells then make their way to the draining cervical lymph nodes where additional infection of CD4+ T cells take place. The virus reaches the blood stream through the lymphatic system and the thoracic duct and begins to disseminate throughout the body.

The virus now has access to the body's largest lymphoid organ, the gut-associated lymphoid tissue (GALT) [23]. The GALT contains a large number of lymphocytes, many of which are CD4+, CCR5+ T cells. These cells are quickly eliminated upon infection by massive viral replication and viral induced Fas-mediated apoptosis [24, 25]. This depletion occurs irrespective of route of infection, i.e. the same events occur for individuals that are infected intravenously [26]. This model of infection represents a major shift from the paradigm of CD4+ T-cell depletion occurring gradually over a period of years; instead, the majority of this cell population may be quickly eliminated within the first three weeks of infection, while the remaining cells in the periphery are depleted over a longer period of time. At this point, the viral load is at a maximum. Although the immune response cannot keep up with viral mutations and clear the infection, it applies selective pressure on the virus, leading to the development of less replicatively fit virus. As a result, the viral load drops to a steady-state level. If untreated, the virus will continue to replicate and the population of CD4+ T-cells will gradually decline.

SIV models suggest that viral replication by itself is not enough to cause AIDS. Natural SIV infections of African non-human primates, e.g. African green monkeys and sooty mangabeys, lead to high viral loads and decreased CD4+ T cell counts, but these animals do not progress to AIDS [27]. On the other hand, Asian non-human primates, e.g. rhesus and pig-tailed macaques, have similar viral loads and CD4+ T cell counts as do African green monkeys, yet these animals will progress to AIDS.

When these two models are compared, there are higher amounts of immune system activation in the animals that progress to AIDS [27]. Elevated inflammation in the GALT can cause destruction of the epithelial barrier. As a result, commensal bacteria in the GALT are no longer separated and can now interact with the body, which leads to further increased inflammation. The resulting state of chronic inflammation could lead to depletion of cells that leads to AIDS. This potential mechanism is further corroborated by the evidence that the epithelial barrier is not disrupted in the animals that do not progress to AIDS. Therefore, it appears chronic immune activation induced indirectly by the virus causes further immune cell depletion and leads to AIDS. Further studies are being performed to determine why high amounts of activation are not seen in African non-human primates asymptomatically infected with SIV.

HIV-1 Treatment and Prevention

Currently, there are over 25 different anti-HIV-1 drugs and single dose drug co-formulations approved for clinical use [28]. Despite the existence of numerous drug options, few people in the developing world have access to any of these medications. The proportion of HIV-1 positive individuals who are receiving anti-retroviral therapy (ART) has increased from a mere 7% in 2003 to 42% in 2008 [4]. However, the individuals that are able to receive ART in developing countries are receiving therapy which is much less effective than the highly active anti-retroviral therapy (HAART) that individuals in the industrialized world receive. With the number of newly infected individuals in 2009 estimated to be almost 2 million, the costs of ART can only be expected to rise [4].

Current studies suggest that initiating HAART therapy earlier in the course of infection will improve long-term survival rates. In addition, the effectiveness of prophylactic drug use and starting HAART therapy immediately after being diagnosed with HIV-1 infection are under investigation. Therefore, it is likely that there will be an increase in the cost of treating HIV-1 infected individuals. In addition, long-term HAART therapy has been associated with accelerated aging and increased incidence of non-AIDS related cancers [29]. Because of the expense and logistical challenges of delivering effective anti-HIV treatments to an increasing number of HIV positive individuals worldwide and the potential side effects of long-term HAART therapy, there is an urgent need to prevent the spread of HIV by developing effective preventative measures.

The ideal way to end the HIV/AIDS epidemic is through the use of protective vaccination. Unfortunately, an effective vaccine for HIV-1 remains out of reach even after nearly three decades of research. The most promising trial to date used two vaccines that were ineffectual on their own: ALVAC and AIDSVAX. However, efficacy of this vaccine was only about 30% [30]. HIV vaccine development is challenging due to a variety of factors, including unknown correlates of immune protection against viral infection, viral diversity, viral evasion from neutralizing antibodies by mutations, and viral shielding caused by high amounts of N-linked glycosylation found on the HIV-1 envelope.

Current preventative measures for sexual HIV-1 transmission include abstinence, maintaining monogamous sexual relationships, male circumcision, and condom use.

Condoms can be effective, but only if they are used every time and used correctly [31, 32]. These preventative means are more in the control of men than women, i.e. women are not always able to enforce condom use or male circumcision [33, 34]. In addition, women are often at greater risk for HIV-1 infection than men and it is estimated that 60% of the infected individuals in sub-Saharan Africa are women [4, 35]. Thus, there is a need to develop agents that not only can block sexual transmission of HIV-1, but whose use can be controlled by women.

A microbicide is a topical agent that is administered prior to sexual intercourse that protects against sexually transmitted pathogens, for instance HIV-1 [36]. These agents can be in various forms such as suppositories, gels, creams, or foams. These agents could be applied vaginally prior to sexual intercourse by women, therefore giving them more control in their safe sex practices.

Unfortunately, the results from microbicide clinical trials have not yet been promising. Two separate trials have been performed for the detergents C31G and nonoxynol-9. These compounds are relatively inexpensive and inhibit HIV-1 by disruption of the viral membrane. C31G (SAVVY) was shown not to inhibit HIV-1 transmission, while the nonoxynol-9 based microbicide was shown to actually increase HIV-1 infection [37, 38]. Two clinical trials have been performed with polyanions, PRO 2000 and Carraguard, which inhibit HIV-1 by binding charged regions on the HIV-1 envelope and prevent subsequent viral attachment and entry. However, none of these compounds have shown any signs of success. Numerous studies have since suggested that dextran sulfate, the

component of the microbicide Carraguard, can lead to increased HIV-1 infection by increasing amounts of HIV-1 that is able to cross the epithelial barrier or by increased attachment to cellular targets [39, 40]. Despite the previous failures, microbicide development is being continued. Current anti-virals that are being tested in microbicide trials include the NNRTI UC781, the NRTI tenofavir, and a charged dendrimer SPL7013 I (VivaGel) [41]. A variety of candidates are being testing in animal models, including several different HIV-1 entry inhibitors, fusion inhibitors, and integrase inhibitors [42].

Arguably, the best way to prevent HIV-1 infection with a microbicide is to prevent the virus from attaching to and entering the cell. Anti-virals with this mechanism include ionic polymers, anti-envelope monoclonal antibodies, small molecules targeting the CD4 or the co-receptors, fusion inhibitors, and lectins. Because the HIV-1 envelope mediates viral attachment and entry into the cell, it is likely that an effective microbicide anti-viral would interact with the envelope to block these processes.

N-linked Glycosylation

One of the difficulties in obtaining antibodies that are able to recognize the HIV-1 envelope protein and can block infection is the large number of N-linked glycosylation sites found on the HIV-1 envelope. N-linked glycosylation is a carbohydrate structure that is attached to the amino acid asparagine. Glycosylation of asparagines can occur when the amino acid is part of an N-X-S/T motif (also called a sequon), where X is any

amino acid except proline [43]. These sites are termed potential N-linked glycosylation sites (PNGS). N-linked glycosylation structures start out with the attachment of a high-mannose structure that contains terminal glucose residues, which occurs while the protein is in the endoplasmic reticulum. The glycosylation structure is then enzymatically modified in the Golgi apparatus. The resulting structure can be categorized into three different groups: high-mannose, complex, and hybrid (figure 1-4). All three structures contain a common core structure but vary in the carbohydrate groups that can be attached to this core structure. The common core consists of two N-acetyglucosamine residues, one of which is attached to the asparagine side chain and the other is attached to a tri-mannose structures. High-mannose (also known as oligomannose) structures exclusively contain mannose residues attached to the common core structure. Complex oligosaccharides can contain a variety of different carbohydrates, such as galactose, sialic acid, and N-acetyl glucosamine. Hybrid structures have one branch that only contains mannose while the other branch contains carbohydrates that make up complex glycans.

It is not easy to predict what type of N-linked glycosylation can occur at a particular PNGS. Variations exist in the glycosylation structures found at a particular site, referred to as microheterogeneity [44]. In addition, N-linked glycosylation may not occur at every PNGS, and their glycosylation state can differ among a population of the same protein [45].

Glycosylation is often required for proper folding of proteins, including the HIV-1 envelope protein. Most proteins that are produced in the E.R. have disulfide bonds, and it

has been shown that removal of a PNGS can impact disulfide bond formation and subsequent protein conformation. Glycosylation can also have roles beyond ensuring the proper folding of a protein, and this is also the case for HIV-1 gp120. Glycosylation is likely indispensible for production of infectious HIV-1 viral particles, and therefore, these glycosylation sites are attractive drug targets, and molecules that recognize N-linked glycosylation structures found on the HIV-1 envelope may be able to inhibit HIV-1 infection.

HIV-1 Envelope Protein gp120

The HIV-1 envelope protein gp160 is cleaved by a cellular furin protease to produce the proteins gp120 and gp41 [46]. These two envelope proteins form a trimeric "spike" consisting of three gp120 molecules non-covalently attached to three gp41 proteins. These complexes move to sites of viral assembly by a mechanism that is not well understood and are incorporated into the budding virion, with approximately ten spikes contained on the surface of each virion [47]. The gp120 protein consists of five variable (V1-5) and five constant (C1-5) domains [48, 49]

There are other functions of the gp120 protein that may have a role in HIV-1 pathogenesis including altering the immune responsiveness of dendritic cells, inducing chemokine production in macrophages, and induction of T-cell apoptosis [12, 50, 51]. The envelope protein also plays a role in cellular transmission of the virus. In the periphery, dendritic cells can bind the virus via interactions with high-mannose, N-linked

glycosylation found on the viral envelope [52]. The cells will then traffic to secondary lymphoid structures that contain numerous CD4+ T cells [12].

As mentioned above, the gp120 protein is extensively glycosylated, and N-linked glycosylation structures are accountable for approximately 50% of the molecular weight of the protein. The number of PNGS for gp120 ranges from 18 to 33 or more with a median of 25, while the median number of sites for gp41 is 4 [53]. The total number of PNGS does not appear to differ among subtypes, nor has this number increased over time. When individual positions are analyzed, there is still no statistical difference in the number of PNGS [53]. There are areas that tend to be more variable in the number of PNGS, and more PNGS are located in the variable regions than in the constant regions. Interestingly, the PNGS that have been found to be predominantly occupied by high-mannose structures are relatively well conserved [54].

The glycosylation of gp120 is thought to have multiple roles. Glycosylation assists in viral evasion from the immune response, since these structures appear to immune cells as self antigen and therefore are poorly immunogenic. In addition, the structures also appear to occlude the binding of antibodies to the envelope protein. Glycosylation also can assist in the affinity of the envelope for cellular receptors, including CD4 and DC-SIGN. The multiple functions of glycosylation make it an attractive target for novel anti-HIV drugs, such as lectins

Plant Lectins

Lectins are multivalent proteins that are able to reversibly bind carbohydrates. Lectins from plants have been the best characterized, and can be organized into five different groups based on structure: jacalin-related lectins (JRLs), legume lectins, monocot mannose-binding lectins, trefoil lectins, and hevein [55]. Plant lectins likely have multiple functions. The ability of plant lectins to recognize glycosylation found on animal proteins suggests a role in defense against plant consuming animals [56].

The affinity of lectins for mono- or disaccharides is relatively weak. Their equilibrium constants (Kd) typically range between 10^{-3} and 10^{-4} M (10^{-6} M is considered weak affinity). However, glycoproteins contain oligosaccharides and the affinity of lectins for these proteins can be increased up to five orders of magnitude through multivalent interactions [57].

The JRL family is named after the lectin jacalin, which was the first lectin found to have a β-prism-I structure. The JRL family is subdivided into their preferred carbohydrate recognition ligands: galactose-specific JRL (gJRL) or mannose-specific JRL (mJRL) [55]. gJRL member lectins are composed of a short and a long amino acid chain that are the products of the cleavage of a common precursor protein, while mJRL members contain a single amino acid chain [58].

The β-prism structure is composed of Greek key structures, which are named after the patterns of β-strands that make up the protein. The JRLs have a common GXXXD amino

acid motif found on a loop located on top of the first Greek key structure. This motif is important for carbohydrate binding, but not solely responsible. An amino acid loop found on the third Greek key structure is thought to play a role in determining the lectin's carbohydrate affinity and specificity [59, 60]. This loop is known as the recognition loop and there is a large amount of variability among JRLs in the composition and length of this region.

Two JRL family members have been shown to have anti-HIV-1 activity. The lectin Jacalin from jack fruit (*Artocarpus integrifolia*), is a member of the gJRL family and has been shown to block HIV-1 infection by binding to CD4, and not to the HIV-1 envelope complex [61]. A more recently discovered JRL family member, griffithsin (GRFT), has been identified as a potent inhibitor of HIV-1 infection [62, 63]. GRFT is currently being evaluated for its potential use as a vaginal microbicide after showing success in several in vitro microbicide models [64, 65]. Although only two JRLs have been reported for anti-HIV-1 activity, other members will likely be identified after further screening and testing.

BanLec

The mannose/glucose binding lectin isolated from bananas, BanLec, was first identified through an investigation looking at antibody responses towards a variety of foods [66]. BanLec is a member of the JRL family [67]. BanLec has been shown to exist as a homodimer with two binding sites per monomer for a total of four potential binding sites (Figure 1-6) [67, 68]. This makes BanLec a rather unusual lectin, since JRL typically

contain one carbohydrate binding site per monomer. The only other JRL lectin with multiple binding sites per monomer is GRFT, which contains three binding sites.

BanLec binds high-mannose structures that exist as N-linked glycans found on mammalian proteins with greater affinity than some other mJRL members [60, 66]. This suggests that BanLec has potential uses in carbohydrate biology. Indeed, it has been shown that BanLec can be used to stimulate CD3+, CD4+ and CD8+ T cell populations and induce the production of different cytokines [69, 70]. In addition, oral administration of BanLec has also been shown to increase hematopoietic stem cell populations in mice, suggesting another potential use for this protein [71]. Although several potential uses of BanLec have been proposed, the potential anti-HIV-1 properties of BanLec had not previously been investigated.

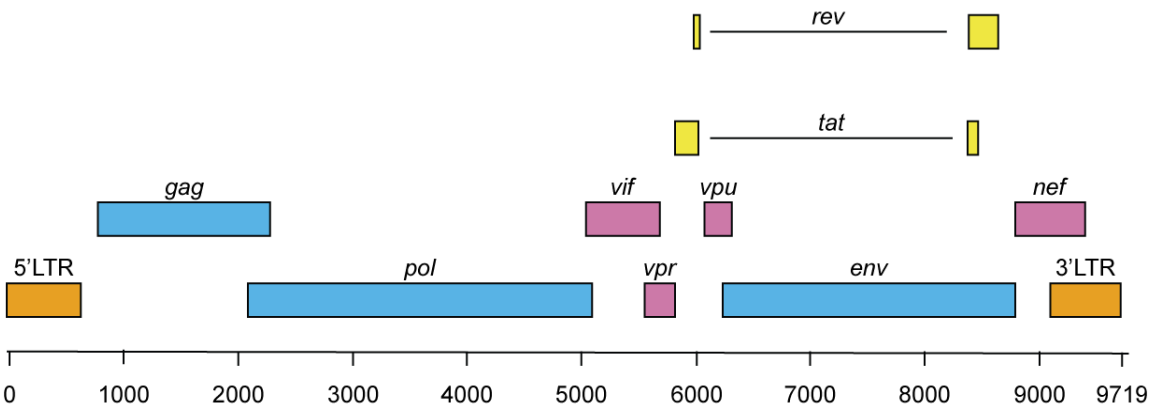

Figure 1-1. Map of the HIV-1 genome. The orange boxes indicate the 5' and 3' long terminal repeats. The blue coded genes encode structural proteins, while the yellow and purple colored genes encode for the regulatory and accessory proteins, respectively.

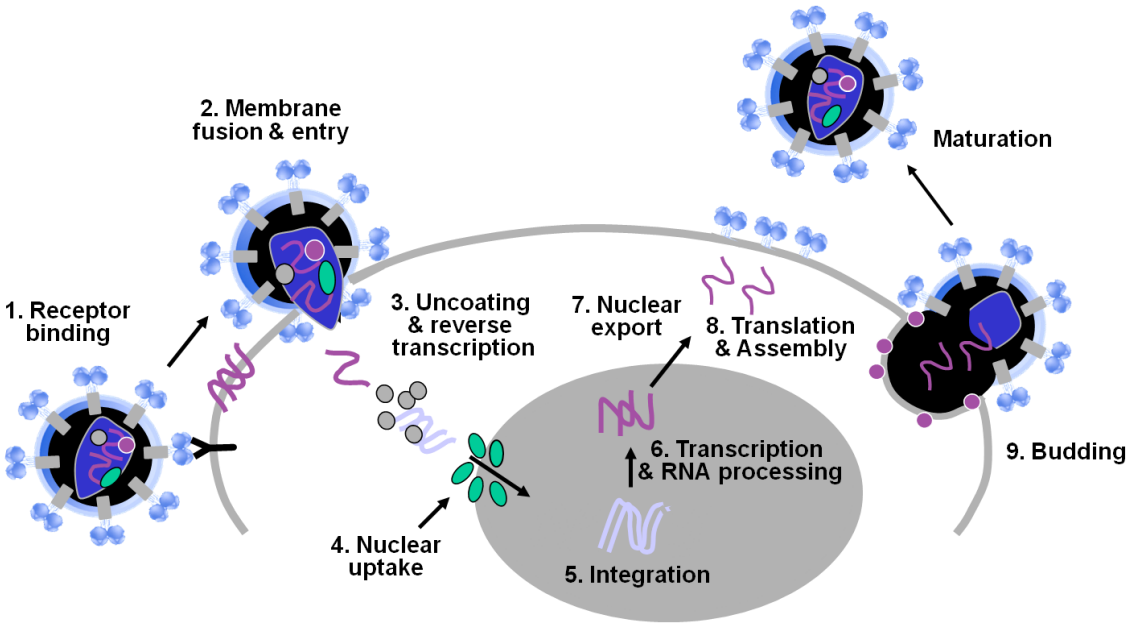

Figure 1-2. The HIV-1 replication cycle. Figure adapted from Doms, www.clinicaloptions.com/HIV.

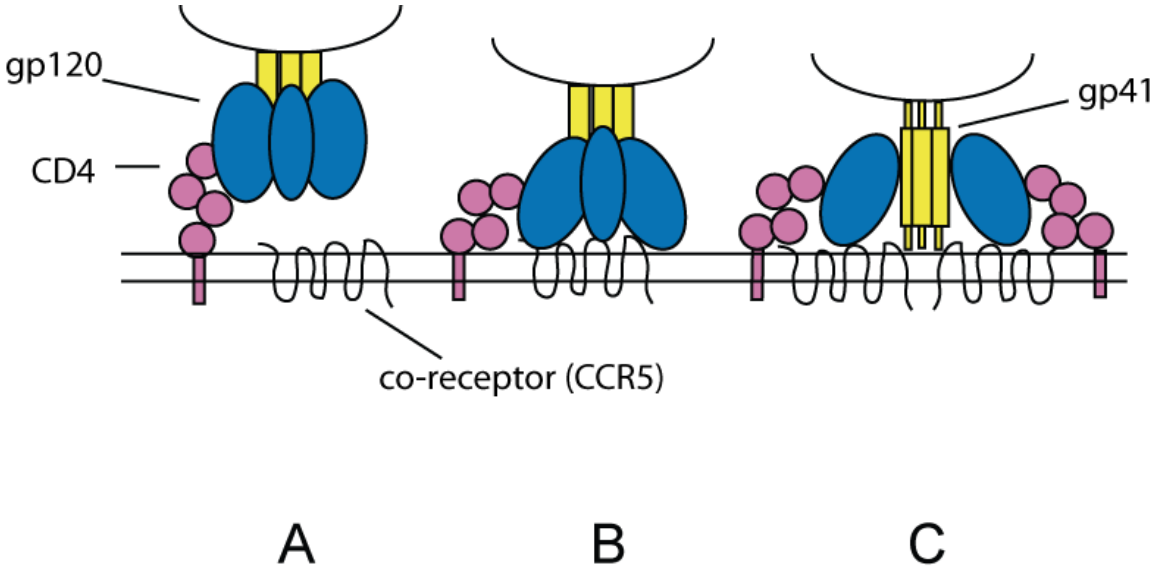

Figure 1-3. The process of HIV-1 entry. A) The HIV-1 envelope protein gp120 binds to CD4. Upon binding, a previously hidden portion of the envelope is exposed. B) This portion of the envelope is able to bind to the HIV-1 co-receptor, typically CCR5. C) The binding to the co-receptor induces further changes which result in the formation of a six-helix bundle of the gp41 protein. This protein then mediates fusion of the viral and target cell membranes.

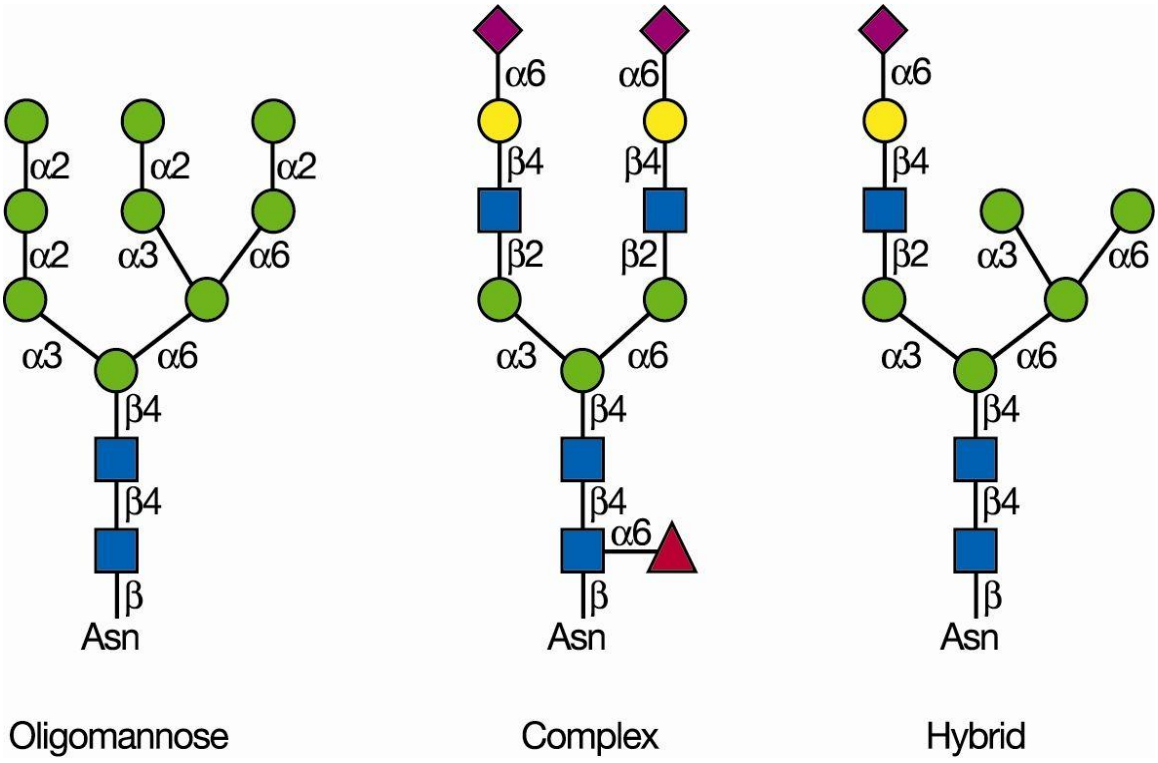

Figure 1-4. Types of N-linked glycosylation. N-linked glycosylation added to a protein at Asn-X-Ser/Thr sequons are of three general types in a mature glycoprotein: oligomannose, complex, and hybrid. All three of these structures contain a common core consisting of Man3GlcNAc2Asn, but they vary in the type of carbohydrates added to this structure. Oligomannose or high-mannose structures contain additional mannose residues, exclusively. Complex structures contain additional non-mannose residues. The color scheme for the carbohydrates is the following: blue = N-acetylglucosamine (GlcNAc), green = mannose (Man), yellow = galactose, purple = N-acetylneuraminic acid, red = fuocse. The covalent bonds between carbohydrates (linkages) are indicated. Source: *Essentials of Glycobiology*, 2nd edition [43].

