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TESI DI DOTTORATO DI RICERCA

# EVALUATION OF ANTI-INFECTIVE ACTIVITY OF GLYCOMIMETIC DC-SIGN LIGANDS

Settore MED/04 - Patologia Generale

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## INDEX

## ABSTRACT

***Background.*** HIV remains one of the leading causes of mortality and morbidity worldwide. As the vast majority of HIV infections occurs via unprotected sexual intercourses, the development of topical microbicides is a new and promising approach to prevent HIV sexual transmission.
DC-SIGN, a C-type Lectin Receptor (CLR), participates in the initial stages of sexually transmitted HIV infection by recognizing highly mannosylated structures displayed in multiple copies on HIV gp120. Dendritic cells located in genital mucosae internalize HIV through DC-SIGN and transmit the virus in *trans* to CD4 T lymphocytes, promoting virus dissemination. Furthermore, binding of HIV to DC-SIGN activates signaling pathways that induce immunosuppression and promote HIV replication and transmission.
Thus, inhibition of HIV interaction with DC-SIGN represents a potential therapeutic approach to prevent HIV infection at the mucosal level. Aim of this study was to evaluate the efficacy in inhibiting HIV infection and the potential toxicity of a multimeric glycomimetic DC-SIGN ligand (dendron **12**).
***Methods.*** In the initial phase of this study, the ability of dendron **12** to block laboratory and primary HIV-1 strains transmission to CD4 T cells was assessed using a *trans* infection assay in *vitro*. Owing the results obtained, the efficacy of dendron **12** in inhibiting HIV-1 infection of mucosal tissue taken from human uterine cervix was evaluated. Cervical explants were treated with the dendron **12** and then exposed to different HIV-1 strains in a non polarized manner, mimicking a condition of compromised epithelial integrity. Infection was determined by measuring p24 HIV-1 core protein concentration in supernatants of cell and explant cultures. β chemokines production following stimulation of monocyte-derived DCs was also analyzed. The selectivity of dendron **12** towards DC-SIGN and Langerin, another CLR that recognize high mannosylated structures, was evaluated by Surface Plasmon Resonance (SPR) studies. Toxicity of the compound was evaluated in both cellular and tissue models.
***Results.*** Dendron **12** prevented *trans* infection of CD4 T lymphocytes and infection of human cervical tissue by multiple clades of R5 and X4 tropic HIV-1 strains, even in presence of elevated viral loads. The compound displayed a prolonged activity and absence of toxicity at the highest concentration tested in infection assays. Treatment with dendron **12** did not interfere with the activity of Langerin that, in contrast to DC-SIGN, prevents HIV transmission promoting the degradation of the virus. Moreover dendron **12** significantly elicited the production of the β chemokines MIP-1α, MIP-1β and RANTES. The dendron **12** was found to be soluble in physiological media and stable at both neutral and acid pH.
***Conclusion.*** Dendron **12** inhibits HIV-1 infection by competition with binding of HIV to DC-SIGN and stimulation of β chemokines production. Furthermore dendron **12** is highly soluble in physiological media, stable at acidic vaginal pH, no toxic and endowed with a long-lasting effect. Thus, dendron **12** represents a promising lead compound for the development of anti-HIV topical microbicides.

## SOMMARIO

**Introduzione**. HIV è tuttora una delle principali cause di mortalità e morbilità nel mondo. Poiché la grande maggioranza delle infezioni da parte di HIV avviene attraverso rapporti sessuali non protetti, lo sviluppo di microbicidi topici è un nuovo e promettente approccio per prevenire la trasmissione sessuale di HIV. DC-SIGN, un recettore della famiglia delle lectine di tipo C (CLR), è coinvolto nelle fasi iniziali della trasmissione sessuale dell'infezione da HIV, grazie alla sua capacità di legare strutture altamente mannosilate presenti in copia multipla sulla glicoproteina dell'envelope di HIV gp120. Le cellule dendritiche situate nelle mucose genitali internalizzano HIV attraverso DC-SIGN e lo trasmettono in *trans* ai linfociti T CD4, promuovendo la diffusione del virus. Inoltre, il legame di HIV a DC-SIGN attiva vie di trasduzione del segnale, che inducono immunosoppressione e favoriscono la replicazione e la trasmissione di HIV. L'inibizione dell'interazione di HIV con DC-SIGN rappresenta quindi un potenziale approccio terapeutico per prevenire l'infezione da parte del virus a livello mucosale. Scopo di questo studio è stato quindi di valutare l'efficacia nell'inibire l'infezione da parte di HIV e la potenziale tossicità di un ligando glicomimetico multimerico di DC-SIGN (dendrone **12**).

**Metodi.** Inizialmente la capacità del dendrone **12** di impedire la trasmissione di ceppi di laboratorio e primari di HIV-1 ai linfociti T CD4 è stata valutata mediante un saggio in *vitro* d'infezione in *trans*. In seguito è stata valutata l'efficacia del dendrone **12** nell'inibire l'infezione di tessuto prelevato dalla cervice uterina umana da parte di HIV-1. Gli espianti cervicali sono stati trattati con il dendrone **12** ed esposti a diversi ceppi di HIV-1 in modo non polarizzato, simulando la condizione in cui l'integrità epiteliale è compromessa. I livelli di infezione sono stati determinati misurando la concentrazione della proteina del capside di HIV p24 nei surnatanti delle colture cellulari e degli espianti. Inoltre è stata analizzata la produzione di β chemochine dopo la stimolazione di cellule dendritiche con il dendrone **12**. La selettività del dendrone **12** per DC-SIGN e Langerina, un altro CLR che riconosce strutture altamente mannosilate, è stata determinata mediante Risonanza Plasmonica di Superficie (SPR). Infine è stata valutata la tossicità del composto sia nel modello cellulare che nel modello tissutale.

**Risultati.** Il dendrone **12** impedisce l'infezione in *trans* dei linfociti T CD4 e l'infezione di tessuto cervicale umano da parte di ceppi di HIV-1 R5 e X4 tropici appartenenti a diverse cladi, anche in presenza di elevata carica virale. Il composto è caratterizzato da un'attività di lunga durata e assenza di tossicità. Il trattamento con dendrone **12** non interferisce con l'attività della Langerina, che a differenza di DC-SIGN, impedisce la trasmissione del virus HIV promuovendo la degradazione del virus. Inoltre il dendrone **12** stimola significativamente la produzione delle β chemochine MIP-1α, MIP-1β e RANTES. Il dendrone **12** mostra elevata solubilità nei medium fisiologici e stabilità sia a pH neutro che acido.

**Conclusioni.** Il dendrone **12** inibisce l'infezione da HIV-1 sia mediante competizione con il legame del virus a DC-SIGN sia inducendo la produzione di β chemochine. Inoltre dendrone **12** è altamente solubile nei medium fisiologici, stabile al pH acido della vagina, privo di tossicità e dotato di un effetto prolungato. Alla luce di questi risultati, il dendrone **12** può essere considerato un promettente composto lead per lo sviluppo di microbicidi topici anti-HIV.

## LIST OF ABBREVIATIONS

**7-AAD:** 7-aminoactinomicyn D
**AIDS:** Acquired Immune Deficiency Syndrome
**BIV:** Bovine Immunodeficiency Virus
**BSA:** Bovine Serum Albumine
**CLR(s):** C-type Lectin Receptors
**CMV:** Cytomegalovirus
**CRD:** Carbohydrate Recognizing Domain
**CRFs:** Circulating Recombinant Form
**Ct:** Threshold Cycle
**CTLs:** Cytotoxic T lymphocytes
**DC(s):** Dendritic Cell(s)
**DCIR:** DC Immunoreceptor
**DC-SIGN:** Dendritic Cell-specific ICAM-3 Grabbing Non-integrin
**DC-SIGNR:**
**EBV:** Epstein-Barr Virus
**ECD:** Extracellular Domain
**ESN:** Exposed Seronegative
**FBS:** Fetal Bovine Serum
**FIV:** Feline Immunodeficiency Virus
**GM-CSF**: Granulocyte-Macrophage-Colony Stimulating Factor
**gp**: Glycoprotein
**HAART:** Highly Active Antiretroviral Therapy
**HCV:** Hepatitis C Virus
**HIV:** Human Immunodeficiency Virus
**HTLV-1:** Human T-cell Lymphotropic Virus Type 1
**ICAM:** Intracellular Adhesion Molecule
**iDC(s):** immature Dendritic Cell(s)
**IL-2:** Interleukin 2
**IL-4:** Interleukin 4
**ITAM:** Immunoreceptor Tyrosine Based Activation Motif
**LC(s):** Langerhans Cell(s)
**LFA:** Lymphocyte function-associated antigen
**LG:** L-glutamine
**LNTPs:** Long term Non Progressor(s)
**$LTR_S$:** Long Term Repeat Sequences
**$Man_9$:** High mannose glycan
**MDDC(s):** Monocyte Derived Dendritic Cell(s)
**MFI:** Mean Fluorescence Intensity
**MHC:** Major Histocompatibility Complex
**MIP-1α and β:** Macrophage Inflammatory Protein-1 α and β
**MK(s):** Megacariocytes
**MR:** Mannose Receptor
**MTT:** tetrazolium salt 1-(4,5-dimethylthiazol-2-yl)-3,5diphenyl formazan
**NNRTIs:** Non-Nucleoside Reverse Transcriptase Inhibitors

**NRTIs:** Nucleoside Reverse Transcriptase Inhibitors
**P/S:** Penicillin and streptomycin
**PBMCs:** Peripheral Blood Mononuclear Cell(s)
**PBS:** Phosphate Buffered Saline
**PCR:** Polymerase Chain Reaction
**PHA:** Phytohemagglutinin
**PI:** Protease Inhibitors
**RANTES:** Regulated on Activation, Normal T cell Expressed and Secreted
**RNAPII:** RNA Polymerase II
**RT:** Reverse Transcriptase
**SDF:** Stromal Derived Factor
**SI and NSI viruses:** Syncytium Inducing and Non Syncytium Inducing Viruses
**SIGNR:** SIGN Related Genes
**SIV:** Simian Immunodeficiency virus
**SPR:** Surface Plasmon Resonance
**$TCID_{50}$:** Tissue Culture Infectious Dose
**TLR(s):** Toll Like Receptor(s)

# *INTRODUCTION*

# 1. HUMAN IMMUNODEFICIENCY VIRUS (HIV)

HIV remains one of the leading causes of mortality and morbidity worldwide. The virus infects 15,000 people every day and causes millions of deaths every year.
According to the latest UNAIDS estimates, nearly 33.3 million people are living with HIV and about 2.6 million people became newly infected with HIV in 2009.
The vast majority of HIV-1 infections occurs via sexual transmission through mucosal surfaces. Other transmission routes are contaminated needles used for intravenous drug delivery, mother to child transmission during perinatal and breast feeding period, transfusion of infected blood or therapeutic use of contaminated blood products. The latter has been almost completely eliminated, expecially in the developed world [1].
HIV belong to lentivirus family of animal retroviruses. Lentiviruses, that also comprises visna virus of sheep, Bovine Immunodeficiency Virus (BIV), Feline Immunodeficiency Virus (FIV) and Simian Immunodeficiency Virus (SIV), cause short term cytopathic effects and long term latent infection of the susceptible cells. All these virus are responsible of slowly progressive fatal diseases.
Two types of HIV: HIV-1 and HIV-2, which are closely related to each other, have been identified. The more virulent HIV-1 is the most common cause of AIDS worldwide, whereas HIV-2 is endemic in West Africa and is now spreading in India.
HIV-1 and HIV-2 differ in genomic structures and antigenicity, but cause a similar clinical syndrome. Both viruses appear to have spread to humans from other primate species. The sequence relationships suggest that HIV-1 has passed to humans on at least three independent occasions from the chimpanzee *(Pan troglodytes)*, and HIV-2 from the sooty mangabey (*Cercocebus atys)* [2,3].

## 1.1 Structure, genome

Each virus particle, or virion, consist of two copies of an RNA genome and associated enzymes (reverse transcriptase, integrase and protese), packaged in the viral core and surrounded by the envelope. The core is constituted of the capsid p24 protein and surrounded by the matrix protein p17. The envelope is a phospholipid bilayer that derived from plasmatic membrane of the infected cell, but contains the viral protein gp41 and gp120 (Fig.1).
The HIV genome is about 9.2 kB long, contains nine genes and, like all retroviruses, presents at both the ends LTRs (long terminal repeat sequences). LTRs are required for the integration of the provirus into the host cell genome and contain binding sites for gene regulatory proteins that control the expression of the viral genes. Like other retroviruses, HIV has three major genes: *gag*, *pol* and *env*. The *gag* gene encodes the structural proteins of the viral core; *pol* encodes reverse transcriptase, integrase and viral protease, involved in viral replication and integration; *env* encodes gp160, precursor of the envelope glycoproteins gp120 and gp41. HIV genome also contains six smaller genes encoding proteins that regulate viral replication and infectivity*: tat*, *rev*, *vif*, *nef*, *vpr and vpu*.

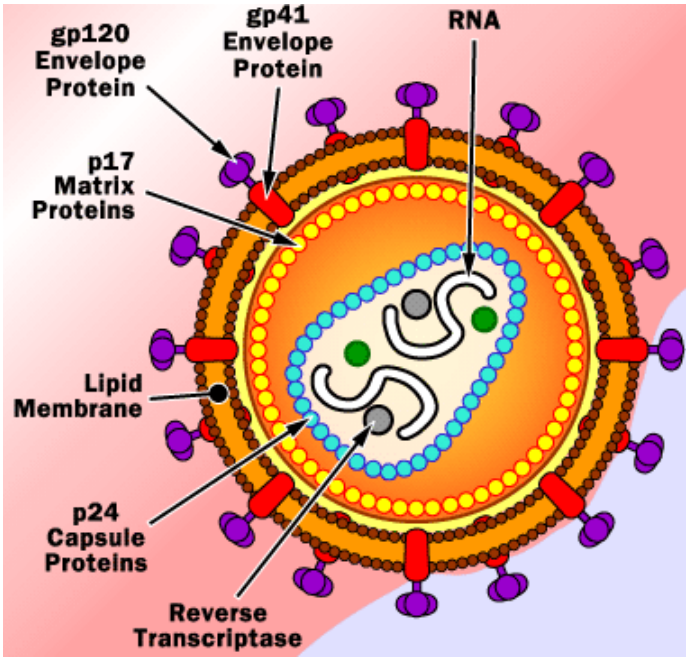



***Figure 1**. HIV structure.*

## 1.2 Infectious cycle

The HIV cycle begins with the entry of the virus in susceptible cells, such as CD4 T cells, monocytes, macrophages and dendritic cells (DCs) that express CD4 and a co-receptor (CCR5 and/or CXCR4, both members of chemokine receptor family).
HIV enters into the cells thanks to Env complex, constituted of non-covalently associated gp120 and gp41. The complex is a trimeric structure made of three gp120/gp41 pairs and mediates fusion of virion envelope with the membrane of target cell. Following the binding to CD4, gp120 undergoes a conformational change that enables gp120 binding to chemokine co-receptor. This binding causes a conformational change of gp41, that exposed a hydrophobic region, defined the fusion peptide. Insertion of the fusion peptide makes possible fusion of the viral envelope with host cell membrane and the release of viral core into the cytoplasm.
Once in the cell, the core releases the RNA genome, which is then transcribed into double-stranded cDNA by reverse transcriptase. The viral cDNA enter the nucleus in association with viral integrase and vpr protein (that promote nuclear import of the viral cDNA) and it is integrated as provirus into host cell genome by the integrase. Cell activation enhances integration events. HIV, like other retroviruses,

can establish a latent infection in which the provirus remains quiescent for months or years.
The LTRs contain a TATA box promoter sequence and binding sites for the cellular transcription factors NFκB and SP1.
Activation of target cells induces the expression of the transcription factor NFκB, which binds to the proviral LTR and initiates the transcription of the HIV genome. Activation by cytokines such as IL-2, TNFα and lymphotoxin, antigens and polyclonal activators induces HIV gene expression and viral replication in CD4 T cells. Exposure to IL-1, IL-3, IL-6 and lymphotoxin induces the same effect in monocytes and macrophages.
Binding of NFκB to the viral LTR, initiates the transcription of viral RNA by the cellular RNA polymerase II (RNAPII). However the transcription of HIV genes by RNAPII is inefficient. The Tat protein binds nascent viral mRNA and enhances RNAPII processivity, allowing the completion of the mRNA transcription.
Recent studies demonstrated that activation by HIV of both TLR-8 and DC-SIGN signaling pathways is required for transcription elongation by RNAPII, resulting in full-length HIV-1 transcripts and DCs productive infection (see chapter 3, paragraph 8). These transcripts are spliced in various ways to produce mRNAs for the viral proteins.
Regulatory genes are early expressed, whereas structural genes, such as *gag*, *pol* and *env* are expressed in a late phase. Tat, Rev and Nef early gene products are translated from multiply spliced mRNA, exported from the nucleus by host cell nuclear transport mechanisms. The Gag and Pol proteins are translated from unspliced mRNA; Vif, Vpr, Vpu and Env are translated from singly spliced viral mRNA. Rev promotes export of singly spliced and unspliced transcripts from the nucleus.
The *pol* mRNAs is translated to give a precursor protein, cleaved by the viral protease into reverse transcriptase, protease, ribonuclease, and integrase.
The gag gene product is a 55kda protein cleaved by viral protease into p24, p17 and p15. The product of the env gene, gp160, is cleaved by cellular protease into gp120 and gp41.
The full length unspliced RNA transcripts are packaged with the viral protein as RNA genome. Then viral particles are enclosed in membrane envelope and are released from the infected cells by budding [2,3,4].

## 1.3 Course of HIV disease

Few days after virus exposure, abundant HIV replication occurs in lymph nodes. This lead to elevated viremia with spreading of the infection throughout the body.
The number of circulating CD4 T cells markedly drops from the normal value (1200 cells $\mu l^{-1}$). Three- six week after infection many infected people develop aspecific symptoms, like fever, headhaches, sore throat with pharingitis, lymphoadenopathy and rashes (Acute HIV Syndrome). However in several cases primary HIV infection is asymptomatic.
The acute viremia is associated with an early immune response. In virtually all patients the activation of CD8 T cells and the anti HIV antibody production partially

control the infection and the viral production. The development of detectable antibodies directed against HIV in the blood (seroconversion) occurs six-nine weeks after the infection. The cytotoxic T lymphocytes (CTLs), which kill HIV-infected cells, are important in controlling viremia, that declines to low but still detectable levels by about 3 months after the virus exposure. Similarly, the CD4 T cell counts rebound to around 800 cells $\mu l^{-1}$.
Most patients will develop AIDS after a period known as clinical latency or the asymptomatic period, during which the immune system can control opportunistic infection and no clinical manifestations of HIV infection are present.
Immune response can control HIV infection, but does not eliminate the virus. Indeed HIV evades host immunity in different ways. HIV is characterized by a high rate of mutations since viral RT lacks of a proof-reading domain. Mutations that occur when HIV replicates can allow variants of the virus to escape recognition by antibody or cytotoxic T cells. HIV may evade CTLs also down-regulating MHC I expression.
During the asymptomatic period persistent replication of the virus occurs; CD4 T cell destruction in spleen and lymph nodes gradually progresses, leading to a gradual decline in the function and numbers of CD4 T cells. Different mechanism causes the loss of CD4 T cells in HIV infection: the virus can exert a direct cytopathic effect towards infected cells; the infected cells are more susceptible to apoptosis or are killed by CTLs. In addition antibody against HIV-1 envelope may bind env expressed on surface of infected CD4 T cells triggering antibody dependent cell mediate cytotoxicity.
Once CD4 T cell count is around 500 cells $\mu l^{-1}$ the disease enters the symptomatic phase and opportunistic infections and other symptoms appear. This can occur between 2 and 15 years, or more, after the primary infection.
When CD4 T cell counts fall below 200 cells $\mu l^{-1}$ cell-mediated immunity is lost, viremia dramatically raises and HIV disease progresses to AIDS. Infections with a variety of opportunistic microbes (oral Candida species, Mycobacterium tuberculosis, herpes zoster) appear. Pneumonia caused by the fungus *Pneumocystis carinii* is common and often fatal. In the final stages of AIDS, infection with cytomegalovirus or *Mycobacterium avium* is more prominent. The patients are also affected by tumors like EBV-induced B-cell lymphomas, Kaposi's sarcoma and cervical carcinoma. Cachexia (AIDS wasting syndrome), kidney degeneration (AIDS nephropathy) and degeneration of central nervous system (AIDS encephalopathy) also occur [2,3,4].
However the course of the disease can vary widely. A small percentage of people, called long-term non progressor (LTNPs) seroconvert, but do not seem to have progressive disease. LNTPs maintain a high CD4 and CD8 T cells count, and have unusually low plasma HIV RNA levels. Viral, genetic and immunological components seem to be involved in the control of HIV infection by LNTPs [5].
Some individuals, called HIV exposed seronegative individuals (ESN), do not seroconvert and remain disease-free and virus-negative despite multiple and documented exposure to HIV. Some of these people have specific cytotoxic lymphocytes and $T_H1$ lymphocytes directed against infected cells. HIV specific IgA,

but not IgG were detected in genital tract, saliva and plasma. Genetic factors, such as deletion and polymorphism in CCR5, chemokine variants, HLA polymorphism and others, are also involved. However so far, none of the identified immunological and/or genetic mechanism has been able to completely explain this phenomenon [6,7].

## 1.4 HIV tropism

HIV-1 strains that use as co-receptor CCR5, receptor of the β chemokines MIP-1α, MIP-1β and RANTES, are referred as R5 tropic. Instead strains that use as co-receptor CXCR4, receptor of CXC chemokine SDF-1, are defined X4 tropic [8].
Env R5 tropic viruses of interacts with the N-terminus of the CCR5 [9], while Env of X4-tropic viruses interacts with the first and second extracellular loops of the CXCR4. Initially R5 tropic strains were named "macrophage-tropic" because they infect macrophage but not T-cell lines in *vitro*. Conversely X4 tropic strains were defined "lymphocyte-tropic", as they infect T cell lines and primary CD4 T cells. Dual tropic R5/X4 strains can use efficiently both co-receptors to enter the cells, interacting with both N-terminal of CCR5 and first and second extracellular loops of CXCR4. However they preferentially exploit CXCR4 to infect CD4 T cells.
R5 strains infect CD4 T cells, dendritic cells and macrophages, that express CCR5, and require only a low level of CD4 on the target cells. These strains are not able to form syncytia and therefore are named non-syncytium (NSI) inducing viruses.
R5 tropic isolates of HIV predominate during the transmission and are the dominant viral phenotype found in newly infected individuals. R5 strains are particularly involved in sexual and mother to child transmission. However the predominance of R5-tropic viruses occurs regardless of the transmission route as they represent the majority of viruses that are found in patients that are infected through intravenous drug injection, blood transfusion, or sexual intercourse.
Infection of CD4 T cells via CCR5 occurs early in the course of infection and continues to occur, with activated CD4 T cells accounting for the major production of HIV throughout infection.
Late in infection, the viral phenotype switches to a X4 in about 50% of individuals and this is followed by a rapid decline in CD4 T cell count and progression to AIDS. To evolve to X4 tropic strains, R5-tropic viruses undergo mutations in the V3-loop of gp120. This strains are able to induce syncytia and are referred as syncytium-inducing (SI) viruses [8].
The β chemokines MIP-1α, MIP-1β and RANTES, natural ligands of CCR5, suppress HIV-1 R5 tropic strain replication, competing directly with the binding of the virus to CCR5 or inducing receptor internalization [10].
Some individuals are homozygous for an allelic, nonfunctional variant of CCR5 caused by a 32-base-pair deletion (CCR5Δ32) from the coding region, which results in a frameshift and truncation of the translated protein that cannot be exported to cell surface. The gene frequency of this mutant allele in Caucasoid populations is 0.09,resulting in about 1% of homozygous carriers and about 10% of heterozygous [11]. The mutant allele has not been found in Japaneses or Africans from Western or Central Africa.

People homozygous for CCR5Δ32 are highly resistant to R5- and R5X4-tropic HIV-1 strains.
Individual heterozygous for CCR5Δ32 express low levels of CCR5 and are partially protected protection against sexual transmission of HIV, and display low viremia reduction in the rate of progression to AIDS. In addition to the structural polymorphism of the gene, variation of the promoter region of the CCR5 gene has been found in both Caucasian and African Americans. Different promoter variants were associated with different rates of progression of disease.
However rare cases of HIV-1 infection by X4-tropic strains were observed in CCR5Δ32 homozygotes [12].
Several studies suggest that other chemokine or chemokine receptor–like orphan receptors are used as co-receptor by one o more HIV-1 strains, but the use of some of these receptors was demonstrated only *in vitro* [8].

## 1.5 HIV-1 classification

Four groups of HIV-1 have been identified so far: M (the "major" group), O (the "outlier"), N ("new") and the recently discovered P. These groups seem to derive from at least three distinct passages of simian immunodeficiency virus (SIV) from chimpanzee to man. Group M is responsible for more than 90% of HIV infections reported and is sub-classified into nine subtypes or clades. Group O is restricted to west and central Africa [13,14]. The group N, phylogenetically equidistant from M and O, was firstly described in 1998 in Cameroun and is very rare [15]. In 2009 a new HIV-1 strain was isolated in a Cameroonian woman. This virus, closely related to gorilla simian immunodeficiency virus (SIVgor) and distinct from HIV-1 groups M, N and O, was designed group P [16].
Group M is subclassified into nine phylogenetically genetically distinct clades (A, B, C, D, F, G, H, J and K). Group M also includes several "circulating recombinant forms" or CRFs, that arise when two viruses of different subtypes meet in the cell of an infected individual and mix together their genetic material, generating a new hybrid virus. Subtype E has never been isolated alone, but CRF A/E exists. Similarly subtype I, has been reclassified as a recombinant form A/G/I.
The HIV-1 subtypes and CRFs have a distinct geographical distribution. Subtype A and CRFs A/G are predominant in West and central Africa. Subtype B is the most common subtype in Europe, the Americas, Japan and Australia. It is the predominant sub-type found among homosexuals and intravenous drugs abusers infected in Europe. However, with increasing immigration and globalization, other subtypes are becoming more frequent and now are responsible for at least 25% of new HIV infections in Europe. Subtype C is the most abundant subtype in countries with the majority of HIV-1 infections. Indeed it Subtype C is present the eastern Africa and predominates in all countries of Southern Africa and in some countries of Asia, such as India and Nepal. It has caused the world's worst HIV epidemics and accounts for around half of all infections. Subtype D is generally limited to East and Central Africa. CRF A/E is prevalent in South-East Asia, but originated in Central Africa. Subtype F has been found in Central Africa, South America and Eastern Europe. Subtype G and CRF A/G have been observed in West and East

Africa and Central Europe. Subtype H is present only in Central Africa, J exclusively in Central America and K has been isolated in the Democratic Republic of Congo and Cameroon. However, due to HIV-1 high rate of mutation, new subtypes will probably discovered in the future.
Clades are classified on the basis of 20-50% differences in the *env* coding sequences. Intra-clade variation of 10-15% is reported. The *pol* sequences are less divergent than *env,* to avoid that excessive mutations render inactive RT and protease. On the contrary *gag* sequences, encoding for structural proteins of the core are quite conserved. Interestingly also LTR display substantial inter-clade variations in the LTR copy number and in sequence of enhancer and promoter structures, whereas SP1 sites, TATA box and TAT responsive elements are more conserved.
Viruses belonging to different clades display difference in co-receptor usage and in capacity to induce syncytia. Clade A and even more Clade C viruses maintain a CCR5/NSI phenotype even at later stages of the disease. Clade D maintain a dual tropic tropism during all the course of the disease [13,14].
AIDS progression may differ in function of infecting subtype. Patients infected by Clade C strains appears to undergo more rapid disease progression than patients infected by other clades [17].

## 1.6 HIV mucosal transmission

Most of HIV infections are acquired through sexual transmission. The type of sexual activity affect the risk of transmission. Initial estimates of transmission rates per coital act have ranged from 0·0003 to 0·008, with insertive vaginal intercourse associated with lower estimates and receptive anal intercourse associated with estimates as high as 0·01 or 1%. Sexual transmission also depends on factors such as circumcision status, genital ulcer disease, and phase of disease [18].
The vagina and the ectocervix are lined by a thick pluristratified squamous epithelium, whereas epithelium of endocervix and rectum consist of single layer of columnar cells that may be more easily traversed by HIV and SIV. Rectal mucosae contains in subepithelial region many HIV cell target. Furthermore, rectal lymphoid follicles contain specialised M cells (microfold cells), which have been shown to bind and present HIV-1 to underlying lymphoid tissue.
Physical abrasion, ulceration, or pre-existing inflammatory conditions, such as sexually transmitted diseases and bacterial vaginosis, cause microulcerations and breaks in the epithelium. Hence thinning and breaches in the mucosal barrier could expose susceptible cells in the submucosa to virus, increasing the likelihood of establishing and rapidly disseminating infection. HIV-1 could traverse the intact stratified squamous epithelium of the vaginal mucosa and the simple columnar epithelium of the cervix by different ways: transcytosis by epithelial cells, infection of intraepithelial lymphocytes, capture or infection of DCs (Fig. 2).
Epithelial cells selectively capture R5 HIV-1 and then transfer infection to CCR5-expressing target cells underneath the epithelia, which could account for the preferential transmission of HIV-1 strain R5. Interestingly epithelial cells express high level of SDF-1 (the CXCR4 natural ligand) providing a barrier for X4 tropic

isolates of HIV-1. Langerans cells are located in vaginal epithelium and immature dendritic cells (iDCs) are present in subepithelial zone of the different mucosal tissues and are among the first cells encountered by the virus.
After crossing the epithelial barrier, HIV-1 and SIV can infect CCR5-expressing DCs, macrophages and T cells in the underlying mucosal tissues to initiate infection. iDCs capture HIV-1 through C-Type lectin receptors (CRLs) (DC-SIGN, Mannose receptor) and are productively infected by a CCR5-dependent mechanism. Captured virus can be internalized (without productive infection) and then rapidly transmitted to nearby CD4 T, most efficiently to CD4 memory subset. Virus carrying DCs, after migration to lymphoid organs, also disseminate infection to large numbers of CD4 T cells (see Chapter 3 for a more detailed discussion).

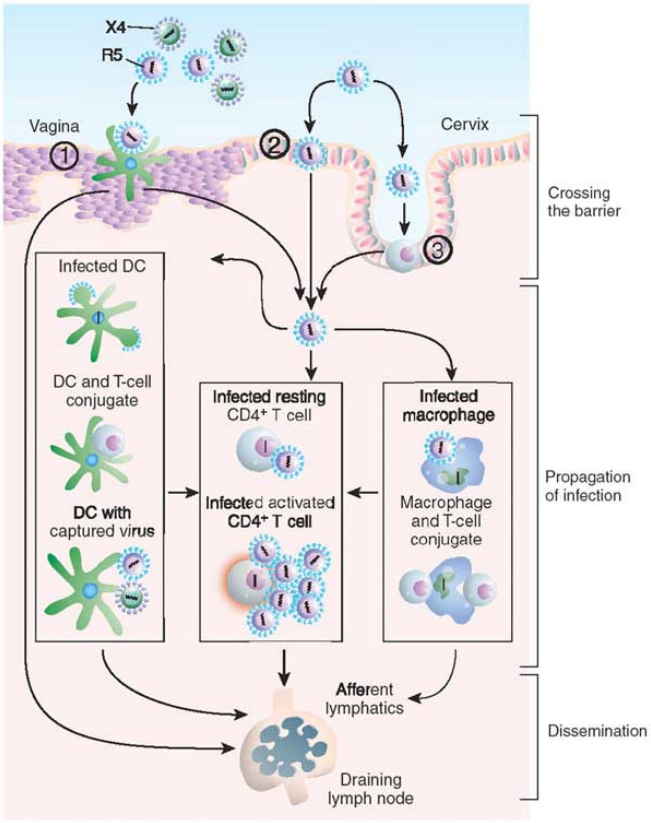



***Figure 2.*** *HIV vaginal transmission*

Also macrophages, that express mannose receptor and DC-SIGN can transmit the virus to CD4 T cells. The acute infection phase is characterized by the infection and subsequently the death of memory $CCR5^+$ CD4 T cells in mucosal tissue. As

mucosae are the principal localization sites of memory T lymphocytes and contains the major reserve of these cells in the body, the loss of CD4 T cells in the mucosae leads to a systemic reduction of CD4 T cells. After infection reaches the lymphoid tissues, the virus gain access to large numbers of susceptible cells in close proximity. In both SIV and HIV infection, although CD4 memory T cells are lost in all lymphoid-tissue compartments, the greatest losses happens in the Gut Associated Lymphoid Tissue (GALT).
HIV transmission across the male genital tract is poorly understood. All major HIV/SIV target cells (CD4 T cells, Langerhans cells, DCs, and macrophages) have been identified in the foreskin and glans penis. However most CD4 T cells reside below the basement membrane in these mucosal tissues, whereas Langerhans cells exclusively reside in the epithelium. Ex *vivo* tissue cultures of human foreskin have shown that the it is susceptible to R5 tropic strain infection. Like the others mucosal tissues, inflammation of the glans penis and foreskin increases susceptibility to HIV infection [19, 20, 21].

## 1.7 HIV infection therapy

Treatment of HIV infection is based on drugs that interfere with the viral cycle. The first drugs developed were the nucleoside reverse transcriptase inhibitors (NRTIs), that interfere with HIV-1 replication by competitively inhibiting this enzyme. These drugs are often effective in reducing HIV levels in the plasma for months or years, but do not stop disease progression as resistances emerge.
Subsequently protease inhibitors (PI), that bind the active site of HIV protease impeding the cleavage of precursor protein into mature viral capsid and core protein, were developed. However when these drugs are used alone resistant HIV mutants quickly emerge.
Protease inhibitors are now being used in combination with two different NRTIs. Different studies proved that this triple combination therapy, or highly active antiretroviral therapy (HAART), was efficacy in inducing viral load suppression, in restoring the immune function and in markedly reducing mortality.
More recently non-nucleoside reverse transcriptase inhibitors (NNRTIs) were developed. NNRTIs are a group of drugs with different structures that bind the viral RT at a region distant from the active site, resulting in conformational changes at the active site and consequently in inhibition of RT. NNRTIs are highly specific inhibitors with potent antiviral activity in *vivo*, but they favour a rapid emergence of resistant viral strains if used as monotherapy.
The introduction of NNRTIs makes feasible the use of other triple combination therapies. Indeed NNRTIs combined with two NRTIs are very effective in suppressing HIV-1 replication [22]. Recently, integrase inhibitors have been successfully introduced into clinical use in combination with others antiretroviral drugs [23]. A CCR5 inhibitor, maraviroc was recently approved by the Food and Drugs Administration and now is used for the management of R5 tropic HIV-1 infection in combination with optimized background therapy regimens [24].
However HAART is not able to eradicate HIV infection, a life-long treatment is required and resistances emerge after long period of treatment. The presence of

viral reservoirs represent major obstacle to HIV eradication. Indeed several cell types (latently infected CD4 cells, macrophages, that are infected but not killed by HIV and Follicular dendritic cells, that trap on their surface large amounts of HIV) or anatomical sites (for example central nervous system) are potential HIV reservoirs. Moreover even in presence of the HAART low levels of viral replication, below the limits of the plasma viral load detection, occur [22].
HAART has serious side-effects linked to metabolic complication such as lactic acidosis, insulin resistance, abnormalities in lipid metabolism, lipodystrophy. These abnormalities in the glucidic and lipidic metabolism may favour the insurgence of diabetes and increase the risk of coronary artery calcification and myocardial infarction [25,26].
Another disadvantage of the HAART, especially for undeveloped countries, is the high cost of the therapy.

## 1.8 HIV vaccines

Although many efforts have been devoted to generating HIV vaccines, to date there is no approved vaccine on the market. One contribution to vaccine failure could be HIV's ability to activate multiple mechanisms of immune downregulation. HIV-associated disease is characterized by blunted immune responses, particularly those that require T cells. Indeed immune responses against antigens to which the host had previously been primed are progressively lost during HIV infection. Evolution of mutant virions, that escape recognition by antibodies and by cytotoxic T lymphocytes, makes extremely difficult the development of therapeutic vaccination strategies for HIV-infected patients .
Strong cytotoxic responses are necessary to provide protection against HIV, but such response might be difficult to develop and sustain through vaccination.
Furthermore the ability of the virus to persist in latent form as a transcriptionally silent provirus, might prevent the immune system from clearing the infection once it has been established [3,27]. Several vaccine strategies failed to protect against HIV-1 infection or to decrease plasma viral load after infection. For example, the efficacy Merk's STEP trial was stopped when early results suggested that not only the vaccine was ineffective at lowering post-infection plasma viremia, but people receiving the vaccine may have increased their chance of becoming infected with HIV-1 [28]. The recent RV144 trial based on a recombinant canarypox vector vaccine (ALVAC-HIV) plus two booster injections of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX) showed only a partial preventative effect.
Really the vaccine efficacy was 31.2%, but the vaccine did not affect the degree of viremia or the CD4 T-cell count in subjects in whom HIV-1 infection was subsequently diagnosed [29].

# 2. TOPICAL MICROBICIDES

## 2.1 Importance of developing microbicide drugs

Thirty years after its discovery, HIV remains one of the leading causes of mortality and morbidity worldwide. Highly active antiretroviral therapy (HAART), has markedly reduced morbidity and mortality of HIV infected individuals, but is not able to eradicate HIV infection and lifelong treatment is needed. Furthermore such treatments are associated with emergence of drug resistance and with serious side effects. In addition people living in developing countries often do not have access to this expensive therapy (see paragraph 1.7).
So far, no candidate vaccine was able to prevent HIV infection, with the exception of results of RV144 trial, which showed only a very limited preventative effect, and no effective vaccine is foreseeable in the immediate future (see paragraph 1.8).
Proper use of condoms are effective in preventing HIV sexually transmitted infection. However, owing to social, ethical, cultural and economic reasons, condom use is not yet sufficiently accepted. In this contest there is an urgent need for the development of new methodologies of prevention [30]. Women are biologically more susceptible to HIV and increasingly bear a disproportionate burden of the pandemic. The proportion of women living with HIV worldwide is slightly less than 52% of the global total, but the HIV-1 epidemic is increasingly affecting woman especially in sub-Saharan Africa, where young women in the age group of 15-24 years are eight times more likely to contract the virus compared with men [1]. Several factors account for major susceptibility to HIV infection difference. Heterosexual intercourse can cause microscopic damage to the lining of a woman's vagina, and these tiny breaches may permit the entry of pathogens. In addition, the female reproductive tract provides more surface area for viruses or bacteria to invade than the male reproductive tract. Indeed in presence of another sexually transmitted disease, the risk of acquiring HIV significantly increases [31]. On the other hand receptive anal intercourse associate with an high risk of transmission.
As the vast majority of HIV-1 infections occurs via sexual transmission through mucosal surfaces, the development of topical microbicides, formulated for vaginal or rectal use, represents a new and promising approach to prevent sexually transmitted HIV infection as well as other sexually transmitted diseases (STDs).
Topical microbicide drugs inhibits early stages of virus infection or replication inactivating virus directly, avoiding infection of susceptible cells or blocking virus dissemination [30].

## 2.2 Characteristic of an ideal anti-HIV topical microbicide

In addition to the potent activity against HIV an ideal microbicide should be:

- safe for daily use over long periods of time
- fast acting and long-lasting in effect

• resistant to acidic vaginal pH
• available in both contraceptive and non-contraceptive formulations
• suitable for both vaginal and rectal application
• preferably active also against others sexually transmitted diseases
• active against HIV and/or other STDs in both ejaculate and cervico-vaginal secretions to provide bi-directional protection for both partners
• stable, even at high temperature
• acceptable to both sexual partners or completely unobtrusive
• colorless, odorless and tasteless
• easy to use, low cost and readily accessible

Furthermore a topical microbicide should not cause epithelial disruption and induce inflammation, conditions that increase the risk of acquiring HIV infection.
It is also important that topical microbicides do not upset the vaginal or rectal microbial ecology, for example by killing Lactobacilli or enhance overgrowth by other pathogens. Systemic absorption should be avoided for safety concerns [32].

## 2.3 Preclinical and clinical test to development of topical microbicides

Preclinical or non-clinical testing of microbicides now comprises numerous study types. The traditional in-*vitro* HIV infection models for microbicide testing include infection of laboratory cell lines, PBMCs, CD4 T lymphocytes, primary macrophages, and dendritic cells by a range of laboratory and clinical virus isolates from different HIV clades and with different co-receptor usage. However, ex-*vivo* human explant studies (human penile, cervico-vaginal and rectal explants) are important to assess efficacy and toxicity as explant models better approximate the in *vivo* condition (see paragraph 2.4). Also animal models are important to determinate efficacy and toxicity. The Rhesus macaque, the pig-tailed macaque, and the Cynomolgus monkey model are most relevant for microbicide studies upon vaginal or rectal inoculation of SIV or SHIV (a SIV/HIV-1 chimeric clone, used to analyze functions of selected HIV-1 genes in *vivo* in nonhuman primates).
Other test required are animal vaginal irritation tests; genetic, general, and reproductive toxicity studies; pharmacokinetic studies; safety pharmacology studies; carcinogenic studies; hypersensitivity/photosensitivity studies; condom integrity studies. Large-scale production capacity, stability, formulation, and costs of the candidate microbicide drug are factors to consider before to test the candidate microbicide in clinical trials.
Clinical testing includes: phase I and phase II dosing, safety and acceptability studies, penile tolerance studies; phase III trials for efficacy. Phase III trials need several thousands of women to be enrolled to give a statistically meaningful result. The lower is incidence of HIV infection the higher is the number of people that are to be enrolled in the study. The clinical evaluation of microbicides requires that sites of the study are able to recruit and retain participants, and can accurately estimate HIV and STI incidence, pregnancy rates and normal ranges for clinical laboratory measures of health in participating populations. The understanding of

women's and men's sexual behavior and their risks for HIV and STI infections can help researchers interpret clinical trial results [18,33, 34].

## 2.4 Cervical explant model

Human cervical mucosal tissues has been studied to define HIV target cells in genital mucosa, interaction with epithelial cell and susceptibility to R5 and X4 tropic strain. Furthermore cervical explants are an useful model to test the efficacy in inhibiting HIV infection and the potential toxicity of candidate microbicide drugs [35,36].

Cervical explants are taken from seronegative pre-menopausal women undergoing planned hysterectomy and comprises both epithelial and stromal tissue. Non polarized and polarized cervical explant model has been described.

*Polarized explants.* In this model the tissue is cultured in a polarized state with the epithelial surface positioned at the air/tissue interface and the submucosa (stroma) submerged in medium. Circular tissue explants are inserted through a hole in a transwell insert with the epithelium oriented upward in the apical chamber. The epithelial surface of the explant is surrounded with agarose to maintain tissue orientation and minimize leakage of microbicides and HIV around the tissue edges. The stroma is cultured in medium culture in the basolateral chamber. This positioning of the cervical tissue allows application of virus and candidate topical microbicides directly to the epithelium, simulating mucosal exposure in *vivo*, and allows access to the cells in the submucosa [37]. However because the tissue deteriorates quickly, an accurate monitoring of cell viability and tissue permeability is essential [35].

*Non polarized explants.* In this system, the tissue is exposed to HIV-1 alone or in presence of a candidate microbicide in a non polarized manner. Then the explants are maintained in culture submerged in medium. Usually two thirds of the medium are changed every two and three days. This model mimics a situation in which genital epithelial integrity is compromised [36,38,39,40]. Indeed breaches in epithelium are associated with an increased risk to acquire HIV infection.

Although immune-activation enhance cervico-vaginal tissue infection, R5 tropic strains such as the laboratory HIV-1 strain BaL can infect cervico-vaginal tissue without immune-activation. On the contrary X4 tropic strain requires immunoactivation (e.g with IL-2 and PHA) for productive infection. This is in accord with the epidemiological evidence of a correlation between STDs (that increase the recruitment and the activation of HIV cell target) and HIV infection. Pre-activation of explants with IL-2 and PHA that could mimic aspects of infection/inflammation, but the tissues are at least 48 hours old when they were exposed to HIV and may be already deteriorated [35,36].

Explant were usually exposed to a viral concentration ranging from $10^3$ to $10^6$ $TCID_{50}$ (50% tissue culture infective dose) to obtain reproducible levels of HIV infection. Eventual toxicity of compound tested is usually assessed by a MTT based assay, that allow to evaluate alteration of the vitality of explants following compound exposure [36-40].

Many HIV microbicides test were conducted on stratified squamous vaginal or ectocervical tissue because of its relative abundance. However the endocervix, lined by monostratified columnar epithelium and rich of HIV cell target in the subepithelial zone, may be more susceptible to HIV infection [35].
Cervical explant models have different limitations. Presurgical hormone therapy and natural hormonal states of women undergone to hysterectomia may influence the results obtained. Numbers, types and activation status of HIV target cells (CD4 T cells, DC-SIGN$^+$ DCs, Langerhans cells and macrophages) can vary enormously within cervical tissue sites (ectocervix, endocervix, and transformation zone) and between individuals. Important physiological variables such as circulatory support, presence of mucus, endogenous normal microflora, are lost.
Despite these limitation previously described, explants may represent a more relevant method of testing topical microbicides than cell-based assays. However cell based assays can be more easily standardized.
Actually explant models present an high intrinsic variability. Furthermore protocols of explant infection vary across laboratories. Therefore the results obtained may be influenced by several factors including tissue type, HIV-1 strain or isolate, culture medium formulation, size of the virus inoculum, culture medium formulation, length of virus incubation, frequency of medium change, concentration of test compound, drug treatment period prior to or after viral exposure and endpoint viral growth measurements.
However with adequate standardization methods, as suggested by a study of Microbicide Quality Assurance Program, explant studies can provide consistent evaluation of anti HIV microbicides efficacy [41].

## 2.5 Classification of topical microbicides

Microbicides can act through different ways and can be classified in different groups according to their characteristics and mechanism of action [30,33,18]:

- Surfactants/membrane disruptors
- Vaginal milieu protectors
- Viral entry inhibitors
- Post entry inhibitors (reverse transcriptase inhibitors)
- Compounds with unknown mechanism of action

***Surfactants*** (nonoxinol-9, C31G, and sodium lauryl sulfate) are the earliest compounds developed and evaluated in clinical trials as topical microbicides. These compounds disrupt membranes aspecifically, displaying activity against HIV and other sexually transmitted pathogens.
Vagina has an acid pH (3.5 - 4.5) maintained by commensal lactobacilli, whereas semen pH is alkaline (around 7.1 - 8). ***Vaginal milieu protectors*** are aimed to maintain or restore the acid pH of the vagina. Acidifying gel as Carbopol 974P (BufferGel) and Acidform belong to this group. A probiotic strategy, involving the use of exogenous lactobacilli for colonization of vaginal canals, was also developed.

***Viral entry inhibitors*** belong to a wide class of topical microbicides that comprises anionic polymers, CCR5 inhibitors, fusion inhibitors and DC-SIGN inhibitors. *Anionic polymers* interact with HIV envelope interfering with the attachment of the virus to susceptible cells. However these compounds are more active against X4 tropic strains, that expose higher level of positive charges on gp120 than R5 tropic strains. Nevertheless some of these compounds, such as Naphtalene sulfate (PRO 2000), Cellulose sulfate (Ushercell) and Cellulose phthalate (CAP), block both R5 and X4 tropic HIV strains. Since R5 tropic strain predominates in the viral transmission, *CCR5 inhibitors* like PSC-RANTES and CMPD167 were developed as topical microbicides. Maraviroc is already used alone or in combination with other antiretroviral for treatment of HIV (see chapter 1, paragraph 7). Now maraviroc is in preclinical development, alone or associated to others antiretroviral drugs [42] and a clinical trial is planned by the International Partnership for Microbicides. *Fusion inhibitors* comprises the clinically approved fusion peptide inhibitor enfuvirtide (T-20) and carbohydrate binding agents (CBAs). Among CBAs the lectin Cyanovirin-N, that binds high mannose glycans on HIV gp120 is a promising compound. *DC-SIGN inhibitors* will be described in the chapter 3 paragraph 9.
Some ***Reverse transcriptase inhibitors*** are currently in clinical trials as topical microbicides. The lipophylic NNRTIs TMC-120 (dapivirine) and UC-781 are advanced in clinical trials. The CAPRISA study, a two-arm, double-blind, placebo controlled trial, recently demonstrated efficacy and safety of a 1% vaginal gel formulation of Tenofovir, a NRTI. Tenofovir reduced HIV infection by 50% in women with high gel adherence after one year of follow up. However the reduction was minor (40%) after 2 years. Low level of tenofovir was detected in the serum, but no tenofovir related mutation or mutation conferring multiple resistance to NRTIs were reported in seroconverted women. However it is not clear if low level of tenofovir absorption are necessary for protection from HIV transmission or if longer periods of treatment may induce resistance mutations [43].

More than 70 preclinical and 50 clinical trials have been performed using compounds that fall in each of the five classes. However, so far, only tenefovir, showed a partial efficacy against HIV-1 infection in a clinical trial.

## 2.6 Potential risk associated with microbicide use

Several microbicide clinical trials were stopped for lack of efficacy or to augmented risk of contracting HIV infection. For example both Nonoxinol-9 gel and Cellulose Sulfate gel showed increased risk of HIV infection compared to placebo.
Different biological reasons may explain this phenomenon. Repeated exposure of the cervico-vaginal mucosa to a microbicide may lead to changes in epithelial integrity and/or permeability, which could facilitate pathogen transmission instead of preventing it. As Nonoxinol-9 disrupts the phospholipid membranes, it can cause non-specifc damage to genital tissue. Indeed upon treatment with Nonoxinol-9 an increase of genital tract lesions with an epithelial breach, which was associated to a higher incidence of HIV seroconversion, was observed. Genital epithelial findings

and clinical signs of inflammation are therefore essential safety endpoints in clinical trials of candidate microbicides. However the individuation of validated biomarkers of genital irritation would be useful for standardization of the studies.
Repeated exposure of the cervico-vaginal mucosa to a candidate microbicide may also lead to a subclinical inflammatory reaction, increased immune activation and/ or decreased innate immunity. Inflammation can recruit and promote activation of HIV target cells. Microbicide may also induce vaginal flora changes. Minor changes in the vaginal flora following Nonoxinol-9, Cellulose Sulfate, and Carbopol 974P have been reported. However even minor changes of the vaginal flora may be associate with bacterial vaginosis and yeast infections, that increases the risk of acquiring HIV infection [33,44].

## 2.7 Future perspective

After mucosal exposure, HIV is able to establishing infection exploiting multiple pathways, that involve a variety of target cells (CD4 T lymphocytes, mucosal DCs DC-SIGN$^+$, Langerhans cells and macrophages), receptors and co-receptors (CD4, CCR5, CXCR4, DC-SIGN and others c-type lectins).
Defining molecular basis and chronology of early steps of HIV infection and identifying the role of different cell types, HIV receptors, co-receptors and host factors in facilitating transmission and spreading of the virus may be crucial for the rational design of candidate topical microbicides. Indeed, new knowledge about early events of HIV infection allows the development of novel molecules that interfere with specific molecular and cellular targets during the different steps of HIV infection process. The ideal microbicide should be able to interfere with different ways of HIV infection mechanisms but should also be able to block HIV replication and release once HIV is entered into the cells. The combination of microbicides with complementary mechanisms of action is expected to increase the potency of the formulation. Indeed, the combinations reported so far were all found to display synergistic activity in infection assays. Indeed, at lower concentrations, double and triple combinations were generally more effective than individual inhibitors. Furthermore the chances of a virus being simultaneously resistant to three compounds is less than to any single inhibitor. [30,34].

## 3. DC-SIGN

DC-SIGN (Dendritic Cell-specific ICAM-3 Grabbing Non-integrin) is a tetrameric calcium-dependent transmembrane protein, mainly expressed by dendritic cells (DCs) and macrophages.
DC-SIGN was firstly identified in 2000 as a DC specific c-type lectin that mediates adhesion to ICAM-3 on resting CD4 T lymphocytes and regulates DC induced T lymphocytes proliferation [45].
Additional studies demonstrated that DC-SIGN was identical to the HIV-1 gp120 binding C-type lectin, previously cloned from a placental c-DNA library, [46] and that it mediated capture of HIV-1 and CD4 T lymphocytes infection through a in *trans* mechanism [47].
The function of DC-SIGN in HIV-1 infection was further investigated and its involvement in the recognition and immune escape of several other pathogens was elucidated.

### 3.1 Cromosomic localization and structure of DC-SIGN

The genes encoding human DC-SIGN (CD209), which contains 7 exons and 6 introns, are located on chromosome 19p13.2-3 and are about 13 kb in length. The gene for DC-SIGN is adjacent to gene of CLR CD23 and next to its closely related gene, DC-SIGNR or L-SIGN [48,49]. DC-SIGN belongs to C-Type lectins, a superfamily of proteins that bind specific sugars in a calcium-dependent manner.
As most C-type lectins, DC-SIGN is a type II transmenbrane protein. DC-SIGN consists of an extracellular domain, a transmembrane region and a cytoplasmic domain (Fig. 3).

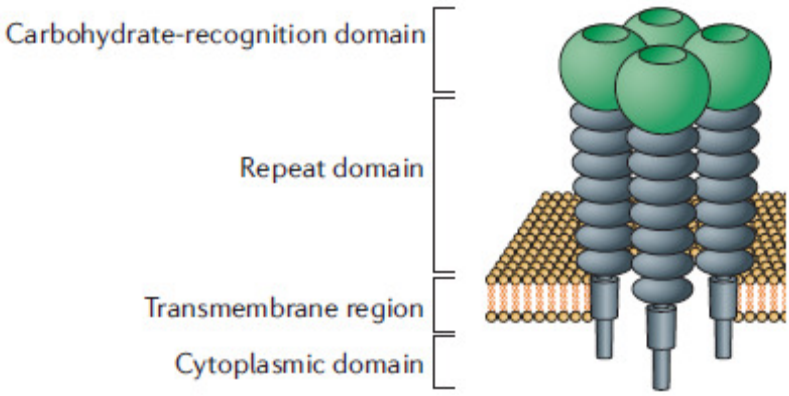



***Figure 3.*** *DC-SIGN tetramer structure.*

The extracellular domain contains a carbohydrate recognition domain (CRD) and a neck domain or hinge domain. CRD could recognize specific carbohydrate residues like high mannose, Lewis$^x$ and mannose-cap (see paragraph 3.8). The CRD of DC-SIGN is a globular structure consisting of 2 α-helices, 12 β-strands, and 3 disulphide bridges. A loop protrudes from the protein surface and forms part of two $Ca_2$+ binding sites (Fig. 4). One of such sites is necessary for the conformation of the CRD, and the other is indispensable for direct coordination of the carbohydrate structures. Four amino acids (Glu$^{347}$, Asn$^{349}$, Glu$^{354}$ and Asn$^{365}$) interact with $Ca_2$+ at this site and determine the recognition of specific carbohydrate structures. Mutations of these sites cause the loss of ligand binding. Carbohydrate ligands interact with $Ca_2^+$ through hydroxyl groups and with Glu and Asn through hydrogen bonds. A valine (Val$^{351}$) residue in the CRD is involved in interaction with some of ligands, suggesting that different ligand have distinct, but overlapping, binding sites [50,51].

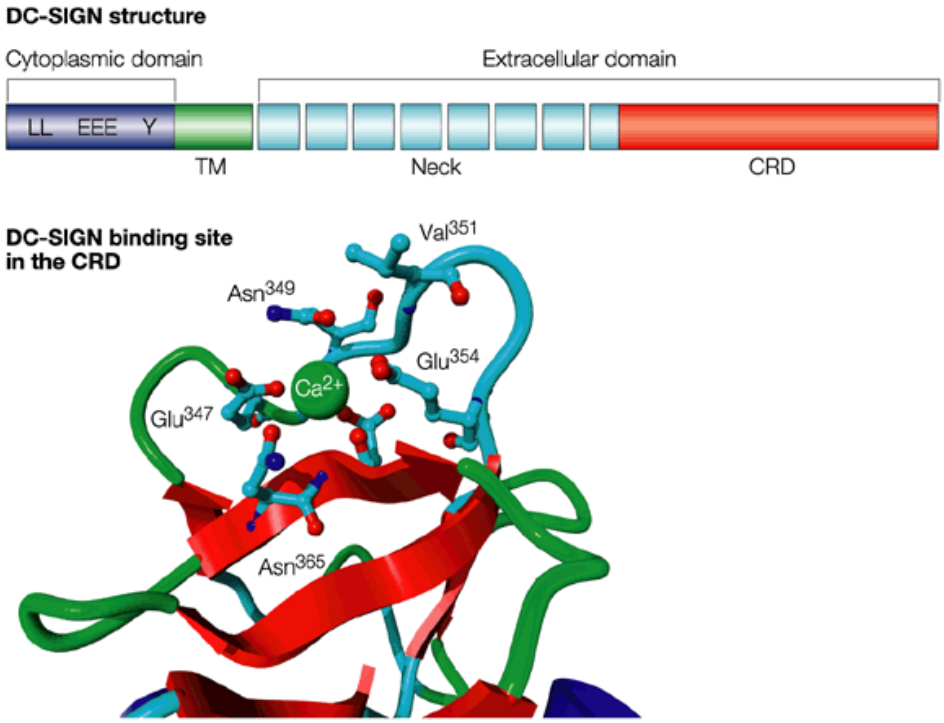



***Figure 4.*** *Structure of DC-SIGN binding site in the CRD.*

The neck domain comprises seven or eight complete IgG like tandem repeats and one incomplete repetitive sequence, that are required for oligomerization and influence carbohydrate specificity. DC-SIGN molecules form tetramers through their neck domain, thus allowing high affinity recognition of specific ligands [52].
Transmembrane region is essential in localization of DC-SIGN on cell surface.
The cytoplasmic region contains internalization motifs, such as di-leucine (LL) motif, tri-acidic (EEE) clusters and an incomplete immunoreceptor tyrosine based activation motif (ITAM). The LL motif and the triacidic clusters are involved in pathogen internalization. ITAM participates in signal transduction [53].
In addition to this prototypical structure, alternative splicing events generate DC-SIGN isoform transcripts whose presence exhibits inter-individual variations. Analysis of DC-SIGN neck polymorphisms indicated that the number of allelic variants is higher than previously thought and that multimerization of the prototypic molecule is modulated in the presence of allelic variants with a different neck structure. It was also demonstrated that the presence of allelic variants or a high level of expression of neck domain splicing isoforms might influence the presence and stability of DC-SIGN multimers on the cell surface [54].
Transcripts lacking transmembrane region were also reported. Recently soluble DC-SIGN variants (sDC-SIGN) were detected in *vitro*-generate DC culture supernatants as cell free secreted tetramers. Furthermore the presence of sDC-SIGN was demonstrated in several human body fluids, such as serum, joint fluids and bronco alveolar lavages [55].

## 3.2 DC-SIGN expression

DC-SIGN was firstly identified as specifically expressed by mature and immature DCs, but further studies found DC-SIGN expression also by other different cell types.
DC-SIGN is expressed by immature myeloid DCs (iDCs) in mucosal tissue of vagina, cervix, rectum, uterus and penis foreskin, in region beneath the epithelium. In skin section DC-SIGN was found express on dermal DCs. On the contrary Langherans cell residing in epidermis and in stratified epithelia of the vagina, ectocervix and foreskin do not express DC-SIGN but a langherans cell specific C-lectine called Langerin. Furthermore DC-SIGN is expressed by iDCs of placenta and lungs. DC-SIGN expressing DCs are present also in T cell areas of lymph nodes, tonsil and spleen and in Peyer's patches. Immature DCs derived from monocytes in *vitro*, a model to study DC function, also express elevated amounts of DC-SIGN [45,47,56,57,58,59,60].
Monocytes are negative for DC-SIGN, but several studies reported DC-SIGN expression by macrophages. DC-SIGN expression was found on macrophages in genital mucosae, in lymph nodes and on alveolar macrophages the lung. Subsets specialized macrophages in the placenta such as the macrophages of deciduas and Hofbauer cells, (fetal macrophages, abundant in chorionic villi) express DC-SIGN [59,61,62,63].