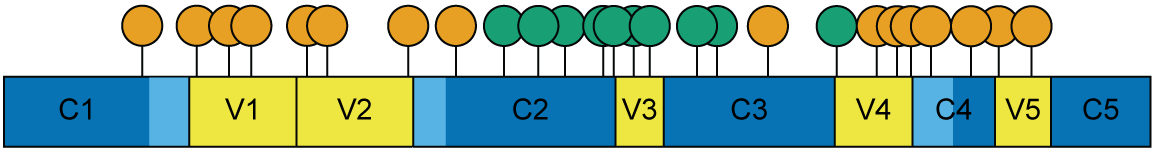

Figure 1-5: The regions of the HIV-1 gp120 protein and its glycosylation sites. The constant and variable regions are colored blue and yellow, respectively. The light blue color represents the amino acids that form the bridging sheet. N-linked glycosylation is represented as circles with orange representing complex or hybrid N-linked structures while blue-green represents high-mannose structures. Adapted fromTilton and Doms [72].

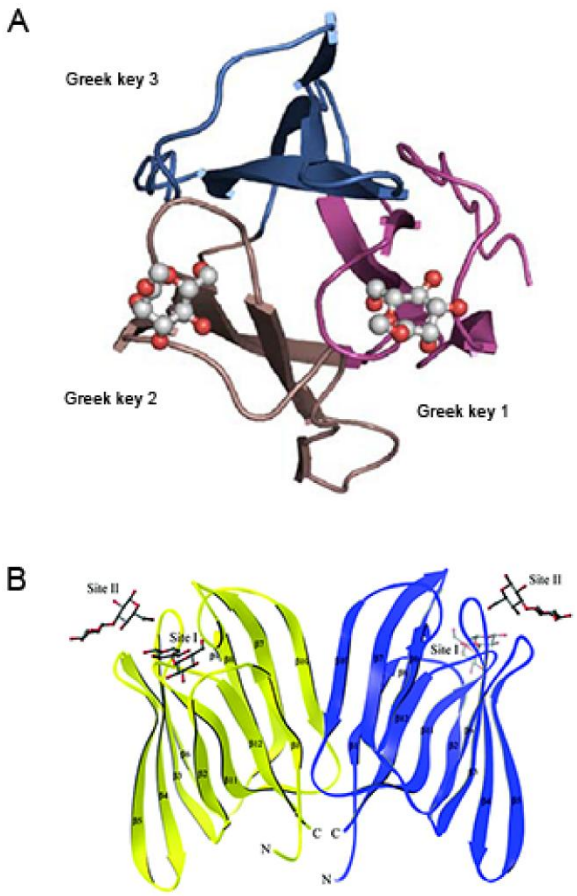

Figure 1-6. Three-dimensional structures of BanLec. A) An overhead view of monomeric BanLec displays the β-prism structure created by the three Greek key structures. Greek keys 1 and 2 contain binding sites 1 and 2, respectively, and are shown bound to carbohydrate. Although the third Greek key lacks a carbohydrate binding site, it plays a role in carbohydrate specificity. B) Side view of dimeric BanLec. BanLec exists as a homodimer and each dimer has a total of four potential carbohydrate binding sites. Figures from Sharma et al. and Meagher et al. [68, 73].

Chapter 2: A lectin isolated from bananas is a potent inhibitor of HIV replication

Introduction

Despite the development of over twenty-five approved anti-HIV drugs and improvements in the availability of antiretroviral drugs in low- and middle-income countries, the rate of new HIV-1 infections is outpacing the rate of new individuals receiving antiretroviral therapy by 2.5:1 [74]. At present, it appears that an efficacious HIV vaccine is still many years away. Therefore, other methods for halting the spread of HIV are vitally needed. This has raised the possibility of developing either intravaginally or intrarectally applied microbicides in order to halt the spread of HIV during sexual intercourse. This type of intervention is particularly needed in the developing world, such as sub-Saharan Africa, where over twenty million people are living with HIV/AIDS [74]. While abstinence has been suggested by some groups, campaigns to encourage this method of halting transmission have not been effective [75]. Although condoms are quite effective against the spread of HIV and some other sexually transmitted diseases, they are only effective if they are used consistently and correctly, which is often not the case [76, 77]. This is particularly true in the developing world, where women have relatively little control over sexual encounters and thus have not been able to enforce condom usage [78], so the development of a long-lasting, self-applied, microbicide is very attractive. In fact, it is estimated that 20% coverage with a microbicide that is only 60% effective against HIV may prevent up to 2.5 million HIV infections over three years [79]. Therefore, even modest success with microbicides could save millions of lives.

Arguably, the compounds with the greatest potential for inhibiting vaginal or rectal HIV transmission are agents that block HIV attachment and fusion, as these agents specifically inhibit HIV from the beginning of its replication cycle [80]. These reagents include long-chain and ionic polymers (such as Pro 2000), as well as dendrimers, lipid membrane modifiers, and anti-CD4 antibodies. HIV-binding peptides and small molecule inhibitors have also been considered, including the fusion inhibitor T-20 (enfuvirtide) and the CCR5 blocker maraviroc, which are already in clinical use for the treatment of HIV infection. In addition, lectins are a growing class of HIV-1 inhibitors under consideration as microbicide candidates [81]. Lectins inhibit HIV-1 entry by binding to carbohydrate structures found on the viral envelope. Examples of anti-HIV lectins include Cyanovirin-N (CV-N) [82], Griffithsin (GRFT) [62], and snowdrop lectin (GNA) [83-85].

The HIV-1 envelope protein gp120 contains 20 to 30 possible N-linked glycosylation sites. These carbohydrate structures make up approximately 50% of the molecular weight of the protein [86-88]. Glycosylation affects aspects of the viral life cycle including protein folding [89], cellular transport, binding to cellular receptors [12, 86, 90], trans-infection by dendritic cells [12], and shielding from the immune response [91]. Because glycosylation is essential to the virus, it presents an attractive therapeutic target.

The lectin termed BanLec, isolated from the ripened fruit of the banana (*Musa acuminata* cultivars), exists as a dimer with a molecular weight of approximately 30 kDa [67]. It is a member of the jacalin-related lectin (JRL) family and can recognize high-mannose

structures [60, 66, 92]. Lectins in this family are characterized by the presence of a β-prism 1 structure composed of three Greek Key turn motifs. Greek Keys 1 and 2 are both involved in binding carbohydrates and contain a GXXXD binding motif, whereas Key 3 does not contain the binding motif [68, 93]. However, this loop can assist ligand binding and determine lectin specificity [59]. Because of its affinity for high-mannose structures, we sought to investigate whether BanLec might bind the mannose-rich envelope of HIV-1 [87], and thereby block HIV infection. The results presented below demonstrate that BanLec is a potent inhibitor of HIV infection that markedly reduces the replication of a range of HIV-1 isolates, and has potential to be further developed for use as a vaginal microbicide.

Materials and Methods

Lectins and Anti-HIV Compounds

BanLec was isolated from bananas by modification of previously described methods [66, 67]1. Snowdrop lectin (GNA) was isolated from crude extracts of snowdrop bulbs and purified over a mannose-agarose column as described previously [94]. Recombinant his-tagged Griffithsin (GRFT), T-20, maraviroc, and CD4-IgG2 were obtained from the NIH AIDS Research & Reference Reagent Program [62].

HIV-1 Production

The HIV-1 isolate Bru was produced in peripheral blood lymphocytes (PBL), while the HIV-1 BaL [95] isolate was produced in macrophages. Primary, dual-tropic isolates ASM44 and ASM54 were expanded in peripheral blood mononuclear cells (PBMC)

containing both lymphocytes and macrophages. Production of pseudotyped HIV-1 was performed by transfecting 293FT cells with plasmids coding for an HIV-1 envelope from either subtype B [96] or C along with an envelope-deleted proviral clone, pSG3Δenv [97]. Proviral plasmid DNA clones pNL4-3 [98], pNL(AD8) [99], p81A-4 [100-102], and p89.6 [103] were transfected into 293FT cells with Lipofectamine 2000 (Invitrogen). The media was changed 24 hours post transfection, and at 48 hours post transfection the supernatants were harvested and frozen at -80° C. The concentration of virus in the stocks was determined by the HIV-1 p24 Antigen Capture Assay ELISA (AIDS & Cancer Virus Program) or by determining the infectious titer. HIV-1 Bru was treated with 10 U/μL of RNAse-free DNAse I (Roche) prior to use in the experiments in which the products of early reverse-transcription were assayed.

HIV-1 Indicator Assays

HIV-1 infection was quantified using TZM-bl cells, which express a luciferase and β-galactosidase gene under the control of the HIV-1 LTR promoter [97, 104-106]. The day before infection, 5000 cells/ml of TZM-bl cells in 100 μl DMEM media containing 10% fetal bovine serum, 25 mM HEPES and 50 μg/ml geneticin were added to the wells of white, opaque, 96-well tissue culture plates (Falcon). Cells were pretreated with BanLec for 30 minutes prior to infection with 100 $TCID_{50}$ units of virus (approximately 15,000 relative luminescence units, RLU) to a final volume of 200 μl per well. Cells were exposed to virus and lectin for either two days with replication competent viruses or for three days with pseudotyped, replication defective virus. Steady-Glo® Luciferase Assay

System (Promega) and a plate reader containing a luminometer (Tecan) were used to measure luminescence, which was indicative of viral infection.

MAGI-CCR5 cells [107, 108] were plated in 24 well tissue culture plates with 40,000 cells per well in DMEM media containing 10% fetal bovine serum, penicillin, and streptomycin. The cells were pretreated with lectin for 30 minutes and then infected with different viral isolates at concentrations that yielded approximately 100 positively infected cells per well. Forty hours post infection, cells were stained for β-galactosidase activity as described within the reagent data sheet and positive cells were counted visually.

Isolation and Culture of Primary Cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by venipuncture. Briefly, blood was drawn into a 60 ml syringe containing 7 ml of 250 mM sodium citrate and 10 ml of 6% dextran solution and mixed by inversion. After 30 minutes, to allow for the sedimentation of red blood cells, the supernatant was separated using hypaque-ficoll, and the buffy coat layer was removed, washed twice with cold PBS containing 0.2% bovine serum albumin, and centrifuged at 350 x g for 10 minutes. The cell pellet was resuspended in RPMI-1640 media at a concentration of 5×10^6 cells per ml and seeded into non-tissue culture treated plates. Peripheral blood lymphocytes (PBL) were removed from the adherent monocytes and each was washed three times with PBS. For the differentiation of monocytes to macrophages for HIV-1 infection, the monocytes

were cultured with Iscove's Modified Dulbecco's Media (IMDM) media containing 10% heat-inactivated human AB sera for seven days.

Infection of Monocyte-derived Macrophages

Monocyte-derived macrophages (MDM) were washed with PBS three times, followed by the addition of fresh media containing BanLec or PBS 30 minutes prior to infection. Cells were infected with approximately 100 $TCID_{50}$ of NL(AD8) for 24 hours, and the residual virus was removed by three PBS washes followed by the addition of fresh media. Every three days, a sample was removed and replaced with fresh media containing the appropriate amount of BanLec for 15 days. The samples were stored at -80° C until viral replication was determined by the HIV-1 p24 Antigen Capture Assay ELISA (AIDS & Cancer Virus Program). A similar experiment was done in which samples were not removed until the end of the experiment on day 7. For both experiments, an MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay was performed on the final day to assess cellular viability.

Detection of Early Products of HIV-1 Reverse Transcription (Strong-Stop DNA) in Peripheral Blood Lymphocytes

PBL were stimulated with PHA for three days in RPMI media containing 10% heat-inactivated fetal bovine serum and IL-2. The cells were washed with PBS and resuspended in RPMI media containing 10% heat-inactivated fetal bovine serum. Lectins were added 30 minutes prior to centrifuge-mediated infection (spin-infection) with DNAse I-treated HIV-1 Bru [109]. Three hours post infection the cells were harvested,

washed with PBS, and then stored at -80° C. Cellular DNA, including genomic and viral DNA products, was isolated using the QIAamp DNA Blood Mini Kit (Qiagen). Strong-stop DNA, the first product of HIV-1 reverse transcription, is used for the assessment of viral entry [85, 110]. This reverse transcription product was quantified by performing real-time PCR with primers specific for strong-stop DNA, and the DNA concentration of the each sample was normalized and equal DNA loading was confirmed with primers for α-Tubulin [110].

Determination of BanLec and Glycosylated HIV-1 gp120 Interaction by ELISA

96-well ELISA plates were coated by adding 50 µl of 5 µg/ml BanLec per well and incubated overnight at room temperature. The next day, plates were blocked for 1.5 hours at room temperature with PBS containing 1% BSA, 5% sucrose, and 0.05% sodium azide, and then rinsed with wash buffer (PBS containing 0.05% Tween-20, pH 7.4) three times prior to addition of recombinant, glycosylated gp120 protein diluted in blocking buffer. After a one hour incubation at room temperature, the plates were washed three times prior to the addition of the detection antibodies. A sheep anti-gp120 antibody (AIDS Research and Reference Reagent Program) was diluted 1:2000 in dilution buffer (wash buffer containing 0.1% BSA) and added to the wells and incubated for one hour. The plate was washed again before a one hour incubation with an anti-sheep antibody conjugated to alkaline phosphatase (Sigma) diluted 1:40,000 in dilution buffer. After the plate was washed, p-nitrophenyl phosphate (Sigma) was added for colorimetric analysis, and the absorbance was measured at 405 nm.

To determine if BanLec could block the recognition of gp120 by the anti-HIV monoclonal antibody 2G12, ELISA plates were coated overnight with 100 ul of a 1 ug/ml solution of recombinant gp120 diluted in PBS. The plates were blocked as described above, washed, and then treated with serial dilutions of BanLec in dilution buffer. After a one hour incubation, the plates were washed to remove unbound BanLec. The 2G12 antibody was added at concentration of 100 ng/ml to allow for binding to the gp120 protein. One hour later, the plates were washed and incubated with biotinylated anti-human antibody (Jackson ImmunoResearch) followed by another series of washes, and the addition of streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch). The alkaline phosphatase dilution buffer contained 500 mM methyl-α-D-mannopyranoside, which was added to reduce any potential non-specific binding of the 2G12 antibody to BanLec. The plates were washed again and the substrate p-nitrophenyl phosphate (Sigma) was added for colorimetric analysis of 2G12 binding. The amount of antibody that remained bound was quantified by comparison to a standard curve consisting of serial two-fold dilutions of 2G12.

Determination of Anti-HIV Activity Post Cellular Attachment

For measuring inhibition of HIV-1 infection post cellular attachment, TZM-bl cells were plated in 96 well plates as described above and spin-infected with a pseudotyped virus with pConCgp160-opt, a consensus subtype C envelope sequence, at 1250 x g for 2 hours at 16° C one day after plating [109, 111]. The cells were placed on ice, the supernatant was removed, and unbound virus was removed by washing two times with PBS containing 0.2% BSA. Cell culture media containing inhibitors (CD4-IgG2, T-20,

maraviroc, or BanLec) was added to the cells and incubated for 30 minutes on ice. The cells were then moved to a 37° C incubator and luciferase activity was measured three days later. These results were compared to cells that were infected with virus that had been pretreated with the same inhibitors described above on ice for 30 minutes and then incubated at 37° C (i.e., standard infection conditions).

Results

BanLec is a Potent Inhibitor of Multiple HIV-1 Isolates

To determine the anti-HIV activity of BanLec, different concentrations of the lectin were incubated with TZM-bl indicator cells prior to infection with various HIV-1 isolates. Because a microbicide would need to inhibit HIV-1 of different tropisms and because glycosylation can play a role in determining viral tropism [90], we tested the ability of BanLec to inhibit several different HIV-1 isolates. The viral clones 81A-4 and NL(AD8) are both derivatives of NL4-3 in which a portion of the envelope is swapped with the envelope region from either the R5 HIV-1 isolates BaL or ADA, respectively. 81A-4 and NL(AD8) use CCR5 as a cellular co-receptor (R5 tropic), while NL4-3 uses CXCR4 (X4 tropic). These isolates allow for the assessment of different HIV-1 envelope sensitivity to BanLec, while keeping the remainder of the NL4-3 viral components unchanged. The dual-tropic isolate 89.6 was also assessed for susceptibility to BanLec. We observed dose-dependent inhibition of viral infection with IC_{50} values calculated in the low nanomolar range against viral isolates with different tropisms (Figure 2-1A). These results suggest that sensitivity of HIV-1 isolates to BanLec is independent of viral tropism.

We further confirmed the anti-HIV activity of BanLec with the HIV-1 indicator cell line, MAGI-CCR5. With this cell line, we tested the ability of BanLec to inhibit infection by the laboratory-adapted isolates BaL (R5) and Bru (X4), and the primary isolates ASM 44 (R5X4) and ASM 54 (R5X4) and determined that all were inhibited by BanLec (Figure 2-1B). The virus used in this experiment was generated by infection of PBMC, while the experiment shown in Figure 2-1A used virus produced by transfection of 293FT cells with a proviral plasmid clone. These results further support our initial studies by showing that BanLec can inhibit HIV isolates both independent of viral tropism and of the cell type used to produce virus.

R5 tropic viruses are the dominant form found in sexually transmitted HIV-1, and therefore would have to be neutralized by microbicides. In addition, anti-HIV microbicides will need to inhibit infection by viral isolates from different subtypes. Although one would assume that all clades could be neutralized due to the conservation of gp120 glycosylation, a difference in the susceptibility of the viral subtypes B and C to the anti-HIV, high-mannose recognizing antibody 2G12 has been observed [112]. To determine if BanLec could inhibit additional primary isolates from different clades, we tested BanLec for inhibition of HIV-1 pseudotyped with envelopes derived from primary isolates of subtypes B and C. These subtypes are commonly found in North and Central America (subtype B) and parts of Africa and India (subtype C). We observed potent, sub-nanomolar inhibition of viral replication by BanLec (Figure 2-2 and Table 2-1), and no significant difference was observed when the average IC_{50} values from the two different

subtypes were compared by Student's t-test ($p < 0.56$). This suggests that BanLec can effectively inhibit infection by viral isolates prominent in regions where a microbicide would be most valuable.

BanLec Blocks Infection of MDM

Macrophages are susceptible to HIV-1 infection and can become viral reservoirs that cannot be eliminated by highly active anti-retroviral therapy (HAART). The role of vaginal macrophages in HIV-1 pathogenesis has not been fully characterized, but recent evidence indicates that these cells are permissive for HIV-1 infection [113]. We tested the ability of BanLec to inhibit HIV-1 infection of MDM. As shown in Figure 2-3A, nanomolar concentrations of BanLec inhibited HIV replication in MDM over a period of 15 days. Furthermore, BanLec had no effect on cellular viability as determined by MTT assay performed on day 15 (data not shown); therefore, this effect was not due to cellular toxicity. When BanLec remained in the culture supernatant for seven days without changing the media or adding additional lectin, the IC_{50} value for BanLec inhibition of HIV-1 replication was 9.72 nM (Figure 2-3B), demonstrating that BanLec remains a potent and stable inhibitor in a long term culture system at 37 ° C.

BanLec Blocks HIV-1 Infection at the Viral Entry Step

Having shown that BanLec can inhibit HIV replication in MDM, we tested the ability of BanLec to block cellular entry of HIV-1 in peripheral blood lymphocytes (PBL). We hypothesized that BanLec binds to high-mannose structures found on the HIV-1 envelope, preventing entry and thus infection. If so, little or none of the strong-stop DNA

product of early HIV-1 reverse transcription (see Materials and Methods section) should be detected when cells are exposed to HIV-1 in the presence of BanLec [110, 114]. To test this hypothesis, we incubated PBL with the HIV-1 Bru isolate in the presence of different concentrations of BanLec. As a positive control, and for comparison, a similar experiment with the lectin GNA was performed in parallel. Real-time PCR was used to detect strong-stop DNA, which is a reverse-transcription product that can be detected early after viral entry, before viral uncoating takes place [115-117]. Strong-stop DNA that may have been present in the virus stock was removed by treatment with DNAse I to eliminate false detection of reverse transcription products. Treatment with BanLec resulted in a marked decrease in strong-stop DNA at low lectin concentrations (Figure 2-4) indicating that, in addition to inhibiting viral replication in MDM, BanLec blocks HIV-1 infection in PBL. Furthermore, this inhibition occurs at a step prior to early replication events, apparently at the level of viral entry.

BanLec Binds to Glycosylated gp120

BanLec is known to bind to mannose, and thus we hypothesized that BanLec binds the high-mannose structures found on the glycosylated gp120 envelope protein and blocks entry of HIV-1 into cells. We prepared a BanLec-based ELISA to measure binding of glycosylated HIV-1 gp120 to BanLec. We observed that BanLec does indeed bind to gp120 in a concentration-dependent manner (Figure 2-5A). Furthermore, a known BanLec ligand, methyl-α-D-mannopyranoside, inhibited such binding in a concentration dependent manner (Figure 2-5B). Not surprisingly, a high concentration of methyl-α-D-mannopyranoside ligand was needed to compete for binding to gp120 because of the high

density of carbohydrate residues on the HIV-1 envelope protein. These results corroborate our hypothesis that BanLec inhibits HIV-1 cellular entry by binding to high-mannose structures found on the virus.

To explore the BanLec binding sites on gp120, we determined the ability of BanLec to block binding by the monoclonal antibody (mAb) 2G12 using the ELISA-based assay. 2G12 recognizes a cluster of N-linked glycosylation structures at positions N295, N332, and N392 (position numbering is of the HXB2 reference sequence), which are crucial for antibody recognition [54, 118, 119]. We found that pretreatment of gp120 with BanLec inhibited recognition by 2G12 in a dose-dependent manner, suggesting that BanLec is capable of binding to this antibody's epitope consisting of high-mannose structures (Figure 2-6).

BanLec Inhibits HIV-1 Infection Prior to Viral Fusion

To further investigate at which point in the viral life cycle BanLec inhibits HIV-1 infection, we tested BanLec for ability to inhibit HIV-1 infection post-attachment. To do so, we tested if BanLec could inhibit HIV-1 that was already bound to the cell, but could not complete fusion due to temperature restriction [120]. As controls, we took advantage of the fact that CD4-IgG2 inhibits HIV-1 infection by blocking attachment while T-20 works by blocking fusion. As anticipated, we observed a large decrease in the inhibitory activity of the HIV-1 attachment inhibitor CD4-IgG2 in the post-attachment assay, while the bound virus was still essentially completely susceptible to the fusion inhibitor T-20 (Figure 2-7 and Table 2-2). This demonstrates that the assay works as expected, with

viral attachment, but not fusion, taking place at 16 °C. Both the CCR5 binding inhibitor maraviroc and BanLec primarily blocked viral replication by inhibiting HIV-1 attachment, but each does appear to also have a modest effect on viral fusion (Figure 2-7). Importantly, when we compare the IC_{50} value of BanLec to those of other anti-HIV compounds, we see that BanLec's potency compares quite well to the clinically approved anti-virals T-20 and maraviroc (Table 2-2). Thus, we conclude that BanLec potently inhibits the attachment of HIV-1 to cells and has a more modest effect on viral fusion.