Although first works [45,47] did not find DC-SIGN expression on B cells, DC-SIGN expression by activated B cells was reported by other studies [64,65].
It was recently reported that megakaryocytes (MKs, the platelet precursors) and the 15% of platelets express DC-SIGN on their surface. The presence of DC-SIGN in MKs and platelets was demonstrated by various technical approaches, such as RT-PCR, Western blot, flow cytometry, immunoelettromicroscopy [66,67].

## 3.3 Role of DC-SIGN in DC-T cells interaction and in DC migration

DC-SIGN binds the self proteins Intracellular Adhesion Molecule 2 and 3 (ICAM-2 and ICAM-3), mediating respectively DC migration and DC-T cell interaction.
Both ICAM-2 and ICAM-3 are glicosylated protein and contain high mannose oligosaccharides and Lewis$^y$ residues. Removal of these oligosaccharides abrogates the binding to DC-SIGN.
By binding ICAM-2 expressed on endothelium, DC-SIGN acts as a rolling receptor and favours migration of precursor DCs from blood. The glycosylation of ICAM-2 contributes to its recognition by DC-SIGN. Cytokines up regulated DC-SIGN and induce migration of precursor DCs from blood into the periphery. Interactions between DC-SIGN and ICAM-2 mediate rolling along endothelial vessels and transmigration of DCs into the periphery.
Interaction of Selectin and DC-SIGN with ICAM-2, in presence of chemokines, activates integrins such as LFA-1 and induces adhesion. Meanwhile, ICAM-1 up-regulated by inflammatory mediators strengthens such adhesion through LFA-1/ICAM-1 interaction. Thus, DC-SIGN/ICAM-2 interaction induces initial adhesion of DCs, while LFA-1/ICAM-1 interaction promotes their *trans*-endothelial migration. On antigen challenge, immature DCs will mature and migrate to the lymphoid tissues, where ICAM-2 is abundantly expressed on the endothelium of both vascular and lymphatic vessels. [68, 69, 70].
Interaction between DC-SIGN and ICAM-3 is important in the initial DC-T cell contact. Indeed, the initial bind of DCs to ICAM-3, expressed by resting T cells, is mediated by DC-SIGN and not by the $\beta_2$ integrins LFA-1 and $\alpha D\beta_2$. The interaction between DC-SIGN and ICAM-3, transiently stabilizes the DC-Tcell membrane contact to provide efficient TCR engagement. Subsequently the interaction mediated by LFA-1 and LFA-3 is required for a more stable DC-T cells contact.
Hence the transient nature of DC-SIGN/ICAM-3 interaction allows the screening of a large number of resting T cells by DCs, until productive TCR engagement is obtained [45,69].
Some studies showed that binding of DC-SIGN to both CEA-related cell adhesion molecule 1 (CEACAM1) and Mac1 is necessary to establish cellular interaction between DCs and neutrophils, and such interaction may promote T cell proliferation and differentiation to $T_H1$ cells. The ligand recognized by DC-SIGN on CEACAM1 is the Lewis$^x$ antigens. Thus, the binding of both Mac-1 and CEACAM1 to DC-SIGN is important for the modulation by neutrophils of T cell responses through interactions with DCs [71].

## 3.4 DC-SIGN interaction with pathogens

DC-SIGN, as well as the others C-type Lectin receptors (CLRs), acts as Pathogen Recognition Receptors (PRRs), recognizing specific carbohydrate structures that are present on surface of several microorganisms and internalizing microrganisms in endosomes and lysosomes through the di-leucine and tri-acidic cluster motifs [72].
DC-SIGN recognizes specific highly-glycosylated structures presented at the surface of viruses and non viral pathogens.
Indeed DC-SIGN recognizes envelope glycoprotein of several virus, such as HIV-1, HIV-2, SIV [47,63], Dengue Virus [73], Ebola Virus [74], cytomegalovirus [75] (CMV), Hepatitis C virus [76] (HCV), Human T-cell lymphotropic virus type 1 (HTLV-1) [77], Herpes virus 8 [65,78], Flebovirus [79] and Adenovirus [80,81]. Furthermore DC-SIGN binds component of cell wall of some bacteria [72] (*Mycobacterium tubercolosis*, *Helicobacter pylori*) and yeast (*Candida albicans* [82]) and recognizes also parasites (*Leihsmania pifanoi*, *Schistosoma mansoni*) [83].
Several of these pathogens have developed different systems to subvert DC functions and to escape immunity through the binding to DC-SIGN. Binding to DC-SIGN may promote pathogen infection and dissemination. These pathogens can also shift the $T_H1/T_H2$ balance towards a $T_H2$ response that favour pathogen persistence. These mechanisms have been described in detail for HIV-1.

## 3.5 DC-SIGN and HIV infection

Mieloid immmature DCs located in mucosal tissues of vagina, cervix, rectum and penis foreskin, site of entry of the HIV virus, express DC-SIGN and are proposed to be among to first cells to encounter sexually transmitted HIV [19].
In the vagina and uterine ectocervix DC-SIGN$^+$ DCs are located in subepithelial zone. The thick squamous epithelium acts as a barrier to HIV access to DC-SIGN. However physical breaches in epithelial integrity, caused by physical abrasion, ulceration, or inflammation, provide access for HIV to subepithelial DC-SIGN$^+$ DCs and other HIV cell target, strongly enhancing HIV transmission. Rectum and endocervix are more susceptible to HIV, being lined by a single layer of columnar epithelium. Interestingly recent studies described a more abundant expression of DC-SIGN in genital tissue of women and man at risk of infection [84,85].
DCs through DC-SIGN, by binding HIV-1 envelope glycoprotein gp120, efficiently capture the virus, without themselves became infected, and transmit the virus to CD4 T cells (Fig. 5) This particular mode of HIV-1 transmission is called infection *in trans* [47,86]. Moreover DC-SIGN can also facilitate DCs in *cis* infection.
In addition, binding of HIV to DC-SIGN induces complex intracellular signal pathways that interfere with Toll Like Receptors (TLRs) signalling and help the virus to evade immune responses and spread to cells (see chapter 3.6).

### 3.5.1 HIV-1 Infection in *trans*

DC-SIGN captures HIV-1 at low titres by binding with high affinity carbohydrate residues on gp120. Captured virion is internalized but escapes, at least in part, degradation into lysosomes.
Indeed a fraction of the virus is degraded in lysosomes and by proteasome and presented to MHCII and MHCI [87,88].

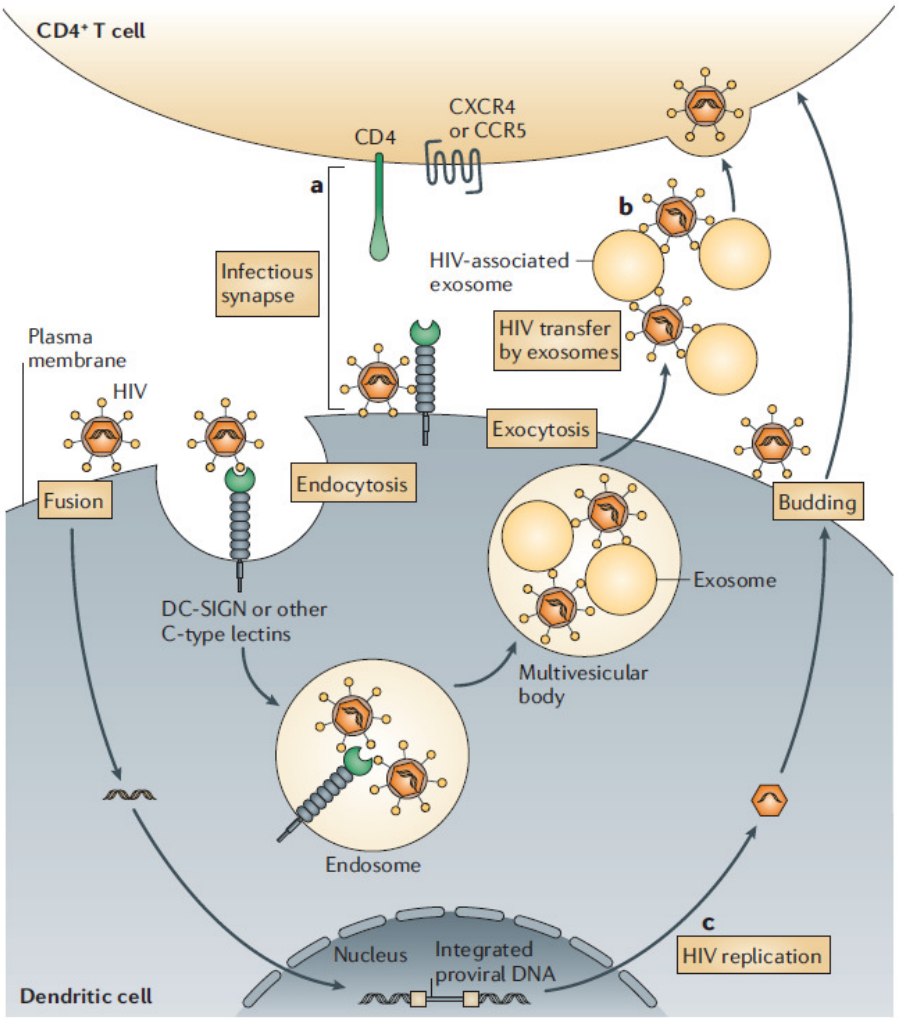



***Figure 5.*** *Mechanism of infection in trans.*

However part of the captured HIV is internalised into the endosomes and multivescicolar bodies, intracellular compartments with low pH, where it is protected from degradation and retained in a high infective state. Some data suggest that DC-SIGN expressed by DCs can retain HIV-1 in a highly infective state for more than 5 days, whereas free virus lost is infectivity after a day [47].
Neutralisation of the pH of endosomes and multivescicular bodies abrogates DC-SIGN mediated *trans* infection [89]. A relation between DC-SIGN ability to bind pathogens and its micro-localisation in plasma membrane was reported. DC-SIGN is concentrated in defined microdomains, with an average diameter of 200 nm. These microdomains are localized in lipid rafts and are important for binding and internalization of pathogens [90]. A clatrin dependent pathway is involved in endocytosis of DC-SIGN and allows recycling back of the receptor to cell surface through the di-leucine internalization motif.
DCs then transmit the virus in *trans* to nearby CD4 T lymphocytes in genital mucosae or, after migration to lymphoid tissue, to CD4 T lymphocytes resident in lymphoid tissue, promoting HIV-1 dissemination.
Viral transmission to target cells occurs across infectious synapses or through HIV associate exosomes.
First studies with MDDCs indicated that cell–cell contact is important for efficient CD4 T cell infection. Further studies have disclosed that HIV-1 transmission can occur across structures called infectious synapses. The structure of the infectious synapse is so called because of similarities to the immunological synapse, which is formed between antigen presenting cell and T cell. The set of cell surface molecules that contributes to the infectious synapse and are involved in the transfer of HIV-1 from DCs to CD4 T cells has not been fully identified.
HIV itself and HIV receptors are found concentrated at the infectious synapse, and DC-SIGN molecules are also detected at the infectious synapse. These data suggest that following contact with CD4 T cells DCs recycle HIV-1 to membrane, facilitating transmission to CD4 T cells. Interestingly, suppression of DC-SIGN expression abrogates the formation of the infectious synapse and inhibits *trans* infection of CD4 T cells with X4 HIV strains [91,92,93]. Recent data demonstrated that DC-SIGN induced signaling is responsible for viral synapse formation between DCs and T cells (see paragraph 3.9).
However other studies indicated that viral transmission to CD4 T cells can occur in the absence of the classically defined synapse.
Studies reported that endocytic vesicles called exosomes can mediate HIV *trans* infection. HIV-1 captured by immature MDDCs is quickly internalized into endosomal multivesicular bodies, endocytic bodies enriched in tetraspanins. Interestingly, a part of the endocytosed HIV-1 virions are constitutively released into the extracellular milieu associated with exosomes. Then exosomes are released in the extracellular space and can fuse with cells target membranes to transfer infectious virus [94,95]. The infectious virus that is associated with cell-free exosomes is only a part of the total virus that can be measured during synaptic transmission. The remaining virus endocytated multivesicular bodies in DCs is thought to enter the lysosomal pathway and be degraded [87,88].

Therefore, the exosome-release pathway might allow HIV to circumvent degradation after capture by DCs. The exosome-associated HIV particles that are released from immature MDDCs have been reported to be 10 fold more infectious than cell-free viruses on a per-particle basis. In the same way of immature MDDCs, HIV trafficking to the infectious synapse between mature MDDCs and CD4 T cells also involves a tetraspanin sorting pathway. HIV also exploits the exosome-release pathway of mature DCs to facilitate viral transmission and immune evasion [93].

In addition to DC-SIGN the proteoglycan Syndecan 3 and different C-type lectin such as Mannose Receptor (MR), DC-immunoreceptor (DCIR), CLEC 2 and the DC-SIGN omologue DC-SIGNR are involved in HIV infection in *trans* (see paragraph 3.6).

Besides DCs other cells types are reported to transmit HIV-1 in *trans* through DC-SIGN and other C-Type Lectins.
Macrophages express DC-SIGN and the mannose receptor and mediate HIV *trans* infection thanks to both these receptors.
Recent studies showed that DC-SIGN was highly expressed on the surface of alternatively activated M2a Monocyte Derived Macrophages (MDMs). Such MDMs were able to efficiently transfer HIV-1 to activated PBMCs and T cells through DC-SIGN. Thus, M2a macrophages expressing DC-SIGN may play an important role in the pathogenesis of mucosal infection and in sexual transmission [96,97].

Intriguily also platelets and megakariocytes were reported to transmit HIV-1 in *trans* (see paragraph 3.5.4).

### 3.5.2 HIV-1 infection in *cis*

DCs are susceptible to HIV infection but, due to low expression of HIV receptor CD4 and of HIV co-receptors CCR5 and CXCR4, efficiency of direct infection of DCs is lower than infection of CD4 T lymphocytes.
DC-SIGN, facilitating HIV interaction with CD4 and co-receptors, enhances DCs infection in *cis*. Indeed it was reported that DC-SIGN, when co-expressed with CD4 and co-receptors, allowed more efficient infection by both HIV and SIV strains. The augmentation of the efficiency of the infection varied from 2- to 40-fold, depending on the virus strain. However presence of CD4 and HIV co-receptor was necessary for productive infection [98].
Different evidences indicate that long-term HIV transmission DCs-mediated depends on viral production by the DCs. DCs infection results in de *novo* production and long term transmission of the virus to CD4 T lymphocytes, a phenomenon called “second phase transmission” [99]. Several days after viral exposure, progeny virions are transmitted to CD4 T cells by infected DCs. After exposure to HIV, pDCs and myeloid DCs from human blood can transfer the virus to CD4 T cells in the absence of a productive infection. However, after the DCs have been productively infected, it is mainly progeny virus that was transmitted to CD4 T cells.

### 3.5.3 DC-SIGN and HIV-1 vertical transmission

Placenta is implicated in a part of cases of HIV-1 vertical transmission from mother to child, expecially during the third trimester of pregnancy. Some studies displayed that DC-SIGN is expressed on specialized macrophages of placenta: the decidual macrophages and the Hofbauer cells (macrophages fetally derived) located in chorionic villi. These studies also showed a DC-SIGN implication in HIV-1 vertical transmission.
During pregnancy DC-SIGN expression by Hofbauer cells in the chorionic villi increases and this expression has been correlated with increased rates of HIV-1 vertical transmission. Thus, DC-SIGN can enhance the binding of HIV-1 on the surface of Hofbauer cells, providing an efficient mechanism by which the virus can be transmitted to target cells in *trans*. Furthermore Hofbauer cells can be infected by HIV-1, expressing CD4, CCR5, CXCR4. Hofbauer cells can entry in contact with maternal virus by contact with trophoblasts cells, that are susceptible to HIV-1 infection. Moreover, breaches in the wall of the trophoblasts can allow a contact between fetal Hofbauer cells and HIV-1 particles adsorbed to the maternal decidual macrophages or DCs expressing DC-SIGN, present in maternal blood. Hofbauer then can transmit the virus to fetus [100,101].

### 3.5.4 DC-SIGN and platelets

Platelets and megacariocytes (MK) can internalize HIV-1 and a considerable amount of HIV-1 in the blood of infected individuals is associated with platelets. DC-SIGN is expressed on platelets and MK, efficiently binds HIV-1 and allows virus internalization. In MK HIV-1 is internalized in multivescicular bodies. In platelets DC-SIGN also accumulates in specific endocytic vescicules and traffics to surface connected canalicular system [66]. Was further demonstrated that captured HIV-1 was maintained in an infectious state over several days and that platelets was able to transmit in *trans* the virus susceptible cells. These data suggest that HIV-1 can escape degradation by platelets and might use these cells to promote its spread. The C-type lectin CLEC2 also expressed by platelets contributes to HIV-1 internalisation and infection in *trans*. HIV-1 remains in a infective state for at least 3 days, a sufficient time frame to allow efficient platelet-mediated HIV-1 dissemination via the bloodstream [67].

## 3.6 DC-SIGN and other virus infection

DC-SIGN mediates the infection by several viruses. Mosquito-cell-derived Dengue Virus primarily targets immature dendritic cells (DCs) after a bite by an infected mosquito vector. DC-SIGN binds envelope glycoprotein E (gE) of Dengue Virus and is essential for productive infection of DCs [73]
DC-SIGN is involved in cellular entry of Ebola virus, by recognizing glycoprotein GP. Furthermore, DC-SIGN on the surface of DCs is able to function as a *trans* receptor, binding Ebola virus-pseudotyped lentiviral particles and transmitting infection to susceptible cells [74].

DC-SIGN is also required for CMV infection of DCs and is implicated in transmission in *trans* of the virus to fibroblast and endothelial cells. CMV envelope glycoprotein B is the ligand of DC-SIGN [75].
DC-SIGN and its liver homologue L-SIGN/DC-SIGNR (see paragraph 3.8) are receptors for HCV envelope glycoproteins E1 and E2. This glycoproteins recognize the same binding site on DC-SIGN as HIV-1. Internalized HCV virus-like particles are targeted to non-lysosomal compartments within immature DCs, where they are protected from lysosomal degradation in similar manner to HIV-1. L-SIGN is largely expressed on endothelial cells in liver sinusoids, whereas DC-SIGN is expressed on dendritic cells. Capture of circulating HCV particles by these cells DC-SIGN and L-SIGN positive facilitates virus infection of proximal hepatocytes and lymphocyte subpopulations and may be essential for the establishment of persistent infection [76,102,103]. The recent described soluble form of DC-SIGN (sDC-SIGN) is able to interact with CMV gB and promotes CMV infection in *cis* of DCs [55].
It was recently demonstrated that DC-SIGN plays a role in the infection of MDDCs as well as model B-cell lines by HTLV-1. Binding of DC-SIGN to HTLV-1 leads to productive infection of B-cell lines and MDDCs. Also blood myeloid DCs, in presence of IL-4 production induced by HTLV-1, express DC-SIGN and are productively infected by the virus. In addition DC-SIGN mediates HTLV-1 infection in *trans* of T lymphocytes [77].

## 3.7 DC-SIGN homologues

DC-SIGN homologues from species used as animal models for HIV and AIDS and from mice, as this species affords an opportunity to study the normal functions of DC-SIGN in *vivo*, were cloned and characterized.
Rhesus macaque, pigtailed macaque and chimpanzee DC-SIGN are highly similar to the human homologue. The in *vivo* expression of DC-SIGN in the non human primates seems largely similar to the expression in humans. The primate homologues are abundantly expressed by DCs in lymphoid tissues such as lymph nodes, as well as in mucosal tissues involved in sexual transmission of HIV-1. DC-SIGN expression on macrophages in both rhesus macaque and chimpanzee lymphoid tissue was also observed.
These homologues are functionally similar to human DC-SIGN. They bind HIV and SIV strains and have a high affinity for the immunological ligands of DC-SIGN: ICAM-2 and ICAM-3 [104,105].
In particular rhesus and pigtailed macaque DC-SIGN have an aminoacidical sequence highly similar to human DC-SIGN and differ from each other by only 5 aminoacids. These homologues share with human DC-SIGN approximately 87% amino acid identify overall. The degree of homology was highest in the lectin binding domain, with 93% amino acid identity. Endocytosis signals present in the cytoplasmic domain and the single N-linked glycosylation site were also conserved. The only significant difference was in the repeat region, where rhesus and pigtailed macaque DC-SIGN contained 6.5 copies of a 23-amino-acid repeat sequence, compared to 7.5 copies in the human protein. However, this do not have significant

functional consequences, since both rhesus DC-SIGN and pigtailed macaque DC-SIGN support HIV-1, HIV-2, and SIV binding and transmission [105].
Indeed the homologues bind the HIV-1 envelope glycoprotein gp120 and can act as a HIV-1 *trans*-receptor in the same way as human DC-SIGN, transmitting the bound virus to infection susceptible cell types. Antibodies against human DC-SIGN cross-react with the primate homologues of DC-SIGN and inhibit the function of these primate homologues.
Thus primate models and in particular the rhesus macaque model represent a potent tool in studying and developing DC-SIGN-based HIV treatment.
Five mouse genes homologues to DC-SIGN and DC-SIGNR, called mouse DC-SIGN, SIGNR1 (SIGN-related gene 1), SIGNR2, SIGNR3, SIGNR4, SIGNR5 were cloned. Mouse DC-SIGN is highly expressed in DCs, but not in macrophages and it was located in chromosome 8 next to CD23 gene.
In contrast to primate DC-SIGN, murine DC-SIGN exhibited only a 68% of homology to human DC-SIGN in the lectin binding domain and in the transmembrane domain of the protein, but the other domains are quite divergent. Murine DC-SIGN binds ICAM-3, HIV and SIV strains. However virus bound to murine DC-SIGN was not transmitted to receptor-positive cells, indicating that binding of virus to a C-type lectin protein does not result in efficient virus transmission [105,106].
DC-SIGN was also reported in other species. For example the cat DC-SIGN homologue is critical to cellular entry of Feline Infectious Peritonitis Virus [107] and the bovine DC-SIGN gene was recently identified and implicated in infection of bovine DCs by *Mycobacterium bovis* [108].

## 3.8 DC-SIGN signalling

Binding of pathogens to DC-SIGN induces a transduction signaling pathway that interferes with TLRs signaling, modulating immune responses. Upon pathogen binding, DC-SIGN induces different pathways converging to activation of serin/threonine kinase Raf-1. Specific DC-SIGN ligands induce distinct intracellular signalling pathways downstream of Raf-1 that lead to transcription of different gene targets and to different immunological responses, depending on the pathogen involved. Mycobacteria component ManLAM binds DC-SIGN and interferes with TLR4-mediated immune responses by impairing DC-maturation and enhancing IL-10 production. Similarly, DC-SIGN binding to the Lewis antigens on LPS from Helicobacter pylori induces IL-10 production and inhibits $T_H1$ polarization. In contrast, LPS from Neisseria meningitidis mutants induces a $T_H1$ response. Binding of HIV-1 to DC-SIGN enhances TLR induced IL-10 production [109, 110].
Upon stimulation, DC-SIGN activates three pathways that converge to activate Raf-1: a) Ras is activated and binds to Raf-1, inducing conformational changes that permit the subsequent phosphorylation of Raf-1 by Src and Pak kinases; b) Src kinases induce the phosphorylation of Raf-1 at residue $Tyr^{340/341}$; c) Rho GTPase-dependent activation of Pak kinases results in phosphorylation of Raf-1 at $Ser^{338}$.

Raf-1 then phosphorylates NF-κB subunit p65 at Ser276, leading to p65-acetylation. Acetylation of p65 both prolongs and increases IL-10 transcription, resulting in increased IL-10 production.
Furthermore, DC-SIGN signaling induced by HIV-1 binding triggers effects independent of TLR activation. Cross-linking of DC-SIGN with an antibody that mimics HIV-1-binding specifically affected the gene expression profile of DCs. Genes of MHCII, Jagged 1 and interferon response molecules are down-regulated. Other genes such as ICAM-1, the transcription factor ATF3 (a negative regulator of TLR4) and the chemokine MIP-1α are up regulated. This indicates that DC-SIGN signaling both modulates and represses TLR4 signaling.
In addition HIV-1 stimulation of DC-SIGN activates the guanine-nucleotide exchange factor LARG, that activates the GTPase RhoA, required for the formation of viral synapses between DCs and CD4 T cells [111].
A recent work showed that HIV-1 exploits the innate signaling pathways induced via TLR8 and DC-SIGN for its replication and productive infection of DCs. HIV-1 ssRNA stimulates TLR8 signaling that activates p65 subunit of NF-κB. Activation of p65 is necessary for transcription initiation of integrated HIV-1 provirus by RNA polymerase II. However a second signal provided by HIV-1 gp120 interaction with DC-SIGN is required for transcription elongation by RNAPII. Raf-1 dependent phosphorylation of p65 at $Ser^{276}$, induced by binding of HIV-1 to DC-SIGN, allows the recruitment of the transcription elongation factor pTEF-b to nascent transcripts. pTEF-b phosphorylates RNAPII at $Ser^{2}$ promoting transcription elongation by RNAPII. Thus, activation of both TLR8 and DC-SIGN signaling pathways results in full-length HIV-1 transcripts and DCs productive infection. Subsequently infected DCs can transmit the virus to CD4 T cells [112].

## 3.9 DC-SIGN ligands and DC-SIGN inhibitors

DC-SIGN through its CRD recognizes with high affinity fucose containing carbohydrates and mannose containing carbohydrates.
Indeed DC-SIGN binds with higher affinity Lewis blood-group antigens ($Le^{x}$, $Le^{y}$, $Le^{a}$, $Le^{b}$), that contain fucose residues in different anomeric linkages. On the contrary DC-SIGN is not able to recognise sialyl-$Le^{x}$, a ligand of L-, E- and P selectin. This indicates that DC-SIGN carbohydrate specificity is distinct from that of the selectins that mediate leukocyte rolling. $Le^{x}$ antigens are expressed by different pathogens. In *H. pylori*, $Le^{x}$ is present on surface-located LPS, whereas in *S.mansoni* $Le^{x}$ is expressed by all stages of the parasite, including soluble egg antigen [113,83].
Mannose containing structures are present on the surface of several pathogens, such as HIV, SIV, Ebola Virus, *Mycobacterium tubercolosis*, and *Leismania pifanoi*. DC-SIGN binds both internal mannose branched structures with a minimum of three mannose (high mannose) and end-standing di-mannose.
The main carbohydrate ligand recognized by DC-SIGN on HIV gp120 as well on other pathogens glycoproteins is the high mannose glycan $(Man)_9(GlcNAc)_2$, also named $Man_9$. High mannose is a branched oligosaccharide, characterized by a

structure quite complex, presented in multiple copies on HIV gp120 (Fig. 6). [114,53].
DC-SIGN is involved in the initial steps of HIV-1 sexually transmitted infection, as well as in infection by others pathogens, and therefore may be considered a new promising therapeutic target.
Owing to the role played by DC-SIGN in immunity and infection processes, different groups are working to design molecules capable of blocking the interaction between DC-SIGN and pathogens, especially HIV-1, in order to prevent pathogen transmission and infection. In particular the design and preparation of carbohydrate inhibitors of DC-SIGN is receiving increasing interest [115].
Because high mannose structure is quite complex, ligands mimicking the whole structure are not suitable for the application in biomedicine.
However $Man_9$ the terminal disaccharides Manα1-2Man present on $Man_9$ arms are probably involved in $Man_9$ recognition by DC-SIGN. For example, the neutralizing monoclonal antibody 2G12, that recognizes the highly glycosylated HIV glycoprotein gp120, interacts with this terminal disaccharide [116]. Furthermore fragments of $Man_9$ arms terminated by a di- or a tri-mannoside (Manα1-2Man or Manα1-6Manα1-2Man) were reported to bind to DC-SIGN almost as efficiently as the entire $Man_9$ [117].

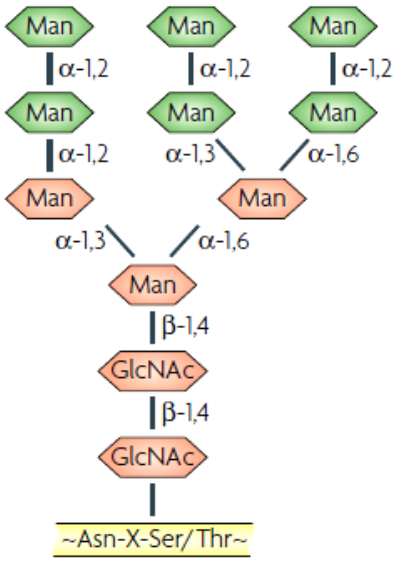



***Figure 6.*** *Structure of High-mannose-type N-glycans that are abundantly present on the envelope glycoprotein gp120 of HIV. Asn, asparagine; Fuc, fucose; Gal, Galactose; GlcNAc, N-acetylglucosamine; Man, mannose; SA, sialic acid; Ser, serine; Thr, threonine; X, any amino acid except proline.*

The design and the synthesis of structural and conformational analogues of $Man_9$ terminal di- and trisaccharides, were recently reported. Such compounds are more resistant to hydrolysis by glycosylases than the corresponding natural oligosaccharides and interact efficiently with DC-SIGN. One of these glycomimetic compounds exhibited anti-infective action against DC-SIGN-mediated infections by Ebola.
Furthermore these glycomimetics compounds can be easily linked to tetravalent (dendron) or polyvalent (dendrimer) scaffolds to augmentate avidity of binding to DC-SIGN [118].
Indeed another study reported that a mannosylated dendrimer was able to bind DC-SIGN and to inhibit interaction with HIV-1gp120-coated chips.
The Wang group reported oligomannose dendrons that display complex oligomannoses in high density and inhibit binding of gp120 to recombinant dimeric DC-SIGN with IC50 in the nanomolar range [119]. Penadés, Alcami and their groups have reported that gold nanoparticles (GNP) displaying various linear and branched mannosyl oligosaccharides are potent inhibitors of DC-SIGN mediated HIV *trans*-infection of human activated peripheral blood mono-nuclear cells [120].
Although most of the work are focused on mannose or mannose analogues, a fucose-based antagonist was recently reported. This new ligand is based on a fucosylamide anchor and inhibits DC-SIGN binding to mannosylated BSA (bovine serum albumin) with slightly higher potency than the natural ligand $Lewis^x$. Hence compounds able to compete with the binding of HIV gp120 to DC-SIGN are promising lead compounds in the perspective of developing new topical microbicides and HIV entry inhibitors.

## 3.10 Other C-type lectin as HIV-1 *trans* Receptor

The mannose receptor (MR, designed also as CD206) is a trimeric protein expressed by macrophages and by MDDCs. MR recognize mannose containing carbohydrates, but with different specificity respect to DC-SIGN. MR can capture HIV-1 and transmit the virus to CD4 T cells. Unlike HIV-1 bound to DC-SIGN, HIV-1 bound to MR has a shorter half-life than free virus. This finding suggest that internalization routes of MR and DC-SIGN are different [121,122].
The recently described DC immunoreceptor (DCIR) is transmembrane molecule constituted by an intracellular domain containing a ITIM motif, a transmembrane domain and an extracellular region that contain a CRD and a neck domain required for the oligomerization. DCIR is expressed on APCs (various DC subsets, monocytes, macrophages, and B cells). As DC-SIGN, it is down-regulated upon maturation of DCs. A recent work showed that DCIR participates in the capture of HIV-1 and promotes infection in *trans* and in *cis* of autologous CD4 T cells from human immature MDDCs [123].
DC-SIGNR (for DCSIGNRI related) or L-SIGN (Liver and lymph node specific) is a homologue of DC-SIGN, firstly described by Soilleux in 2000 [49]. The genes for DC-SIGN and DC-SIGNR are highly similar and are localised within a 30-kb region on chromosome 19p13.2-3, indicating that duplication of an ancestral gene may have happened. DC-SIGNR displays 77% amino acid identity with DC-SIGN. Like

DC-SIGN, DC-SIGNR binds to ICAM-3 and is able to bind and transmit different primate lentiviruses (HIV-1, HIV-2, and SIV strains) to susceptible cells.
DC-SIGNR is expressed by sinusoidal endothelial cells in the liver, endothelial cells present in lymph node sinuses, and a significant proportion of capillary endothelial cells in term placenta. Expression of DC-SIGNR on DCs or in other tissues has not yet been demonstrated [76,102,103].
Together DC-SIGN, DC-SIGNR is involved in vertical transmission of HIV from mother to child [100,101] (see paragraph 3.5.3). Furthermore, like DC-SIGN binds HCV glycoprotein E2 and mediated *trans* infection of liver cell by HCV and is involved in Ebola Virus infection.

## 3.11 Langerin

Langerin is a member of C-type lectins superfamily specifically expressed by Langherans cells (LCs). Similar to DC-SIGN, Langerin is a type II transmembrane protein endowed with an extracellular domain that contains a neck region for multimerization and a carboydrate recognizing domain. Like DC-SIGN, Langerin recognizes structures with high mannose glycan on HIV-1 gp120, but with different specificity. In contrast to DC-SIGN, the murine homologue of Langerin shows large similarities to human Langerin, allowing studies on Langerin function in the mouse model [124].
Recent data demonstrate that Langerin exerts a protective role against HIV infection, at least in the presence of low concentration of the virus, promoting rapid degradation and clearance of the virus. HIV-1 captured by Langerin was internalized into Birbeck granules, subdomains of the endosomal recycling compartment. Moreover Langerin protects LCs, that express CD4 and CCR5, from HIV infection and thus prevents LC-mediated HIV-1 transmission. Nevertheless, in the presence of high HIV concentration, LCs lose their protective function and can mediate infection in *trans* like DC-SIGN positive DCs or become themselves infected [125].
LCs are located in the stratified mucosal epithelia of the vagina, ectocervix and foreskin, in the epidermis of foreskin, glans penis and skin, but are absent in the columnar epithelium of the rectum and the endocervix. LCs protective function may explain the low rate of HIV-1 transmission when the mucosal barrier is intact. Damage to epithelium allows the contact of HIV with DC-SIGN$^+$ DCs increasing the risk of HIV infection. Furthermore rectal epithelium contains only DC-SIGN$^+$ DCs, and this may be one of the factors that contribute to the higher transmission rate of the virus in rectal intercourses.

# *MATERIALS AND METHODS*

# 1. VIRUSES

## 1.1 HIV-1 strains

The following laboratory-adapted HIV-1 strains were used in the experiments: the R5 tropic HIV-1 BaL (contributed by Drs. S. Gartner, M. Popovic and R. Gallo, courtesy of the National Institutes of Health AIDS Research and Reference Reagent Program), the R4 tropic HIV-1 IIIB (contributed by Drs. M. Popovic and R. Gallo), the dual tropic strain HIV-1 89.6 (contributed by Dr R. Collman) the R5 tropic DU174 (Source: Dr. L Morris). All these strains were provided through the EU programme EVA centre for AIDS Reagents (The National Institute for Biological Standards and Control NIBSC, Potter Bars, UK). Primary isolates HIV-1 V6 (CCR5 tropic) and V17 (CXCR4 tropic) were kindly provided by Dr. M. Andreoni (University of Rome Tor Vergata, Italy). The R5 tropic primary isolate 8g and the R4 tropic primary isolate DPMVF were kindly provided by Prof. Stefano Aquaro (University of Rome Tor Vergata, Italy). The HIV-1 strains were grown to high titer in Peripheral Blood Mononuclear Cells (PBMCs) from healthy donors.

## 1.2 Expansion of HIV-1 strains in PBMCs

PBMCs from healthy donors (PBMCs isolation procedure from blood will be described in the paragraph 2.3) were seeded at density of $2\times10^6$ cells/ml and were activated by culturing them in complete RPMI [RPMI 160 with 20% Fetal Bovine serum (FBS), penicillin and streptomycin (P/S) and L-Glutamine (LG); all from Euroclone (Siziano, Italy)] in presence of IL-2 (15 ng/ml; R&D Systems Minneapolis, USA) and PHA (7.5 µg/ml; Sigma Aldrich) for two days.
$60\times10^6$ activated PBMCs were incubated with different HIV-1 strains 3 hours at 37°C. After washing PBMC were maintained in culture at density of $10^6$ cells/ml in complete RPMI supplemented with IL-2 (15 ng/ml) for 14 days.
Two times per week the 50% of medium and of the cells was removed and replaced with fresh RPMI medium and pre-activated PBMCs.
Supernatants were collected, aliquoted and stored in liquid nitrogen. Viral concentration in supernatants was monitored by p24 ELISA. HIV-1 titer in supernatants were subsequently measured by $TCID_{50}$ quantitation.

## 1.3 Determination of HIV-1 titer by $TCID_{50}$ quantitation

Virus load of supernatants of HIV-1 expansion cultures, expressed as tissue culture infectious doses ($TCID_{50}$) were calculated by the accumulative method (Reed-Muench equation).
$TCID_{50}$ is defined as the median tissue culture infective dose; the amount of a pathogenic agent that will produce pathological change in 50% of cell cultures inoculated.

### 1.3.1 Procedure

PBMCs from 3 healthy donors were activated with IL-2 and PHA as described above.

At day 0 activated PBMCs were counted. Six fivefold dilutions of supernatants to test were prepared starting with a 1:5 dilution. $10^5$ activated PBMCs (pool of 3 donors) were seeded in a 96 well plate and were exposed to each viral dilution (in quintuplicate). The negative virus controls consisted in:

(I) 3 wells containing virus (at the lowest dilution) but without PBMCs

(II) 3 wells with activated PBMCs but no virus.

Then the plate was placed in incubator at 37°C and 5% $CO_2$. Two thirds of the medium were changed at day 1 and at day 3 post infection. At day 7 post infection supernatant were harvested for HIV-1 p24 antigen determination.

# 2. ISOLATION OF CD4 T LYMPHOCYTES AND MONOCYTES FROM PERIPHERAL BLOOD

## 2.1 Isolation of PBMCs

Peripheral blood was collected from buffycoat of healthy donors (provided by Transfusional Unit of Vimercate Hospital, Vimercate, Italy) following informed consent.
PBMCs were isolated by centrifugation 25 minutes at 2300 RPM, without brake, on a Ficoll discontinuous density gradient (Lympholyte-H, Cederlane Laboratories, Burlington, NC, USA).
After the centrifugation step, the blood sample was separated showing the following layers from top to bottom (Fig.1): plasma and platelets, the PBMCs band situated at plasma/Ficoll interface, Ficoll and red blood cells, covered by a granulocyte layer.
The PBMCs layer were carefully removed from the tube and transferred to a new conical tube. The PBMCs were then washed with Phosphate buffered saline (PBS) and cell number and cellular vitality were determined.

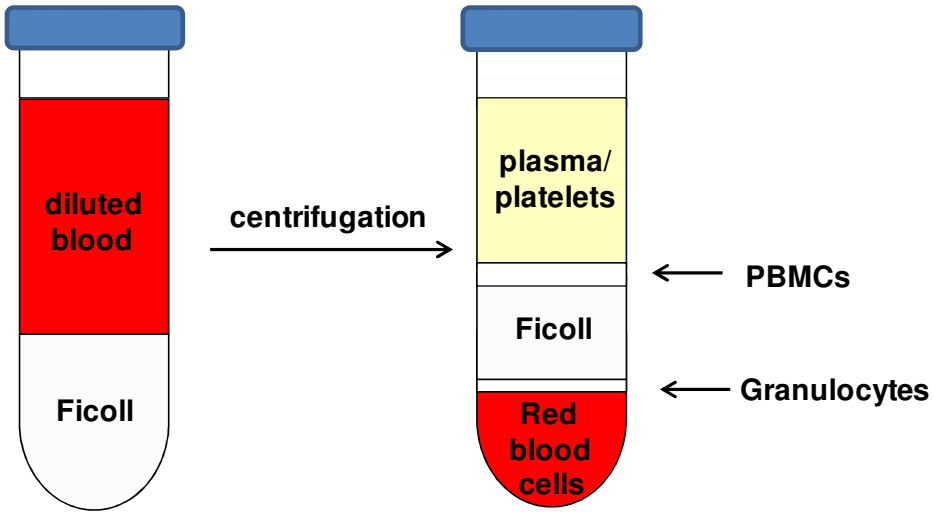



***Figure 1.*** *Isolation of PBMCs from pheripheral blood*

## 2.2 Cell count

Cell count was performed with the automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc, Corea). ADAM system provides two kind of staining solution:
1) AccuStain solution T for total cell counting constituted of Propidium Iodide (PI), a fluorescent nucleic acid dye, and a lysis solution.
2) AccuStain Solution N for non-viable cells counting, constituted of PI and PBS
The lysis solution of AccuStain Solution T disrupts cell membranes and allows staining with PI to measure total concentration of the cells.
In AccuStain solution N, live cells remained intact and were not stained. Only death cells were stained by PI and were detected. After staining the prepared cells were loaded into a chip and inserted into ADAM for cell counting. A 532 nm green laser was automatically focused onto the chip and cells that had been stained were recorded by a sensitive CCD camera. The image results were automatically processed, generating the count of non-viable and viable cells and the percentage of viability, which was displayed on the front of the instrument.

## 2.3 CD4 T lymphocytes and monocytes magnetic separation

CD4 T lymphocytes and monocytes were separated from PBMC by direct magnetic labelling using the CD4 microbeads or CD14 microbeads respectively (Miltenyi Biotech, Bergisch Gladbach, Germany), according to manufacturer's protocol.
After determination of PBMCs number, cell suspension were centrifuged at 300×g. Pellet was resupended in a specific buffer (containing PBS, 0.5% bovine serum albumin and EDTA 2mM) and magnetically labeled with CD4 or CD14 microbeads in ice.
After a 15 minutes incubation at 4°C cell were washed and resuspended in the specific buffer. MS columns were placed in the magnetic field of a MACS Separator (both from Miltenyi Biotech).
After rinsing of MS columns with the buffer, cell suspension were applied onto columns for positive selection. Unlabeled cells passed through the column. After 3 washing steps columns were removed from magnetic separator.
The magnetically labeled $CD4^+$ lymphocytes or $CD14^+$ monocytes retained in the columns were eluted as positively selected cell fraction. Purity of cell population was determined by staining with anti CD4 or anti CD14 antibody and Flow Cytometric analysis.
Cell number and vitality were determined and cells were used for subsequent analysis.

# 3. HIV-1 *TRANS* INFECTION ASSAY

## 3.1 B-THP1/DC-SIGN cells and DC-SIGN ligands

Human B cell line B-THP1 is derived from Raji B cells and is Epstein Barr virus (EBV) positive. B-THP1/DC-SIGN were obtained by transducing B-THP1 with the MLV vector MX-DC-SIGN and sorted by Fluorescence Activated Cell Sorting (FACS) as a population for high levels of DC-SIGN expression. Parental B-THP1 are used as negative control in DC-SIGN mediated HIV-1 transmission assay.
B-THP1 and B-THP1/DC-SIGN (contributed by Drs. Li Wu and Vinet N.KevalRamani; courtesy of the National Institutes of Health AIDS Research and Reference Reagent Program) were provided through the EU programme EVA centre for AIDS Reagents (NIBSC, UK). B-THP1 and B-THP1/DC-SIGN were cultured in RPMI supplemented with 10% FBS, P/S and L-glutamine LG. Both cell lines growth in suspension and have a doubling time of approximately one day.

## 3.2 Inhibition of *Trans* infection

CD4 T cells, (purified as described in paragraph 2.3), were seeded at density of $2\times10^6$ cells/ml and activated by culturing them in RPMI with 20% FBS, P/S and LG in presence of IL-2 (15 ng/ml) and PHA (7.5 µg/ml) for two days. DC-SIGN ligands, synthesized by the Department of Organic and Industrial Chemistry of the University of Milan, were diluted to desired concentration into culture medium. B-THP1/DC-SIGN cells or B-THP1 cells ($10^6$ cells/ml) were pre-incubated with different concentration of compounds **2b**, **11**, **12**, **14** and mannan (Sigma), or with culture medium alone (negative control) for 30 minutes prior to exposure to HIV-1 strains BaL, IIIB, 89.6, V6 or V17 (final concentration of all HIV-1 strain corresponding to 10 $TCID_{50}$) in the continued presence of the inhibitors for 3 hours at 37 °C and 5% $CO_2$. After extensive washing, to remove unbound virus and inhibitors, B-THP1/DC-SIGN cells were co-cultured with activated CD4 T cells in culture medium supplemented with IL-2 (15 ng/ml) at 37 °C and 5% $CO_2$ for three days. Cellular concentration of both B-THP1/DC-SIGN cells and CD4 T lymphocytes was $2\times10^6$ cells/ml and the ratio B-THP1/DC-SIGN: CD4 T lymphocytes was 1:4. To evaluate dendron **12** effectiveness in inhibiting infection in presence of increasing viral loads B-THP1/DC-SIGN cells or B-THP1 cells ($10^6$ cells/ml) were pre-incubated with dendron **12** (250 µM), or culture medium alone for 30 minutes prior to exposure to HIV-1 BaL (virus titer ranging from 5 $TCID_{50}$ to 80 $TCID_{50}$), in the continued presence of the inhibitor for 3 hours. Subsequently B-THP1/DC-SIGN cells were co-cultured with activated CD4 T cells, as described above. For some experiments B-THP-1/DC-SIGN cells were treated 30 minutes or 2 hours with the inhibitor or medium alone and washed with PBS to eliminate unbound compounds. After wash B-THP-1/DC-SIGN cells were put in culture medium and then were exposed 3 hours to HIV-1 BaL (10 $TCID_{50}$) immediately or after 6 or 12 hours. After incubation with the virus the cells were the co-cultured with CD4 T lymphocytes, as previously described.

## 4. HUMAN CERVICAL EXPLANTS INFECTION

Cervical tissue was obtained from premenopausal women (following written informed consent), HIV, HBV and HCV seronegative and without current genital infection, undergoing planned therapeutic hysterectomy at Sacco hospital (Milan, Italy). Endocervical 3 mm x 3 mm explants biopsies, comprising both epithelium and stromal tissue, was produced using a 3 mm diameter biopsy punches.
Tissues were transported in HBSS with phenol red medium (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (FBS), penicillin and streptomycin, L-glutamine (all from EuroClone, Italy) and 62,5 µg/ml gentamycin (Sigma, Saint Louis, Missouri, USA).
After washing the explants were either immediately treated with dendron **12** and infected with R5 tropic strains or were pre-stimulated for 2 days in presence of IL-2 (15ng/ml) and PHA (7.5 µg/ml) prior to X4 tropic HIV-1 DPMVF exposure in a non polarized way. More in detail, explants were pre-treated 30 minutes at 37 °C with different concentration of tetravalent dendron **12** diluted into culture medium or with culture medium alone (control). Afterwards explants were exposed to HIV-1 BaL ($2.6 \times 10^4$ $TCID_{50}$), 8g ($10^4 TCID_{50}$), DPMVF ($10^4$ $TCID_{50}$) or DU174 ($10^4$ $TCID_{50}$) in the continued presence of tetravalent dendron **12** for 3 hours at 37 °C. Subsequently explants were washed with RPMI to remove residual virus and compounds.
Unstimulated explants were cultured in RPMI medium supplemented with 20% FBS, penicillin and streptomycin(P/S), L-glutamine (LG) (all from Euroclone) and gentamycin (Sigma) at 37 °C and 5% $CO_2$. Stimulated explants were cultured under the same conditions in presence of IL-2 (15 ng/ml). Two third of the medium culture were repaced with fresh medium at day 3.
Supernatants of tissue culture were collected at day 3 and 7 post infection and assayed for p24 concentration.

## 5. DIFFERENTIATION OF IMMATURE DENDRITIC CELLS FROM MONOCYTES

CD14 positive monocytes were isolated from healthy donors peripheral blood as previously described (see paragraph 2.3).
Purified monocytes ($10^6$ cells/ml) were differentiated into immature Dendritic Cells (iDCs) by culturing them in RPMI with 10% FBS, PS, LG in presence of recombinant human IL-4 (20 ng/ml) and GM-CSF (20 ng/ml) (both from R&D Systems) for 6 days.
The 50% of culture medium was replaced at day 3 with fresh medium containing IL-4 and GM-CSF.
At day 6 the Monocyte Derived Dendritic Cells (MDDCs) were analyzed for the expression of DC-SIGN and of others surface markers typical of myeloid DCs. The absence of monocyte marker CD14 expression was also confirmed. The analysis was performed by flow cytometry (see chapter 7).
MDDCs were plated at $2\times10^6$ cells/ml in RPMI plus P/S, LG and 10% FBS in presence or absence (control) of dendron **12** (250μM) for 3, 24 or 48 hours.
RNA was extracted from MDDCs culture to evaluation of chemokine expression (see chapter 8). Supernatants were harvested and MIP-1α, MIP-1β and RANTES concentration in was evaluated by ELISA (see chapter 6).

# 6. ELISA

## 6.1 p24 ELISA

Co-culture supernatants were collected at day 3 post infection for *trans* infection assay and at day 3 and day 7 for cervical explant infection assay. p24 concentration, as a measure of HIV infection, was assayed using the Alliance HIV-1 p24 Antigen kit (Perkin Elmer, Boston, USA) following manufacturer's instructions. Microplate wells are already pre-coated with a monoclonal antibody endowed with a high specificity and affinity to HIV-1 p24. Standards and samples (when needed) were diluted in inoculated culture medium. A five point standard curve using two-fold serial dilution and an high standard of 100 pg/ml were prepared. A linear standard curve was generated. Firstly Triton X-100 was added to all microplate wells (except substrate blank) to disaggregate HIV-1 virion, then the standards and samples were added to designated wells and the plate were incubated 2 hours at 37 °C. Plate were washed with wash buffer (phosphate buffer plus 1% Tween-20). Biotinylated polyclonal antibody to HIV-1 p24 was added to all wells except substrate blank at 37 °C for 1 hours. After washes Streptavidine conjugated with the horseradish peroxidase was added and the microplate was incubated 30 minutes at room temperature (RT). After washing the chromogenic substrate ortho-phenilenediamine-HCL (OPD) were added 30 minutes to all wells at RT. Following incubation with OPD, yellow color directly proportional to amount of p24 captured was generated. Reaction was stopped by adding stop solution (4N sulfuric acid) to all wells. Plates were immediately read at 490 nm, with the correction wavelength set at 655 nm, using the IMark microplate reader equipped with Microplate Manager ® 6 software (both from Biorad, Segrate, Italy).

## 6.2 β Chemokine ELISA

MIP-1α, MIP-1β and RANTES concentration in supernatants of iDCs culture at 24 and 48 hours was evaluated using specific DuoSet kit (all from R&D Systems).
Plates were coated with Capture antibody specific to MIP-1α, MIP-1β or RANTES overnight at room temperature. Plate were then washed 3 times with wash buffer (0.05% Tween-20 in PBS) and blocked with Reagent diluent (1% Bovine Serum Albumine, BSA, in PBS) for 1 hour. Subsequently standard and samples were added to designated well. A seven point standard curve using 2-fold serial dilutions was used. The high standard was of 500 pg/ml for MIP-1α and MIP-1β and of 1000 pg/ml for RANTES. A 4 Parameter Logistic (4-PL) standard curve was generated. After washing biotinylated detector antibody was added and incubated 2 hours at RT. Plate was washed as described and Streptavidine was added to all wells 20 minutes. After washing substrate solution (1:1 mixture of Color Reagent A, $H_2O_2$, and Color Reagent B, Tetramethylbenzidine) was added to each well. After a 20 minutes incubation stop solution (2N was added sulfuric acid). The optical density of each well was read at 450 nm, with the correction wavelength set at 595 nm. Plates were read using the IMark microplate reader.

## 7. FLOW CYTOMETRY

DC-SIGN expression in the cell line B-THP1/DC-SIGN was checked by staining with the anti-human DC-SIGN PE monoclonal antibody (clone AZND1, Beckman Coulter, Miami, USA).
To confirm differentiation of monocytes to iDCs and DC-SIGN expression $2\times10^5$ cells were stained with the following anti- human monoclonal antibodies:
CD14 PE-cy7 (mouse IgG2a, clone RMO52), CD11c PE-cy5 (mouse IgG1, clone BU15), CD86 PE (mouse IgG2b, clone HA5.2B7) CD83FITC (mouse IgG2b, clone HB15a), DRII PE (mouse IgG1, clone Immu357), DC-SIGN PE (all from Beckman Coulter).
Cells were resuspended in PBS and stained with the indicated antibodies. After 15 minutes of incubation at room temperature in the dark cells were washed with PBS and fixed in 1% of formaldehyde (Sigma).
To evaluate the toxicity of dendron **12** B-THP-1/DC-SIGN cells were incubated with different concentrations of the compound for 3 hours and 30 minutes. To evaluate the toxicity of **12** PBMC were incubated with different concentrations of **12** (0.25 mM, 0.5 mM, 1 mM) for 3 or 7 days. After the incubation period with the dendron **12**, the cells were stained with DNA incorporating dye 7-aminoactinomicyn D (7-AAD, Beckman Coulter) for 15 minutes at room temperature in the dark. After that 500µl of PBS were added and preparation were analyzed by flow cytometry.
The apoptosis was monitored evaluating the number of dead cells according to forward and side scatters of flow cytometric analysis and the staining with 7-AAD. All flow cytometric analyses were performed using a CYTOMICS FC-500 flow cytometer equipped with a double 15-mV argon ion laser operating at 456 and 488, interfaced with CXP 21 software (Beckman Coulter). Green fluorescence from FITC was collected through a 525 nm band pass filter (FL1), orange-red fluorescence from PE was collected through a 575 nm band pass filter (FL2), red fluorescence from 7-AAD was collected through a 620 nm band pass (FL-3), red fluorescence from PE- Cy5 was collected through a 670 nm band pass filter (FL4), deep red fluorescence from PE- Cy7 was collected through a 755 nm band pass filter (FL-5). Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL-1, FL-2, FL-3, FL-4 and FL-5.

# 8. RNA EXTRACTION AND REAL TIME PCR

## 8.1 RNA extraction

RNA was extracted from cultured iMDDCs by using the acid guanidium thiocyanate–phenol–chloroform method. RNAzol B reagent (TEL-TEST, Inc., Duotech, Milan, Italy), a monophase solution containing phenol and guanidine thiocyanate, was used.
MDDCs were lysed in RNAzol B and the lysate were separated into aqueous and organic phase by the addition of chloroform (20% of RNAzol B initial volume used). Samples were centifuged (at 12,000g 15 minutes at 4°C) to efficiently remove DNA and proteins from the aqueous phase containing RNA. The undegraded, pure RNA is obtained from the aqueous phase by the isopropanol precipitation and washing with 75% ethanol.

## 8.2 DNAse treatment and retrotranscription

The RNA was dissolved in RNase-free water, and purified from genomic DNA with TURBO DNAse (Applied Biosystems/Ambion, Austin, TX), a genetically engineered form of bovine DNase I with greater catalytic efficiency than conventional DNase I at higher salt concentrations and lower DNA concentrations. A reaction mixture, containing 1µg of RNA, Turbo DNasi 1U and TURBO DNasi Buffer, were incubated 30 minutes at 37 °C. Then DNase was inactivated by DNase inactivation reagent (Applied Biosystems/Ambion), that binds and removes the divalent cations from DNase.
1 µg of RNA was reverse transcribed into first-strand cDNA in a 20-µl final volume. A reaction mixture, containing 1 µM random hexanucleotide primers, 1 µM oligo dT and the RNA, was heated at 70 °C for 5 minutes to melt secondary structure within the template. The mixture was immediately cooled on ice to prevent secondary structure from reforming. A dNTPs mix, 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 20 U Recombinant RNasi inhibitor and M-MLV 5X reaction buffer were added (all from Promega). The reaction mix were incubated 60 minutes at 42 °C and then heated 5 minutes at 95 °C to inactivate the RT.

## 8.3 Real time PCR

cDNA quantification for MIP-1α, MIP-1β, RANTES and GAPDH was performed by real-time PCR (DNA Engine Opticon 2; MJ Research, Ramsey, MN). Reactions were performed using a SYBR Green PCR mix (Finnzymes, Espoo, Finland). SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. Reactions were performed according with the following thermal profile: an initial denaturation (95 °C ,15 minutes) followed by 40 cycles of 15 sec at 95 °C (denaturation) and 1 min at 60 °C (annealing) and 20 seconds at 72 °C (extension). By recording the amount of fluorescence emission at each

cycle, the PCR reaction was monitored during exponential phase, where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. Melting point or dissociation curve analysis for amplicon identification, was performed.
The threshold line is the level of the detection or the point at which a reaction the reaction reaches a fluorescent intensity above background (the mean of fluorescence values detected from to third to tenth cycle, when target amplification it is no appreciable yet) . The threshold was set placed above baseline activity and in the exponential increase phase of the amplification for the most accurate reading. The parameter Ct (Threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the Ct value. A Ct value of 40 or higher means no amplification and this value was not included in the calculations.
Results were expressed as ΔΔCt and presented as ratios between the target gene and the GAPDH housekeeping mRNA. All the samples were analyzed in triplicate.

## 9. MTT ASSAY

Toxicity of dendron **12** was determined by a MTT based assay (In *vitro* toxicology assay kit MTT based, Sigma). The MTT system is a method to measure the activity of living cells or tissues via mitochondrial dehydrogenases activity.
Viable explants reduce the yellow tetrazolium salt 1-(4,5-dimethylthiazol-2-yl)-3,5diphenyl formazan (MTT) to purple formazan insoluble crystals.
Explants were cultured in medium culture alone (RPMI medium supplemented with 20% FBS, penicillin and streptomycin, L-glutamine and gentamycin) or with increasing concentration (0.25mM, 0.5 mM, 1 mM) of the compound **12**, diluted in medium culture, for 3 and 7 days. Regarding explants treated 7 days, the 50% of medium culture was changed at day 3 with fresh medium containing dendron **12** at the concentrations above indicated.
Media and salt solutions with phenol red contribute to higher background absorbance and can decrease sensitivity. Furthermore medium containing more than 10% of FBS may form a precipitate when MTT Solubilization Solution is added.
Therefore, after culturing, explants were washed five times in RPMI without phenol red (Euroclone). Explants were then incubated in medium RPMI without phenol red plus 10% FBS containing MTT (500 µg/ml) for a period of 4 hours.
Insoluble formazan product was dissolved by incubation with an amount of an acidified Solubilisation Solution (Sigma) equal to original culture media volume.
The MTT Solubilisation Solution consisted of 10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol.
Finally formazan absorbance was measured at 595 nm (IMark microplate reader).
Tissue viability was established by dividing the absorbance reading of the formazan by the dry weight of explants. Toxicity was determined by dividing viability of the dendron **12** treated explants by viability of untreated explant control.

# 10. SURFACE PLASMON RESONANCE (SPR)

Since the development of the first biosensor based on surface plasmon resonance (SPR), the use of this technique has progressively increased. Although there are several SPR-based systems, certainly the most widely used one is the BIAcore, (produced by BIAcore AB) which has developed into a range of instruments.
The BIAcore presents considerable advantages for analysis of weak macromolecular interactions, permitting measurements that are not possible using other techniques. The small sample volumes required for BIAcore injections (<20 µL) make it feasible to inject the very high concentrations (>500 µM) of protein required to saturate low affinity interactions.
SPR-based instruments use an optical method to measure the refractive index near (within ~300 nm) a sensor surface. In the BIAcore this surface forms the floor of a small flow cell, 20-60 nL in volume, through which an aqueous solution (the running buffer) passes under continuous flow (1-100 µL.min-1). In order to detect an interaction one molecule (the ligand) is immobilised onto the sensor surface. Its binding partner (the analyte) is injected in aqueous solution (sample buffer) through the flow cell, also under continuous flow. As the analyte binds to the ligand the accumulation of protein on the surface results in an increase in the refractive index. This change in refractive index is measured in real time, and the result plotted as response or resonance units (RUs) versus time (a sensorgram).
The BIAcore presents considerable advantages for analysis of weak macromolecular interactions, permitting measurements that are not possible using other techniques.
SPR technique has different application. One of these, exploited in this study, is is the measurement of equilibrium affinity.
The small sample volumes required for BIAcore injections (<20 µL) make it feasible to inject the very high concentrations of protein required to saturate low affinity interactions. Furthermore equilibrium affinity measurements on the BIAcore are highly reproducible [126].