The Anti-HIV Activity of BanLec Compares Well to Other Anti-HIV Lectins

Several different lectins have been found to inhibit HIV-1 infection. However, they vary in their degree of anti-viral activity. To assess the relative molar-based potency of BanLec, we compared the anti-HIV activity of BanLec to that of two previously described anti-HIV lectins: GNA and GRFT. Upon comparison, all three lectins showed activity in the nanomolar range (Figure 2-8). Taken together, our data suggest that BanLec inhibits infection with a broad range of HIV-1 isolates by blocking viral entry, compares favorably with the potency of previously described lectins and clinically available anti-HIV drugs, and is a potential component for future anti-HIV vaginal microbicides.

Discussion

The primary mechanism of inhibition by BanLec appears to be blocking cellular attachment of HIV and thus viral entry. Our conclusion is based on the findings from our ELISA assays that BanLec can bind to high mannose structures found on HIV-1 gp120,

including the high-mannose structures that are recognized by the monoclonal antibody 2G12. This was corroborated by the finding that cells treated with BanLec had decreased amounts of an early HIV reverse transcription product, strong-stop DNA, that can be detected shortly after cellular entry of the virus and before viral uncoating. In addition, we performed an assay that took advantage of a temperature arrested state (16°C) that prevents HIV-1 fusion and compared the inhibitory activity of BanLec and other anti-HIV drugs pre- and post-cellular attachment of the virus, finding that most of the BanLec inhibitory activity of BanLec comes from blocking viral attachment. Interestingly, while most of the inhibitory activity of the CCR5 blocker maraviroc, used as a control in these experiments, was due to blocking viral attachment, when maraviroc was added post-attachment, we still observed inhibition of HIV, albeit at a reduced level. A similar result was also seen with BanLec, suggesting that these two compounds could have additional inhibitory activity at a post-attachment step, such as fusion of the virus to the cell.

Our studies indicate that BanLec is a new and promising member of the group of lectins that are able to inhibit HIV-1 infection through interactions with glycosylation sites found on the viral envelope. The inhibitory activity of BanLec against HIV-1 was broad, independent of tropism, and effective against several subtype B and C envelope sequences. HIV-1 pseudotyped with envelopes derived from primary isolates was inhibited by BanLec in the low nanomolar range. BanLec was also able to inhibit HIV-1 infection of primary cells and thus our results are not limited to cell lines.

Based on our findings, it is likely that BanLec will be able to inhibit other HIV-1 subtypes, as they all contain glycosylation sites in their envelope sequences. The isolates used in our experiments differed in the number of predicted N-linked glycosylation sites, supporting the likelihood that BanLec will be effective against most HIV subtypes found in both the developing and developed world. Since glycosylation is not specific to HIV-1, lectins have the potential to inhibit the replication of a broad spectrum of viruses. Indeed, it has been shown that lectins can inhibit other enveloped viruses including Ebola [121, 122], Marburg [122], influenza [123], severe acute respiratory syndrome (SARS) coronavirus [124], and hepatitis C virus (HCV) [125, 126].

One potential benefit of the use of lectins as anti-HIV agents is their ability to target multiple different glycosylation sites on the virus, thus making it more difficult for resistance to develop. In support of this prediction, previous studies that determined the resistance profiles of HIV-1 treated with lectin showed that multiple mutations in the envelope sequence were needed for the development of resistance [127]. Furthermore, different mutations in N-linked glycosylation sites are required for the development of resistance to different lectins. This suggests that the combinatorial or simultaneous use of multiple lectins can reduce the likelihood of failure of a lectin based anti-viral therapy due to resistance. If a population of virus develops resistance to BanLec or other anti-HIV lectins, one interesting possible consequence is that the virus will then be more susceptible to neutralization by the human immune response, as the carbohydrate structures found on the HIV-1 envelope are thought to act as a shield against neutralizing antibody responses [128]. This glycan shield works by blocking access of epitopes to

potentially neutralizing antibodies. Previously published data demonstrate that alterations in glycosylation that result in resistance to lectins can make the virus vulnerable to neutralizing antibody responses (18,19).

Although several anti-HIV lectins have been described, it is highly unlikely that a majority of them can be developed for therapeutic use. Like all potential drugs, lectins can vary in their degrees of potency and toxicity [129, 130]. Also, it has been shown that two anti-HIV lectins can significantly differ in their ability to block attachment of HIV to epithelial cells [131]. Concerns have been raised about the potential toxicity of lectins, for example CV-N. This lectin has shown success as a microbicide in in vivo macaque vaginal and rectal transmission models [132, 133], but safety concerns exist. CV-N was found to have mitogenic activity when PBMC cultures were exposed to the lectin for three days [130, 134]. However, recombinant therapeutic proteins can be PEGylated to change bioavailability and reduce toxicity. This modification of CV-N has been shown to be effective in reducing mitogenicity in vitro [135]. Although BanLec has also been reported to possess mitogenic activity [69], the relationship between mitogenicity in vitro and microbicide efficacy has not been elucidated, so it remains possible that recombinant versions of BanLec and other lectins could be developed that retain efficacy but have minimal mitogenicity. The anti-HIV lectin GRFT has recently been reported not to have a mitogenic effect when added to human PBMCs [65]. This observation is of interest, as GRFT is in the same JRL family and has a similar structure to BanLec [63]. GRFT has also been shown to be non-inflammatory, non-toxic and capable of being manufactured on a large scale. Although clinical testing of these newer lectins has yet to be performed,

it appears that lectins have potential to be used as an anti-HIV agent [65]. Since the binding, toxicity, and anti-HIV activity of lectins vary, the identification of novel anti-viral lectins, such as BanLec, will further increase the possibility of successful development of a lectin-based anti-HIV microbicide.

Table 2-1. Summary of the calculated IC_{50} values for BanLec inhibition of HIV-1 pseudotyped with HIV-1 envelopes from subtypes B and C.

Subtype B	Envelope	SVPB5	SVPB6	SVPB11	SVPB17
	IC_{50} (nM)	0.30	0.85	0.71	0.33
	95% CI	0.27 to 0.34	0.74 to 0.98	0.65 to 0.77	0.26 to 0.41

Subtype C	Envelope	SVPC3	SVPC5	SVPC6	SVPC7
	IC_{50} (nM)	0.57	0.30	0.28	2.3
	95% CI	0.51 to 0.63	0.21 to 0.42	0.25 to 0.31	1.8 to 2.9

Table 2-2. Summary of the calculated IC_{50} values for inhibition of HIV-1 infection with addition of drug either pre- or post-attachment to TZM-bl cells.

Post-attachment	Compound	CD4-IgG2	T-20	Maraviroc	BanLec
	IC_{50} (nM)	*	2.78	10.2	13.1
	95% CI†	-	1.96 to 3.95	7.5 to 13.8	9.61 to 17.7

Pre-attachment	Compound	CD4-IgG2	T-20	Maraviroc	BanLec
	IC_{50} (nM)	1.39	1.90	1.23	0.224
	95% CI	0.67 to 2.89	0.96 to 3.77	0.5/ to 2.58	0.17 to 0.300

* = Too high to calculate

† = Confidence intervals

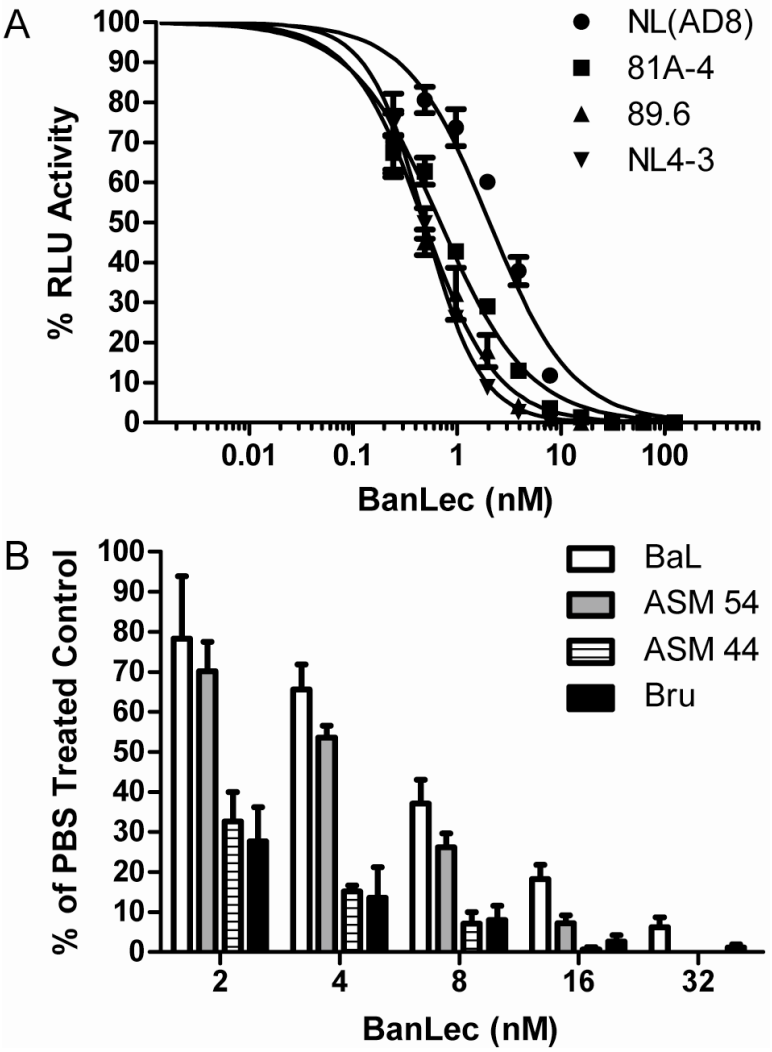

Figure 2-1. BanLec has antiviral activity against multiple HIV-1 isolates with different tropisms. A) TZM-bl cells were pretreated with different concentrations of BanLec prior to infection with the R5 tropic isolates NL(AD8) and 81A-4, dual tropic 89.6, and X4 tropic NL4-3. Forty-eight hours after exposure to virus, luciferase activity was determined by measuring relative luminescent units (RLU). The averages from three separate experiments were used for the calculation of IC_{50} values, which were determined by non-linear regression. The IC_{50} for viral inhibition were as follows: NL(AD8) = 2.06 nM, 81A-4 = 0.69 nM, 89.6 = 0.48 nM, NL4-3 = 0.49 nM. B) Magi-CCR5 indicator cells were used to determine anti-viral activity of BanLec against multiple strains of HIV-1. Forty hours after exposure to virus, infected cells were quantified by staining for β-galactosidase activity. Infectivity of BanLec-treated virus is presented as a percent of positively infected cells as compared to the PBS control. Error bars represent standard deviation from three separate experiments.

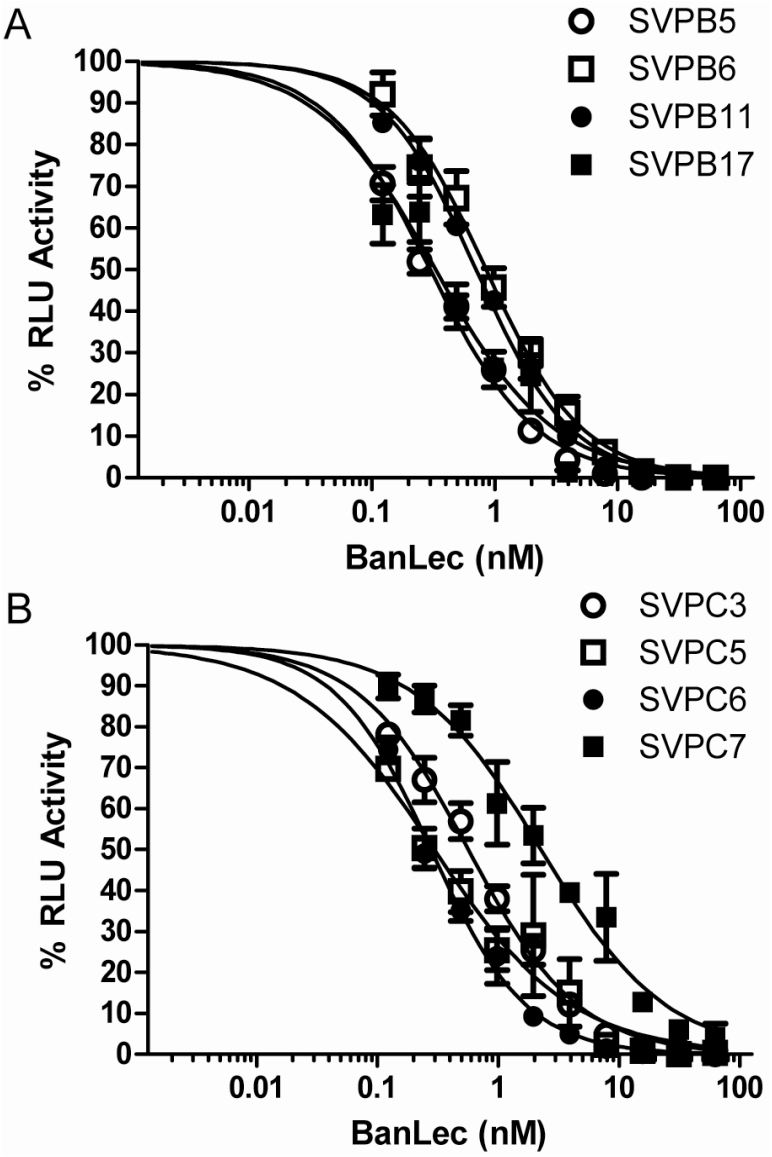

Figure 2-2. BanLec inhibits infection of HIV-1 pseudotyped with envelopes from multiple primary isolates. TZM-bl cells were infected with HIV-1 pseudotyped with primary HIV-1 envelope proteins from subtype B (A) and subtype C (B) in the presence of different concentrations of BanLec. Forty-eight hours later, luciferase activity was assessed. The IC_{50} values were determined as in Figure 1 and are shown in Table 1. Results shown are the average of three independent experiments and error bars represent the standard deviation.

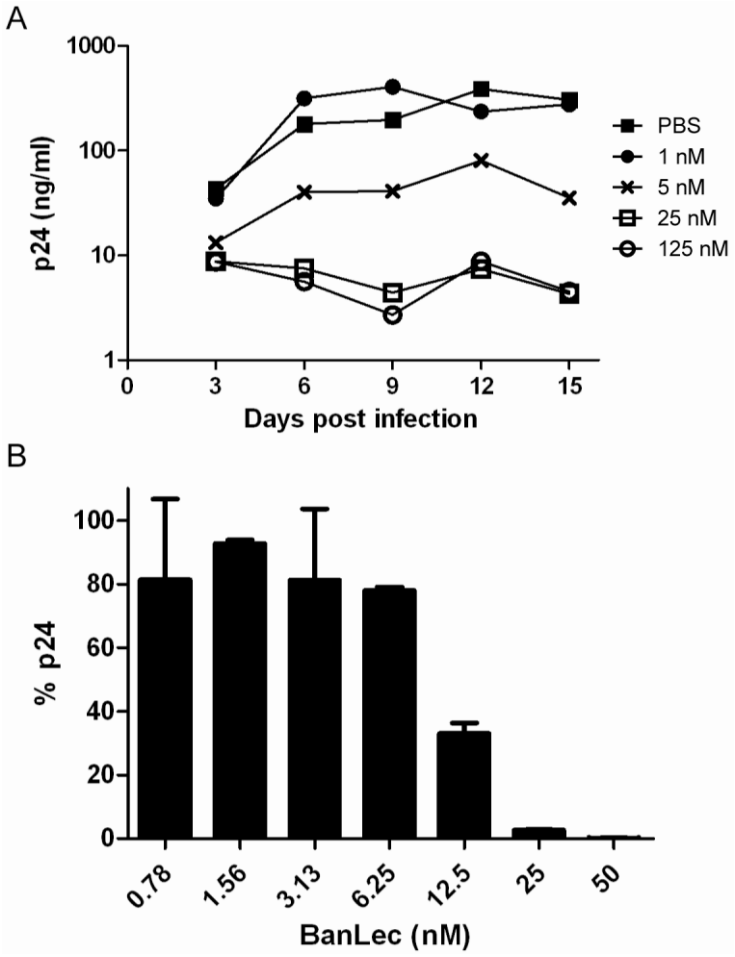

Figure 2-3. BanLec inhibits HIV-1 infection of MDM. A) MDM were pretreated with BanLec for 30 minutes prior to addition of 100 $TCID_{50}$ of HIV-1 NL(AD8). Twenty-four hours later, the media was removed and the cells were washed with PBS to eliminate remaining virus. Fresh media containing BanLec or PBS was added to the cells. A sample of culture supernatant was taken every three days for p24 quantification by ELISA and replaced with new media containing lectin in PBS or PBS alone as a control. On day 15, viability was assessed by an MTT assay, which indicated no cellular toxicity (data not shown). B) MDM were pretreated and infected with HIV-1 as described above. 24 hours post-infection the cells were washed with PBS to remove residual virus and cultured in media containing BanLec or PBS. Seven days post-infection, supernatants were removed for determination of p24 antigen as detected by ELISA. The concentration for a 50% reduction in p24 production was calculated to be 9.72 nM. Cellular viability was assessed by an MTT assay and no toxicity was observed (data not shown). Results shown in parts A and B are representative of three and two separate experiments, respectively.

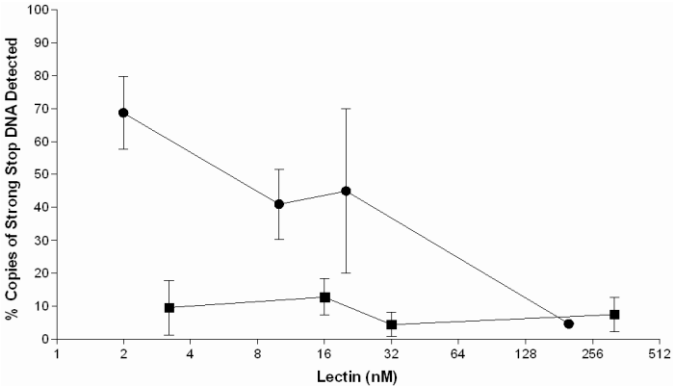

Figure 2-4. BanLec inhibits production of early HIV-1 reverse transcription products in peripheral blood lymphocytes. Peripheral blood lymphocytes were treated with different lectin concentrations thirty minutes prior to infection with HIV-1 Bru. Three hours post infection, cellular DNA of the infected cells was harvested and strong-stop DNA was quantified by real-time PCR. The number of copies was normalized to a PBS-treated control (100%). The known anti-HIV lectin GNA (circles) was used as a positive control and to assess the relative molar potency of BanLec (squares).

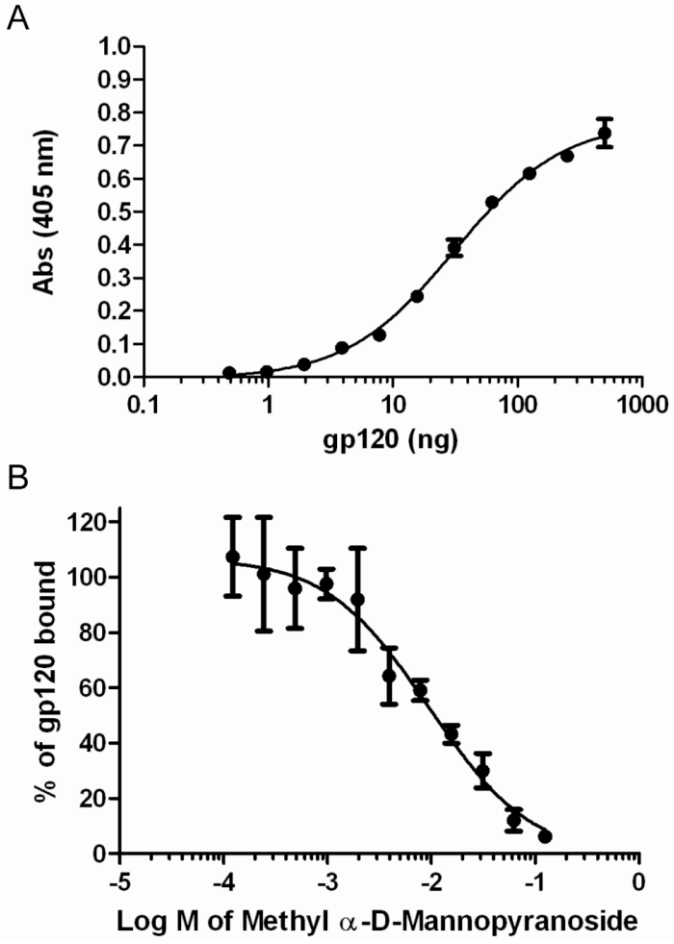

Figure 2-5. BanLec binds to glycosylated gp120. A) Dose-dependent binding of BanLec to glycosylated gp120. BanLec was used to coat a 96 well ELISA plate. Serial dilutions of gp120 were added in duplicate to the wells. gp120 was detected with an anti-gp120 antibody. Results are representative of four independent experiments. B) Methyl α-D-mannopyranoside inhibits interaction of BanLec with gp120. ELISA plates were coated with BanLec as in part A. Serial dilutions of methyl α-D-mannopyranoside were added to wells along with a constant amount of gp120. The amount of gp120 bound was determined using the standard curve produced in part A.

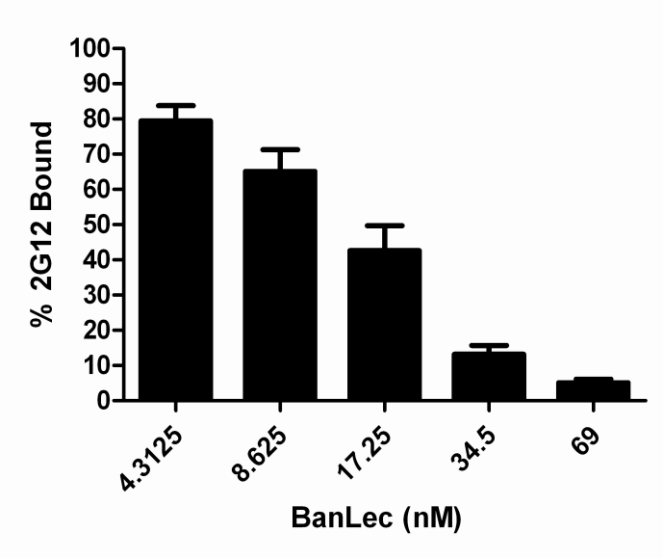

Figure 2-6. Binding of gp120 by BanLec blocks access to the anti-HIV monoclonal antibody 2G12. Recombinant gp120-coated ELISA plates were treated with different concentrations of BanLec prior to incubation with the 2G12 antibody, which recognizes the high mannose structures found at positions N295, N332, and N392 of the gp120 protein. Unbound antibody was removed and the amount of antibody remaining was determined by comparison to a standard curve. The results shown represent the average from three separate experiments. Error bars represent standard error of the mean. The effect of increasing amounts of BanLec was determined to be significant by 1-way ANOVA testing ($p < 0.01$).