## 10.1 Monovalent ligands affinity to DC-SIGN

The experiments were performed in a collaboration with the Institut de Biologie Structurale (Université Joseph Fourier, Grenoble, France).
DC-SIGN ECD protein (residue 66-404) has been overexpressed and purified as described previously [127] All experiments were performed on a BIAcore 3000 using functionalized CM4 chips and the corresponding reagents from BIAcore. Two flow cells were activated as previously described [75]. Flow cell one was then blocked with 50 µL of 1 M ethanolamine and served as control surface. The second one was treated with BSA-Manα1-3[Manα1-6]Man (BSA-Mannotriose, Dextra) (60 µg/mL) in 10 mM acetate buffer, pH 4. Remaining activated groups were blocked with 50 µL of 1 M ethanolamine. The final density immobilized on the surface of the second flow cell was 1200 RU. The BSA-Mannotriose used to functionalize CM4 chip harbours 15 glycosylation sites according to manufacturer. The affinity of the

various compounds was then estimated through a DC-SIGN ECD binding inhibition assay. The ECD of DC-SIGN was injected onto the BSA-Mannotriose surface, at a concentration of 20 µM, alone or in presence of an increasing concentration of the sugar derivatives. Injection were performed at 20 µL/min using 25 mM Tris-HCl, pH 8, 150 mM NaCl, 4mM CaCl2, and 0,005 % of P20 surfactant as running buffer.
To determine $IC_{50}$ values for sugar derivatives, the steady state binding responses of DC-SIGN ECD to BSA-Mannotriose surface were obtained from sensorgrams and converted to relative residual activity values. Relative $IC_{50}$ values were determined from the plots of sugar derivative concentration vs relative residual DC-SIGN ECD activity as previoulsly described [128].

## 10.2 Selectivity to DC-SIGN and Langerin

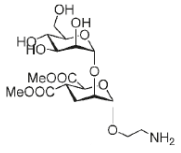
Extracellular domain (ECD) of Langerin (residue 68-328) were overexpressed, as inclusion BL21(DE3), refolded and purified to homogeneity in a functional form as described [129] DC-SIGN ECD protein (residue 66-404) were overexpressed and purified as described. All experiments were performed on a BIAcore 3000 using functionalized CM4 chips and the corresponding reagents from BIAcore. Two flow cells were activated. Flow cell one was blocked with ethanolamine and used as a control surface. The second one was treated with BSA-Manα1-3[Manα1-6]Man (Man-BSA, Dextra) (60 µg/mL) in 10 mM acetate buffer, pH 4. Remaining activated groups were blocked with ethanolamine.
The final density immobilized on the surface of the second flow cell was 5200 RU. The Man-BSA used to functionalize CM4 chip harbours 12 glycosylation sites according to manufacturer.
The affinities for DC-SIGN ECD and Langerin ECD of pseudo-mannotrioside and D-mannose were estimated by an inhibition assay, as described [128,130]. Each lectin was injected onto the Man-BSA surface, at 20 µM alone or in presence of an increasing concentration of compounds. Injections were performed at 5 µL/min using 25 mM Tris-HCl, pH 8, 150 mM NaCl, 4 mM CaCl2, and 0.005% of P20 surfactant as running buffer. The surface was regenerated by 1’ injection of 50 mM EDTA, pH 8. The $IC_{50}$ values were determined as previously described [128,130].

# *RESULTS AND DISCUSSION*

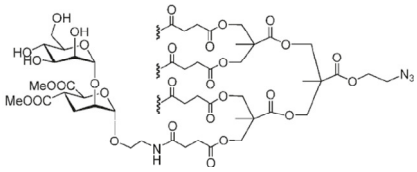
## 1. COMPOUNDS

The following glycomimetics compounds were assayed to evaluate affinity towards DC-SIGN and ability to block HIV-1 infection (Fig. 1):

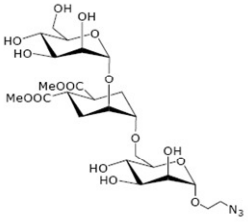
- Compound **1**: a pseudo-disaccharide that shares the three-dimensional structure and conformational behavior of Manα1-2Man terminal of $Man_9$. The molecule was built by connecting a mannose unit to a conformationally locked diol
- Compound **2b**: a pseudo-trisaccharide mimicking the linear mannotrioside Manα1-2Manα1-6Manα of $Man_9$ by replacing central mannose unit with a carbocyclic diole
- Compound (or dendron) **11**: a tetravalent dendron containing four copies of the monovalent compound **1**
- Compound (or dendron)**12**: a tetravalent dendron containing four copies of the monovalent compound **2b**
- Compound (or dendron) **14**: a tetravalent dendron containing four copies of mannose



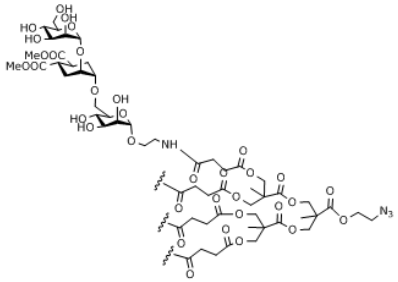
**Compound 1**



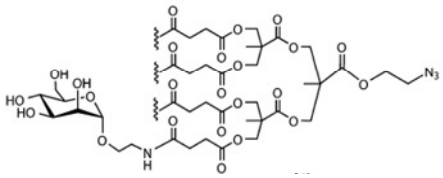
**Compound 11**



**Compound 2b**



**Compound 12**



**Compound 14**

***Figure1.*** *Chemical structure of the synthesized compounds*

## 2. EVALUATION OF MONOVALENT GLYCOMIMETICS AFFINITY TO DC-SIGN

Surface plasmon resonance (SPR) was used to compare the DC-SIGN recognition properties of the two monovalent ligands **1** and **2b** showed in Fig. 1. As previously described, because of the natural low affinity of monovalent compounds to DC-SIGN, evaluation of their relative affinity cannot be obtained in a direct interaction mode, but can only be accessed through a competition assay recently reported [131].

The experiments were performed in a collaboration with the Institut de Biologie Structurale of the Université Joseph Fourier (Grenoble, France). For this assay, a CM4 SPR chip functionalized with Man-BSA containing 15 glycosylation sites displaying the Manα1-3[Manα1-6]Man trisaccharide was used. The DC-SIGN extra-cellular domain (ECD) exhibited good affinity (in the µM range) for this surface. Inhibition studies were then performed using DC-SIGN ECD, at a fixed concentration, injected alone or in the presence of an increasing amount of the different ligands. As a reference, a competition experiment has been performed with D-mannose.

The efficiency of inhibition as a function of compounds concentration is directly related to the ligand affinity towards DC-SIGN ECD (Fig. 2). An $IC_{50}$ of 2.51 mM was estimated for mannose. Indeed, within experimental deviations, the $IC_{50}$ of D-mannose was in the range of previously reported experiments [132], thus validating this test.

As displayed in the Figure 2, the $IC_{50}$ of the compounds **1** and **2b** was determined to be 1.01 mM and 125 µM, respectively. Hence the monovalent ligands **1** and **2b** displayed an affinity to DC-SIGN much more higher than D-mannose, thus confirming a direct contribution of the additional carbocyclic-diol in the binding to DC-SIGN CRD. While the interaction properties of the pseudo-disaccharide **1** with DC-SIGN have already been demonstrated by NMR [118], it was shown here for the first time that its affinity is superior than that of mannose.

In compound **2b**, the addition of a second mannose unit mimicking an α1-6 link and resulting in a linear trimannoside mimic, improves the affinity by one order of magnitude relative to mannose.

Both glycomimetic compounds discussed here represented therefore interesting ligands to pursue with multivalent versions. Thus, considering affinity to DC-SIGN of the pseudo-disaccharide **1** and of the trimannoside mimic **2b**, these compounds and the corresponding tetravalent versions were selected to test its capability to inhibit the HIV-1 infection in *trans* infection assay.

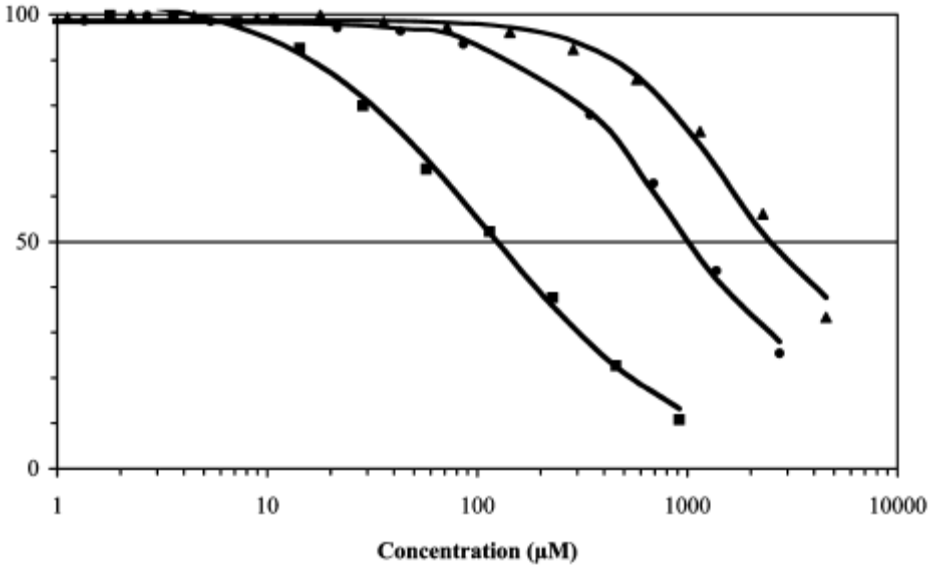



**Figure 2.** *Comparison of inhibitory properties of different compounds on interaction between DC-SIGN and BSA-mannotriose. DC-SIGN ECD at 20 M was incubated with mannose (▲), **1** (●), or **2b** (■) and injected on a BSA-mannotriose functionalized surface. $IC_{50}$ values are 2.51 mM for mannose, 1.01mM for compound **1**, and 125 µM for compound **2b**.*

# 3. EVALUATION OF GLYCOMIMETICS COMPOUNDS ABILITY TO INHIBIT HIV-1 *TRANS* INFECTION DC-SIGN MEDIATED

## 3.1 Description of In *vitro trans* Infection assay

Synthesized compounds were tested for the ability to inhibit HIV-1 transmission in an in *vitro trans* infection assay. B-THP-1/DC-SIGN cells are derived from B-THP-1 human B cell line by transfection of DC-SIGN expression vector in order to express high levels of the DC-SIGN receptor. This cell line supports efficient DC-SIGN mediated HIV transmission and is a widely-used model system to mimic HIV capture and transmission to T-lymphocytes by dendritic cells [133]. In a first series of experiments, B-THP-1/DC-SIGN cells pre-incubated for 30 minutes in the presence or in the absence of the DC-SIGN inhibitors were subsequently exposed to (the Clade B R5 tropic laboratory-adapted strain HIV-1 BaL) in the continued presence of inhibitors. Mannan is known to inhibit DC-SIGN mediated viral infection and was used as positive control (0.25 mg/mL). Non transfected B-THP-1 cells were used as a negative control and, as expected, did not transmit infection (Figure 3). After washing, to eliminate unbound virus and compounds, the B-THP-1/DC-SIGN cells were co-cultured with activated CD4 T lymphocytes from healthy volunteer donors. Viral infection of CD4 T lymphocytes was assessed by measuring the concentration of the HIV core protein p24 in the co-culture supernatants by ELISA. p24, immunologically distinct from the protein of most other retrovirus, is a major structural core component of HIV-1and is estimated to be present at 2000-4000 molecules in each virion. Furthermore p24, unlike envelope glycoproteins, is well conserved [13]. The measurement of p24 levels is therefore a commonly exploited method to verify the successful infection by the virus. Each point was obtained in triplicate using CD4 T lymphocytes from at least three different healthy donors.

## 3.2 Comparison of the monovalent compound 2b and the dendron 12

Initially the monovalent pseudo-disaccharide **1**, the monovalent pseudo-trisaccharide **2b** and mannose were assayed. At 1mM the compound **2b** inhibited HIV-1 BaL (a R5 tropic laboratory-adapted strain) transmission to CD4 T by over the 90%. On the contrary, the compound **1** inhibited only partially the infection and mannose failed to inhibit infection even at concentration of 1M (not shown).
Such results are in accord with the SPR studies described above. Indeed the compound **2b** displayed the lowest $IC_{50}$ and so the higher affinity to DC-SIGN ECD among the compounds tested in SPR assay.
Due to these results, the monovalent **2b** was further tested and compared to corresponding tetravalent dendron **12**. The results obtained showed that the tetravalent compound **12** at 50 µM reduced the *trans* infection of CD4 T lymphocytes by over 90%; moreover, at 100 µM and at 250 µM inhibition was almost complete. Although the uncertainties deriving from the involvement of three

different donors are high in these experiments, the data suggest that higher concentrations of the monovalent compound **2b** (1 mM and 0.5 mM) are necessary to obtain a comparable inhibitory effect. Each point was obtained in triplicate using CD4 T lymphocytes from at least three different healthy donors (Fig. 3).

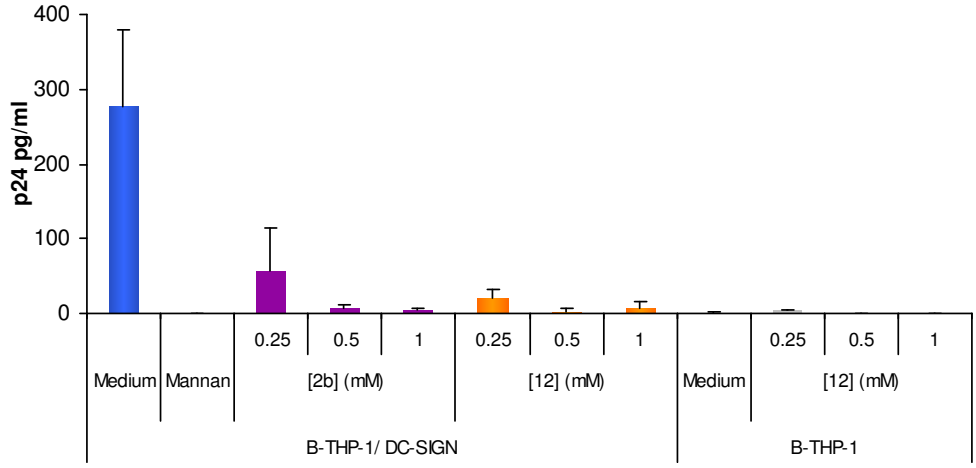



***Figure 3.*** *Inhibition of HIV transmission after treatment with* ***2b*** *and* ***12****. After 30' of preincubation with* ***2b*** *and* ***12****, B-THP-1/ DC-SIGN cells or B-THP-1 cells were incubated for 3 h with HIV-1 BaL in the presence of the indicated concentrations of compounds. Mannan (0.25 mg/ml) was used as positive control. Infection was assessed by measuring the concentration of p24 in the co-culture supernatants. Data were obtained in triplicate, from 3 different healthy donors. Values are mean ± SD (Standard deviation).*

In a second set of experiments the B-THP-1/DC-SIGN cells were first incubated with the inhibitors and then washed with PBS prior to exposure to HIV-1 BaL. In this case (Fig. 4) the tetravalent system **12** displayed highly efficient anti-infective properties with >94 % of inhibition at 100 µM. On the contrary, the monovalent compound **2b**, even when used at 5 mM concentration (data not shown), did not show anti-infective activity. Although alternative explanations cannot be discarded, these data suggest that the tetravalent system possesses significant affinity to DC-SIGN, which allows it to deploy its antiviral activity and to prevent the interaction between HIV gp120 and DC-SIGN even after a washing cycle. On the contrary, the affinity of **2b** is not sufficient for the DC-SIGN / **2b** complex to survive the washing step, hence viral transmission can occur when the cells are challenged with BaL. A quantitative assessment of the relative potency of **12** and **2b** was obtained in dose response experiments which will be described below (paragraph 3.3).

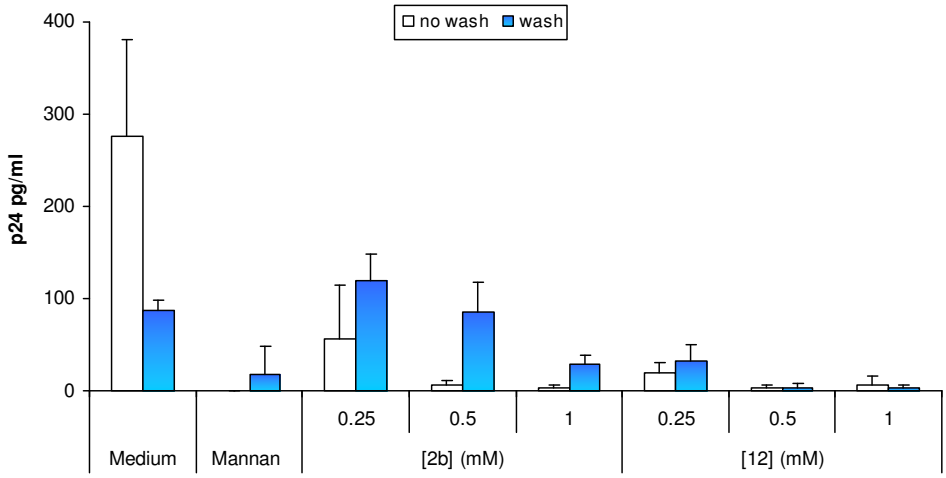



***Figure 4.*** *Effect of cell washing after treatment with the inhibitors. B-THP- 1/DC-SIGN cells were washed after 30 minutes of incubation with the inhibitor, before exposure to HIV-1 BaL. Results obtained with or without intermediate wash step are compared. Different concentrations of* ***2b*** *and* ***12*** *were assayed. Mannan (0.25 mg/ml) was included as a control. Values are mean ± SD.*

The distance spanned by two ligand units of **12** at full extension of the dendron arm is 28–30 Å at best, too short to allow simultaneous binding to two CRDs of the same DC-SIGN tetramer (from the recently published tetrameric model of DC-SIGN, derived from small-angle X-ray scattering studies, the distance between two adjacent $Ca_2^+$ sites is close to 40 Å) [127]. Although, depending on protein density on the cell surface, simultaneous binding to two CRDs on different tetramers could occur. Most probably, the ligand presentation on dendrons allows achievement of a high local concentration of recognition elements. With the use of dendrons, that are highly soluble in aqueous medium, this effect is obtained without increasing the viscosity of the system. Thus the dendrons are fully compatible with use in physiological fluids.

## 3.3 Dose reponse curves of monovalent 2b and tetravalent 12

Owing the previous results the properties of **12** were further analyzed. The protocol in which inhibitors were not removed by washing was applied in the following *trans* infection experiments, unless otherwise specified. Dose response curves were obtained for the compound **12** using CD4 T lymphocytes from healthy donors B-THP-1/DC-SIGN cells were challenged with HIV-1 BaL in the presence of increasing concentrations of **12**. After washing and co-culture with the CD4 T lymphocytes viral infection was assessed using analysis of p24 concentration in the supernatants. At 100 µM the inhibition of infection was complete for all donors and an $IC_{50}$ of 5 µM could be estimated (Fig. 5).

Dose response data were also obtained for **2b** and allowed to estimate an $IC_{50}$ of ca. 100 µM for the monovalent ligand (Fig. 5).

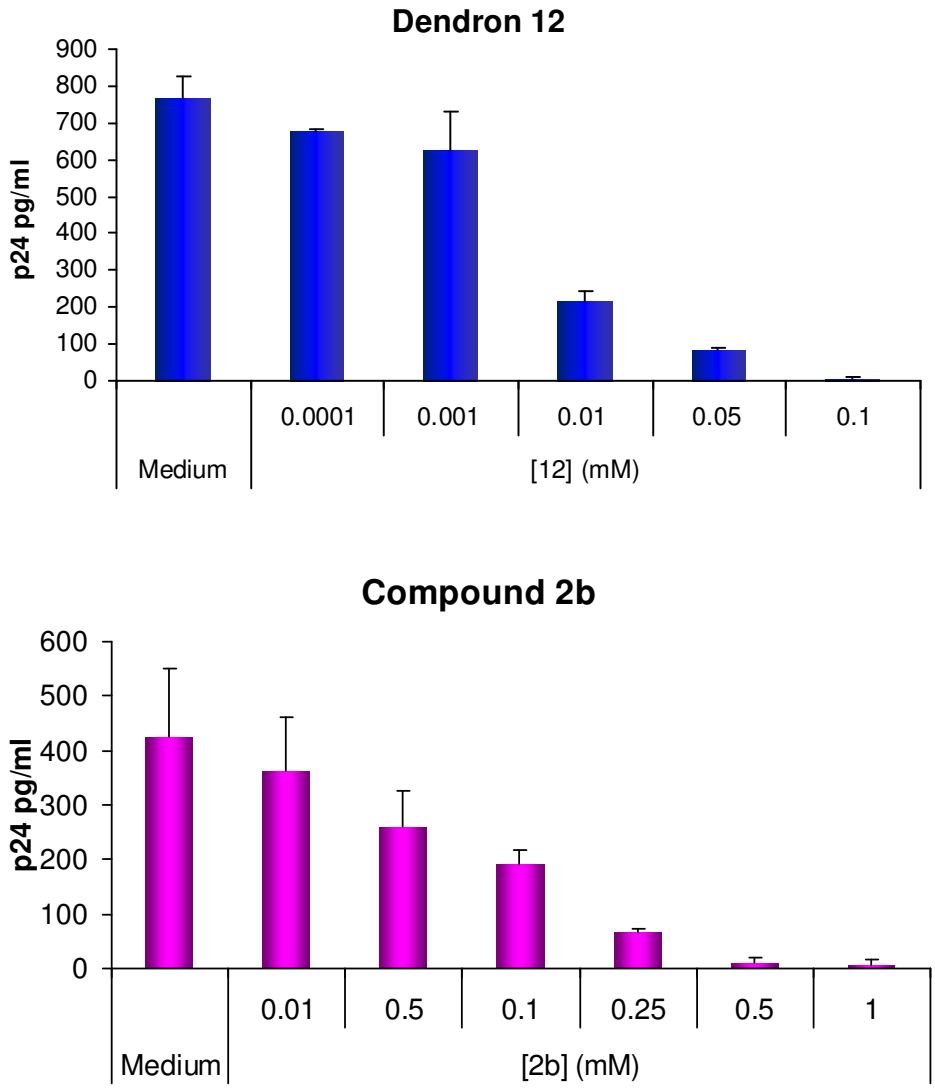



***Figure 5**. Dose-response results for dendron **12** and the monovalent compound **2b**. After a pre-tratment with different concentrations of **12** or **2b**, B-THP1/DC-SIGN cells were exposed for 3 h to HIV-1 BaL in the continued presence of the compounds. Experiments were performed on CD4 T cells of 3 healthy donors. Each donor was tested in duplicate. Values are mean ± SD.*

## 3.4 Comparison of tetravalent dendrons

The tetravalent dendron **12** was compared with dendron **11**, the tetravalent system presenting four **1b** units, and with dendron **14**, that contains four mannose units (see Fig. 1). Results showed that dendron **12** displayed a stronger inhibitory activity at both concentrations tested (100 and 10 µM). Notably, at 100 µM the compound **12** abrogated almost totally HIV-1 BaL transmission to CD4 T lymphocytes. The dendron **11** reduced the infection by about about the 85% of the infection, whereas the inhibition provided by dendron **14** was only partial (approximately 65%) (Fig.6). The results obtained are consistent with the affinity difference estimated by SPR studies for the corresponding monovalent binding elements (the linear trimannoside mimic **2b** contained in **12**, the pseudodi-saccharide contained in dendron **11**, and mannose contained in dendron **14**).

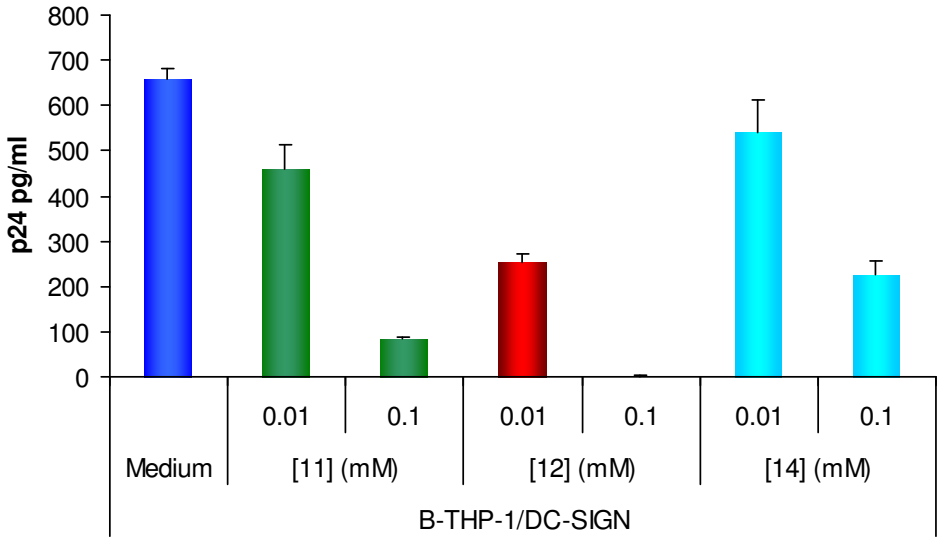



***Figure 6.*** *Comparison between dendrons **11**, **12** and **14**. Compounds ability to inhibit HIV-1 Bal trans infection was evaluated. Experiments were performed on CD4 T cells of 3 healthy donors. Each donor was tested in duplicate.*
*Values are mean ± SD.*

## 3.5 The dendron 12 inhibits the HIV-1 infection induced by X4 tropic and dual tropic strain

DC-SIGN can mediate *trans* infection by both R5 tropic and X4 tropic HIV-1 strains. Thus, we next verified the capability of dendron **12** to block transmission of the HIV-1 laboratory strain IIIB (X4 tropic) and 89.6 (dual tropic, R5/X4).

Results showed that transmission of virus to CD4 T lymphocytes was almost completely prevented at 250 and 100 µM and about 90% at 50 µM, when B-THP-1/DC-SIGN cells were challenged with either viral strains (Fig. 7).

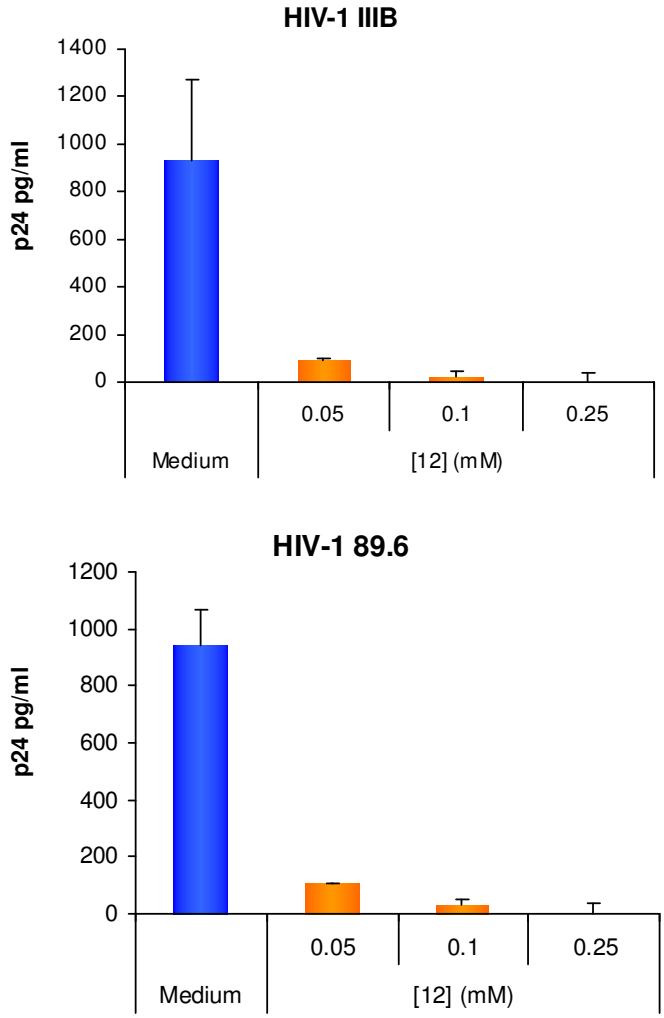



**Figure 7.** *Inhibition of trans infection mediated by laboratory-adapted HIV-1 strain (IIIB and 86.9). Virus transmission was determined by measuring the concentration of p24 in the supernatants. Experiments were performed on 3 healthy donors CD4 T cells. Values are mean ±SD.*

## 3.6 The dendron 12 inhibits the HIV-1 infection induced by primary HIV-1 isolates

In subsequent analyses the primary HIV-1 isolates V6 (R5 tropic) and V17 (X4 tropic) were utilized; also in this case results indicated an inhibition of HIV-1 infection with both types of virus > 99% at all concentration tested (Fig. 8).

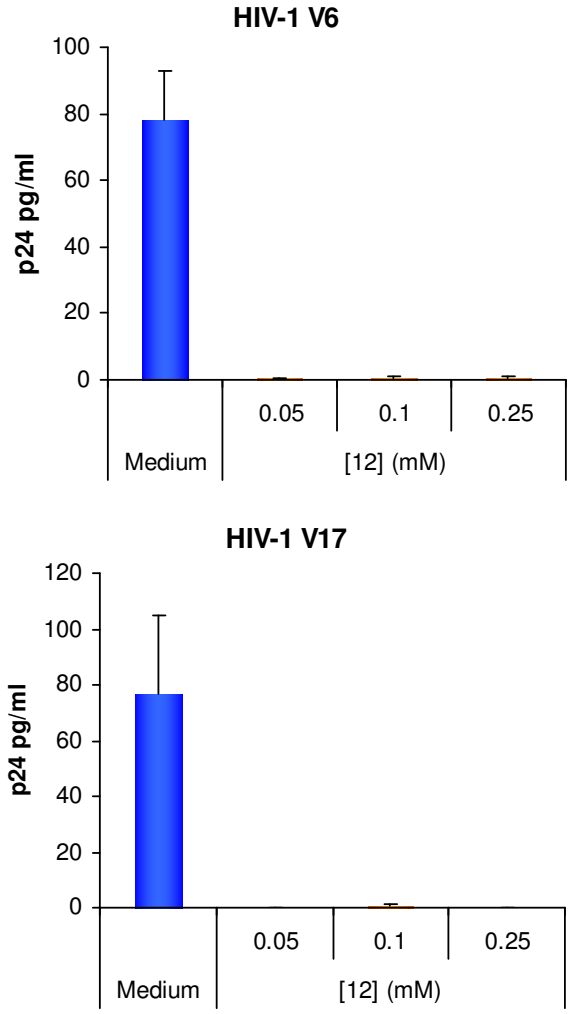



**Figure 8.** *Inhibition of trans infection mediated by primary viral isolates (V6 and V17). Experiments were performed on 3 healthy donors CD4 T cells. Values are mean ±SD.*

The anti-infective action is therefore independent of viral tropism as shown by inhibition of laboratory-adapted strains and primary isolates with different tropism.

## 3.7 Evaluation of dendron 12 antiviral effect duration

To evaluate the duration of the inhibitory properties of tetravalent dendron **12**, B-THP-1/DC-SIGN cells were treated for either 30 minutes or 2 hours with the compound at 250 µM. Dendron **12** was subsequently removed and cells were exposed to HIV-1 BaL either immediately thereafter, or after 6 and 12 hours. Subsequently B-THP-1/DC-SIGN cells were washed and co-cultured with CD4 T lymphocytes as described above. Virus transmission to CD4 T lymphocytes was almost completely abrogated at 0 and 6 hours post compound removal; notably, infection was still reduced by over 80% after 12 hours. Two hour pre-treatment did not appear to prolong inhibitory effect of the compound **12** compared to 30 minutes pre-treatment (Fig. 9).

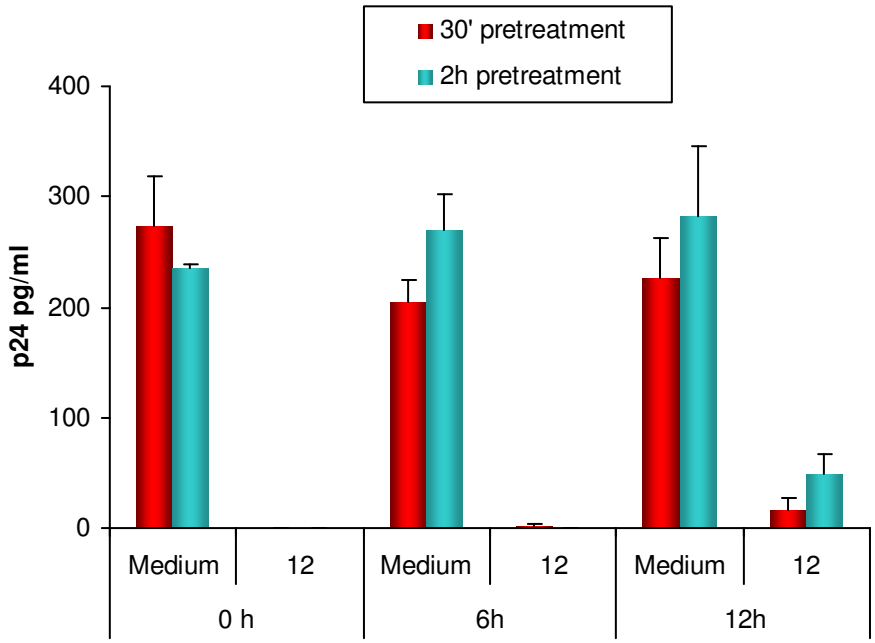



***Figure 9.*** *Persistence of inhibitory effect of **12** after compound removal.B-THP-1/DC-SIGN cells were incubated with 12 (0.25 mM) for 30' or 2 h. After extensive washing, cells were pulsed with HIV-1 BaL following 0, 6, or 12 h. Levels of infection (determined by p24 concentration in co-culture supernatants) 3 days post infection were shown. Experiment was performed on two donors. Each condition was tested in duplicate. Values are mean ± SD.*

The experiments shown in Fig.4 and the time-course studies described in this paragraph demonstrate that the antiviral effect of **12** persists after the cells have been washed, even for hours (Fig.9). The mechanism of this inhibition after removal could be based on the persistency of the multivalent ligand on the receptor binding site (slow off-rate of the tetravalent compound from the protein). However others mechanism can contribute to this effect (see chapter 4).

## 3.8 Dendron 12 ability to inhibit *trans* infection in presence of increasing concentration of HIV-1

The experiments carried out previously showed that the tetravalent dendron **12**, blocked almost completely infection in *trans* of CD4 T lymphocytes when a HIV-1 Bal concentration corresponding to 10 $TCID_{50}$ was used. Thus, we examined whether the dendron **12** was still able to inhibit the *trans* infection in the presence of increasing viral load. B-THP1/DC-SIGN were used as model to mimic HIV capture and transmission to CD4 T cells as above described. B-THP1/DC-SIGN cells were pre-incubated 30 minutes in presence or in absence of compound **12** (250 µM) and afterwards were exposed 3 hours to different inoculums of HIV-1 BaL, without removing the dendron. Indicated viral load (Fig.10), ranging from 5 to 80 $TCID_{50}$ (Tissue Culture Infective Dose $TCID_{50}$), were assayed.

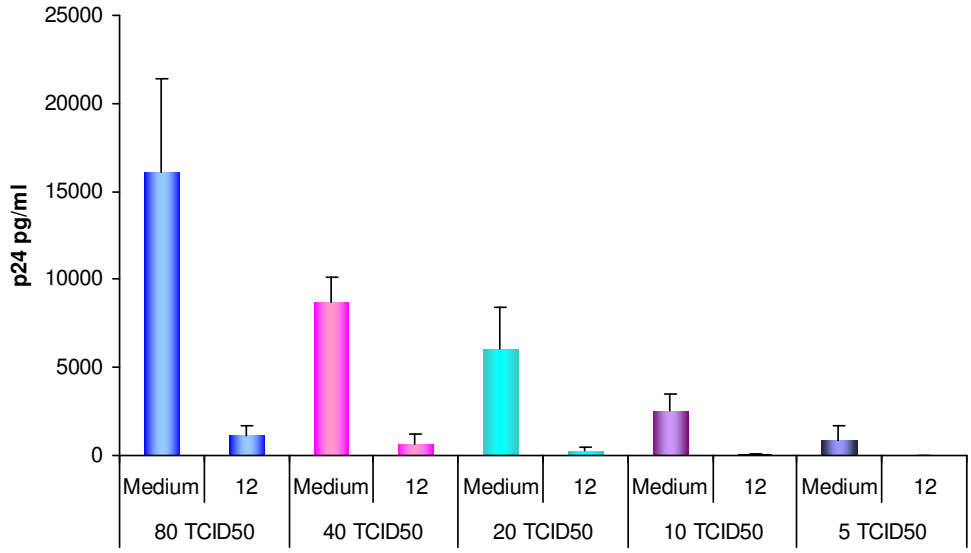



***Figure10.*** *Evaluation of dendron **12** ability to inhibit HIV-1 Bal trans infection in presence of increasing amounts of the virus. Data were obtained from 3 different healthy donors. Each donor was tested in duplicate. Values are mean ± SD.*

After BaI exposure B-THP-1/DC-SIGN cells were washed, to remove unbound BaL and dendron, and then co-cultured with activated CD4 T cells from 3 different healthy donors. Levels of viral infection were quantified by measuring amounts of HIV core protein p24 in the supernatants of co-cultures by ELISA.
Non transfected B-THP1 cell (negative control) did not transmit the infection (not shown). As expected, when B-THP-1/DC-SIGN cells were exposed to increasing viral load infection level raised of CD4 T lymphocytes. At lower viral concentration (5 $TCID_{50}$ and 10 $TCID_{50}$) inhibition of BaL transmission to CD4 T cells was almost complete (more than 99% and more than 97% respectively). Even at higher viral load, corresponding to 40 and 80 $TCID_{50}$ the dendron was able to counteract the transmission of the virus to CD4 T lymphocytes. Indeed, more than 92% inhibition was observed (Fig.10).

This potent inhibitory activity is due both to elevate affinity for DC-SIGN of dendron **12** pseudo-trisaccaride units and to high avidity of binding, guaranteed by the tetravalent presentation on the dendron scaffold.
Others DC-SIGN inhibitors endowed with high affinity to DC-SIGN have been described so far. Dendrons which display complex oligomannoses in high density was reported to inhibit binding of gp120 to DC-SIGN with $IC_{50}$ in the nanomolar range [119]. Nevertheless the complexity of the oligosaccharides used limits the possibility of a therapeutic application. Gold nanoparticles displaying various linear and branched mannosyl oligosaccharides (Manno-GNPs) are potent inhibitors of DC-SIGN mediated HIV-1 *trans* infection of human activated PBMCs [120]. However, GNPs may have toxic effects in consequence of gold accumulation.

A recent study demonstrate that a vaginal gel formulation of the NRTI tenofovir reduced HIV infection by 50% (see paragraph 2.5 of introduction). Tenofovir and other topical microbicides can prevent localized infection of target cells in genital mucosae. However DCs DC-SIGN+ internalize and transport HIV to secondary lymphoid organs, rendering the virus inaccessible to inhibitory effect of the microbicides. Therefore inhibition of DC-SIGN is essential to block HIV-1 uptake and dissemination from migratory DCs.

## 3.9 Evaluation of compounds toxicity towards B-THP-1/DC-SIGN cells

The B-THP-1/DC-SIGN were incubated with dendron **12** 30 minutes or 3 hours and 30 minutes: the same incubation time utilized in *trans* infection assays.
7-aminoactinomicyn D (7-AAD) labeling of the cells after the incubation period with the dendron **12** showed that the anti-infective properties of the inhibitors are not an epiphenomenon due to cell death. The data obtained for **12** indicate that the percentage of 7-AAD positive cells (non viable cells) was below 1% and did not change significantly in the absence of the compound or in its presence up to 250 µM (Fig.11).

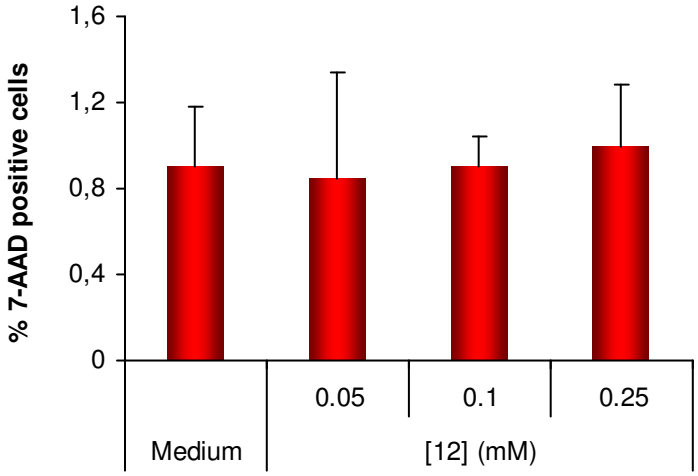



***Figure 11****.Toxicity of compounds. Percentage of 7-AAD positive B-THP1/DC-SIGN cells after 3 h and 30' of incubation with different concentrations of dendron* ***12****, or in the absence of inhibitor (Medium). Percentage of 7-AAD was determined by flow cytometry. Mean of two different experiments is reported. Values are mean ± SD.*

## 4. EFFECT OF COMPOUNDS 2b AND 12 ON DC-SIGN EXPRESSION

Compounds **2b** and **12** could exert their effect secondarily to the suppression of DC-SIGN expression. To examine this possibility, DC-SIGN expression was studied by flow cytometry experiments; B-THP1/DC-SIGN cells were stained with anti DC-SIGN antibody and both percentage of DC-SIGN positive cells and Mean Fluorescence Intensity (MFI) were evaluated after 3 hours and 30 minutes of incubation with different concentrations of monovalent compound **2b** and tetravalent dendron **12**, or in the absence of inhibitor. Results showed that incubation of cells with the compounds did not modify percentages of DC-SIGN positive cells (data not shown). A reduction of MFI was observed after treatment of the cells with **12**. The effect was dose-dependent: ca 20% reduction of Mean Fluorescence Intensity was observed for a 50 µM concentration of **12**, up to a 52% reduction for a 250 µM concentration of **12**. The monovalent ligand **2b** was tested at higher concentrations (Figure 11).
The effects observed may be at least partially due to increased internalization of DC-SIGN receptors after binding of the dendron. However, the concentration required to exert a noticeable effect in the flow cytometry studies seems somewhat higher than the infection inhibition concentration.

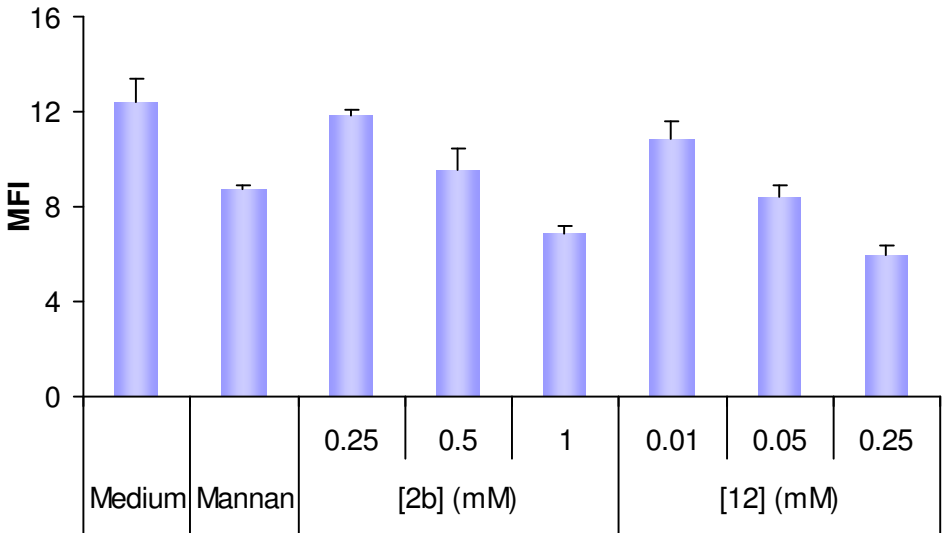



***Figure 12.*** *Mean Fluorescence Intensity. B-THP1/DC-SIGN cells were stained with anti DC-SIGN antibody or 3 hours and 30 minutes of incubation with different concentrations of **2b** and **12**, or in the absence of inhibitors (Medium). Mannan at 0.25 mg/mL is included as a reference. MFI was determined by flow cytometry.*

The dose-dependent reduction in MFI observed in the flow cytometry studies suggests that exposure to **12** may also alter somewhat significantly the observed cell surface concentration of DC-SIGN, possibly by induced endocytosis.
Depletion of receptor membrane concentration is an interesting feature of dendron **12** and could at least partially account for its antiviral activity. Complex mechanisms can be involved, and for this reason further studies are in progress to quantitatively understand the correlation between ant-infectivity and modification of cell-surface receptor concentration in this system, as well as to establish the underlying mechanisms.

## 5. SELECTIVITY OF DENDRON 12 TOWARDS DC-SIGN AND LANGERIN

Unlike DC-SIGN, the C-Type lectin receptor Langerin, expressed on Langerhans cells (LCs) appears to play a protective role against HIV infection. LCs are located in the stratified mucosal epithelia of female and male genital tissue. Both DC-SIGN and Langerin recognise high mannose glycan on gp120 through their CRD. Recent data demonstrate that Langerin prevent HIV transmission by LCs, at least in the presence of low concentration of the virus, promoting rapid degradation and clearance of HIV-1 [124,125].

Therefore, compounds designed to inhibit DC-SIGN could be of valuable interest if they were specific enough to avoid Langerin inhibition.

Both Langerin and DC-SIGN are able to recognized high mannose present on gp120, but have distinct specificities towards complex oligosaccharides.

Thus, specificity of pseudo-mannotrioside ligand for Langerin and DC-SIGN has been tested. Competition experiments using surface plasmon resonance (SPR) have been performed as previously described [131]. A CM4 sensorchip has been functionalized with BSA-Mannotriose and a fixed amount of the extracellular domain (ECD) of both lectins, DC-SIGN and Langerin, were injected over the surface in the presence or absence of pseudo-mannotrioside or mannose as a control. From the inhibition curves (Fig. 13a), an $IC_{50}$ of the two ligands towards each lectins can be evaluated and compared (Fig. 13b). A limited, if any, difference in favour of DC-SIGN is observed for mannose with an $IC_{50}$ of 3.4 mM towards DC-SIGN vs 10.3 mM towards Langerin (only a factor of 3). On the contrary, for the pseudo-mannotriose, $IC_{50}$ of 0.25 mM and 5 mM has been measured towards DC-SIGN and Langerin respectively.

So, while natural D-mannose does not discriminate between both lectin, the pseudo-mannotrioside, contained in dendron **12**, is 20 times more potent toward DC-SIGN than against Langerin. Moreover, from D-mannose to pseudo-mannotrioside the inhibitory potency towards DC-SIGN is increased by a factor 14.

Indeed, the use of allows real improvement in affinity but more importantly in selectivity for its use onto multivalent presenting scaffold to target more specifically $DC\text{-}SIGN^{+}$ cells within a complex human cervical tissue. Thus, the fact that the dendron **12** does not interfere with the function of Langerin is a remarkable advantage in view of developing new microbicide compounds.

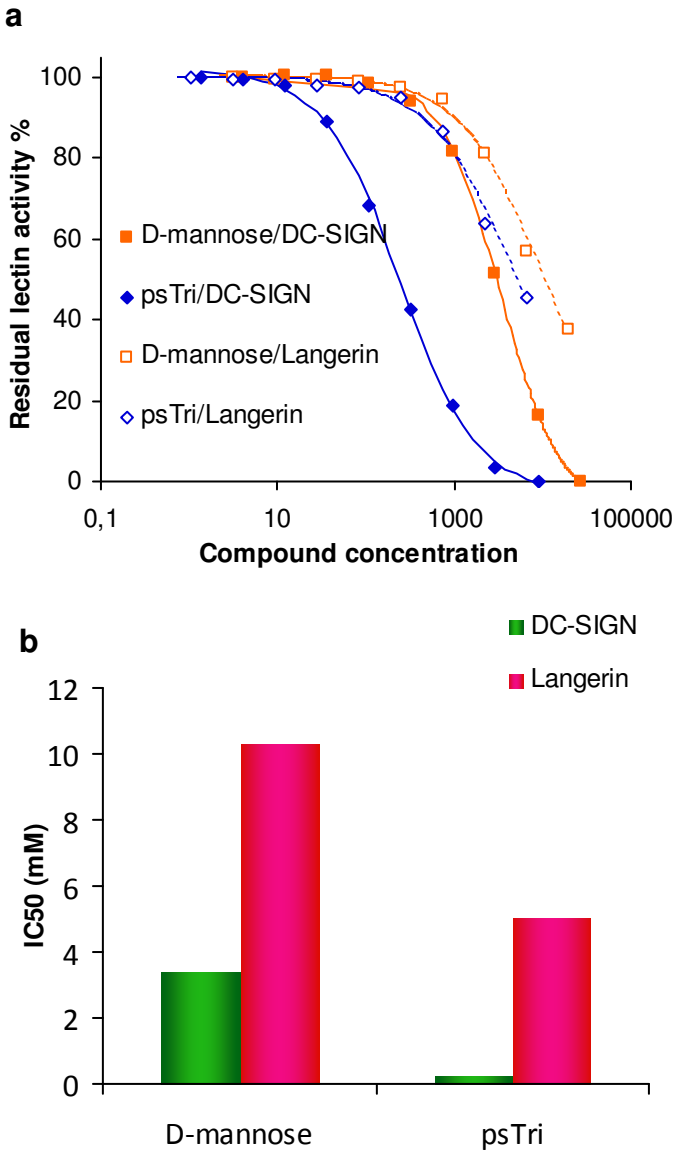



***Figure 13.*** *SPR experiment results of DC-SIGN ECD and Langerin ECD binding to Man-BSA/dextran surface inhibition by pseudo-mannotrioside (psTri) and D-mannose. (**a**) inhibition curves, the compound concentration is expressed in μM. (**b**) lectin selectivity histograms.*

# 6. INHIBITION OF HIV-1 INFECTION OF HUMAN CERVICAL TISSUE BY DENDRON 12

Experimental models based on infection with HIV of explants taken from human uterine cervix, albeit with some limitation, allows a better approximation of the conditions in *vivo* compared to cellular models. Therefore these models are used to evaluate the effectiveness in inhibiting infection by the virus and the potential toxicity of candidate compounds to develop as vaginal microbicides [36,37,39].
Thus, owing to the results obtained in *trans* infection assay, a cervical explant model was exploited to assess the efficacy of the tetravalent dendron **12** in inhibiting HIV infection and its potential toxicity, with the purpose of evaluating if the compound is a suitable candidate for the development as topical microbicide.
Endocervical tissue was obtained from premenopausal women, HIV, HBV and HCV seronegative, undergoing planned therapeutic hysterectomy.
Endocervix is more susceptible to HIV-1 being lined by a single layer of columnar epithelium and contains DC-SIGN$^+$ DCs in the subepithelial region [19,35,45]. Explants were extensively washed to eliminate traces of Betadine, a broad spectrum topical microbicide applied before hysterectomy, that could interfere with HIV infection of cervical explants [35]. In a preliminary phase, to develop the infection assay, immuno-activated explants (with IL-2 and PHA) or explants not activated were infected with a range of concentrations of HIV-1 different strains, in order to determine the optimal conditions of infection. The choice of the range of concentrations tested was based on data in the literature.
The explants were placed in contact with the virus in a non-polarized manner, thus mimicking the situation where the integrity of genital epithelium is compromised in *vivo* (a condition that significantly increases the risk of becoming infected with HIV).
The results, obtained by determining the p24 protein in the supernatant of cultures of the explants, allowed to individuate the optimal concentration of virus to use. The highest infection levels were reached in the third and seventh day post infection and then tended to decline, probably due to the deterioration of the explants following a prolonged culture. As regarding R5 tropic strains (BaL, DU 174 and 8g) were found that levels of infection are similar in explants stimulated and not stimulated (not shown) According with the literature data [36]. On the contrary the X4 tropic strain required pre-activation.

## 6.1 Inhibition of HIV-1 BaL infection

As the laboratory adapted R5 strain HIV-1BaL is able to infect resting tissue, explants were not activated with IL-2 and PHA to mimic physiological conditions.
Explants were pre-treated 30 minutes in absence or in presence of different concentration of dendron **12** and then were exposed to HIV-1 BaL ($2.6 \times 10^4$ $TCID_{50}$) in the continued presence of **12**. After washing to remove unbound dendron and virus, explants were maintained in culture up to 7 days. Supernatants of tissue culture were collected at day 3 and 7 post infection and p24

concentration, as measure of HIV-1 BaL infection, was determined by ELISA (Fig.14).

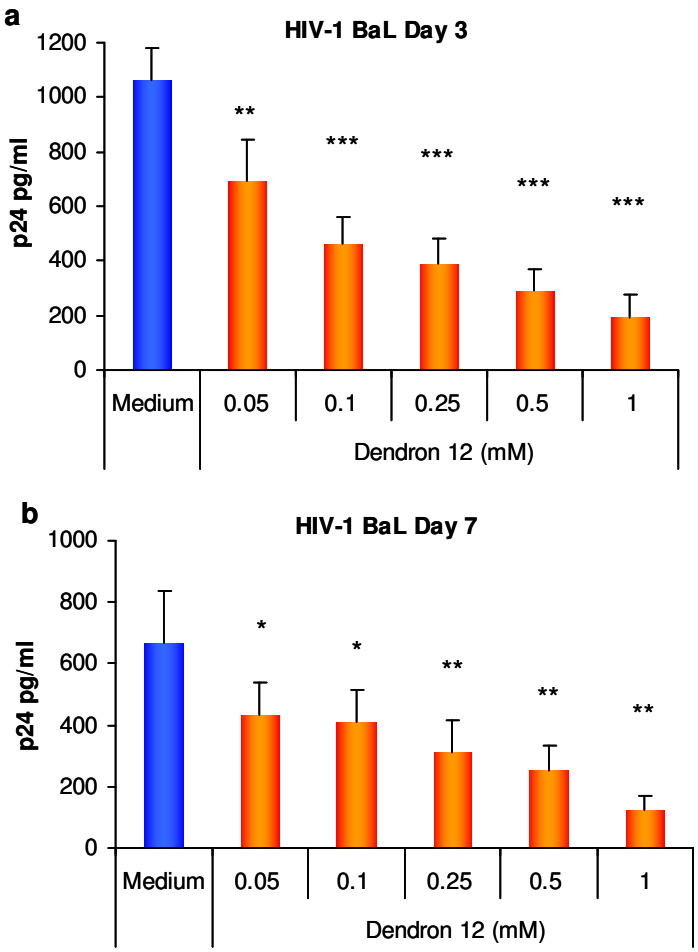



***Figure 14.*** *Inhibition of endocervical tissue infection mediated by HIV-1 Bal. Infection was monitored by ELISA measurement of p24 in explant culture supernatants at day 3 (a) and 7 (b) post infection. Values represent the mean ±SD of 5 independent experiments. ***p values < 0.001, **p values<0.01, *p values<0.05 (Student's T Test).*

To obtain dose-response curves 5 different concentration of dendron **12** were assayed (1mM, 0.5 mM, 0.25 mM, 0.1mM and 0.01 mM). Data represent p24 levels 3 and 7 days after infection and are presented as the mean of 5 independent experiments, using explants from separate donors. The dendron **12** inhibited

cervical explants HIV-1 BaL infection in dose dependent manner. At the higher concentration tested (1mM), the compound reduced the infection by about 80%, both at 3 and 7 days post infection. An $IC_{50}$ of 100 µM could be estimated.

## 6.2 Inhibition of primary HIV-1 isolates infection

The ability of dendron **12** to avert explant infection mediated by from primary HIV-1 isolates was also verified. (Fig.15 and Fig.16).

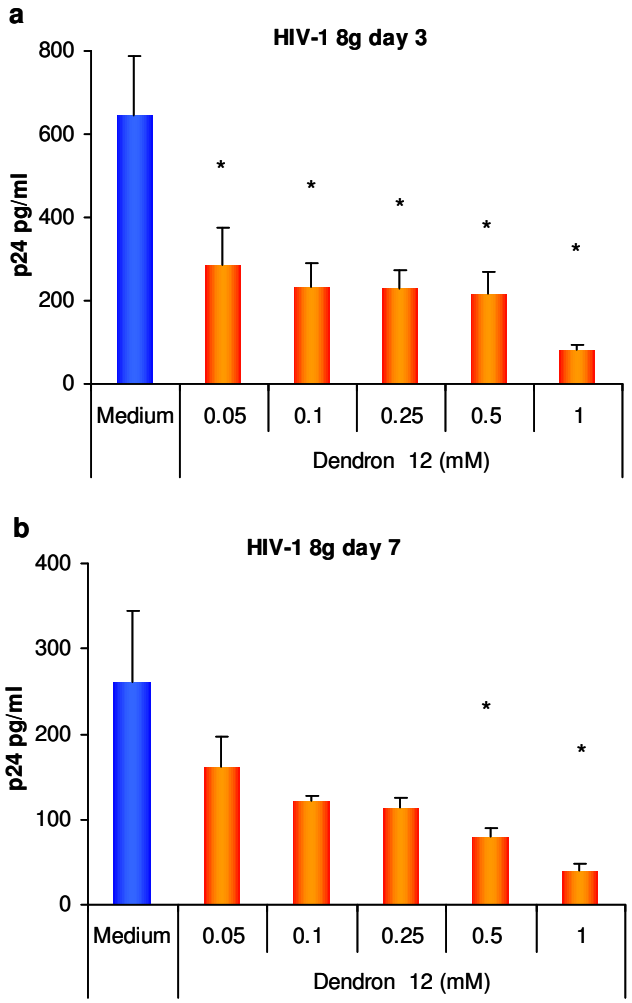



***Figure 15.*** *Inhibition of endocervical infection induced by HIV-1 primary isolate 8g at 3 (a) and 7 days (b) post infection. Values represent the mean ±SD of 3 independent experiments performed on cervical explants of 3 separate donors, *p values<0.05 (Student's T Test).*

R5 tropic isolate 8g was able to infect unstimulated tissue. On the contrary X4 tropic isolate DPMVF needed pre-activation with IL-2 and PHA to induce consistent infection levels (not shown). Explants unstimulated or stimulated 2 days with IL-2 and PHA were pre-treated with increasing concentration of dendron **12** and infected with 8g or DPMVF respectively, as described before.