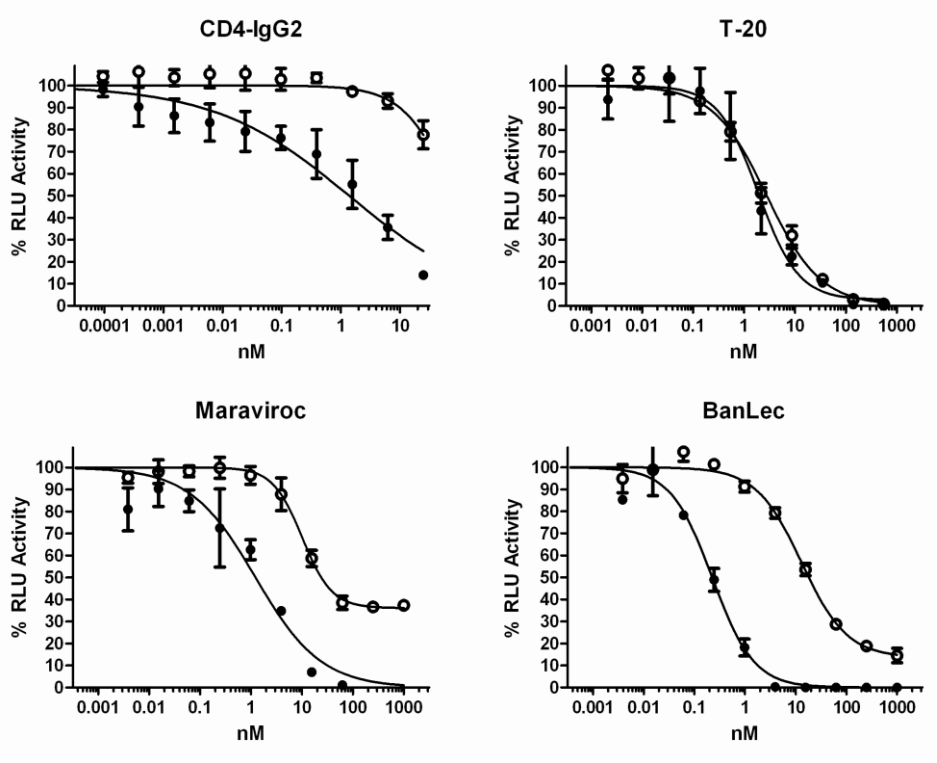

Figure 2-7. BanLec primarily inhibits binding of HIV to the cellular membrane. TZM-bl cells were spin-infected at 16 °C, a temperature that allows for attachment of virus but does not allow fusion events to occur. The unbound virus was removed and the cells were incubated with media containing inhibitors (CD4-IgG2, T-20, maraviroc, or BanLec) on ice for 30 minutes and then the plates were shifted to 37 °C to allow for fusion and infection to be completed (○). The results were compared to a standard infection procedure (pre-attachment) in which the virus and inhibitors were incubated together on ice for 30 minutes and then added to TZM-bl cells and incubated at 37 °C (●). The results shown are the averages from three separate experiments. Non-linear regression analysis was used for curve fitting and calculation of IC_{50} values (Table 2-2).

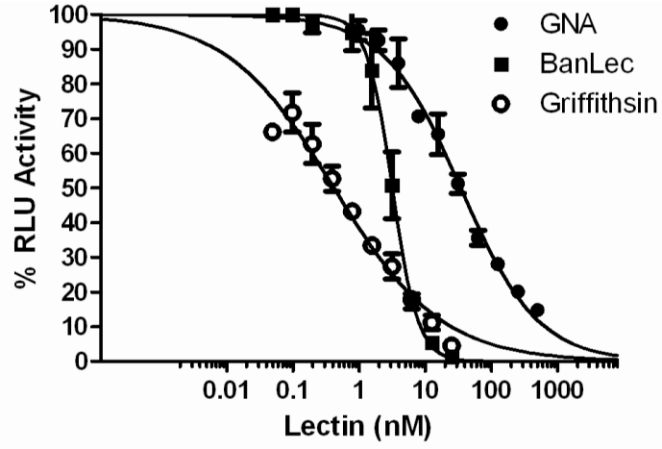

Fig. 2-8. Comparison of the anti-HIV activity of BanLec to the anti-HIV lectins GNA and GRFT. TZM-bl cells were pretreated with BanLec, GRFT, or GNA diluted in PBS, or PBS alone, as a control, for thirty minutes prior to infection by the R5 tropic HIV-1 virus 81-A. Forty-eight hours later, luciferase activity was measured. The results are normalized to infected cells treated with PBS alone. The average of three separate experiments is shown and was used to calculate IC_{50} values by non-linear regression. The calculated IC_{50} values are the following: GNA 34.3 nM, BanLec 3.18 nM, and GRFT 0.42 nM.

Chapter 3: Molecular Engineering of BanLec to Reduce its Mitogenic Activity

Introduction

Heterosexual intercourse is the primary mode of HIV-1 transmission, with a disproportionate number of newly infected individuals being women [4, 136]. Because women have a difficult time enforcing condom use, vaginal microbicides have been proposed as an HIV-1 prevention method that would give women more control over their safe sex practices. The microbicide trials performed so far have been unsuccessful, and several trials may have actually increased the rate of HIV-1 transmission [37, 137, 138]. Therefore, microbicide development is proceeding with caution. Currently approved anti-retrovirals have shown promise when formulated for microbicides, both in preclinical studies and in phase I and II testing [139]. However, the inclusion of small molecule anti-retrovirals might make microbicides prohibitively expensive. In addition, viruses can become resistant to anti-retrovirals, and thus the efficacy of anti-retroviral microbicides would likely decrease over time [140].

Plant lectins are able to inhibit HIV-1 infection by binding glycosylation sites found on the envelope protein, gp120 [62, 141-143]. Lectins possess properties that make them attractive as vaginal microbicide component candidates. These properties include the ability to inhibit viruses from all clades, a high genetic barrier to resistance, the ability to block DC-SIGN transmission, and the potential to block other sexually transmitted diseases such as HCV. GRFT and CV-N are two lectin proteins with demonstrated ability

to protect macaques from infection with SIV. Other lectins are effective at blocking viral infection *in vitro*, and we have recently reported that a lectin isolated from bananas, BanLec, is a potent inhibitor of HIV-1 infection [143]. Thus, lectins such as BanLec have great potential for development as components of vaginal microbicides.

Despite their promise as HIV inhibitors, the mitogenic properties of many lectins suggest that they may not be effective for *in vivo* use. Addition of certain lectins, such as PHA and ConA, to lymphocytes can lead to an increase in proliferation, i.e. they possess mitogenic activity [144, 145]. This can lead to an increase in HIV-1 replication. Lectins can also induce the production of pro-inflammatory cytokines, which has been shown to increase HIV-1 infection and replication [146]. It is thought that the recent microbicide failures were due to an increase in inflammatory cytokine production and destruction of the epithelial barrier [37, 137]. Therefore, it has been suggested that lectins with mitogenic activity may lead to an increase in HIV-1 infection. This has created wariness towards further development of lectins as microbicides.

We previously reported that BanLec is a potent inhibitor of HIV-1 replication, but its mitogenic activity might prohibit its use as a microbicide. As noted before, not all lectins are mitogenic, including a lectin that is of the same category and has a similar structure to BanLec, GRFT. We sought to determine whether BanLec could be molecularly engineered to reduce the unwanted effect of mitogenicity while maintaining potent anti-HIV activity. Other groups have been able to alter the specificity and affinity of a lectin by amino acid substitution. Single amino acid mutations of the PA-IIL lectin from

Pseudomonas aeruginosa resulted in enhancing the lectin's affinity for the monosaccharide methyl α-L-fucopyranoside resulted in decreased affinity for methyl-α-fucose, while improving affinity for methyl α-D-mannopyranoside approximately twenty-five fold [147]. Here we describe the properties of different BanLec mutants, including those that have decreased mitogenic activity, but retain anti-HIV activity.

Methods

Lectins and Carbohydrates

Methyl-α-D-mannopyranoside and PHA-L lectin were obtained from Sigma. The lectin GRFT was obtained from the NIH AIDS Reagent and Reference Program. The lectins GNA and BanLec were isolated through previously described methods [148]. Isolation of BanLec from *E. coli* is described below.

Virus production

Virus was produced using previously described methods. Briefly, production of pseudo-typed virus was performed by co-transfecting 293FT cells with a plasmid containing a proviral genome with a deletion in the envelope gene along with a plasmid that expresses an HIV-1 envelope gene. The following morning, the media was changed. Forty-eight hours post transfection, the supernatant was collected and centrifuged at approximately 300 x g for 5 minutes to remove any contaminating cells. For NL4-3 virus production, 293FT cells were transfected with the pNL4-3 plasmid. Virus was harvested as described above. The viruses were quantified by on TZM-bl cells or by measuring p24 antigen by ELISA (AIDS & Cancer Virus Program).

Inhibition of DC-SIGN Mediated Transmission

One day prior to the experiment, TZM-bl cells in DMEM media with 25 mM HEPES and 10% FBS were added to white, opaque 96-well tissue-culture plates at a density of 1 x10^4 cells per well. Raji B cells expressing dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) were obtained from NIH AIDS Reference and Reagent Program. The day of the experiment, Raji-DC-SIGN cells at a concentration of 5 x 10^6 cells were incubated with HIV-1 virus stock with a concentration of 250 ng of p24 per ml of isolate NL4-3, with a final volume 5 ml. The virus was allowed to bind to the cells for 1 hour. Next, the cells were washed twice with 50 ml of PBS and then resuspended in 2.5 ml of RPMI media containing 10% fetal bovine serum and penicillin and streptomycin (RPMI-10). The cells containing bound virus were then added to TZM-bl cells, which were cultured in 100 µl of RPMI-10 containing BanLec or PBS. After 48 hours of incubation at 37° C, 100 µl of media was removed from each well and replaced with 100 µl of One-Glo reagent (Promega) for determination of luciferase expression.

Assessment of Mitogenic Activity

PBLs were isolated as previously described (Chapter 2), and resuspended in IMDM media containing 10% FBS (IMDM-10) at a concentration of 2 x 10^6 cells/ml. 50 µl of cells were added per well of a white 96-well plate followed by 50 µl of IMDM-10 containing lectin at various concentrations or PBS. The cells were incubated at 37° C for 3 days prior to an 18 hour addition of BrdU. Proliferation was measured by BrdU incorporation, which was detected via a chemiluminescent-ELISA (Cell Proliferation

ELISA (chemiluminescent), Roche) as per manufacturer's instructions. Mitogenic activity was quantified as a stimulation index, which is the signal of the stimulated cells divided by the signal of the non-treated cells (RLU of treated PBL / RLU of untreated PBL).

Construction and Mutation of BanLec Expression Vectors

A cDNA encoding a codon-optimized BanLec for expression of *E. coli* was generated for the protein sequence gi71042661 by Genscript. The cDNA was then cloned into the *E. coli* expression vector pET24b (Novagen) to be in frame with the 6 x His tag. Site-directed mutations were introduced by the QuikChange Multi Site-Directed Mutagenesis Kit or by the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene). PCR primers for introduction of the desired mutations were designed using the QuikChange Primer Design Program; available at http://www.stratagene.com/sdmdesigner/default.aspx.

Purification of Recombinant BanLec and Mutants

A plasmid containing either the wild-type or a mutant form of BanLec was used to transform MDS 42 T7 or RosettaBlue pLysS *E. coli* cells. An overnight culture was used to inoculate 2 x YT media. When the OD_{600} nm of the culture reached 0.7 -1.0, protein expression was induced with IPTG at a final concentration of 1mM. Five hours post induction, the cultures were harvested and the cell pellets were frozen at -20° C until further processing. Recombinant protein was isolated by resuspending the pellet in 5 ml of 50 mM Tris, 0.5 M NaCl, and 0.02% NaN_3 pH 8.0 (the buffers will be referred to

hereafter as IMAC-#, where # represents the amount of imidazole in mM) per 100 ml of culture grown. Lysozyme and DNAse I were added at concentrations of 1 mg/ml and 5 µg/ml, respectively. The mixture was incubated at room temperature for 30 minutes with constant stirring. After the incubation, an equal volume of IMAC-50 buffer was added and the mixture was chilled on ice. Cells were further lysed with four rounds of 30 seconds of pulsed sonication at the 50% duty at power level 5 while on ice followed by a one minute rest period between each 30 second of sonication. The insoluble material was pelletted by centrifugation at 10,000 x g for 20 minutes.

The resulting cleared lysate was added to Ni-NTA agarose (Qiagen) that had been equilibrated with IMAC-25 buffer. The lysate and the resin were incubated for one hour at 4° C with orbital rotation. The column was returned to room temperature and the lysate was allowed to pass through the column via gravity. The column was then washed with IMAC-25 buffer until the flow-through had an absorbance value at 280_{nm} less than 0.05 (approximately 20 volumes; this would vary with the size of the column and flow rates). Elution of the protein was then performed with IMAC-250 buffer. The protein was then dialyzed against PBS using Slide-alyzer dialysis cassettes with a 10 kDa molecular weight cutoff (Pierce). Two two-hour dialysis procedures were performed at 4° C against a volume greater than 200 times that of protein sample followed by overnight dialysis. The protein was then sterile-filtered through a 0.22 µm filter. The protein was aliquoted and stored at -80° C prior to use, where it could then be stored again at 4° C for short-term use. Protein was quantified by BCA (Pierce) using bovine serum albumin protein as a standard.

Assessment of anti-HIV activity

To each well of a white 96-well plate, 100 µl of TZM-bl cells resuspended at 1 x 10^5 cells/ml in DMEM media with 25 mM HEPES and 10% FBS were added to each well of a 96-well plate. The next day, the media was removed by aspiration and fresh media containing lectins or PBS as a control was added to the plate at a concentration two times more than the final concentration. After 30 minutes of incubation, virus diluted with media was added and the cells were incubated for 48 hours at 37° C. After the incubation, 100 µl of media was removed and replaced with 100 µl of One-Glo reagent (Promega) for determination of luciferase expression.

Rabbit Red Blood Cell Agglutination Assay

The haemagglutinating activity of the lectins was determined by a 2-fold serial dilution procedure using formaldehyde-treated 2% rabbit erythrocytes as described previously [149]. The lowest concentration of lectin that resulted in agglutination was used for comparison.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was performed to determine the lectins' affinity for Methyl-α-D-mannopyranoside using previously described methods [92].

Results

Purification of recombinant BanLec

We synthesized a BanLec cDNA sequence that is codon-optimized for expression in *E. coli*. The sequence was sub-cloned into an expression vector that introduced a C-terminal His-tag. We were able to successfully express and purify recombinant BanLec, similar to what has been previously described (figure 3-1) [69]. We compared the anti-HIV activity of the recombinant BanLec (rBanLec) to BanLec isolated from bananas, and found that they had similar anti-HIV activity (figure 3-2). ITC was used to determine the affinity of wild-type and recombinant BanLec for the ligand methyl-α-D-mannopyranoside, and their affinities were the same (data not shown).

rBanLec Inhibits Viruses Containing Consensus Subtype B and Subtype C Envelopes.

We had previously shown that BanLec was able to potently inhibit HIV-1 isolates of subtype B and subtype C. To determine how well rBanLec might be able to inhibit a broad range of subtype B and subtype C envelopes, we tested the ability of rBanLec to inhibit pseudotyped HIV-1 that contained an envelope derived from a consensus sequence for clade B or for clade C. rBanLec was able to potently inhibit both isolates with IC_{50} values in the low nanomolar range (Figure 3-3). These results agree with our previous data in which we tested several different envelopes from clades B and C with natural BanLec. It also suggests that rBanLec would be able to inhibit the majority of isolates from subtypes B and C [143].

BanLec and GRFT differ in the ability to inhibit different isolates

If two lectins were able to recognize different glycans, then it may be possible that they differ in their ability to neutralize virus. We tested the ability of BanLec and GRFT to inhibit several different isolates from additional clades. We observed differences in the neutralizing activity of these lectins for different isolates (Table 3-1). Thus, it appears that BanLec and GRFT can recognize different glycosylation structures found on the HIV-1 envelope protein. Other groups have reported similar results for GRFT and CV-N [150]. These results suggest that a lectin-based microbicide composed of multiple lectins may be more effective than a single lectin-based microbicide.

BanLec inhibits HIV-1 infection by DC-SIGN bound virus

Dendritic cells are able to utilize the receptor DC-SIGN to non-specifically bind HIV-1 through the high mannose glycosylation structures found on gp120. It has been postulated that dendritic cells found in the vaginal epithelium bind HIV-1 and then migrate to the lymph node, thus delivering the virus to its target cells. Therefore, inhibition of this method of transmission would be a beneficial feature of a vaginal microbicide. Using cell lines, we were able to show that the transmission of HIV-1 bound by DC-SIGN could be blocked by rBanLec (Figure 3-4). After allowing binding of the viruses to DC-SIGN, the cells were then washed to remove unbound virus. Cells with bound virus were then added to the TZM-bl indicator cell line with different amounts of BanLec. Total inhibition was achieved in the nanomolar range, further demonstrating another property of rBanLec that would be desirable in a microbicide candidate.

Comparison of the mitogenic activity of BanLec to other lectins

BanLec's potential for therapeutic use could be limited by its mitogenic activity. Other groups have reported BanLec to induce proliferation of human and mouse lymphocytes [66, 69, 70]. Naturally produced BanLec from bananas was assessed for mitogenic activity by another group investigating potential anti-HIV microbicide components (the International Partnership for Microbicides). They compared the mitogenic activity of BanLec to another anti-HIV lectin, CV-N. The degree of cellular proliferation was assessed by measuring the amount of ^{3}H-labeled thymidine that was incorporated by the stimulated cells. For relative comparison, the stimulation index for each lectin was calcuted. This value is the ratio of signal from the treated cells divided by the signal from the untreated cells. Traditionally, a lectin is considered mitogenic if it has a stimulation index greater than 3. BanLec produced a stimulation index greater than three at the lowest concentration tested (3.2 nM) (Figure 3-5). It appears to be more mitogenic than CV-N, since higher concentrations of this latter lectin were required to yield a stimulation index greater than three.

We verified these results in our own hands by comparing the mitogenic activity of BanLec and rBanLec to other lectins using a similar assay. Our method for proliferation measurement differed slightly in that we used BrdU to label proliferating cells instead of ^{3}H labeled thymidine. We treated PBL with different lectins for three days, and then quantified proliferation by using ELISA to measure the amount of incorporated BrdU (Figure 3-6). The lectin PHA-L was used as a positive control in this assay since it is highly mitogenic [144]. Two lectins that have been reported to be non-mitogenic, GNA

and GRFT, were used as negative controls [65, 85]. GNA and GRFT yielded stimulation index values greater than three at the higher concentrations tested (6 and 9, respectively) (Figure 3-6). Therefore, we set our stimulation index cutoff at ten, because the non-mitogenic lectins we assayed never exceeded an index of ten. As expected, PHA produced a high stimulation index value at a low concentration. BanLec also had mitogenic activity, but appeared to have a lower maximum stimulation value than PHA (Figure 3-6). Surprisingly, although rBanLec and BanLec both surpass the threshold for mitogenic activity at a similar concentration, they appear to differ in their maximum stimulation index values (Figure 3-6). Unlike BanLec isolated from bananas, the stimulation index for rBanLec did not continue to rise with increasing concentration, but rather attained a peak value and subsequently decreased. This is similar to the mitogenic activity of another mannose specific jacalin-related lectin, Morniga M., that caused activation induced cellular death (AICD) [151]. BanLec purified from bananas may include a mixture of isoforms, which might explain why its mitogenic activity differs from that of the recombinant form. The results of our assay, along with previous data, show that BanLec has mitogenic effects on lymphocytes.

It is unknown exactly how mitogenic activity *in vitro* correlates with detrimental effects *in vivo*; that is, we cannot define a stimulation index value that corresponds to a cutoff for safe use *in vivo*. However, the mitogenic activity of BanLec must be acknowledged when further considering it as a microbicide component. Interestingly, the anti-HIV lectin and non-mitogenic control in our assay, GRFT, belong to the same lectin family (JRL) and has a structure that is very similar to BanLec. We wondered how two molecules with

highly similar structures could have a drastic difference in regards to mitogenic activity; in particular, we hypothesized that the slight structural differences between these two lectins might reflect important functional differences that are involved in mitogenic interactions. Furthermore, we reasoned that it may be possible to mutate BanLec to alter its structure and reduce its mitogenic activity.

Strategies to Reduce the Mitogenic Activity of BanLec

Cross-linking of cellular receptors can result in signal transduction, which can also initiate cellular proliferation. It has been suggested that the cross-linking of multiple receptors is required to generate a sufficient signal to induce the cells to divide. Experiments performed with antibodies and antibody derived fragments support this idea. An anti-CD3 (T cell receptor or TCR) monoclonal antibody that is capable of cross-linking possesses more mitogenic activity than the monovalent, non-cross-linking Fab portion derived from the same antibody [152]. Oligomeric forms of soybean agglutinin (SGA) and peanut agglutinin (PNA) had much higher mitogenic activity as compared to monomeric forms [153]. BanLec is able to cross-link carbohydrates, as evidenced by its agglutination of sheep red blood cells. It could be possible that the valency of the lectin is more important for lectin-mediated mitogenic effects than it is for anti-HIV activity. Therefore, we hypothesized that reducing the valency of BanLec would decrease its ability to cross-link cellular receptors and mitogenic activity, while the anti-HIV-1 activity would remain relatively unaffected.

Reducing the Valency of BanLec for Reducing its Mitogenic Activity

We sought to determine if both binding sites of rBanLec are required for mitogenic and/or anti-HIV activity. BanLec has two binding sites per monomer and exists as a homodimer and therefore has a total of four potential binding sites [68]. In theory, reducing the valency, i.e. the number of binding sites, found on BanLec should decrease its cross-linking ability. Due to its dimeric nature, eliminating one of the binding sites of BanLec would reduce the valency of the lectin from four to two. It is not known if both binding sites are required for BanLec binding activity. However, the majority of lectins belonging to the JRL family have only one binding site per monomer. Other groups have removed a single binding site of anti-HIV lectin and determined its effect on anti-viral activity. The lectin actinohivin (AH) still has the ability to inhibit HIV-1 when one of its three binding sites is mutated [154], while the inhibitory activity of CV-N is decreased when one particular binding site is mutated [155]. However, these mutants have not been tested for mitogenic activity. Thus, it may be possible to reduce the valency of BanLec and maintain anti-HIV activity.

We mutated the conserved aspartic acid to a glycine in either the first or second binding site (D133G and D38G, respectively), as well as mutating them simultaneously (D38G/D133G). The side chain of the aspartic acid has been implicated in carbohydrate recognition and is a part of the carbohydrate binding GXXXD motif that is found in the JRL family [68, 93]. Therefore, we reasoned that mutation of this glycine residue would render the binding site inactive. We sought to determine if D38G or D133G could bind

carbohydrates with their unaltered binding sites and if binding had indeed been abolished at the mutated sites. However, our method for determination of the affinity of lectins for carbohydrates, ITC, could not be performed on these mutants. This is due to a reduction in protein solubility caused by the mutations. The resulting concentrations are not high enough for this assay. The reduced solubility is not unexpected, since the mutations changed a charged amino acid that was solvent exposed to a more hydrophobic amino acid.

We then tested these mutants for agglutination activity. Mannose binding lectins, including BanLec, have agglutination activity for rabbit red blood cells [66]. A decrease in agglutination activity would suggest that the mutant has either altered carbohydrate specificity, carbohydrate affinity, or an impaired ability to cross-link carbohydrate structures.

We found that D133G required approximately 66 times more protein than wild-type rBanLec for agglutination activity (rBanLec: 3 μg/ml, D133G: 200 μg/ml), while the D38G and the D38G/D133G double mutant did not have any observable agglutination activity. D133G's reduction in agglutination activity may be due to a decrease in the number of binding sites, i.e. valency, from four to two, or a lower affinity for the ligand. One would expect the same from the D38G mutant, but this was not the case. The D38G mutant might lack agglutination activity due to protein misfolding, or perhaps the remaining binding sites are not compatible for cross-linking. As we expected, there was no activity for the double mutant. However we cannot rule out that this lack of activity is

due to protein misfolding rather than alteration of the binding sites. However, overall these results suggest that our mutations had altered the binding activity of rBanLec.

Since D133G was found to have some agglutination activity, we decided to determine if it still had mitogenic activity. However, no mitogenic activity was observed (Figure 3-7A). Similar results were also observed with the D38G/D133G mutant (Figure 3-7B) and we observed no mitogenic activity with a 1 μM concentration of D38G (data not shown). We next tested the D38G and D133G mutations for anti-HIV-1 activity. No inhibition was observed at the highest concentration tested (1 μM) (Figure 3-8).

From these results, we conclude that both binding sites found in BanLec are required for its mitogenic and anti-HIV activity. These results are in agreement with what was observed when one of the two binding sites of a monomeric form of CV-N was inactivated through amino acid substitutions [155]. Thus, this approach to reduce mitogenic activity of BanLec while retaining anti-HIV-1 activity was unsuccessful. However, these results did provide a better understating of the binding site requirements of BanLec. In addition, the mitogenic activity of the recombinant lectins produced in *E. coli* is from the lectin itself and not from protein purification byproducts such as lipopolysaccharides. Since none of the three binding site mutants produced any mitogenic activity, the contribution of co-contaminants to mitogenic activity is negligible.

Mutation of the Binding Loop Found on the Third Greek Key

It has been suggested that a loop found on the third Greek key of members of the JRL family (known as the carbohydrate recognition loop) is important for ligand specificity [59]. The length of the loop appears to play a role in determining the size of the binding pocket. Longer loops can restrict the size of the binding site to short oligosaccharides, while shorter loops have a more open binding pocket that can accommodate larger structures such as those of the high-mannose variety. While the loop lengths do not appear to correlate with specificity or affinity for different monosaccharides, the specificity for larger oligosaccharides with different components, e.g. mannose versus galactose, are likely determined by this loop [59, 60, 156]. We hypothesized that the ligand specificity of BanLec could be influenced by mutating amino acids found in the loop.

We targeted the histidine found at amino acid position 84 of BanLec (H84) since it is found in the carbohydrate recognition loop and has been predicted to play a role in the recognition of oligosaccharides [68, 93]. We made several different amino acid substitutions at random and tested for a decrease in mitogenic activity. The decrease in mitogenic activity was determined as above, comparing the highest mutant concentrations that still yielded a stimulation index value less than ten to rBanLec. The H84Y and the H84A mutants appeared to have no change in mitogenic activity (Figure 3-9). In fact, H84Y may have increased activity. At higher concentrations, we observed a drop in the stimulation index for this mutant. This is likely from activation-induced cellular death. Other mutants such as H84S, H84M and H84T were found to have

markedly decreased mitogenic activity (Figures 3-10 and 3- 11). One of the most promising mutants was H84T, which did not induce a stimulation index greater than ten in the three donors tested (Figure 3-11).

We next evaluated several of these mutants for anti-HIV-1 activity. Since we have shown that rBanLec has anti-HIV activity in the lower nanomolar range, we reasoned that a mutant form of BanLec with up to a 100 fold decrease in anti-HIV activity would maintain IC_{50} values in the nanomolar range. For the mutants with no change in mitogenic activity, we observed a small decrease in anti-HIV-1 activity for H84A and no decrease in activity for H84Y (Table 3-2). We observed decreased IC_{50} values for H84S, H84T, and H84M (Table 3-2). We found that the IC_{50} values of the H84M mutant decreased over-time, which suggest a potential problem with stability (data not shown). Since this mutant is not stable and the H84S mutant appears to have mitogenic activity, we further focused our efforts on the H84T mutant, which was not found to be mitogenic with the donors tested. Although this mutant still had approximately a 23 fold decrease in anti-HIV-1 activity, its IC_{50} remained in the low nanomolar range, 7 nM, which is excellent for a potential microbicide.

Drs. Winter and Goldstein next used the rabbit RBC agglutination assay to determine if H84T had impaired cross-linking ability, binding specificity, or binding affinity. Interestingly, we found H84T's agglutination activity was reduced when compared to rBanLec (Table 3-3). We also tested the agglutination activity of the H84S mutant and found that it to be lower than rBanLec, but greater than H84T (Table 3-3). These results

suggest that suggest that the H84T or other mutants may have altered cross-linking ability or carbohydrate affinity, which may contribute to the decrease in mitogenic activity. In addition, this agglutination assay may be useful for predicting the mitogenic activity of additional H84 mutants.

Analysis of the Binding Affinity and Structure of the H84T Mutant

Drs. Winter and Goldstein next tested the affinity of H84T and H84S for the mannose like ligand D-methy-α-mannopyranoside by ITC. Interestingly, no striking differences were observed (Table 3-3). These results suggest that the H84T mutant's decrease in mitogenic activity is not due to a change in its affinity for D-methy-α-mannopyranoside, and that the protein is properly folded, and that the decrease in agglutination activity is not due to a proportion of the protein being non-functional.

Next, we determined if there was a change in the three-dimensional structure of the protein. We hypothesized that the histidine to threonine mutation altered the confirmation of the protein, such as the amino acid loop that contains H84. As mentioned above, this loop is thought to be important for carbohydrate recognition and specificity. Through X-ray crystallography, the structure of the protein was determined. When an overlay of H84T and natural BanLec's three-dimensional structures was performed, there was no observable difference (Figure 3-12).

Discussion

The clinical use of lectins is appealing since they possess several properties desired in a vaginal microbicide. Many lectins are potent inhibitors of HIV. Lectins block HIV infection by binding multiple glycosylation sites on the HIV envelope, thus presenting a high genetic barrier for viral resistance. Furthermore, virus that is resistant to lectins has been shown to be more susceptible to neutralizing antibody responses. Nevertheless, the mitogenic activity of lectins presents a significant challenge to their development as vaginal microbicide components.

We have shown that a recombinant version of BanLec that is expressed in *E. coli* is a potent inhibitor of HIV-1 infection. It has multiple properties that make it desirable as a component of an anti-HIV-1 microbicide, including inhibition of HIV-1 isolates from a variety of subtypes and inhibition of DC-SIGN transmission. However, BanLec is a mitogenic lectin, which could lead to an increase in HIV-1 replication *in vivo*.

It is not well understood how lectins in general induce mitogenic activity. Lectins are multivalent and are capable of cross-linking, and therefore one obvious mechanism for mitogenic stimulation would be the cross-linking of cellular receptors that induce cell signaling and proliferation. Mitogenic activity is not entirely dependent on cross-linking, but cross-linking appears to make the response more potent, i.e. a lower concentration of the stimulant leads to more proliferation [152].

It seems likely that rBanLec can bind multiple cell surface proteins since N-linked glycosylation is a common feature of many extracellular proteins. Receptors that are part of the T-cell receptor complex, such as CD2, CD3, and CD5 all contain high-mannose structures [157-161]. Cross-linking of these molecules could mimic the immune synapse and lead to activation of cells. Accessory molecules could be bound as well. The interleukin 2 receptor alpha chain (IL-2Rα also known as CD25) contains high-mannose structures which are used by IL-2 for binding [162]. Other receptors not related to the immune response may also be targets for BanLec; for example, BanLec is able to bind to insulin-like growth factor receptor β (IGF-2R) [71]. The source of BanLec's mitogenic activity may be more than one cellular receptor, since BanLec is able to stimulate multiple cell populations that include CD4+ T cells, CD8+ T cells, B cells, and NK cells [69]. Further studies utilizing mass spectrometry could help determine the receptors bound by BanLec, and perhaps allow us to identify common glycosylation structures shared by all these receptors.

Although the binding target of BanLec is unknown, the mitogenic activity of BanLec likely requires recognition of a specific glycosylation structure found on the receptor and the cross-linking of this receptor. Therefore, we sought to reduce the mitogenic activity by reducing BanLec's carbohydrate recognition properties or by reducing its ability to cross-link.

When one of the two different binding sites of BanLec was mutated to eliminate carbohydrate binding activity, we did not observe mitogenic or anti-HIV-1 activity.

These data suggest that BanLec's mitogenic and anti-HIV activities require both binding sites to be functional. In addition, these results and those obtained with a mutant with both binding sites eliminated strongly suggest these activities are not independent of carbohydrate binding. These results agree with a previous report indicating that the mitogenic activity of BanLec was through lectin-carbohydrate interactions [69].

We hypothesized that mutating an amino acid found in BanLec's carbohydrate recognition loop (H84) would eliminate mitogenic activity while maintaining anti-HIV activity in the nanomolar range. Mutating this amino acid resulted in several BanLec mutants with the desired phenotype, including H84T. In addition, we found that mitogenic activity of the H84 mutants correlated with their ability to agglutinate rabbit RBCs, which may be useful for screening additional mutants for mitogenic activity.

The decrease in agglutination activity may result from altered carbohydrate binding properties or decreased cross-linking ability. We would predict that a decrease in cross-linking would be unlikely since the mutation was made outside of the two primary binding loops of BanLec. We determined if H84T had a decreased affinity of methyl-α-D-mannopyranoside, but observed no difference. However, this monosaccharide does not resemble the high-mannose structures found on glycosylated proteins. Thus, differences may be observed when comparing rBanLec's and H84T's affinity for oligomannose, which better resembles the native structures.

We also determined the three-dimensional structure of H84T and compared it to that of wild-type BanLec. We anticipated seeing a different conformation of the amino acid loop containing the H84T mutation. However, no differences were observed that may explain the decrease in mitogenic activity of this mutant.

To our knowledge, we are the first to report that the mitogenic and anti-HIV activities of lectins can be separated. The modified form of BanLec shows promise as a microbicide component and further studies, including a vaginal challenge model of HIV-1 infection, should be performed. These and other considerations are further discussed in Chapter 4.

Table 3-1. Comparison of the anti-HIV-1 activity of BanLec and GRFT.

Envelope I.D. #	Subtype	PNGS*	BanLec IC_{50}	GRFT IC_{50}
11538	D/A	30	0.33	0.007
11539	D/A	32	1	0.013
11545	A	21	0.94	1.47
11546	A	19	3.09	0.044
11499	C	19	2.3	5.75
11407 (ConC)	C	25	0.23	0.173

* Potential N-linked glycosylation sites

TZM-bl cells were pretreated with BanLec or GRFT prior to infection with pseudotyped HIV-1, was produced as described in the Materials and Methods section.

Table 3-2. Comparison of the anti-HIV and mitogenic activity of H84 mutants to rBanLec.

BanLec Mutant	Fold Decrease in Mitogenic Activity*	IC_{50} (nM)†	Fold Decrease in Anti-HIV Activity
H84T	256 1031 4167	7.0	23
H84S	4167 64	1.6	4.1
H84M	4167 1031	7.7	21
H84A	1 1	0.7	2.5
H84Y	1 1	0.29	-

* The values presented are from individual donors.
† Results are the average of two separate experiments.

Table 3-3. Comparison of the agglutination and carbohydrate binding activities of rBanLec to H84T and H84S. Results provided by Harry Winter.

Lectin	**MAC† (µg/ml)**	**Ka* (mM^{-1})**
rBanLec	3	383
H84T	437	358
H84S	237	343

† minimum agglutination concentration
* affinity constant

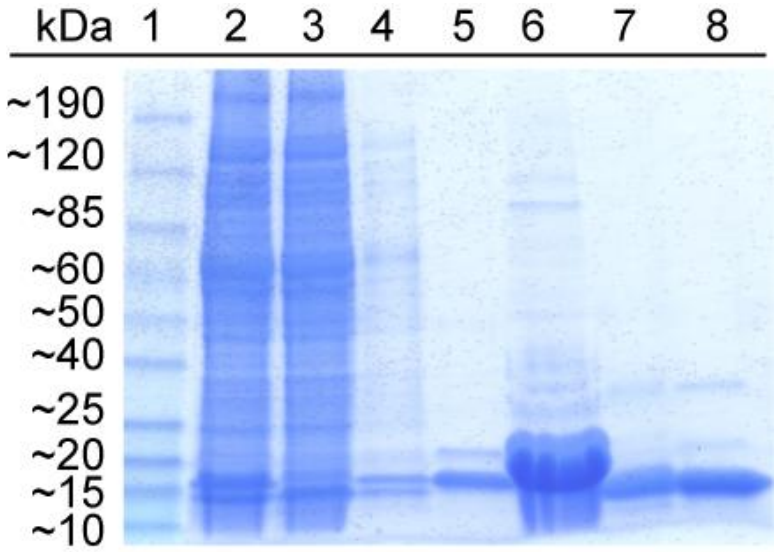

Figure 3-1: Expression of Recombinant BanLec. Coomassie-stained SDS-PAGE gel of the purification of recombinant BanLec. The gel lanes were loaded as follows: 1: Molecular weight marker 2: lysate containing recombinant BanLec 3: column load flow-through 4: 25 mM imidazole wash 5: first 250 mM imidazole elution 6: 2nd 250 mM imidazole elution 7: Banana-derived BanLec 8: lysozyme protein that was used for the cellular lysis step.

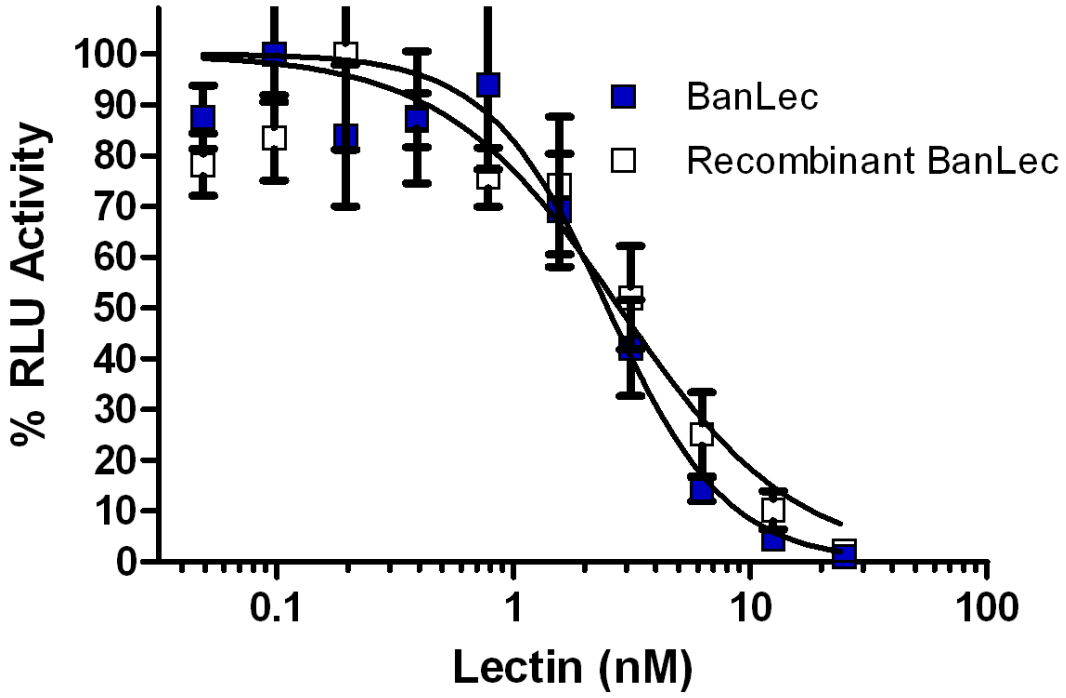

Figure 3-2: Comparison of the anti-HIV-1 activities of natural and recombinant BanLec. Natural BanLec isolated from the fruit of bananas or recombinant BanLec isolated from *E. coli* was added to TZM-bl cells 30 minutes prior to infection with the HIV-1 isolate NL(AD8). Forty-eight hours post infection, infection was quantified by measuring luciferase activity. Results are representative of two separate experiments and error bars represent SEM. Non-linear regression was used to calculate the IC_{50} values for natural BanLec and rBanLec, which were calculated to be 3.28 nM and 4.76 nM with 95% CI intervals of 2.50 to 4.30 nM and 3.27 to 6.94 nM, respectively.

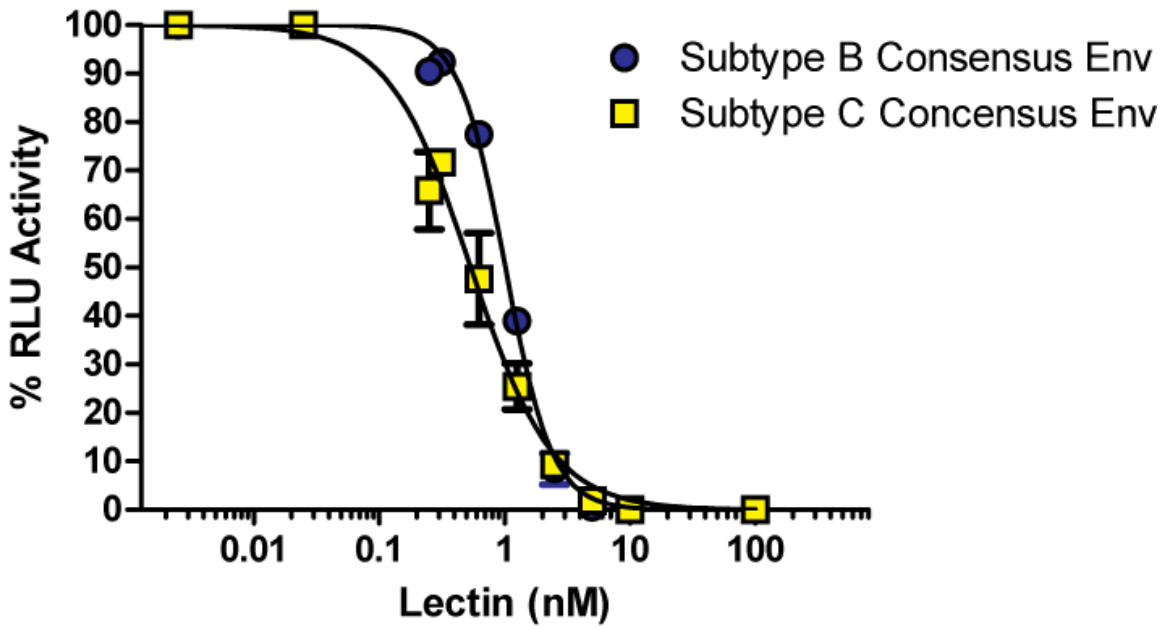

Figure 3-3. BanLec inhibits HIV-1 containing consensus subtype B and subtype C envelopes. TZM-bl cells were pretreated with different concentrations of BanLec for thirty minutes prior to infection with HIV-1 pseudotyped virus containing an envelope representing the consensus envelope sequence for either subtype B or subtype C. Forty-eight hours post infection, the amount of virus inhibition was determined by measurement of luciferase activity. Results are representative of three separate experiments and error bars represent SEM. Non-linear regression was used to calculate the $IC_{50,}$ which were calculated to be 1.0 nM and 0.6 nM for subtypes B and C, respectively.

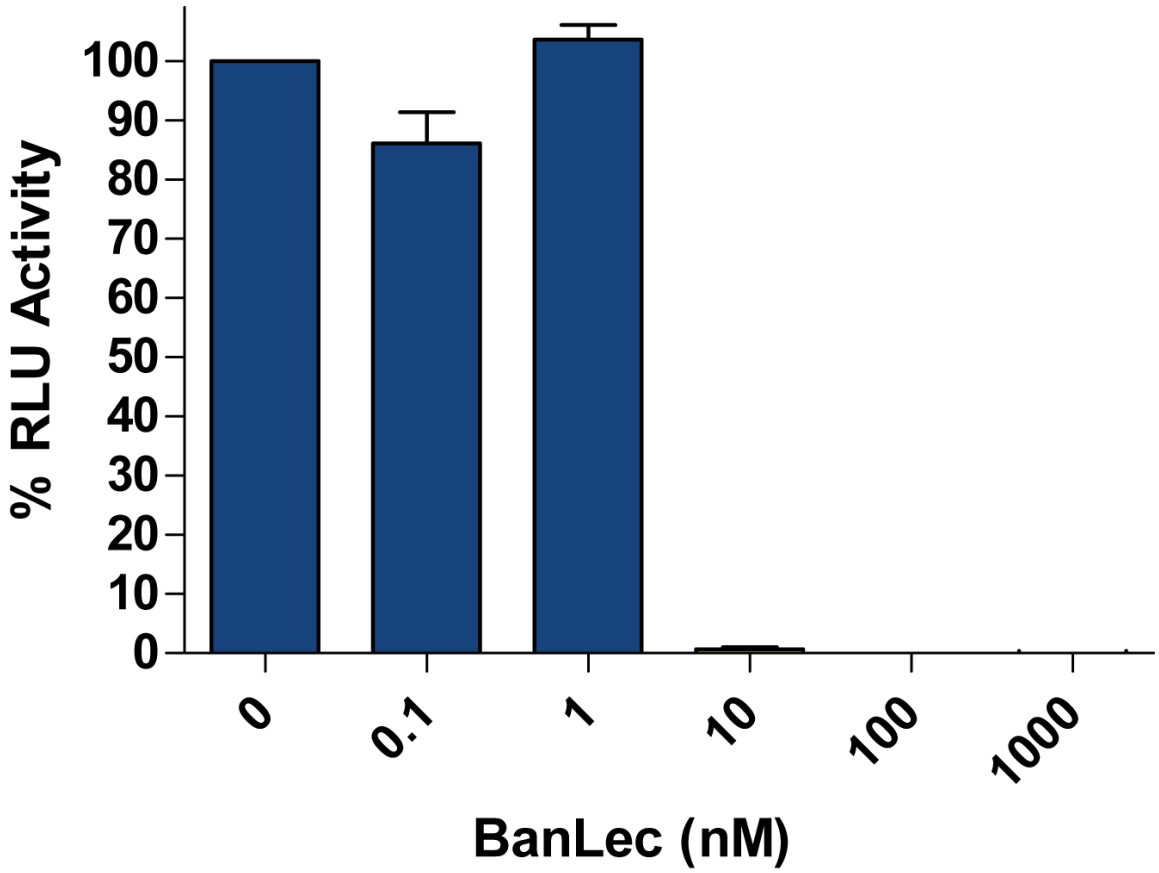

Figure 3-4: BanLec inhibits infection by DC-SIGN-bound HIV-1. Raji B-cells expressing DC-SIGN were incubated with HIV-1 NL4-3. Unbound virus was removed and the cells were resuspended in fresh cell-culture media. The cells were then added to TZM-bl cells that had been pretreated with different concentrations of BanLec. Infection by DC-SIGN-bound virus was assessed 48 hours later by measurement of luciferase activity.