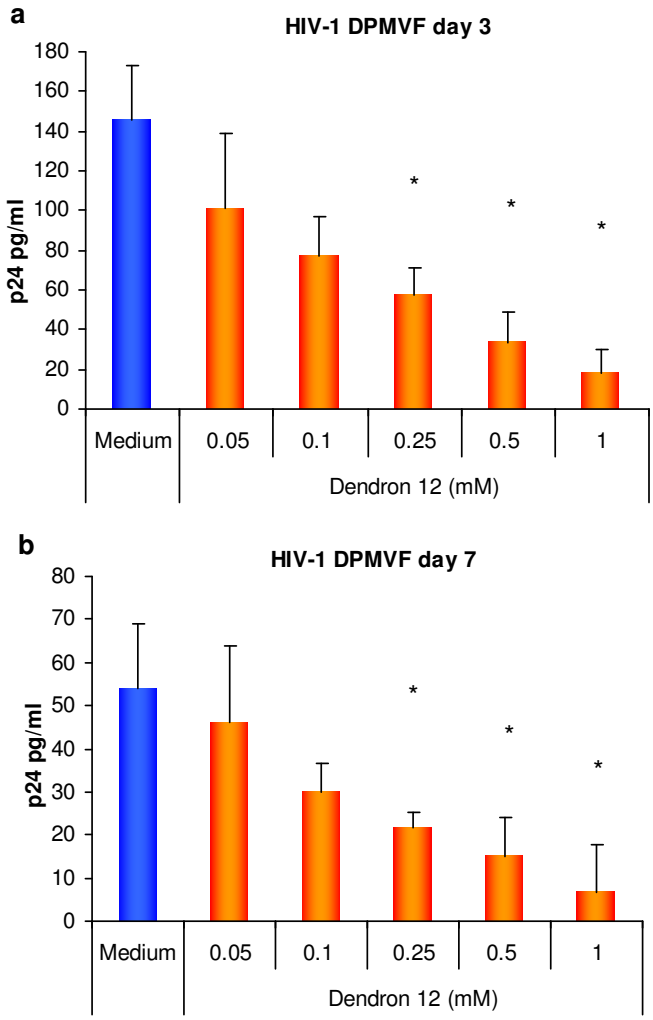



***Figure 16.*** *Inhibition of endocervical infection induced by HIV-1 primary isolate DPMVF at 3 (a) and 7 days (b) post infection. Values represent the mean ±SD of 3 independent experiments performed on cervical explants of 3 separate donors, *p values<0.05 (Student's T Test).*

Infection inhibition was dose dependent both at 3 and at 7 days post infection. Regarding 8g, at the concentration of 1mM of dendron **12** infection was reduced by about 90% at 3 days and 85% at 7 days after the infection. At 0.05 mM a decrease of 45% (day 3 post infection) and of 60% (day 3 post infection) of p24 production was still observed (Fig. 15). The compound **12** decreased DPMVF infection by over 85% at the higher concentration assayed (1mM), but at 0.05 mM the inhibitory effect was largely lost (Fig 16).

## 6.3 Inhibition of HIV-1 DU174 infection

The HIV-1 Subtype C largely predominates in Southern and East Africa and in areas of Asia, where the great majority of infected people reside, and it has caused the world's worst HIV epidemics.

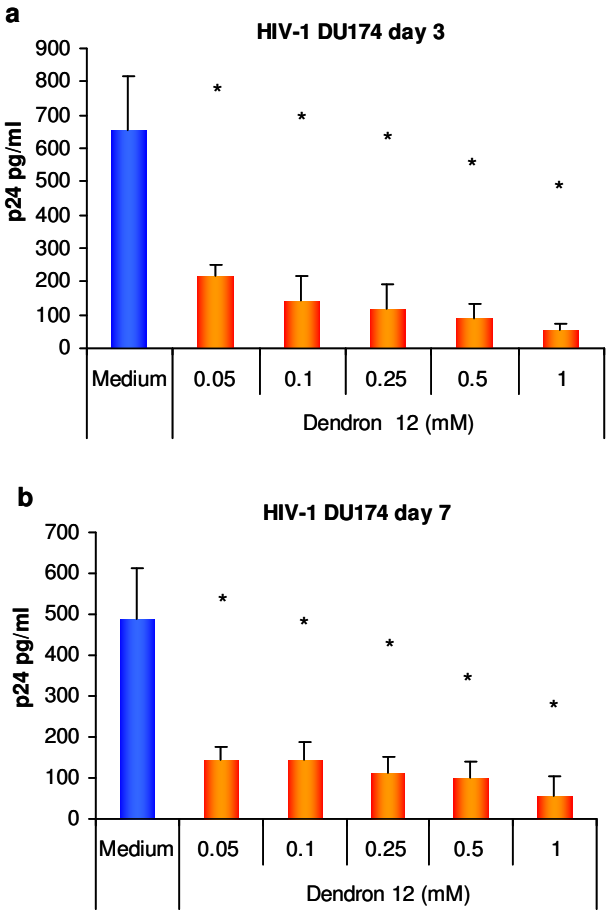



***Figure 17.*** *Dendron **12** activity against HIV-1 Clade C DU174. Values represent the mean ±SD of 3 independent experiments. *p values<0.05 (Student's T Test).*

Thus, we evaluated the capability of **12** to block endocervical explant infection induced by Clade C R5 tropic strain DU174. Unstimulated explants were pre-treated with the dendron, exposed to HIV-1 DU 174 ($2x10^4$ $TCID_{50}$) and cultured as described above. The results obtained showed that the compound 12, at 1 mM, reduced DU 174 infection by about 90% Inhibition was dose dependent and $IC_{50}$ was less than 50 µM both at 3 and 7 days post infection (Fig. 17).

R5-tropic viruses strains predominate during HIV-1 transmission in *vivo* and cause the majority of new infections [8]. The results showed in this chapter demonstrated that the dendron **12** strongly reduced the infection of cervical explants by different HIV-1 R5 tropic strains. In effect the compound inhibited infection by the clade B laboratory adapted strain BaL and by the primary isolate 8g in dose dependent manner. Remarkably the dendron, at the higher concentration assayed, decreased the infection induced by the clade C strain DU 174 by about the 90%. This may have a great impact, considering that clade C is the most abundant subtype in countries with the majority of HIV-1 infections. Subtype C is present the eastern Africa and predominates in all countries of Southern Africa and in some countries of Asia [13,14]. Rare cases of HIV-1 infection by X4-tropic strains were observed in CCR5Δ32 homozygotes [11] and X4 tropic strain can infect immune activate human cervical tissue [36]. Our experiments indicate that the clinical isolate X4 tropic DPMVF can infect explants stimulated with IL-2 and PHA. Dendron **12** was able to inhibit DPMVF primary X4 tropic strain infection at higher concentration assayed, but this effect was in part lost at lower concentrations of the compound.

Vaginal epithelium has limited permeability to particles greater than 30 nm [2]. However the diameter of dendron **12** is certainly below that threshold, so the compound could enter and diffuse into intact mucosal tissue. Furthermore the dendron 12 scaffold can be easily modified to improve absorption of the compound, without decreasing affinity to DC-SIGN.

## 7. INDUCTION OF β1 CHEMOKINES PRODUCTION BY DENDRON 12

We wonder if, in addition to competitive inhibition of DC-SIGN, other mechanisms account for the antiviral effect of the compound **12**. So we investigate if the dendron **12** stimulates the production of factors interfering with HIV infection.
Because of the paucity of submucosal DCs in cervical explants, immature monocytes-derived dendritic cells (iMDDCs) from healthy donors were used as a model. Experiments were performed on iMDDCs differentiated by culturing $CD14^+$ monocytes, isolated from peripheral blood of donors, for 6 days in presence of IL-4 and GM-CSF.
iMDDCs were treated with dendron **12** (250μM) for 3, 24 and 72 hours. Expression and production of β chemokines, MIP-1α, MIP-1β, and RANTES, following stimulation was analyzed by quantitative real time PCR and ELISA.
Treatment with **12** increased expression level of mRNA specific for MIP-1α, MIP-1β and RANTES about 3, 5 and 4 times respectively (Fig. 18 a-c).
MIP-1α production increased by about four times and MIP-1β by about 2 times after 24 and 72 hours of stimulation (Fig. 19 a, b). RANTES production augmented by about 7 and 5 times at 24 and 72 hours respectively (Fig. 19 c).
These data suggest that dendron **12** might have additional mechanisms of action besides competitive inhibition of HIV-1 binding to DC-SIGN and downregulation of DC-SIGN expression. MIP-1α, MIP-1β and RANTES are the natural ligands of CCR5, a chemokine receptor and co-receptor of HIV-1 R5 tropic strain. These β chemokines suppress HIV-1 R5 tropic strain replication, directly competing with the binding of the virus to CCR5 or inducing receptor internalization [9,10,11].
The compound **12**, enhancing β-chemokines production, could interfere, at least partially, with the direct infection of $CCR5^+$ CD4 T lymphocytes and macrophages located in genital mucosae.
As a result of difficulty of isolating sufficient amounts of primary mucosal DCs, iMMDCs were used as model. Although MDCCs share with immature mucosal DCs similar features and the expression of DC-SIGN and other markers, they do not completely mimic function of primary DCs. Therefore we intend to isolate mucosal DCs to further investigate the effect of the compound.

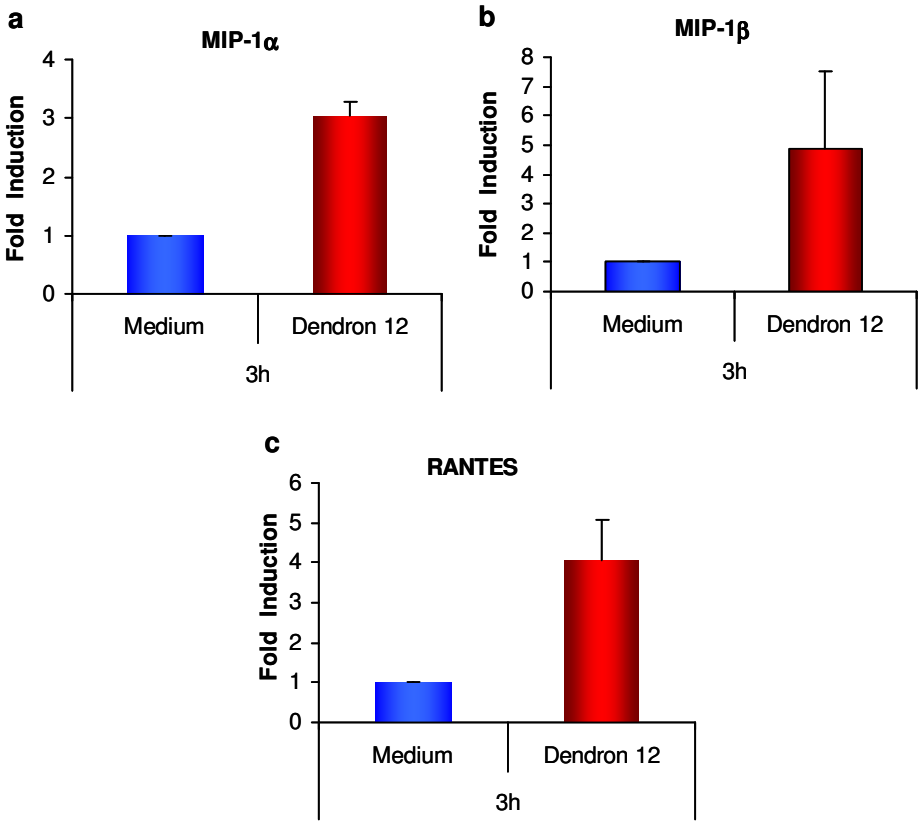



***Figure 18.*** *(a-c) β chemokines expression following 3h dendron* ***12*** *(250 μM) treatment. mRNA levels of MIP-1α (a), MIP-1β (b) and RANTES (c) were assessed by quantitative real time PCR. Expression is normalized to GAPDH expression and shown as fold changes expression from the unstimulated sample, set as 1. Experiments were performed on iMDDCs from 2 healthy donors. Values represent the mean ±SD.*

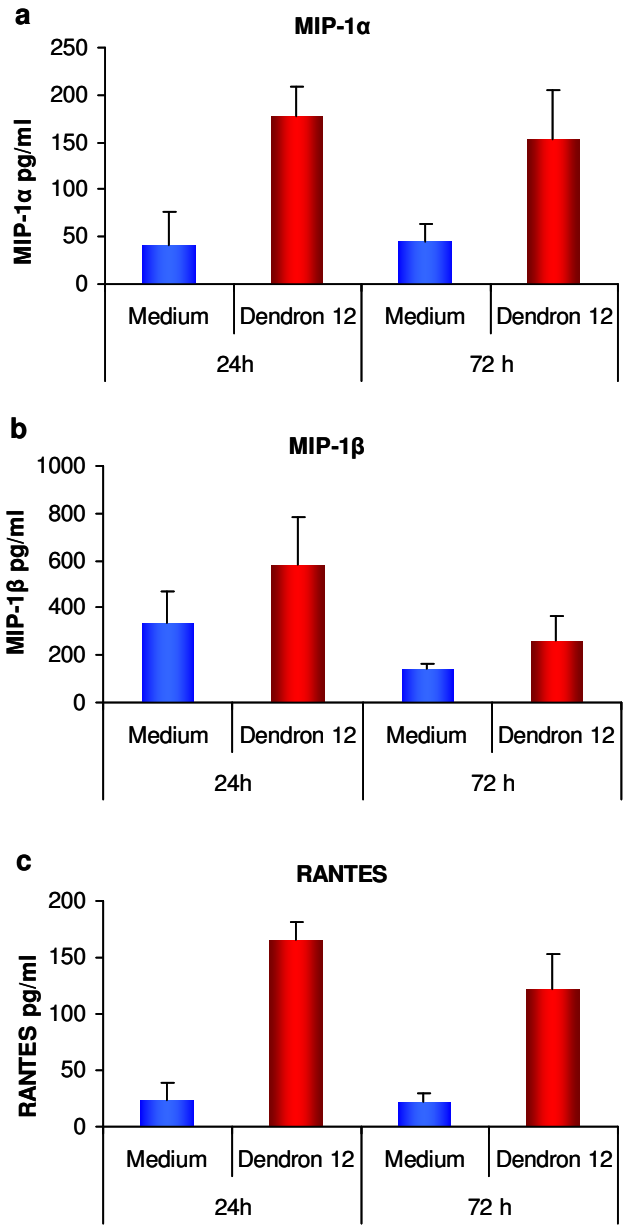



***Figure 19.*** *(a-c) β chemokines protein production after stimulation with dendron* ***12*** *(250 µM). The concentration of MIP-1α (a), MIP-1β (b) and RANTES (c) in the culture supernatants was assayed by ELISA at 24 and 72h. Experiments were performed on iMDDCs from 2 healthy donors. Values represent the mean ±SD.*

## 8. EVALUATION OF DENDRON 12 TOXICITY

Cytotoxicity against PBMCs was evaluated by labelling with 7-Amino-Actinomycin D (7-AAD), that identifies non-viable cells. The results obtained indicate that the dendron **12** exposure for 3 and 7 days did not alter significantly the viability of PBMCs at the concentrations assayed (Fig. 20).

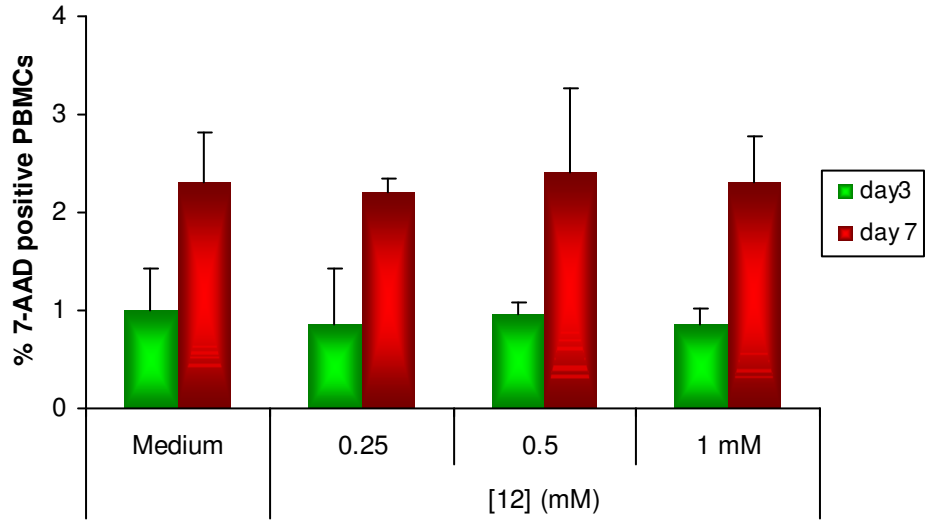



***Figure 20**. Toxicity of compound **12**. Percentage of 7-AAD positive (non viable) PBMCs after 3 or 7 days of incubation with different concentrations of **12** (0.25 mM, 0.5 mM and 0.1 mM), or in the absence of the inhibitor (Medium). Percentage of 7-AAD was determined by flow cytometry. Experiments were performed on PBMCs from 3 healthy donors. Values are mean ± SD.*

To assess potential toxicity of dendron **12** towards cervical tissue, effect of non polarized exposure of the compound on cervical explants viability was monitored (Fig. 21). After 3 and 7 days treatment in absence or in presence of different concentration of compound **12**, explants viability was evaluated by a MTT based assay. Formazan absorbance reading was normalized to explant weight. Viability of the compound treated explants were compared to viability of untreated control. The results showed no significant difference between control and treated explants up to a maximal concentration of 1mM (the higher concentration tested in assessment of dendron **12** efficacy against HIV-1 infection).
The compound **12** do not reduce the vitality of PBMCs and of cervical explants.
However additional experiments, such as rabbit vaginal irritation (RVI) assay or studies in primate models [134], would be needed for a more accurate evaluation of potential toxic effects.

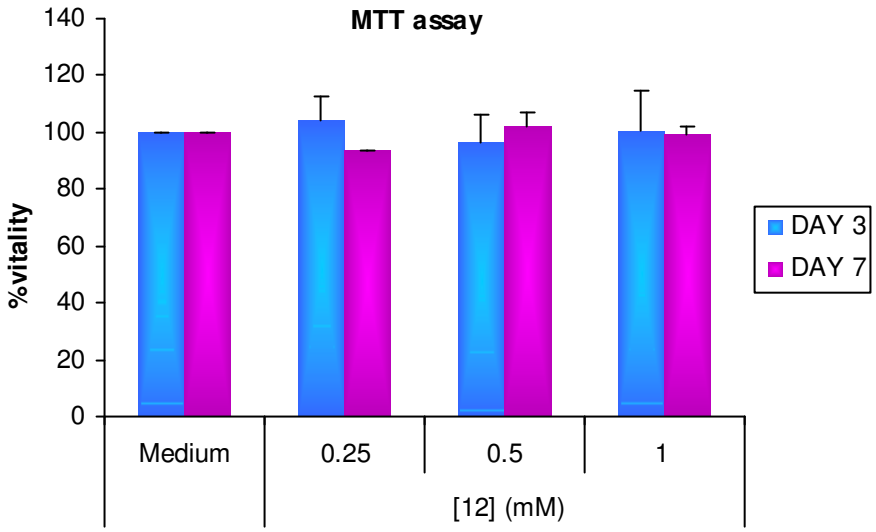



***Figure 21.*** *Effect of dendron* ***12*** *on cervical explants viability. Endocervical explants, derived from the same donors, were exposed in non polarised manner to* ***12*** *or culture medium (control) for 3 and 7 days. Effect of dendron* ***12*** *on tissue viability was determined by the MTT assay. Explants were weighted to normalize optical density of formazan yielded. Viability was expressed as percentage, dividing viability of compound* ***12*** *treated explants by viability of control explant. Experiments were performed on explants from 3 donors. Data are reported as the average percent viability (± SD).*

# *CONCLUSIONS*

In the absence of an effective protective vaccine against HIV-1, topical microbicides represent a possible and promising alternate way to prevent sexually transmitted HIV-1 infection. Considering the role of the C-type lectin receptor DC-SIGN in the first stages of sexually-transmitted HIV infection, the most frequent route of HIV-1 transmission, the possibility of inhibiting this receptor in order to prevent HIV-1 infection was explored.

The ligand recognized by DC-SIGN on the HIV surface is the high mannose glycan, a branched oligosaccharide expressed in multiple copies on HIV gp120.
A series of monovalent and polyvalent pseudo-mannosylated compounds was designed in the attempt to compete with the binding of HIV gp120 to DC-SIGN.

The binding affinity of the synthesized compounds to DC-SIGN was evaluated by Surface Plasmon resonance studies (SPR). The ability of such compounds to inhibit HIV infection was assessed in a in *vitro* model of *trans* infection. Productive HIV infection was monitored by measuring HIV core protein p24 in the supernatants of cell culture.

One of these compounds, a tetravalent pseudo-mannosylated compound (dendron **12**), competitively inhibited DC-SIGN mediated HIV *trans* infection of CD4 T cells at low micromolar range, regardless of viral strain, even in the presence of elevated viral loads. The time-course studies, described in the results section, demonstrated that the antiviral effect of dendron **12** persists for hours even after the compound have been removed. This long-lasting inhibition after removal could be due to the persistency of the dendron **12** binding to DC-SIGN. The reduction of DC-SIGN expression following the binding of dendron **12** may also contribute to this effect.

In the attempt to clarify the mechanisms of action of this compound a biosensor with a SPR detection method was employed. Results showed that compound **12** is much more selective for DC-SIGN than for the C-type lectin Langerin, expressed by Langheran's cells. Considering the similarity between the carbohydrate recognition domain of DC-SIGN and Langerin (that prevents HIV transmission promoting the rapid degradation and clearance of this virus), the fact that the dendron **12** does not interfere with the function of Langerin is a remarkable advantage in view of developing new microbicide compounds.

To further assess the ability of the compound to inhibit HIV-1 infection, a model based on the infection with different HIV-1 strains of explants taken from human uterine cervix was utilized. Explants were obtained from HIV-, HBV-, and HCV-seronegative premenopausal women, undergoing planned therapeutic hysterectomy. After dendron **12** pretreatment, explants were exposed to different HIV-1 strains in a non polarised manner, mimicking a condition of compromised epithelium in *vivo* (a condition that highly increase the risk of HIV infection). Viral infection was quantified by measuring HIV core protein p24 concentration in explant culture supernatans at different time points. As R5-tropic viruses predominate during HIV-1 transmission in *vivo* and cause the majority of new infections, the ability of dendron **12** to avert the infection of cervical explants by different HIV-1 R5 tropic strains was evaluated. Results showed that the compound strongly inhibits infection by the clade B laboratory adapted strain BaL and by a R5

primary isolate in a dose dependent manner. Remarkably the dendron decreased the infection induced by the clade C strain DU 174 by more than 90%. This may have a great impact, considering that clade C is the most abundant subtype in Southern Africa and in areas of East Africa and Asia, where the great majority of HIV infected people reside.
Rare cases of HIV-1 infection by X4-tropic strains were observed in CCR5Δ32 homozygotes and X4 tropic strain can infect immune activate human cervical tissue. Experiments performed indicate that dendron **12** was able to inhibit DPMVF primary X4 tropic strain infection at higher concentration, but this effect was in part lost at lower concentrations of the compound.

The competitive inhibition of HIV-1 binding to DC-SIGN is one of the mechanisms by which the compound exerts its effect. To verify whether dendron **12** might have additional mechanisms of action besides competitive inhibition of HIV-1 binding to DC-SIGN additional experiments were performed. Results showed that dendron **12** elicits production of the β chemokines MIP-1α, MIP-1β and RANTES by monocyte derived DCs. These chemokines suppress HIV-1 R5 tropic strain replication, directly competing with the binding of the virus to CCR5 or inducing receptor internalization. Therefore compound **12** likely interferes with the infection of $CCR5^+$ CD4 lymphocytes, DCs and macrophages located in genital mucosae by enhancing β-chemokines production.

Thus, this work shows that the tetravalent dendron **12** is able to prevent HIV *trans* infection of CD4 T lymphocytes at low micromolar range, even in presence of elevated viral load, is devoid of toxicity and inhibits in a dose dependent manner the HIV-1 infection of human cervical explants. Furthermore the compound is highly soluble in physiological media and is stable at acidic vaginal pH. These features make the dendron **12** an optimal compound in the design of new microbicidal drugs. Indeed the development of innovative topical microbicide drugs, such as dendron **12**, may have a considerable impact on human health.

However the compound **12** is able to inhibit 80-90% of cervical explants HIV-1 infection, but cannot block it completely. To overcome these limitations, different lines of study are in progress in our laboratories.
Structure of dendron **12** can be improved in both the scaffold and the active pseudo-saccharide ligand to develop of new ligands of DC-SIGN even more effective in inhibiting infection and easier to synthesize than the original lead. Furthermore, dendron **12** (or its derivatives) can be used in combination with other molecules directed against different HIV targets. Indeed, the combinations reported so far were all found to display synergistic activity in infection assays. In particular mixed multivalent structures, presenting on the same scaffold multiple copies of DC-SIGN and HIV co-receptors ligands, simultaneously blocking different HIV target, could protect against different routes of HIV transmission. Hence such structures could represent a highly promising strategy to prevent the first phases HIV infection.

# *REFERENCES*

***References***


[1] UNAIDS REPORT ON THE GLOBAL AIDS EPIDEMIC, 2010. http://www.unaids.org/globalreport/documents/20101123_GlobalReport_full_en.pdf

[2] Abbas A. K., Lichtman A. H., Pillai S., **Cellular and Molecular Immunology**, Elsevier 6th Edition, 2007.

[3] Ken Murphy, Paul Travers, Mark Walport, **Janeway's Immunobiology**, Garland Sciences, 7h edition, 2007.

[4] Gerald B Pier, Jeffrey B Lyczak and Lee M Wetzler. **Immunology, Infection, and Immunity**, American Society for Microbiology Press, 2004.

[5] Poropatich K., Sullivan D.J., "Human immunodeficiency virus type 1 long-term non-progressors: the viral, genetic and immunological basis for disease non-progression". *Journal of General Virology*, Vol. 92, 2011, pp. 247-268.

[6] Piacentini L., Biasin M., Fenizia C., Clerici M., "Genetic correlates of protection against HIV infection: the ally within", *Journal of Internal Medicine,* Vol. 265, No.1, 2009, pp.110-124.

[7] Miyazawa M., Lopalco L., Mazzotta F., Lo Caputo S., Veas F., Clerici M., ESN Study Group, "The 'immunologic advantage' of HIV-exposed seronegative individuals", *AIDS*, 2009 Vol. 14; No. 23, pp. 161-175.

[8] Nazari R., Joshi S., "CCR5 as Target for HIV-1 Gene Therapy", *Current Gene Therapy*, 2008, Vol. 8, No. 4, pp. 1-9.

[9] Wu L., LaRosa G., Kassam N., Gordon C.J., Heath H., Ruffing N., Chen H., Humblias J., Samson M., Parmentier M., Moore JP., Mackay CR., "Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding", *Journal of Experimental Medicine*, Vol.186, No. 8 1997, pp.1373-1381,

[10] Cocchi F., DeVico A.L., Garzino-Demo A., Arya S.K., Gallo R.C., Lusso P. "Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells", *Science*, 1995, Vol. 270, pp. 1811-1815.

[11] Samson M., Libert F, Doranz B.J., Rucker J., Liesnard C., Farber C.M., Saragosti S., Lapoumeroulie C., Cognaux J., Forceille C., Muyldermans G., Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman R.G., Doms R.W., Vassart G., Parmentier M., "Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene", *Nature*, 1996, Vol. 382, pp. 722-725.

[12] O'Brien T.R., Winkler C., Dean M., Nelson J.A., Carrington M., Michael N.L., White G.C, "HIV-1 infection in a man homozygous for CCR5Δ32", *Lancet*, 1997, Vol. 349, pp. 1219.

[13] Spira S., Wainberg M.A, Loemba H., Turner D., Brenner B.G., "Impact of clade diversity on HIV-1 virulence, antiretroviral drug sensitivity and drug resistance", *Journal of Antimicrobial Chemotherapy*, Vol. 51, 2003, pp. 229–240.

[14] Peeters M., Toure-Kane C., Nkengasong J.N., "Genetic diversity of HIV in Africa: impact on diagnosis, treatment, vaccine development and trials". *AIDS*, 2003, Vol. 17, No.18, pp. 2547-2560.

[15] Simon F., Mauclère P., Roques P., Loussert-Ajaka I., Müller-Trutwin M.C., Saragosti S., Georges-Courbot M.C., Barré-Sinoussi F., Brun-Vézinet F., "Identification of a new human immunodeficiency virus type 1 distinct from group M and group O", *Nature Medicine*, 1998, Vol. 4, No. 9, pp.1032-1037.

[16] Plantier J.C., Leoz M., Dickerson J.E., De Oliveira F., Cordonnier F., Lemée V., Damond F., Robertson D.L., Simon F., "A new human immunodeficiency virus derived from gorillas", *Nature Medicine*, 2009, Vol. 15, No. 8, 871-872.

[17] Kanki P.J., Hamel D.J., Sankalé J.L., Hsieh C., Thior I., Barin F., Woodcock S.A., Guèye-Ndiaye A., Zhang E., Montano M., Siby T., Marlink R., Ndoye I., Essex M.E., MBoup S., "Human immunodeficiency virus type 1 subtypes differ in disease progression", *The Journal of Infectious Dis*eases, 1999, Vol. 179,No. 1, pp.68-73.

[18] Cutler B., Justman J., "Vaginal microbicides and the prevention of HIV transmission", *Lancet Infectious Diseases.*, 2008, Vol 8, No.11, pp.685-697.

[19] Pope M., Haase A.T., "Transmission, cute HIV infection and the quest for strategies to prevent infection", *Nature Medicine*, 2003, Vol.9, pp. 847-852.

[20] Haase A.T., "Perils at mucosal front lines for HIV and SIV and their hosts", *Nature Reviews Immunology*, 2005, Vol.5, No. 10, pp. 783-792.

[21] Keele B.F., Estes J.D., "Barriers to mucosal transmission of immunodeficiency viruses", *Blood*, 2011, Vol.118, No. 4 , pp. 839-846.

[22] Pomerantz R.J., Horn D.L., "Twenty years of therapy for HIV-1 infection", *Nature Medicine*, 2003, Vol. 9, No. 7, pp: 867-873.

[23] Powderly W,G. "Integrase inhibitors in the treatment of HIV-1 infection", *Journal of Antimicrobial Chemotherapy*, 2010, Vol.65, No. 12, pp: 2485-2488.

[24] Perry C,M., "Maraviroc: a review of its use in the management of CCR5-tropic HIV-1 infection", *Drugs*, 2010, Vol. 70, No. 9, pp: 1189-1213.

[25] Jericó C., Knobel H., Montero M., Ordoñez-Llanos J., Guelar A., Gimeno J.L., Saballs P., López-Colomés J.L., Pedro-Botet J., "Metabolic syndrome among HIV-infected patients: prevalence, characteristics, and related factors", *Diabetes Care*, 2005, Vol.28, No.1, pp: 132-137.