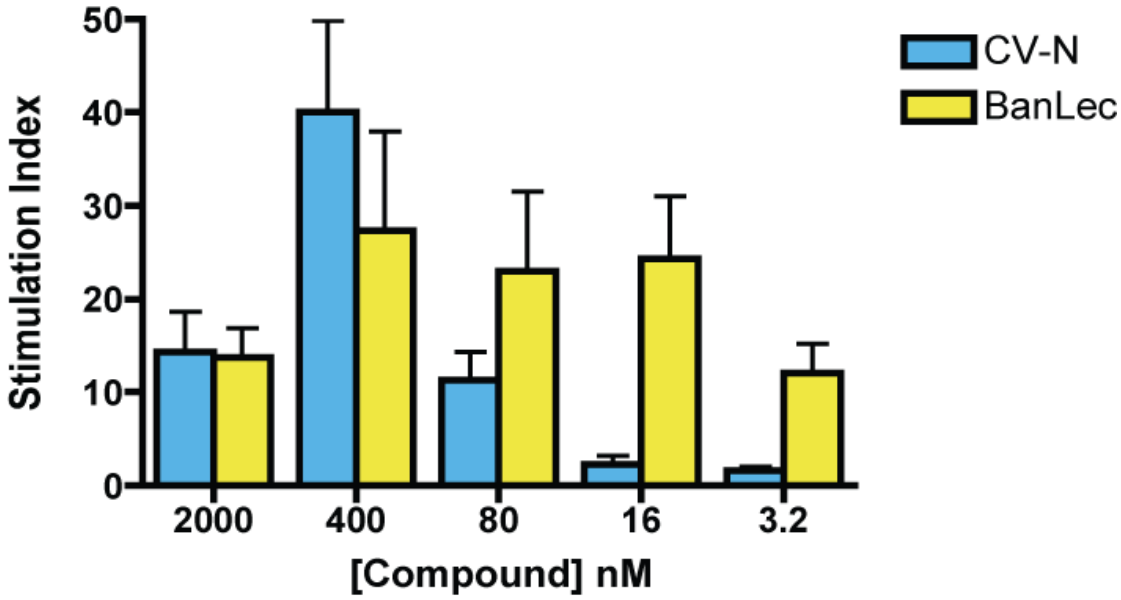

Figure 3-5. Comparison of the mitogenic activies of BanLec and CV-N. The potential for BanLec to induce cell proliferation was determined using freshly isolated, single donor peripheral blood mononuclear cells (PBMC). Cells were exposed to compound for 3 days prior to the addition of ^{3}H-Thymidine overnight. Cells were then harvested and counted to determine the amount of incorporated ^{3}H-Thymidine. PHA (phytohaemagglutin, 2 μg/ml) was included as a positive control to ensure that the cells were able to respond to stimulation (data not shown). Data represent the mean ± SD of 3 independent PBMC donors, where each condition was tested in triplicate. Results were generated by the International Partnership for Microbicides.

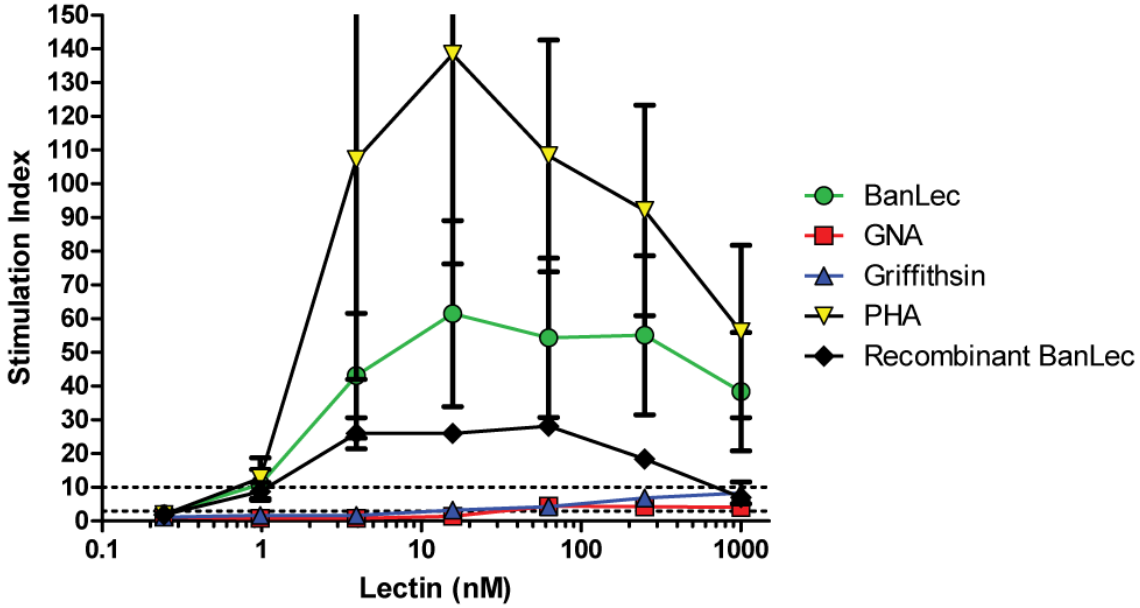

Figure 3-6. Comparison of the mitogenic activity of BanLec to other lectins. PBLs were incubated with lectin for three days prior to labeling with BrdU. BrdU incorporation by proliferating cells was determined by ELISA. The amount of proliferation, i.e. mitogenic activity, is quantified as stimulation index (described above). The results shown are the averages of three separate experiments, each with a different donor, and the error bars represent SEM.

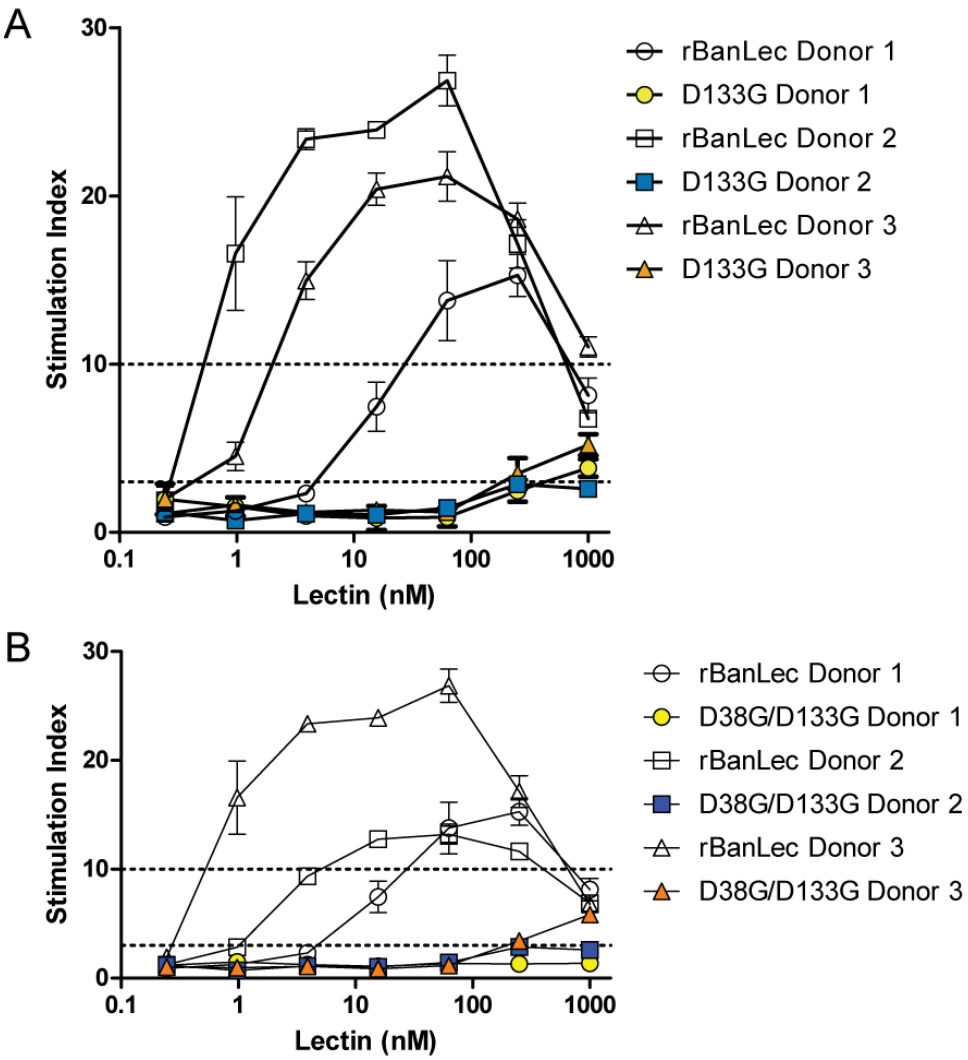

Figure 3-7. Mitogenic activity of BanLec binding site mutants. Peripheral blood lymphocytes were treated with rBanLec, D133G (A), or D38G/D133G (B) for three days prior to the addition to BrdU. The next day, BrdU incorporation was measured by ELISA. Different donors are represented by different shapes, with non-colored shapes representing rBanLec and colored shapes representing the binding site mutants. The samples for each donor were analyzed in triplicate and error bars represent SEM.

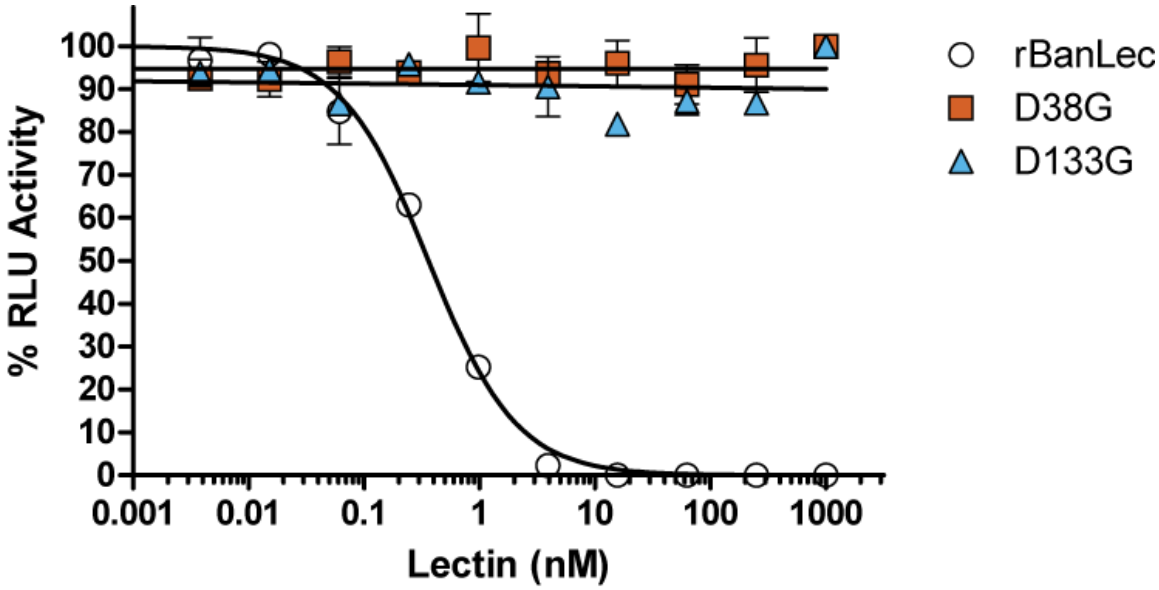

Figure 3-8. Anti-HIV-1 activity of binding site mutants of BanLec. Anti-HIV-1 activity of rBanLec with mutations to the first (D133G) or second (D38G) binding site was compared to wild-type lectin (rBanLec). Lectins were added to TZM-bl cells 30 minutes prior to addition of HIV-1 pseudotyped with a consensus subtype C envelope. Forty-eight hours post infection, HIV-1 infection was assessed by measurement of luciferase activity. Results are representative of two independent experiments. Error bars represent SEM.

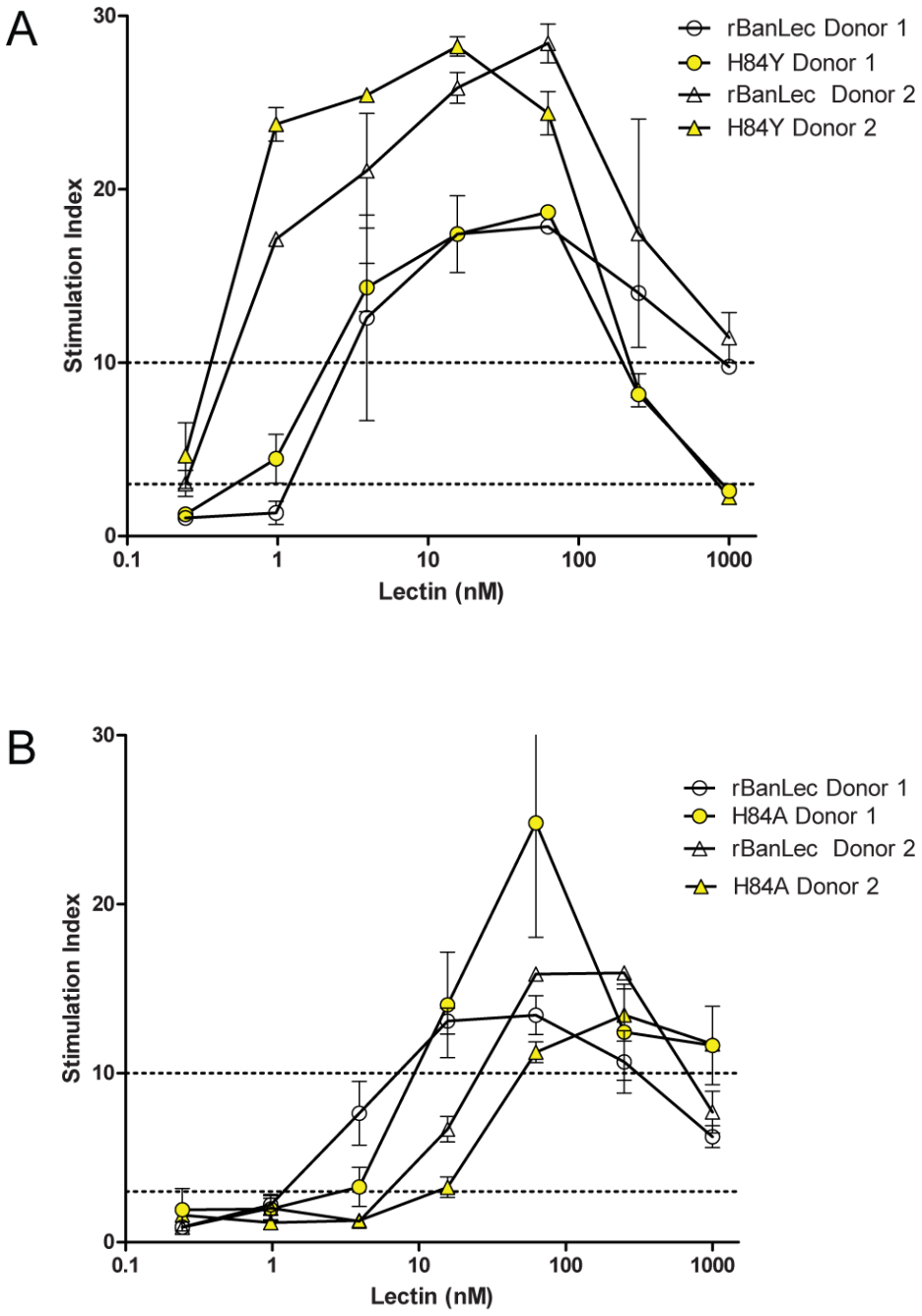

Figure 3-9. Mitogenic activity of H84Y and H84A relative to rBanLec. Peripheral blood lymphocytes were treated with rBanLec, H84Y (A), or H84A (B) for three days prior to the addition to BrdU. The next day, BrdU incorporation was measured by ELISA. Different donors are represented by different shapes, with non-colored shapes representing rBanLec and colored shapes representing the H84 mutants. The samples for each donor were analyzed in triplicate and error bars represent SEM.

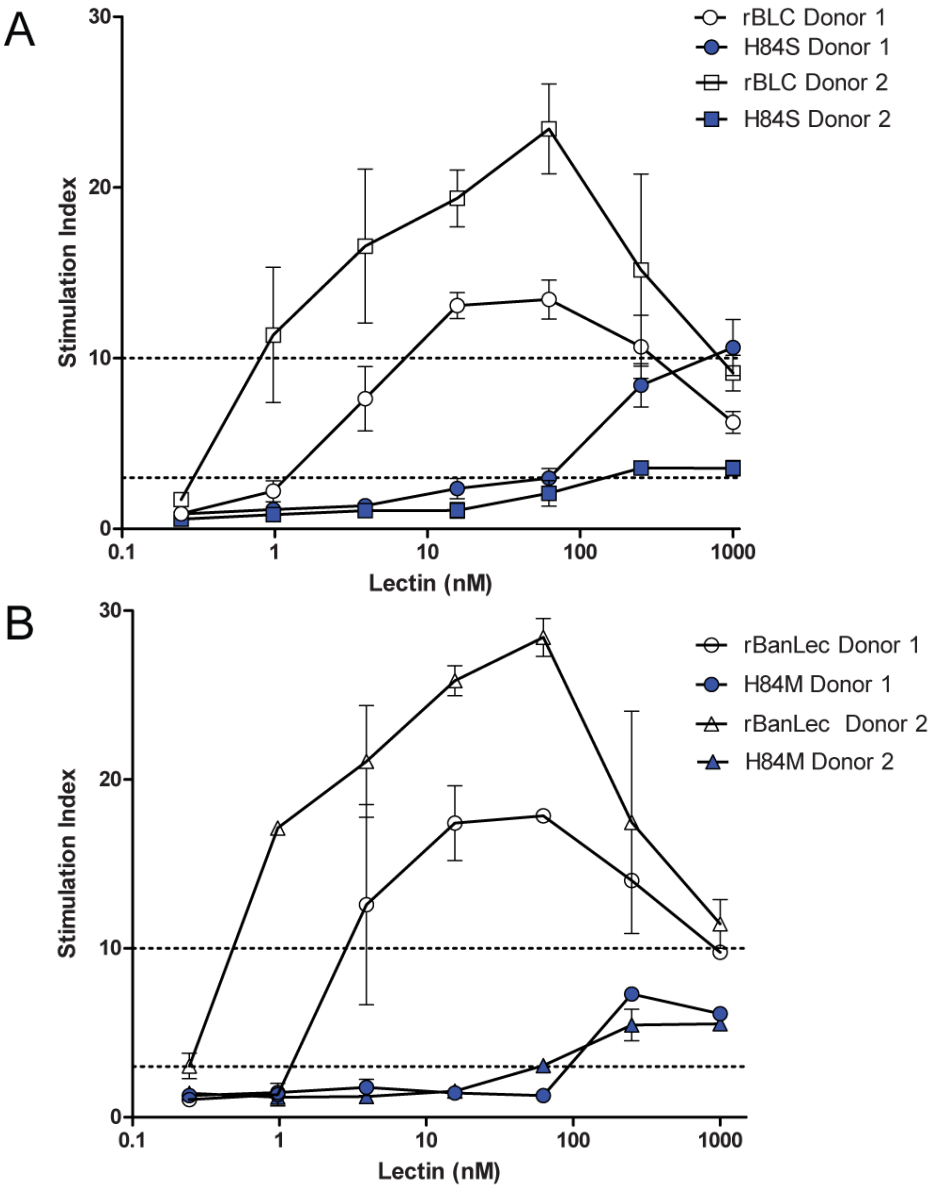

Figure 3-10. Mitogenic activity of H84S and H84M relative to rBanLec. Peripheral blood lymphocytes were treated with rBanLec, H84S (A), or H84M (B) for three days prior to the addition to BrdU. The next day, BrdU incorporation was measured by ELISA. Different donors are represented by different shapes, with non-colored shapes representing rBanLec and colored shapes representing the H84 mutants. The samples for each donor were analyzed in triplicate and error bars represent SEM.

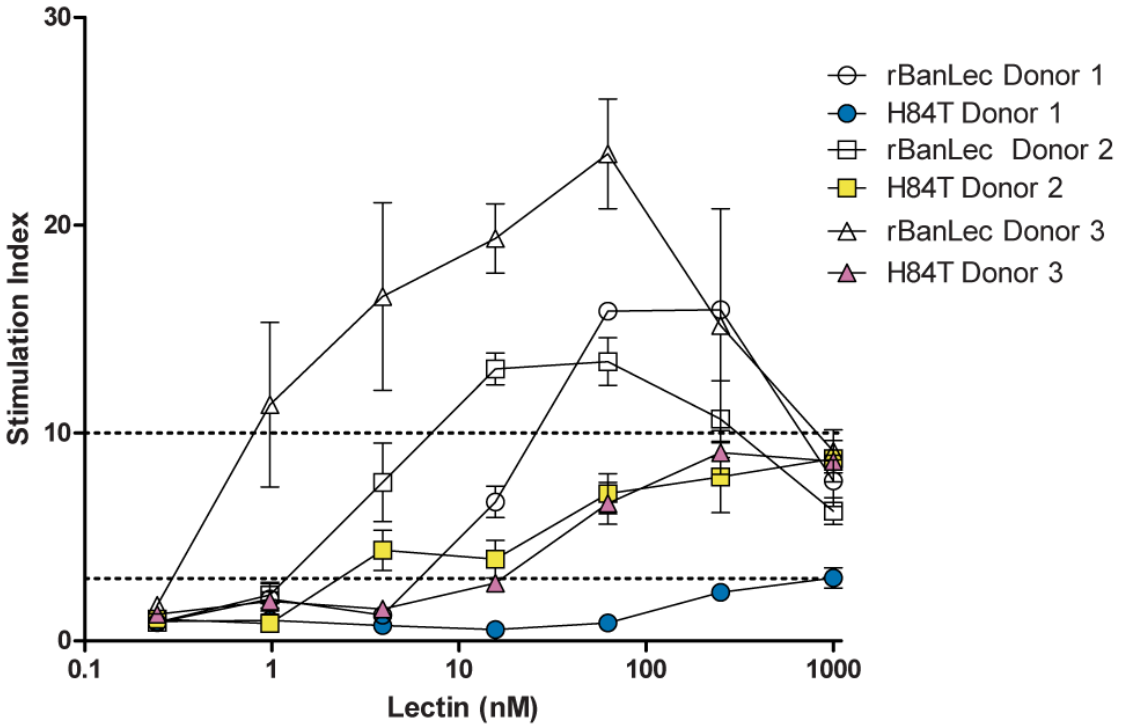

Figure 3-11. Comparison of the mitogenic activity of H84T to rBanLec. Peripheral blood lymphocytes were incubated with different concentrations of rBanLec or the H84T mutant for three day prior to addition of BrdU. The amount of BrdU incorporation by proliferating cells was determined by ELISA. Different donors are represented by different shapes, with non-colored shapes representing rBanLec and colored shapes representing the H84T mutants. The samples for each donor were analyzed in triplicate and error bars represent SEM.

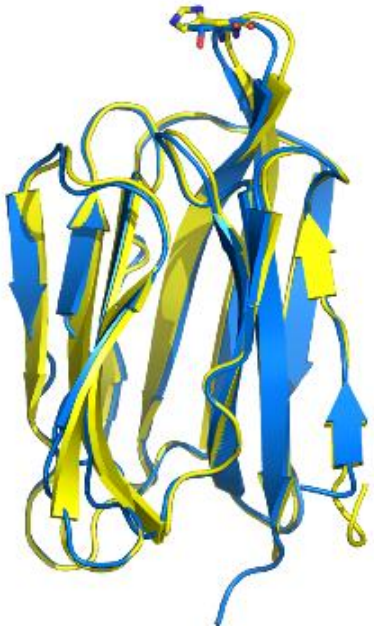

Figure 3-12 Comparison of the three-dimensional structures of BanLec and BanLec H84T. Structure overlays of the BanLec H84T structure (blue) and wild-type BanLec structure (yellow) were performed using Pymol. No significant difference in the two structures was observed. X-ray crystallography was performed by Drs. Jennifer Meagher and Jeanne Stuckey.

Chapter 4: Conclusions

BanLec is a Potent Inhibitor of HIV-1 Replication

We have thoroughly characterized the anti-HIV-1 activity of BanLec *in vitro*. We demonstrated that BanLec is able to inhibit R5-tropic, X4-tropic and R5/X4-tropic HIV-1. Not all entry inhibitors are capable of this, e.g. polyanions inhibit X4 tropic viruses much more than R5 tropic viruses [163]. Anionic polymers and small molecule antagonists of CXCR4 preferentially inhibit X4 viruses over R5, while the R5 viruses are preferentially inhibited by CCR5 antagonists. We also demonstrated that BanLec is able to inhibit HIV-1 isolates from a variety of clades. This is important, since the HIV-1 epidemic is made up of a diverse viral population, and this genetic diversity has presented a great challenge to the development of effective vaccines and neutralizing antibodies against HIV-1.

Mechanisms of Inhibition of HIV Infection by BanLec

Our results strongly suggest that the anti-HIV-1 mechanism of BanLec is mediated by binding to the viral envelope protein gp120 and preventing viral attachment and subsequent entry into the cell. We found that BanLec is able to compete with a monoclonal antibody that recognizes a specific cluster of high-mannose structures for binding to the envelope protein, suggesting that these glycans can be recognized by the lectin. However, it is likely that BanLec is able to bind other high-mannose structures found on the envelope. In a collaborative effort with another laboratory, we are

identifying the sites on gp120 that are targeted by BanLec. HIV-1 will be cultured with increasing concentrations of BanLec, which will promote the selection of mutations that lead to increased BanLec resistance. Molecular cloning of the envelope sequence from the resistant virus will allow us to determine the specific glycosylation sites that are eliminated, thus suggesting that BanLec is able to inhibit replication by binding to these sites. This method has been used with other anti-HIV lectins to identify their N-linked glycosylation target sites, and it appears that different lectins can bind to different sites [164, 165]. We hypothesize that resistance will be due to deletions of functionally important N-linked glycosylation sites, but we should also consider alternative possibilities. If we observe gain of novel glycosylation sites or a change in site locations, these sites could potentially be bound by BanLec without leading to inhibition of HIV-1 replication. Thus, they could act like an antagonist or a decoy for disruption of BanLec-mediated inhibition. In addition, we can evaluate the binding affinity of BanLec for the envelope proteins of the resistant molecular clones we isolate. This will allow us to express the envelope protein and determine whether BanLec can bind to the mutant glycosylated envelope, but not prevent infection.

We have shown that BanLec binds to the HIV-1 gp120 envelope protein to block HIV attachment, thus preventing infection. However, there remains a possibility that BanLec is able to bind to the other component of the HIV-1 envelope complex: protein gp41. It has been shown that CV-N and other lectins can bind gp41 using *in vitro* ELISA based methods [166]. Gp41 contains four PNGS, including one located in the HR2 region that is targeted by the HIV-1 inhibitor T-20 [97]. It has been shown that glycosylation of the

SIV gp41 protein can help shield against neutralizing antibodies and this is likely the case for HIV-1 as well [167]. Since this site is targeted by the HIV-1 fusion inhibiting peptide T-20 and it neighbors a region that is targeted by the neutralizing mAb 2F5, we hypothesize that BanLec would be able to bind to this glycosylation site. One would expect this mode of BanLec inhibition to be less potent, since there are fewer targets compared to the high amount of glycosylation found on the gp120 protein. In addition, it has been suggested that the glycosylation structures at this site are not of the high-mannose variety. Therefore, although we have not eliminated the possibility that BanLec can bind gp41 to block HIV-1 infection, we believe that the majority of BanLec's anti-HIV activity is based on binding to high-mannose glycosylation sites on gp120.

Possible Mechanisms for the Mitogenic Activity of BanLec

The mechanism for lectin-induced mitogenic activity is not clearly understood. It has been shown that mitogenic lectins can bind to multiple cellular surface receptors. This is because lectins can recognize a common glycosylation structure that is present on a variety of proteins. Because BanLec can bind cellular receptors, it is likely that BanLec activates these receptors to trigger downstream signaling events. Activation of cellular signaling could promote proliferation, providing a mechanism for BanLec's mitogenic effects. BanLec is able to stimulate multiple cell types, including CD4+ and CD8+ T and B cells. These cells may have a common receptor that is bound by BanLec or the lectin may bind different receptors for each cell-type [168]. BanLec was previously shown to bind the insulin-like growth factor type two receptor (IGF-IIR) [169]. IGF-IIR has a high-mannose N-linked glycosylation structure, which is likely how it is able to be bound by

BanLec [169]. Also, binding of this receptor can lead to cellular proliferation and it is found on multiple different cell-types, including lymphocytes [170]. Therefore, this receptor may be a ligand for BanLec that mediates mitogenic activity. Pull-down experiments performed with biotinylated BanLec and membrane protein-containing preparations from peripheral blood lymphocytes can be performed to isolate potential receptors for BanLec. The identity of these proteins can be determined through mass-spectrometry or we can determine the presence of predicted receptors through western blotting.

We observed that the BanLec mutants with reduced mitogenic activity also had reduced ability to agglutinate rabbit red blood cells. These results suggest that this mutant has impaired cross-linking ability of glycosylated surface proteins of the rabbit red blood cells. The loss of cross-linking ability for H84T could be due to a change in its carbohydrate recognition properties or ability to bind two or more separate carbohydrate structures. However, the non-mitogenic GNA is capable of agglutinating rabbit red blood cells. Thus, the mitogenic activity of non-BanLec related lectins in this assay may not be correlated with their mitogenic activity.

It has been demonstrated that mannose-binding lectins can recognize different linkages of high-mannose structures. When we compared binding preferences between several non-mitogenic and mitogenic lectins, we found differences in their preference for different mannose linkages. The non-mitogenic lectin GNA has a preference for Man α(1-3) > α(1-6) > α(1-2) & α(1-4) [171]. Another non-mitogenic lectin belonging to the same family,

hippeastrum hybrid agglutinin (HHA), prefers binding of either terminal or internal Man α(1-3)- and α(1-6)-linked branches. However, the mitogenic lectins CV-N and ConA recognize Man α(1-2) structures. BanLec has been shown to bind Man α(1-2), Man α(1-3), and Man α(1-6) disaccharides with relatively the same affinity [92]. However, high mannose glycans containing terminal Man α(1-2) structures are preferred over terminal Man α(1-3) [60].

Based on these observations, the receptors that are responsible for mitogenic activity may contain high-mannose structures, which have terminal Man α(1-2) linkages. The Man α(1-3) structures preferred by GNA and HHA may be less accessible that the Man α(1-2) linkages preferred by the mitogenic lectins. Thus, one potential explanation for the decreased mitogenic activity of the H84T BanLec mutant is that the lectin has altered affinity for different mannose linkages. Future studies are underway to determine if the non-mitogenic BanLec mutant H84T has a decreased affinity for α(1-2) mannose structures or a high affinity for Man α(1-3) or Man α (1-6) linked branches. In the case that we do observe a difference for the H84T mutant, we will compare the structures of the bound mutant and the wild-type proteins by NMR as part of an ongoing collaboration with the laboratory of Dr. Hashim Al-Hashimi. Evaluating the bound forms may allow us to determine the role of the histidine to threonine mutation in carbohydrate recognition.

Future considerations for the use of BanLec as a microbicide

An anti-HIV microbicide should meet all of the following requirements: efficacy, affordability, acceptability, and safety [172]. Here we consider how a BanLec-based

microbicide can fulfill these requirements, and what further experiments and strategies should be considered to promote its continued development.

Efficacy

BanLec's IC_{50} value for anti-HIV-1 activity is in the low nanomolar range, and in our assays, this value compares favorably to two anti-HIV-1 drugs that are currently on the market, T-20 and maraviroc. These compounds are also being investigated for use as a microbicide. Several previous microbicide trials evaluated the use of polyanions. Since these compounds are less effective against R5 viruses, which make up the majority of sexually transmitted HIV-1, this could be an explanation for their lack of efficacy [40, 163]. We demonstrated that BanLec is able to inhibit both R5 and X4 tropic viruses, thus suggesting it may be more efficacious than polyanions. Efficacy in animal models, such as humanized-mice or non-human primates, will need to be demonstrated prior to human trials.

Cost

It is difficult to estimate the cost of a BanLec-based microbicide at this time. It is not known how much lectin would be required for *in vivo* use. Not surprisingly, it has been shown that higher amounts of anti-HIV-1 compounds are needed for *in vivo* assays as compared to *in vitro* assays [42, 133]. Regardless of how much BanLec would be needed, the source of the lectin could not be from bananas itself. However, another plant may be a convenient source. Several groups have proposed that large scale production of lectins may be accomplished. The lectins CV-N and GRFT have been successfully purified from

transgenic tobacco plants, which can express high amounts of these proteins [65, 173]. Another possible method of production of BanLec is by genetically modified lactobacillus. These microbes can be engineered to secrete lectins and then be used to colonize the vagina, where they would continuously produce the anti-viral compound. One caveat of this method is that it would be not applicable for use as a rectal microbicide.

We have shown that BanLec can be expressed and purified from *E. coli*. With relatively little optimization, we and others have been able to obtain approximately 50 mg of BanLec per liter of culture. Further optimization and the effect of economies of scale could improve protein yield and purification costs. In our experiments, recombinant protein was purified using immobilized metal affinity chromatography, and therapeutic use would require the removal of the 6 x His tag. However, we have successfully purified an untagged version of BanLec from *E. coli* (data not shown). In addition, other purification methods such as ion exchange chromatography, could be developed for isolation of BanLec from *E. coli* or from tobacco. These multiple options for production could very well result in a cost-effective production method for a BanLec-based microbicide.

Safety

The safety of the wild-type BanLec is a concern due to its mitogenic activity as demonstrated by ourselves and others. To circumvent this potential obstacle in development, we created a mutant form of BanLec that has reduced mitogenic activity

yet maintains potent anti-HIV activity, H84T. Future *in vitro* experiments using cells and cell lines along with *in vivo* experiments can be performed to assess the safety of this BanLec variant. Although *in vitro* models are inexpensive, there is a lack of knowledge regarding the *in vivo* relevance of experiments performed *in vitro*. For example, the standard assay for mitogenic activity of lectins involves exposure of cells to the lectin for over three days, while the concentration of a vaginally-applied lectin should decrease over time. Such discrepancies need to be addressed, and therefore it would be valuable to conduct thorough experiments evaluating microbicides *in vivo* for comparison to available *in vitro* methods. The microbicide development field would greatly benefit from a standardized screening algorithm to reduce false positives and negatives.

Although we have developed a non-mitogenic form of BanLec, it may still cause pro-inflammatory cytokine production *in vivo*. The mitogenic lectin CV-N has been found to cause an increase in several inflammatory cytokines [130]. HIV-1 replication can be influenced by cytokines and chemokines through signal transduction. This signaling cascade results in the activation of enhancers that can further activate the HIV-1 LTR. In addition, these signals may recruit activated CD4+ T cells and thus lead to more infected cells. Therefore, the ability of BanLec and the H84T mutant to induce pro-inflammatory cytokine and chemokine production should be evaluated. Several different cell types relevant to microbicides, including lymphocytes, macrophages, epithelial cells, or perhaps cervical tissue explants, should be evaluated for cytokine or chemokine production upon exposure to lectin.

One concern for protein based therapeutics is that the recipient may stimulate an immune response to the product. It has been demonstrated that people have IgG4 antibodies against BanLec [174]. Encouragingly, IgG4 antibodies stand out from other subclasses as having anti-inflammatory activity. These antibodies do not efficiently induce complement or cell activation. In addition, it has been shown that IgG4 antibodies can exchange Fab (fragment, antigen binding) arms with a different IgG4 antibody. The resulting antibodies are bispecific, and should be incapable of cross-linking and forming immune complexes. In fact, they may actually inhibit cross-linking and complex formation caused by non-IgG4 antibodies specific for the same antigen. IgG4 antibodies are produced in response to chronic exposure to an antigen and may be a mechanism for tolerance to an antigen [175]. Thus, IgG4 specific antibodies could potentially increase the tolerability and effectiveness of BanLec based anti-HIV therapeutics.

Taken together, the mitogenic effects of BanLec pose a potential safety problem. However, our H84T mutant with markedly reduced mitogenicity may be safe for use, and the presence of BanLec-specific IgG4 antibodies in humans suggests BanLec could be well tolerated *in vivo*. Although we can hypothesize the possible safety issues with BanLec, a better understanding of its toxicity or tolerability can only be determined with future animal studies.

Acceptance

The acceptance of a microbicide can be hard for bench scientists to understand. A microbicide that is highly effective when used correctly may not be accepted and used

100% of the time, thereby reducing its efficacy. Although condoms are highly effective at preventing HIV-1 transmission, they are not always used [31, 32]. One reason for not using condoms is that men and women find sexual intercourse more pleasurable without a condom than with one [76]. This could also be a limiting factor for microbicides.

An alternative to using a vaginally applied microbicide is the use of orally administered anti-virals as post- or pre-exposure prophylaxis therapy. Oral administration of the HIV-1 entry inhibitors were shown to protect rhesus macaques in a vaginal challenge model [42]. In addition, antiretrovirals pre-exposure prophylaxis has been effective in preventing vaginal transmission in humanized mouse models of HIV-1 infection [176]. It is unclear if orally administered BanLec would have anti-HIV activity. Other orally administered lectins have been found in the serum of mice and appear to retain their carbohydrate binding activity [177]. Oral administration of BanLec has been performed in mouse models without deleterious effects [71]. Thus, it may be possible to use humanized mouse models of HIV-1 transmission to determine the effectiveness of BanLec-mediated inhibition of HIV-1 replication through different routes of administration.

One disadvantage of oral delivery is that it would likely require higher amounts of lectin compared to a vaginally administered form, and it would likely require daily use. Some people have questioned whether consuming bananas could prevent HIV-1 infection. This seems unlikely given since a large amount of bananas would likely be needed to receive sufficient amounts of BanLec, and that most of the lectin in bananas is bound to starches

and may not be effective in that form. Therefore, bananas would not be a good source for oral administration of BanLec.

Summary

We think that the data presented not only describes the properties and mechanism of a new anti-HIV-1 lectin, but identifies a reagent that may lead to the development of an effective anti-HIV microbicide. In addition, our work opens a door that will shed new light on the relatively unknown mechanism of the mitogenic activity of lectins. If we are able to determine a particular carbohydrate structure associated with mitogenic activity and determine if there is a requirement for cross-linking, less expensive and higher-throughput assays can be used for screening the potential mitogenic activity of lectins being investigated for therapeutic potential.

References

1. Gao, F., et al., *Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes.* Nature, 1999. **397**(6718): p. 436-41.
2. Zhu, T., et al., *An African HIV-1 sequence from 1959 and implications for the origin of the epidemic.* Nature, 1998. **391**(6667): p. 594-7.
3. Worobey, M., et al., *Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960.* Nature, 2008. **455**(7213): p. 661-4.
4. Joint United Nations Programme on HIV/AIDS. and World Health Organization., *AIDS epidemic updated : November 2009*. 2009, UNAIDS: Geneva, Switzerland.
5. Taylor, B.S., et al., *The challenge of HIV-1 subtype diversity.* N Engl J Med, 2008. **358**(15): p. 1590-602.
6. Preston, B.D., B.J. Poiesz, and L.A. Loeb, *Fidelity of HIV-1 reverse transcriptase.* Science, 1988. **242**(4882): p. 1168-71.
7. Roberts, J.D., K. Bebenek, and T.A. Kunkel, *The accuracy of reverse transcriptase from HIV-1.* Science, 1988. **242**(4882): p. 1171-3.
8. Korber, B., et al., *Evolutionary and immunological implications of contemporary HIV-1 variation.* Br Med Bull, 2001. **58**: p. 19-42.
9. Robertson, D.L., et al., *HIV-1 nomenclature proposal.* Science, 2000. **288**(5463): p. 55-6.
10. Buonaguro, L., M.L. Tornesello, and F.M. Buonaguro, *Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications.* J Virol, 2007. **81**(19): p. 10209-19.
11. Arthos, J., et al., *HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells.* Nat Immunol, 2008. **9**(3): p. 301-9.
12. Geijtenbeek, T.B., et al., *DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells.* Cell, 2000. **100**(5): p. 587-97.
13. Bobardt, M.D., et al., *Syndecan captures, protects, and transmits HIV to T lymphocytes.* Immunity, 2003. **18**(1): p. 27-39.
14. Kwong, P.D., et al., *Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody.* Nature, 1998. **393**(6686): p. 648-59.
15. Rizzuto, C.D., et al., *A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding.* Science, 1998. **280**(5371): p. 1949-53.
16. Yi, Y., et al., *An unusual syncytia-inducing human immunodeficiency virus type 1 primary isolate from the central nervous system that is restricted to CXCR4, replicates efficiently in macrophages, and induces neuronal apoptosis.* J Neurovirol, 2003. **9**(4): p. 432-41.
17. Yi, Y., et al., *Role of CXCR4 in cell-cell fusion and infection of monocyte-derived macrophages by primary human immunodeficiency virus type 1 (HIV-1) strains: two distinct mechanisms of HIV-1 dual tropism.* J Virol, 1999. **73**(9): p. 7117-25.
18. Salazar-Gonzalez, J.F., et al., *Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection.* J Exp Med, 2009. **206**(6): p. 1273-89.

19. Liu, R., et al., *Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection.* Cell, 1996. **86**(3): p. 367-77.
20. Samson, M., et al., *Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene.* Nature, 1996. **382**(6593): p. 722-5.
21. Hutter, G., et al., *Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation.* N Engl J Med, 2009. **360**(7): p. 692-8.
22. Doms, R.W. and J.P. Moore, *HIV-1 membrane fusion: targets of opportunity.* J Cell Biol, 2000. **151**(2): p. F9-14.
23. Haase, A.T., *Perils at mucosal front lines for HIV and SIV and their hosts.* Nat Rev Immunol, 2005. **5**(10): p. 783-92.
24. Li, Q., et al., *Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells.* Nature, 2005. **434**(7037): p. 1148-52.
25. Brenchley, J.M., et al., *CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract.* J Exp Med, 2004. **200**(6): p. 749-59.
26. Brenchley, J.M., D.A. Price, and D.C. Douek, *HIV disease: fallout from a mucosal catastrophe?* Nat Immunol, 2006. **7**(3): p. 235-9.
27. Silvestri, G., et al., *Understanding the benign nature of SIV infection in natural hosts.* J Clin Invest, 2007. **117**(11): p. 3148-54.
28. Tsibris, A.M. and M.S. Hirsch, *Antiretroviral therapy in the clinic.* J Virol, 2010. **84**(11): p. 5458-64.
29. Powles, T., et al., *Highly active antiretroviral therapy and the incidence of non-AIDS-defining cancers in people with HIV infection.* J Clin Oncol, 2009. **27**(6): p. 884-90.
30. Rerks-Ngarm, S., et al., *Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand.* N Engl J Med, 2009. **361**(23): p. 2209-20.
31. Saracco, A., et al., *Man-to-woman sexual transmission of HIV: longitudinal study of 343 steady partners of infected men.* J Acquir Immune Defic Syndr, 1993. **6**(5): p. 497-502.
32. de Vincenzi, I., *A longitudinal study of human immunodeficiency virus transmission by heterosexual partners. European Study Group on Heterosexual Transmission of HIV.* N Engl J Med, 1994. **331**(6): p. 341-6.
33. Brito, M.O., et al., *Acceptability of male circumcision for the prevention of HIV/AIDS in the Dominican Republic.* PLoS One, 2009. **4**(11): p. e7687.
34. Pool, R., et al., *Men's attitudes to condoms and female controlled means of protection against HIV and STDs in south-western Uganda.* Cult Health Sex, 2000. **2**(2): p. 197-211.
35. Joint United Nations Programme on HIV/AIDS. and World Health Organization., *Report on the global AIDS epidemic 2008*. 2008, UNAIDS: Geneva, Switzerland.
36. Stone, A., *Microbicides: a new approach to preventing HIV and other sexually transmitted infections.* Nat Rev Drug Discov, 2002. **1**(12): p. 977-85.
37. Hillier, S.L., et al., *In vitro and in vivo: the story of nonoxynol 9.* J Acquir Immune Defic Syndr, 2005. **39**(1): p. 1-8.

38. Feldblum, P.J., et al., *SAVVY vaginal gel (C31G) for prevention of HIV infection: a randomized controlled trial in Nigeria.* PLoS One, 2008. **3**(1): p. e1474.
39. Mesquita, P.M., et al., *Disruption of tight junctions by cellulose sulfate facilitates HIV infection: model of microbicide safety.* J Infect Dis, 2009. **200**(4): p. 599-608.
40. Tao, W., C. Richards, and D. Hamer, *Enhancement of HIV infection by cellulose sulfate.* AIDS Res Hum Retroviruses, 2008. **24**(7): p. 925-9.
41. McCarthy, T.D., et al., *Dendrimers as drugs: discovery and preclinical and clinical development of dendrimer-based microbicides for HIV and STI prevention.* Mol Pharm, 2005. **2**(4): p. 312-8.
42. Veazey, R.S., et al., *Protection of macaques from vaginal SHIV challenge by vaginally delivered inhibitors of virus-cell fusion.* Nature, 2005. **438**(7064): p. 99-102.
43. Varki, A., *Essentials of glycobiology*. 2nd ed. 2009, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. xxix, 784 p.
44. Jones, J., S.S. Krag, and M.J. Betenbaugh, *Controlling N-linked glycan site occupancy.* Biochim Biophys Acta, 2005. **1726**(2): p. 121-37.
45. Jenkins, N., R.B. Parekh, and D.C. James, *Getting the glycosylation right: implications for the biotechnology industry.* Nat Biotechnol, 1996. **14**(8): p. 975-81.
46. Decroly, E., et al., *The convertases furin and PC1 can both cleave the human immunodeficiency virus (HIV)-1 envelope glycoprotein gp160 into gp120 (HIV-1 SU) and gp41 (HIV-I TM).* J Biol Chem, 1994. **269**(16): p. 12240-7.
47. Zhu, P., et al., *Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15812-7.
48. Leonard, C.K., et al., *Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells.* J Biol Chem, 1990. **265**(18): p. 10373-82.
49. Starcich, B.R., et al., *Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS.* Cell, 1986. **45**(5): p. 637-48.
50. Lane, B.R., et al., *Human immunodeficiency virus type 1 (HIV-1)-induced GRO-alpha production stimulates HIV-1 replication in macrophages and T lymphocytes.* J Virol, 2001. **75**(13): p. 5812-22.
51. Trushin, S.A., et al., *Glycoprotein 120 binding to CXCR4 causes p38-dependent primary T cell death that is facilitated by, but does not require cell-associated CD4.* J Immunol, 2007. **178**(8): p. 4846-53.
52. Hong, P.W., et al., *Identification of the optimal DC-SIGN binding site on human immunodeficiency virus type 1 gp120.* J Virol, 2007. **81**(15): p. 8325-36.
53. Zhang, M., et al., *Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin.* Glycobiology, 2004. **14**(12): p. 1229-46.
54. Sanders, R.W., et al., *The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120.* J Virol, 2002. **76**(14): p. 7293-305.

55. Raval, S., et al., *A database analysis of jacalin-like lectins: sequence-structure-function relationships.* Glycobiology, 2004. **14**(12): p. 1247-63.
56. Van Damme, E.J., et al., *Cytoplasmic/nuclear plant lectins: a new story.* Trends Plant Sci, 2004. **9**(10): p. 484-9.
57. Shinohara, Y., et al., *Elucidation of the mechanism enhancing the avidity of lectin with oligosaccharides on the solid phase surface.* Glycobiology, 1997. **7**(8): p. 1201-8.
58. Yang, H. and T.H. Czapla, *Isolation and characterization of cDNA clones encoding jacalin isolectins.* J Biol Chem, 1993. **268**(8): p. 5905-10.
59. Jeyaprakash, A.A., et al., *Structural basis for the carbohydrate specificities of artocarpin: variation in the length of a loop as a strategy for generating ligand specificity.* J Mol Biol, 2004. **338**(4): p. 757-70.
60. Nakamura-Tsuruta, S., et al., *Analysis of the sugar-binding specificity of mannose-binding-type Jacalin-related lectins by frontal affinity chromatography--an approach to functional classification.* Febs J, 2008. **275**(6): p. 1227-39.
61. Favero, J., et al., *Inhibition of human immunodeficiency virus infection by the lectin jacalin and by a derived peptide showing a sequence similarity with gp120.* Eur J Immunol, 1993. **23**(1): p. 179-85.
62. Mori, T., et al., *Isolation and characterization of griffithsin, a novel HIV-inactivating protein, from the red alga Griffithsia sp.* J Biol Chem, 2005. **280**(10): p. 9345-53.
63. Ziolkowska, N.E., et al., *Domain-swapped structure of the potent antiviral protein griffithsin and its mode of carbohydrate binding.* Structure, 2006. **14**(7): p. 1127-35.
64. Emau, P., et al., *Griffithsin, a potent HIV entry inhibitor, is an excellent candidate for anti-HIV microbicide.* J Med Primatol, 2007. **36**(4-5): p. 244-53.
65. O'Keefe, B.R., et al., *Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component.* Proc Natl Acad Sci U S A, 2009. **106**(15): p. 6099-104.
66. Koshte, V.L., et al., *Isolation and characterization of BanLec-I, a mannoside-binding lectin from Musa paradisiac (banana).* Biochem J, 1990. **272**(3): p. 721-6.
67. Peumans, W.J., et al., *Fruit-specific lectins from banana and plantain.* Planta, 2000. **211**(4): p. 546-54.
68. Meagher, J.L., et al., *Crystal structure of banana lectin reveals a novel second sugar binding site.* Glycobiology, 2005. **15**(10): p. 1033-42.
69. Gavrovic-Jankulovic, M., et al., *A novel recombinantly produced banana lectin isoform is a valuable tool for glycoproteomics and a potent modulator of the proliferation response in CD3+, CD4+, and CD8+ populations of human PBMCs.* Int J Biochem Cell Biol, 2008. **40**(5): p. 929-41.
70. Stojanovic, M.M., et al., *In vitro stimulation of Balb/c and C57 BL/6 splenocytes by a recombinantly produced banana lectin isoform results in both a proliferation of T cells and an increased secretion of interferon-gamma.* Int Immunopharmacol, 2010. **10**(1): p. 120-9.

71. Hinge, A., et al., *Oral administration of insulin receptor-interacting lectins leads to an enhancement in the hematopoietic stem and progenitor cell pool of mice.* Stem Cells Dev, 2010. **19**(2): p. 163-74.
72. Tilton, J.C. and R.W. Doms, *Entry inhibitors in the treatment of HIV-1 infection.* Antiviral Res, 2010. **85**(1): p. 91-100.
73. Sharma, A., et al., *Multiplicity of carbohydrate-binding sites in beta-prism fold lectins: occurrence and possible evolutionary implications.* J Biosci, 2007. **32**(6): p. 1089-110.
74. *Report on the global AIDS epidemic - Executive Summary* 2008, United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO)
75. Underhill, K., P. Montgomery, and D. Operario, *Sexual abstinence only programmes to prevent HIV infection in high income countries: systematic review.* Bmj, 2007. **335**(7613): p. 248.
76. Randolph, M.E., et al., *Sexual pleasure and condom use.* Arch Sex Behav, 2007. **36**(6): p. 844-8.
77. Crosby, R., et al., *Condom discomfort and associated problems with their use among university students.* J Am Coll Health, 2005. **54**(3): p. 143-7.
78. Gupta, G.R., *How men's power over women fuels the HIV epidemic.* Bmj, 2002. **324**(7331): p. 183-4.
79. Watts, C. *The public health impact of microbicides: model projections*. in *Microbicides*. 2002. Antwerp, Belgium.
80. Balzarini, J. and L. Van Damme, *Microbicide drug candidates to prevent HIV infection.* Lancet, 2007. **369**(9563): p. 787-97.
81. Balzarini, J., *Targeting the glycans of gp120: a novel approach aimed at the Achilles heel of HIV.* Lancet Infect Dis, 2005. **5**(11): p. 726-31.
82. Boyd, M.R., et al., *Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development.* Antimicrob Agents Chemother, 1997. **41**(7): p. 1521-30.
83. Balzarini, J., *Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy.* Nat Rev Microbiol, 2007. **5**(8): p. 583-97.
84. Balzarini, J., et al., *Alpha-(1-3)- and alpha-(1-6)-D-mannose-specific plant lectins are markedly inhibitory to human immunodeficiency virus and cytomegalovirus infections in vitro.* Antimicrob Agents Chemother, 1991. **35**(3): p. 410-6.
85. Balzarini, J., et al., *Mannose-specific plant lectins from the Amaryllidaceae family qualify as efficient microbicides for prevention of human immunodeficiency virus infection.* Antimicrob Agents Chemother, 2004. **48**(10): p. 3858-70.
86. Matthews, T.J., et al., *Interaction between the human T-cell lymphotropic virus type IIIB envelope glycoprotein gp120 and the surface antigen CD4: role of carbohydrate in binding and cell fusion.* Proc Natl Acad Sci U S A, 1987. **84**(15): p. 5424-8.
87. Geyer, H., et al., *Carbohydrates of human immunodeficiency virus. Structures of oligosaccharides linked to the envelope glycoprotein 120.* J Biol Chem, 1988. **263**(24): p. 11760-7.

88. Allan, J.S., et al., *Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III.* Science, 1985. **228**(4703): p. 1091-4.
89. Li, Y., et al., *Glycosylation is necessary for the correct folding of human immunodeficiency virus gp120 in CD4 binding.* J Virol, 1993. **67**(1): p. 584-8.
90. Clevestig, P., et al., *CCR5 use by human immunodeficiency virus type 1 is associated closely with the gp120 V3 loop N-linked glycosylation site.* J Gen Virol, 2006. **87**(Pt 3): p. 607-12.
91. Back, N.K., et al., *An N-glycan within the human immunodeficiency virus type 1 gp120 V3 loop affects virus neutralization.* Virology, 1994. **199**(2): p. 431-8.
92. Mo, H., et al., *Carbohydrate binding properties of banana (Musa acuminata) lectin I. Novel recognition of internal alpha1,3-linked glucosyl residues.* Eur J Biochem, 2001. **268**(9): p. 2609-15.
93. Singh, D.D., et al., *Unusual sugar specificity of banana lectin from Musa paradisiaca and its probable evolutionary origin. Crystallographic and modelling studies.* Glycobiology, 2005. **15**(10): p. 1025-32.
94. Vandamme, E.J.M., A.K. Allen, and W.J. Peumans, *Isolation and Characterization of a Lectin with Exclusive Specificity Towards Mannose from Snowdrop (Galanthus-Nivalis) Bulbs.* Febs Letters, 1987. **215**(1): p. 140-144.
95. Gartner, S., et al., *The role of mononuclear phagocytes in HTLV-III/LAV infection.* Science, 1986. **233**(4760): p. 215-9.
96. Li, M., et al., *Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies.* J Virol, 2005. **79**(16): p. 10108-25.
97. Wei, X., et al., *Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy.* Antimicrob Agents Chemother, 2002. **46**(6): p. 1896-905.
98. Adachi, A., et al., *Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone.* J Virol, 1986. **59**(2): p. 284-91.
99. Freed, E.O., G. Englund, and M.A. Martin, *Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection.* J Virol, 1995. **69**(6): p. 3949-54.
100. Toohey, K., et al., *Human immunodeficiency virus envelope V1 and V2 regions influence replication efficiency in macrophages by affecting virus spread.* Virology, 1995. **213**(1): p. 70-9.
101. Chesebro, B., et al., *Identification of human immunodeficiency virus envelope gene sequences influencing viral entry into CD4-positive HeLa cells, T-leukemia cells, and macrophages.* J Virol, 1991. **65**(11): p. 5782-9.
102. Chesebro, B., et al., *Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell-tropic isolates: definition of critical amino acids involved in cell tropism.* J Virol, 1992. **66**(11): p. 6547-54.
103. Collman, R., et al., *An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1.* J Virol, 1992. **66**(12): p. 7517-21.

104. Derdeyn, C.A., et al., *Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120.* J Virol, 2000. **74**(18): p. 8358-67.
105. Platt, E.J., et al., *Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1.* J Virol, 1998. **72**(4): p. 2855-64.
106. Takeuchi, Y., M.O. McClure, and M. Pizzato, *Identification of gammaretroviruses constitutively released from cell lines used for human immunodeficiency virus research.* J Virol, 2008. **82**(24): p. 12585-8.
107. Chesebro, B., et al., *Use of a new CD4-positive HeLa cell clone for direct quantitation of infectious human immunodeficiency virus from blood cells of AIDS patients.* J Infect Dis, 1991. **163**(1): p. 64-70.
108. Chackerian, B., et al., *Human immunodeficiency virus type 1 coreceptors participate in postentry stages in the virus replication cycle and function in simian immunodeficiency virus infection.* J Virol, 1997. **71**(5): p. 3932-9.
109. O'Doherty, U., W.J. Swiggard, and M.H. Malim, *Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding.* J Virol, 2000. **74**(21): p. 10074-80.
110. Schmidtmayerova, H., et al., *Human immunodeficiency virus type 1 T-lymphotropic strains enter macrophages via a CD4- and CXCR4-mediated pathway: replication is restricted at a postentry level.* J Virol, 1998. **72**(6): p. 4633-42.
111. Kothe, D.L., et al., *Ancestral and consensus envelope immunogens for HIV-1 subtype C.* Virology, 2006. **352**(2): p. 438-49.
112. Gray, E.S., et al., *N-linked glycan modifications in gp120 of human immunodeficiency virus type 1 subtype C render partial sensitivity to 2G12 antibody neutralization.* J Virol, 2007. **81**(19): p. 10769-76.
113. Shen, R., et al., *Macrophages in vaginal but not intestinal mucosa are monocyte-like and permissive to human immunodeficiency virus type 1 infection.* J Virol, 2009. **83**(7): p. 3258-67.
114. Li, B.Q., et al., *Flavonoid baicalin inhibits HIV-1 infection at the level of viral entry.* Biochem Biophys Res Commun, 2000. **276**(2): p. 534-8.
115. Warrilow, D., D. Stenzel, and D. Harrich, *Isolated HIV-1 core is active for reverse transcription.* Retrovirology, 2007. **4**: p. 77.
116. Warrilow, D., et al., *Cell factors stimulate human immunodeficiency virus type 1 reverse transcription in vitro.* J Virol, 2008. **82**(3): p. 1425-37.
117. Arhel, N.J., et al., *HIV-1 DNA Flap formation promotes uncoating of the pre-integration complex at the nuclear pore.* Embo J, 2007. **26**(12): p. 3025-37.
118. Trkola, A., et al., *Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1.* J Virol, 1996. **70**(2): p. 1100-8.
119. Scanlan, C.N., et al., *The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of alpha1-->2 mannose residues on the outer face of gp120.* J Virol, 2002. **76**(14): p. 7306-21.

120. Mkrtchyan, S.R., et al., *Ternary complex formation of human immunodeficiency virus type 1 Env, CD4, and chemokine receptor captured as an intermediate of membrane fusion.* J Virol, 2005. **79**(17): p. 11161-9.
121. Barrientos, L.G., et al., *Cyanovirin-N binds to the viral surface glycoprotein, GP1,2 and inhibits infectivity of Ebola virus.* Antiviral Res, 2003. **58**(1): p. 47-56.
122. Barrientos, L.G., et al., *In vitro evaluation of cyanovirin-N antiviral activity, by use of lentiviral vectors pseudotyped with filovirus envelope glycoproteins.* J Infect Dis, 2004. **189**(8): p. 1440-3.
123. Smee, D.F., et al., *Treatment of influenza A (H1N1) virus infections in mice and ferrets with cyanovirin-N.* Antiviral Res, 2008. **80**(3): p. 266-71.
124. Keyaerts, E., et al., *Plant lectins are potent inhibitors of coronaviruses by interfering with two targets in the viral replication cycle.* Antiviral Res, 2007. **75**(3): p. 179-87.
125. Helle, F., et al., *Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans.* J Biol Chem, 2006. **281**(35): p. 25177-83.
126. Bertaux, C., et al., *Entry of hepatitis C virus and human immunodeficiency virus is selectively inhibited by carbohydrate-binding agents but not by polyanions.* Virology, 2007. **366**(1): p. 40-50.
127. Witvrouw, M., et al., *Resistance of human immunodeficiency virus type 1 to the high-mannose binding agents cyanovirin N and concanavalin A.* J Virol, 2005. **79**(12): p. 7777-84.
128. Wei, X., et al., *Antibody neutralization and escape by HIV-1.* Nature, 2003. **422**(6929): p. 307-12.
129. Lis, H. and N. Sharon, *Lectins*. 2 ed. 2003: Kluwer Acadmeic Publishers.
130. Huskens, D., et al., *Safety concerns for the potential use of cyanovirin-N as a microbicidal anti-HIV agent.* Int J Biochem Cell Biol, 2008. **40**(12): p. 2802-14.
131. Saidi, H., et al., *Differential in vitro inhibitory activity against HIV-1 of alpha-(1-3)- and alpha-(1-6)-D-mannose specific plant lectins: implication for microbicide development.* J Transl Med, 2007. **5**: p. 28.
132. Tsai, C.C., et al., *Cyanovirin-N gel as a topical microbicide prevents rectal transmission of SHIV89.6P in macaques.* AIDS Res Hum Retroviruses, 2003. **19**(7): p. 535-41.
133. Tsai, C.C., et al., *Cyanovirin-N inhibits AIDS virus infections in vaginal transmission models.* AIDS Res Hum Retroviruses, 2004. **20**(1): p. 11-8.
134. Buffa, V., et al., *Cyanovirin-N potently inhibits human immunodeficiency virus type 1 infection in cellular and cervical explant models.* J Gen Virol, 2009. **90**(Pt 1): p. 234-43.
135. Zappe, H., M.E. Snell, and M.J. Bossard, *PEGylation of cyanovirin-N, an entry inhibitor of HIV.* Adv Drug Deliv Rev, 2008. **60**(1): p. 79-87.
136. Quinn, T.C. and J. Overbaugh, *HIV/AIDS in women: an expanding epidemic.* Science, 2005. **308**(5728): p. 1582-3.
137. Weir, S.S., et al., *Nonoxynol-9 use, genital ulcers, and HIV infection in a cohort of sex workers.* Genitourin Med, 1995. **71**(2): p. 78-81.
138. Grant, R.M., et al., *Whither or wither microbicides?* Science, 2008. **321**(5888): p. 532-4.

139. Rohan, L.C., et al., *In vitro and ex vivo testing of tenofovir shows it is effective as an HIV-1 microbicide.* PLoS One, 2010. **5**(2): p. e9310.
140. Lodwick, R., et al., *Triple-class virologic failure in HIV-infected patients undergoing antiretroviral therapy for up to 10 years.* Arch Intern Med, 2010. **170**(5): p. 410-9.
141. Tanaka, H., et al., *Mechanism by which the lectin actinohivin blocks HIV infection of target cells.* Proc Natl Acad Sci U S A, 2009. **106**(37): p. 15633-8.
142. Esser, M.T., et al., *Cyanovirin-N binds to gp120 to interfere with CD4-dependent human immunodeficiency virus type 1 virion binding, fusion, and infectivity but does not affect the CD4 binding site on gp120 or soluble CD4-induced conformational changes in gp120.* J Virol, 1999. **73**(5): p. 4360-71.
143. Swanson, M.D., et al., *A lectin isolated from bananas is a potent inhibitor of HIV replication.* J Biol Chem, 2010. **285**(12): p. 8646-55.
144. Nowell, P.C., *Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes.* Cancer Res, 1960. **20**: p. 462-6.
145. Powell, A.E. and M.A. Leon, *Reversible interaction of human lymphocytes with the mitogen concanavalin A.* Exp Cell Res, 1970. **62**(2): p. 315-25.
146. Shier, W.T., J.T. Trotter, 3rd, and C.L. Reading, *Inflammation induced by concanavalin A and other lectins.* Proc Soc Exp Biol Med, 1974. **146**(2): p. 590-3.
147. Adam, J., et al., *Engineering of PA-IIL lectin from Pseudomonas aeruginosa - Unravelling the role of the specificity loop for sugar preference.* BMC Struct Biol, 2007. **7**: p. 36.
148. Van Damme, E.J.M., A.K. Allen, and W.J. Peumans, *Isolation and Characterization of a Lectin with Exclusive Specificity Towards Mannose from Snowdrop (Galanthus-Nivalis) Bulbs.* FEBS Letters, 1987. **215**(1): p. 140-144.
149. Winter, H.C., K. Mostafapour, and I.J. Goldstein, *The mushroom Marasmius oreades lectin is a blood group type B agglutinin that recognizes the Galalpha 1,3Gal and Galalpha 1,3Galbeta 1,4GlcNAc porcine xenotransplantation epitopes with high affinity.* J Biol Chem, 2002. **277**(17): p. 14996-5001.
150. Alexandre, K.B., et al., *Mannose-rich glycosylation patterns on HIV-1 subtype C gp120 and sensitivity to the lectins, Griffithsin, Cyanovirin-N and Scytovirin.* Virology, 2010.
151. Benoist, H., et al., *Two structurally identical mannose-specific jacalin-related lectins display different effects on human T lymphocyte activation and cell death.* J Leukoc Biol, 2009. **86**(1): p. 103-14.
152. Van Wauwe, J.P., J.R. De Mey, and J.G. Goossens, *OKT3: a monoclonal anti-human T lymphocyte antibody with potent mitogenic properties.* J Immunol, 1980. **124**(6): p. 2708-13.
153. Prujansky, A., A. Ravid, and N. Sharon, *Cooperativity of lectin binding to lymphocytes, and its relevance to mitogenic stimulation.* Biochim Biophys Acta, 1978. **508**(1): p. 137-46.
154. Takahashi, A., et al., *Essential regions for antiviral activities of actinohivin, a sugar-binding anti-human immunodeficiency virus protein from an actinomycete.* Arch Biochem Biophys, 2005. **437**(2): p. 233-40.

155. Barrientos, L.G., et al., *Dissecting carbohydrate-Cyanovirin-N binding by structure-guided mutagenesis: functional implications for viral entry inhibition.* Protein Eng Des Sel, 2006. **19**(12): p. 525-35.
156. Houles Astoul, C., et al., *The size, shape and specificity of the sugar-binding site of the jacalin-related lectins is profoundly affected by the proteolytic cleavage of the subunits.* Biochem J, 2002. **367**(Pt 3): p. 817-24.
157. Wyss, D.F., et al., *Conformation and function of the N-linked glycan in the adhesion domain of human CD2.* Science, 1995. **269**(5228): p. 1273-8.
158. Jones, N.H., et al., *Isolation of complementary DNA clones encoding the human lymphocyte glycoprotein T1/Leu-1.* Nature, 1986. **323**(6086): p. 346-9.
159. McAlister, M.S., et al., *Structural analysis of the CD5 antigen--expression, disulphide bond analysis and physical characterisation of CD5 scavenger receptor superfamily domain 1.* Eur J Biochem, 1998. **257**(1): p. 131-41.
160. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan, *T cell activation.* Annu Rev Immunol, 2009. **27**: p. 591-619.
161. Kuball, J., et al., *Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain.* J Exp Med, 2009. **206**(2): p. 463-75.
162. Sherblom, A.P., et al., *IL-2, a lectin with specificity for high mannose glycopeptides.* J Immunol, 1989. **143**(3): p. 939-44.
163. Moulard, M., et al., *Selective interactions of polyanions with basic surfaces on human immunodeficiency virus type 1 gp120.* J Virol, 2000. **74**(4): p. 1948-60.
164. Balzarini, J., et al., *Carbohydrate-binding agents cause deletions of highly conserved glycosylation sites in HIV GP120: a new therapeutic concept to hit the achilles heel of HIV.* J Biol Chem, 2005. **280**(49): p. 41005-14.
165. Balzarini, J., et al., *Mutational pathways, resistance profile, and side effects of cyanovirin relative to human immunodeficiency virus type 1 strains with N-glycan deletions in their gp120 envelopes.* J Virol, 2006. **80**(17): p. 8411-21.
166. Shenoy, S.R., et al., *Selective interactions of the human immunodeficiency virus-inactivating protein cyanovirin-N with high-mannose oligosaccharides on gp120 and other glycoproteins.* J Pharmacol Exp Ther, 2001. **297**(2): p. 704-10.
167. Yuste, E., et al., *Glycosylation of gp41 of simian immunodeficiency virus shields epitopes that can be targets for neutralizing antibodies.* J Virol, 2008. **82**(24): p. 12472-86.
168. Rudd, P.M., et al., *Roles for glycosylation of cell surface receptors involved in cellular immune recognition.* J Mol Biol, 1999. **293**(2): p. 351-66.
169. Masnikosa, R., et al., *Characterisation of insulin-like growth factor receptors and insulin receptors in the human placenta using lectin affinity methods.* Growth Horm IGF Res, 2006. **16**(3): p. 174-84.
170. Johnson, E.W., L.A. Jones, and R.W. Kozak, *Expression and function of insulin-like growth factor receptors on anti-CD3-activated human T lymphocytes.* J Immunol, 1992. **148**(1): p. 63-71.
171. Shibuya, N., et al., *Binding properties of a mannose-specific lectin from the snowdrop (Galanthus nivalis) bulb.* J Biol Chem, 1988. **263**(2): p. 728-34.

172. Klasse, P.J., R.J. Shattock, and J.P. Moore, *Which topical microbicides for blocking HIV-1 transmission will work in the real world?* PLoS Med, 2006. **3**(9): p. e351.
173. Sexton, A., et al., *Transgenic plant production of Cyanovirin-N, an HIV microbicide.* Faseb J, 2006. **20**(2): p. 356-8.
174. Koshte, V.L., et al., *The potent IgG4-inducing antigen in banana is a mannose-binding lectin, BanLec-I.* Int Arch Allergy Immunol, 1992. **97**(1): p. 17-24.
175. van der Neut Kolfschoten, M., et al., *Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange.* Science, 2007. **317**(5844): p. 1554-7.
176. Denton, P.W., et al., *Systemic administration of antiretrovirals prior to exposure prevents rectal and intravenous HIV-1 transmission in humanized BLT mice.* PLoS One, 2010. **5**(1): p. e8829.
177. Pusztai, A., S. Bardocz, and S.W. Ewen, *Uses of plant lectins in bioscience and biomedicine.* Front Biosci, 2008. **13**: p. 1130-40.