[26] Pao V., Lee G.A., Grunfeld C., "HIV therapy, metabolic syndrome, and cardiovascular risk", *Current Atherosclerosis Reports,* 2008 Vol.10, No.1, pp: 61-70.

[27] Boasso A., Shearer G.M., Clerici M., "The hunt for an HIV vaccine: time to rethink recent failures", *Lancet*, 2008, Vol. 371, No. 9628, pp. 1897-1898.

[28] Buchbinder S.P., Mehrotra D.V., Duerr A., Fitzgerald D.W., Mogg R., Li D., Gilbert P.B., Lama J.R., Marmor M., Del Rio C., McElrath M.J., Casimiro D.R., Gottesdiener K.M., Chodakewitz J.A., Corey L., Robertson M.N., Step Study Protocol Team, "Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial", *Lancet*, 2008, Vol. 372, No. 9653, pp.1881-1893.

[29] Rerks-Ngarm S., Pitisuttithum P., Nitayaphan S., Kaewkungwal J., Chiu J., Paris R., Premsri N., Namwat C., de Souza M., Adams E., Benenson M., Gurunathan S., Tartaglia J., McNeil J.G., Francis D.P., Stablein. D., Birx D.L., Chunsuttiwat S., Khamboonruang C., Thongcharoen P., Robb M.L., Michael N.L., Kunasol P., Kim J.H.; MOPH-TAVEG Investigators, "Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand", *New England Journal of Medicine,* 2009, Vol.361, No. 23, pp. 2209–2220.

[30] Reina J.J., Bernardi A., Clerici M., Rojo J., "HIV microbicides: state-of-the-art and new perspectives on the development of entry inhibitors", *Future Medicinal Chemistry*, 2010, Vol. 2, No. 7, pp.1141-1159

[31] "Topical microbicides preventing sexually transmitted diseases". *NIH Publication*, No. 03-5316, 2003.

[32] "NIAID Topical Microbicide Strategic Plan", *NIH Publication*, No. 03-7138, 2003

[33] Balzarini J., Van Damme L. "Microbicide drug candidates to prevent HIV infection", *Lancet*, 2007, Vol.369, No. 9563, pp. 787-797

[34] MDS Working Groups. "The Microbicide Development Strategy", *Silver Spring, MD, USA: Alliance for Microbicide Development*, 2006.

[35] Anderson D.J., Pudney J., Schust D,J., "Caveats associated with the use of human cervical tissue for HIV and microbicide research. *AIDS*, 2010, Vol. 24, No.1, pp.1-4.

[36] Greenhead P., Hayes P., Watts P.S., Laing K.G., Griffin G.E., Shattock R.J., "Parameters of human immunodeficiency virus infection of human cervical tissue and inhibition by vaginal virucides", *Journal of Virology*, 2000 ,Vol. 74, No.12, pp. 5577-5586.

[37] Cummins J.E., Guarner J., Flowers L., Guenthner P.C., Bartlett J., Morken T., Grohskopf L.A., Paxton L., Dezzutti C.S., "Preclinical testing of candidate topical microbicides for anti-human immunodeficiency virus type 1 activity and tissue toxicity in a human cervical explant culture", *Antimicrobial Agents and Chemotherapy*, 2007 Vol. 51, No. 5, pp.1770-1779.

[38] Fletcher P., Kiselyeva Y., Wallace G., Romano J., Griffin G., Margolis L., Shattock R., "The nonnucleoside reverse transcriptase inhibitor UC-781 inhibits human immunodeficiency virus type 1 infection of human cervical tissue and dissemination by migratory cells", *Journal of Virology*, 2005, Vol.79, No17, pp.11179-11186.

[39] Wallace G.S., Cheng-Mayer C., Schito M.L., Fletcher P., Miller Jenkins L.M., Hayashi R., Neurath A.R., Appella E., Shattock R.J., "Human immunodeficiency virus type 1 nucleocapsid inhibitors impede trans infection in cellular and explant models and protect nonhuman primates from infection", *Journal of Virology*, 2009, Vol. 83, No. 18, pp:9175-9182.

[40] O'Keefe B.R., Vojdani F., Buffa V., Shattock R.J., Montefiori D.C., Bakke J., Mirsalis J., d'Andrea A.L., Hume S.D., Bratcher B., Saucedo C.J., McMahon J.B., Pogue G.P., Palmer K.E, "Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component", *Proceedings of the National Academy of Sciences USA*, 2009, Vol.106, No.15, pp. 6099-6104.

[41] Richardson-Harman N., Lackman-Smith C., Fletcher P.S., Anton P.A., Bremer J.W., Dezzutti C.S., Elliott J., Grivel J.C., Guenthner P., Gupta P., Jones M., Lurain N.S., Margolis L.B., Mohan S., Ratner D., Reichelderfer P., Roberts P., Shattock R.J., Cummins J.E., "Multisite Comparison of Anti-Human Immunodeficiency Virus Microbicide Activity in Explant Assays Using a Novel Endpoint Analysis", *Journal of Clinical Microbiology*, 2009, Vol. 47, No.11, pp. 3530-3539.

[42] Neff C.P., Kurisu T., Ndolo T., Fox K., Akkina R., "A topical microbicide gel formulation of CCR5 antagonist maraviroc prevents HIV-1 vaginal transmission in humanized RAG-hu mice", *PLoS One*, 2011, Vol.6, No. 6, e 20269, pp 1-6.

[43] Abdool Karim Q., Abdool Karim S.S., Frohlich J.A., Grobler A.C., Baxter C., Mansoor L.E., Kharsany A.B., Sibeko S., Mlisana K.P., Omar Z., Gengiah T.N., Maarschalk S., Arulappan N., Mlotshwa M., Morris L., Taylor D. CAPRISA 004 Trial

Group, "Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women", *Science*, 2010, Vol. 329, pp. 1168-1174.

[44] van de Wijgert J.H., Shattock R.J., "Vaginal microbicides: moving ahead after an unexpected setback", *AIDS*, 2007, Vol. 21, No.18, pp. 2369-2376.

[45] Geijtenbeek T.B.H., Torensma R., van Vliet S.J., van Duijnhoven G.C.F, Adema G.J., van Kooyk Y., Figdor C.G., "Identification of DC-SIGN, a novel Dendritic Cell-Specific ICAM-3 receptor that supports primary immune responses", *Cell*, 2000, Vol. 100, No.5, pp. 575-585,

[46] Curtis B.M, Scharnowske S., Watson A.J, "Sequence and expression of a membrane-associated C-type lectin that exhibits CD4-independent binding of human immunodeficiency virus envelope glycoprotein gp120", 1992, *Proceedings of the National Academy of Sciences USA,* Vol.89, No. 17, pp.8356-8360.

[47] Geijtenbeek T.B.H, Kwon D.S, Torensma R., van Vliet S.J., van Duijnhoven G.C., Middel J., Cornelissen I.L., Nottet H.S., KewalRamani V.N., Littman D.R., Figdor C.G., van Kooyk Y., "DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells", *Cell*, 2000, Vol.100, No.5, pp.587-597.

[48] Bashirova A.A., Wu L., Cheng J., Martin T.D., Martin M.P., Benveniste R.E., Lifson J.D., KewalRamani V.N., Hughes A., Carrington M., "Novel member of the CD209 (DC-SIGN) gene family in primates", *Journal of Virology,* 2003, No.77, No.1, pp. 217-227.

[49] Soilleux E.J., Barten R., Trowsdale J., "DC-SIGN; a related gene, DC-SIGNR; and CD23 form a cluster on 19p13", *Journal of Immunology*, 2000, Vol.165, No.6, pp. 2937-2942.

[50] Geitenbeck T.B.H., van Duijnhoven G.C., van Vliet S.J., Krieger E., Vriend G., Figdor C.G., van Kooyk Y., "Identification of different binding sites in the dendritic cell- specific receptor DC-SIGN for intercellular adhesion molecule 3 and HIV-1", *Journal of Biological Chemistry*, 2002, 277(13):11314-11320.

[51] Mitchell D. A., Fadden A. J., Drickamer K., "A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. Subunit organization and binding to multivalent ligands", *Journal of Biological Chemistry*, 2001, Vol. 276, pp. 28939–28945

[52] Feinberg H., Guo Y., Mitchell D. A., Drickamer, K., Weis W. I., "Extended neck regions stabilize tetramers of the receptors DC-SIGN and DC-SIGNR". *Journal of Biological Chemistry*, 2005, Vol. 280, No.2, pp.1327-35.

[53] Feinberg H., Mitchell D.A., Drickamer, K., Weis W.I., "Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR", *Science*, 2001,Vol. 294, pp. 2163–2166.

[54] Serrano-Gómez D., Sierra-Filardi E., Martínez-Nuñez R.T., Caparrós E., Delgado R., Muñoz-Fernández M.A., Abad M.A., Jimenez-Barbero J., Leal M., Corbí A.L. "Structural Requirements for Multimerization of the Pathogen Receptor Dendritic Cell-specific ICAM3-grabbing Non-integrin (CD209) on the Cell Surface" Journal of Biological Chemistry, 2008, Vol.283, No.7, pp. 3889-3903.

[55] Plazolles N., Humbert J.M., Vachot L., Verrier B., Hocke C., Halary F., "Pivotal advance: The promotion of soluble DC-SIGN release by inflammatory signals and its enhancement of cytomegalovirus-mediated *cis*-infection of myeloid dendritic cells", *Journal of Leukocyte Biology*, 2011, Vol. 89, No.3, pp. 329-342.

[56] Engering A., Van Vliet S. J., Geijtenbeek T. B.H., Van Kooyk Y., "Subset of DC-SIGN+ dendritic cells in human blood transmits HIV-1 to T lymphocytes", *Blood*, 2002, Vol. 100, pp. 1780–1786.

[57] Gurney K.B., Elliott J., Nassanian H., Song C., Soilleux E., McGowan I., Anton PA, Lee B., "Binding and transfer of human immunodeficiency virus by DC-SIGN+ cells in human rectal mucosa", *Journal of Virology*, 2005, Vol. 79, No. 9, pp. 5762–5773.

[58] Jameson B., Baribaud F., Pöhlmann S., Ghavimi D., Mortari F., Doms R.W., Iwasaki A., "Expression of DC-SIGN by dendritic cells of intestinal and genital mucosae in humans and rhesus macaques*", Journal of Virology*, 2002, Vol.76,No. 4, pp. 1866–1875.

[59] Soilleux E.J., Morris L.S., Leslie G., Chehimi J., Luo Q., Levroney E., Trowsdale J., Montaner L.J., Doms R.W., Weissman D., Coleman N., Lee B, "Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations in situ and in vitro", *Journal of Leukocyte Biology*, 2002, Vol. 71, No. 3, pp. 445–457.

[60] Engering A., van Vliet S.J., Hebeda K., Jackson D.G., Prevo R., Singh S.K., Geijtenbeek T.B.H., van Krieken H., van Kooyk Y. "Dynamic populations of dendritic cell-specific ICAM-3 grabbing nonintegrin-positive immature dendritic cells and liver/lymph node-specific ICAM-3 grabbing nonintegrin-positive endothelial cells in the outer zones of the paracortex of human lymph nodes", *American Journal of Pathology*, 2004, Vol.164, No. 5, pp. 1587–1595.

[61] Granelli-Piperno A., Pritsker A., Pack M., Shimeliovich I., Arrighi J.F., Park C.G., Trumpfheller C., Piguet V., Moran T.M., Steinman R.M., "Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin/CD209 is abundant on macrophages in the normal human lymph node and is not required for dendritic cell

stimulation of the mixed leukocyte" reaction, 2005, *Journal of Immunology*, Vol. 175, pp. 4265–4273.

[62] Krutzik S.R., Tan B., Li H., Ochoa M.T., Liu P.T., Sharfstein S.E, Graeber T.G., Sieling P.A., Liu Y.J., Rea T.H., Bloom B.R., Modlin R.L., "TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells", *Nature Med,* 2005, Vol. 11, 653–660.

[63] Lee B., Leslie G., Soilleux E., O'Doherty U., Baik S., Levroney E., Flummerfelt K., Swiggard W., Coleman N., Malim M., Doms R.W., "*Cis* expression of DC-SIGN allows for more efficient entry of human and simian immunodeficiency viruses via CD4 and a coreceptor", *Journal of Virology*, 2001, Vol. 75, No. 24, pp. 12028–12038.

[64] Rappocciolo G., Piazza P., Fuller C.L., Reinhart TA., Watkins S.C., Rowe D.T., Jais M., Gupta P., Rinaldo C.R., "DC-SIGN on B lymphocytes is required for transmission of HIV-1 to T lymphocytes", *PLoS Pathogen*, 2006, Vol. 2, No. 7, pp. 691-704.

[65] Rappocciolo G., Hensler H.R., Jais M., Reinhart T.A., Pegu A., Jenkins F.J., Rinaldo C.R., "Human herpesvirus 8 infects and replicates in primary cultures of activated B lymphocytes through DC-SIGN", *Journal of Virology*, 2008, Vol. 82, No.10, pp. 4793-4806.

[66] Boukour S., Massé J.M., Bénit L., Dubart-Kupperschmitt A., Cramer E.M., "Lentivirus degradation and DC-SIGN expression by human platelets and megakaryocytes", *Journal of Thrombosis and Haemostasis*, 2006, Vol. 4, No. 2, pp. 426-435.

[67] Chaipan C., Soilleux E.J., Simpson P., Hofmann H., Gramberg T., Marzi A., Geier M., Stewart E.A., Eisemann J., Steinkasserer A., Suzuki-Inoue K., Fuller G.L, Pearce A.C., Watson S.P., Hoxie J.A., Baribaud F., Pöhlmann S., "DC-SIGN and CLEC-2 mediate human immunodeficiency virus type 1 capture by platelets", *Journal of Virology*, 2006, Vol. 80, No.18, pp. 8951-8960.

[68] Van Kooyk Y., Geijtenbeek T.B.H., "A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions*", Immunological Reviews*, 2002, Vol. 186, pp. 47-56.

[69] Zhou T., Chen Y., Hao L., Zhang Y. "DC-SIGN and Immunoregulation*", Cellular & Molecular Immunology*, 2006, Vol. 3, No.4, pp. 279-283.

[70] García-Vallejo J.J., van Liempt E., da Costa Martins P., Beckers C., van het Hof B., Gringhuis S.I., Zwaginga J.J., van Dijk W., Geijtenbeek T.B.H, van Kooyk Y., van Die I., "DC-SIGN mediates adhesion and rolling of dendritic cells on primary

human umbilical vein endothelial cells through LewisY antigen expressed on ICAM-2", *Molecular Immunology*, 2008, Vol. 45, No. 8, pp. 2359-2369.

[71] van Gisbergen K.P., Ludwig I.S., Geijtenbeek T.B.H, van Kooyk Y., "Interactions of DC-SIGN with Mac-1 and CEACAM1 regulate contact between dendritic cells and neutrophils", *FEBS Letters,* 2005, Vol. 579,No. 27, pp. 6159-6168.

[72] van Kooyk Y., Geijtenbeek T.B.H., "DC-SIGN: escape mechanism for pathogens", *Nature Reviews Immunology*, 2003, Vol. 3, No. 9,pp. 697-709.

[73] Lozach P.Y., Burleigh L., Staropoli I., Navarro-Sanchez E., Harriague J., Virelizier J.L., Rey F.A., Desprès P., Arenzana-Seisdedos F., Amara A., "Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals", *Journal of Biological Chemistry*, 2005, Vol. 280, No. 25, pp. 23698-23708.

[74] Alvarez C.P., Lasala F., Carrillo J., Muñiz O., Corbí A.L., Delgado R.l, "C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in *cis* and in trans", *Journal of Virology*, 2002, Vol. 76, No. 13, pp. 6841-6844.

[75] Halary F., Amara A., Lortat-Jacob H., Messerle M., Delaunay T., Houlès C., Fieschi F., Arenzana-Seisdedos F., Moreau J.F., Déchanet-Merville J., "Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell trans-infection", *Immunity,* 2002, Vol. 17, No. 5,pp. 653-664.

[76] Cormier E.G., Durso R.J., Tsamis F., Boussemart L., Manix C., Olson W.C., Gardner J.P., Dragic T., "L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus", *Proceedings of the National Academy of Sciences USA*, 2004, Vol. 101, No. 39, pp.14067-14072.

[77] Jain P., Manuel S.L., Khan Z.K., Ahuja J., Quann K., Wigdahl B., "DC-SIGN Mediates Cell-Free Infection and Transmission of Human T-Cell Lymphotropic Virus Type 1 by Dendritic Cells", *Journal of Virology*, 2009, Vol. 83,No. 21 pp.10908-10921.

[78] de Jong M.A., de Witte L., Bolmstedt A., van Kooyk Y., Geijtenbeek T.B.H., "Dendritic cells mediate herpes simplex virus infection and transmission through the C-type lectin DC-SIGN", *Journal of General Virology*, 2008, Vol. 89, No. 10, pp. 2398-2409.

[79] Lozach P.Y., Kühbacher A., Meier R., Mancini R., Bitto D., Bouloy M., Helenius A., "DC-SIGN as a Receptor for Phleboviruses", *Cell Host & Microbe*, 2011, Vol. 10, No.1, pp. 75-88.

[80] Adams W.C., Bond E., Havenga M.J., Holterman L., Goudsmit J., Karlsson Hedestam G.B., Koup R.A., Loré K., "Adenovirus serotype 5 infects human dendritic cells via a coxsackievirus-adenovirus receptor-independent receptor pathway mediated by lactoferrin and DC-SIGN", *Journal of General Virology*, 2009, Vol.90, Pt 7, pp. 1600-1610.

[81] Günther P.S., Mikeler E., Hamprecht K., Schneider-Schaulies J., Jahn G., Dennehy K.M., "CD209/DC-SIGN mediates efficient infection of monocyte-derived dendritic cells by clinical adenovirus 2C isolates in the presence of bovine lactoferrin", *Journal of General Virology*, 2011Vol. 92, Pt 8, pp.1754-1759.

[82] Cambi A., Gijzen K., de Vries J.M., Torensma R., Joosten B., Adema G.J., Netea M.G., Kullberg B.J., Romani L., Figdor C.G., "The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells", *European Journal of Immunology*, 2003,Vol. 33, No. 2, pp. 532-538.

[83] van Die I., van Vliet S.J., Nyame A.K., Cummings R.D., Bank C.M., Appelmelk B., Geijtenbeek T.B.H, van Kooyk Y., "The dendritic cell-specific C-type lectin DC-SIGN is a receptor for Schistosoma mansoni egg antigens and recognizes the glycan antigen Lewis x", *Glycobiology*, 2003, Vol. 13,No. 6, pp. 471-478.

[84] Hirbod T., Kaldensjö T., Lopalco .L, Klareskog E., Andersson S., Uberti-Foppa C., Ferrari D., Manghi M., Andersson J., Loré K., Broliden K., "Abundant and superficial expression of C-type lectin receptors in ectocervix of women at risk of HIV infection", *Journal of Acquired Immune Deficience Syndrome*, 2009, Vol. 51, No.3, pp. 239-247.

[85] Hirbod T., Bailey R.C., Agot K., Moses S., Ndinya-Achola J., Murugu R., Andersson J., Nilsson J., Broliden K., "Abundant expression of HIV target cells and C-type lectin receptors in the foreskin tissue of young Kenyan men", *American Journal of Pathology*, 2010, Vol. 176, No.6, pp. 2798-2805.

[86] Wu L., Wu L., KevalRamani V.L., "Dendritic-cell interactions with HIV: infection and viral dissemination", *Nature review of Immunology*, 2006, Vol. 6, No.11, pp. 859-868.

[87] Moris A., Nobile C., Buseyne F., Porrot F., Abastado J.P., Schwartz O., "DC-SIGN promotes exogenous MHC-I-restricted HIV-1 antigen presentation", *Blood*, 2004, Vol. 103, No. 7, pp. 2648–2654.

[88] Moris A., Pajot A., Blanchet F., Guivel-Benhassine F., Salcedo M., Schwartz O., "Dendritic cells and HIV-specific CD4 T cells: HIV antigen presentation, T-cell activation, and viral transfer", *Blood*, Vol. 108, 2006, No. 5, pp. 1643–1651.

[89] Kwon D.S., Gregorio G., Bitton N., Hendrikson W.A., Littman D.R., "DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection", *Immunity*, 2002, Vol. 16, No.1, pp.135-144.

[90] Cambi A., de Lange F., van Maarseveen N.M., Nijhuis M., Joosten B., van Dijk E.M., de Bakker B..I, Fransen J.A., Bovee-Geurts P.H., van Leeuwen F.N., Van Hulst N.F., Figdor C.G., "Microdomains of the C-type lectin DC-SIGN are portals for virus entry into dendritic cells", *Journal of Cellular Biology*, 2004, Vol. 164, No.1, pp.145-155.

[91] McDonald D., Wu L., Bohks S.M., KewalRamani V.N., Unutmaz D., Hope T.J., "Recruitment of HIV and its receptors to dendritic cell–T cell junctions", *Science*, 2003, Vol. 300, 1295–1297.

[92] Arrighi J.F., Pion M., Garcia E., Escola J.M., van Kooyk Y., Geijtenbeek T.B.H., Piguet V., "DC-SIGN-mediated infectious synapse formation enhances X4 HIV-1 transmission from dendritic cells to T cells", *Journal of Experimental Medicine*, 2004, Vol. 200, No. 10, pp. 1279–1288.

[93] Garcia E., Pion M., Pelchen-Matthews A., Collinson L., Arrighi J.F., Blot G., Leuba F., Escola J.M., Demaurex N., Marsh M., Piguet V., "HIV-1 trafficking to the dendritic cell–T-cell infectious synapse uses a pathway of tetraspanin sorting to the immunological synapse", *Traffic*, 2005, Vol. 6, No. 6, pp. 488–501.

[94] Wiley R.D., Gummuluru S., "Immature dendritic cells derived exosomes can mediate HIV-1 trans infection", *Proc. Natl. Acad. Sci. USA*, 2006, Vol. 103, No.3, pp.738-743.

[95] Izquierdo-Useros N., Naranjo-Gómez M., Archer J., Hatch S.C., Erkizia I., Blanco J., Borràs F.E., Puertas M.C., Connor J.H., Fernández-Figueras M.T., Moore L., Clotet B., Gummuluru S., Martinez-Picado J., "Capture and transfer of HIV-1 particles by mature dendritic cells converges with the exosome-dissemination pathway", *Blood*, 2009, Vol. 113, No.12, pp. 2732-2741.

[96] Saïdi H., Magri G., Carbonneil C., Bouhlal H., Hocini H., Belec L., "Apical interactions of HIV type 1 with polarized HEC-1 cell monolayer modulate R5-HIV type 1 spread by submucosal macrophages", *AIDS Research Human Retroviruses*, 2009 Vol. 25, No.5, pp. 497-509.

[97] Cassol E., Cassetta L., Alfano M., Poli G., "Macrophage polarization and HIV-1 infection", *Journal of Leukocyte Biology*, 2010, Vol 87, No. 4, pp. 599-608.

[98] Lee B., Leslie G., Soilleux E., O'Doherty U., Baik S., Levroney E., Flummerfelt K., Swiggard W., Coleman N., Malim M., Doms R.W., "*Cis* expression of DC-SIGN allows for more efficient entry of human and simian immunodeficiency viruses via

CD4 and a co-receptor", *Journal of Virology*, 2001,Vol. 75., Vol. 24, pp. 12028-12038.

[99] Turville S.G., Santos J.J, Frank I., Cameron P.U., Wilkinson J., Miranda-Saksena M., Dable J., Stössel H., Romani N., Piatak M., Lifson J.D., Pope M., Cunningham A.L., "Immunodeficiency virus uptake, turnover, and 2-phase transfer in human dendritic cells", *Blood*, 2004, Vol. 103, No.6,pp. 2170-2179.

[100] Soilleux E.J., Morris L.S., Lee B., Pöhlmann S., Trowsdale J., Doms R.W., Coleman N., "Placental expression of DC-SIGN may mediate intrauterine vertical transmission of HIV", *Journal of Pathology*, 2001, Vol. 195, No.5, pp. 586-92.

[101] Da Silva R.C., Segat L., Crovella S., "Role of DC-SIGN and L-SIGN receptors in HIV-1 vertical transmission", *Human Immunology*, 2011, Vol. 72, No. 4, pp. 305-311.

[102] Ludwig I.S., Lekkerkerker A.N., Depla E., Bosman F., Musters R.J., Depraetere S., van Kooyk Y., Geijtenbeek T.B.H, "Hepatitis C Virus Targets DC-SIGN and L-SIGN To Escape Lysosomal Degradation", *Journal of Virology*, 2004, Vol.78, No.15, pp. 8322-8332.

[103] Lai W.K., Sun P.J., Zhang J., Jennings A., Lalor P.F., Hubscher S., McKeating J.A., Adams D.H., "Expression of DC-SIGN and DC-SIGNR on Human Sinusoidal Endothelium A Role for Capturing Hepatitis C Virus Particles", *American Journal of Pathology*, 2006, Vol. 169, No.1, pp.200-208.

[104] Geijtenbeek T.B.H, Koopman G., van Duijnhoven G.C., van Vliet S.J., van Schijndel A.C., Engering A., Heeney J.L., van Kooyk Y., "Rhesus macaque and chimpanzee DC-SIGN act as HIV/SIV gp120 trans-receptors, similar to human DC-SIGN", *Immunol Lett*., 2001, Vol. 79, No.1-2, pp. 101-107.

[105] Baribaud F., Pöhlmann S., Sparwasser T., Kimata M.T., Choi Y.K., Haggarty B.S., Ahmad N., Macfarlan T., Edwards T.G., Leslie G.J., Arnason J., Reinhart T.A., Kimata J.T., Littman D.R., Hoxie J.A., Doms R.W., "Functional and antigenic characterization of human, rhesus macaque, pigtailed macaque, and murine DC-SIGN", *Journal of Virology*, 2001, Vol. 75, No. 21, pp.10281-10289.

[106] Park C.G., Takahara K., Umemoto E., Yashima Y., Matsubara K, Matsuda Y, Clausen BE, Inaba K, Steinman RM., "Five mouse homologues of the human dendritic cell C-type lectin, DC-SIGN", *International Immunology,* 2001, Vol.13, No. 10, pp.1283-1290.

[107] Regan A.D., Ousterout D.G., Whittaker G.R., "Feline lectin activity is critical for the cellular entry of feline infectious peritonitis virus", *Journal of Virology*, 2010, Vol. 84, No.15, pp. 7917-7921.

[108] Yamakawa Y., Pennelegion C., Willcocks S., Stalker A., Machugh N., Burt D., Coffey T.J., Werling, "Identification and functional characterization of a bovine orthologue to DC-SIGN", *Journal of Leukocyte Biology*, 2008, Vol.83, No.6, pp.1396-1403.

[109] den Dunnen J., Gringhuis S.I., Geijtenbeek T.B.H, "Innate signaling by the C-type lectin DC-SIGN dictates immune responses", *Cancer Immunology, Immunotherapy*, 2009, Vol. 58, No.7, pp.1149-1157.

[110] Gringhuis S.I., den Dunnen J., Litjens M., van Het Hof B., van Kooyk Y., Geijtenbeek T.B.H, "C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dipendent acetylation of transcription factor NF-κB", *Immunity*, 2007, Vol. 26, pp. 605-616.

[111] Hodges A., Sharrocks K., Edelmann M. Baban D., Moris A., Schwartz O, Drakesmith H., Davies K., Kessler B., Mc Michael A., Simmons A., "Activation of the lectin DC-SIGN induces an immature dendritic cell phenotype triggering Rho-GTPase activity required for HIV-1 replication", *Nature Immunology*, 2007, Vol.8, No.6, pp. 569–577.

[112] Gringhuis S.I., van der Vlist M., van den Berg L.M., den Dunnen J., Litjens M., Geijtenbeek T.B.H, "HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells", *Nature Immunology*, 2010, Vol. 11, No. 5, pp. 363-365.

[113] Appelmelk B.J, van Die I., van Vliet S.J., Vandenbroucke-Grauls C.M., Geijtenbeek T.B.H, van Kooyk Y., "Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells", *Journal of Immunology*, 2003, Vol. 170, No.4, pp. 1635-1639.

[114] Balzarini J. "Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy", *Nature Reviews Microbiology*, 2007, Vol.5, No. 8, pp. 583-597.

[115] Reina J.J., Bernardi A., Clerici M., Rojo J., "HIV microbicides: state-of-the-art and new perspectives on the development of entry inhibitors", *Future Medicine Chemistry,* 2010, Vol. 2, No.7, pp.1141-1159.

[116] Wang S.K., Liang P.H., Astronomo R.D., Hsu T.L, Hsieh S. L, Burton, Wong C.H., "Targeting the carbohydrates on HIV-1: Interaction of oligomannose dendrons with human monoclonal antibody 2G12 and DC-SIGN", *Proceedings of the National Academy of Sciences USA*, 2008, Vol. 105, No.10, pp.3690-3695.

[117] Adams E.W., Ratner D.M., Bokesch H.R., McMahon J.B., O'Keefe B.R., Seeberger P.H., Oligosaccharide and glycoprotein microarrays as tools in HIV

glycobiology; glycan-dependent gp120/protein interactions, *Chemistry & Biology,* 2004 Vol. 11, No.6, pp. 875-881.

[118] Reina J.J., Sattin S., Invernizzi D., Mari S., Martínez-Prats L., Tabarani G., Fieschi F., Delgado R., Nieto P.M., Rojo J., Bernardi A., "1,2-Mannobioside mimic: synthesis, DC-SIGN interaction by NMR and docking, and antiviral activity", *ChemMedChem*, 2007, Vol. 2, pp. 1030- 1036.

[119] Wang S.K. et al. "Targeting the carbohydrates on HIV-1: Interaction of oligomannose dendrons with human monoclonal antibody 2G12 and DC-SIGN", *Proc. Natl. Acad. Sci. USA*, 2008,Vol. 105, No 10, pp.3690-3695.

[120] Martinez-Avila O., Bedoya L. M., Marradi M., Clavel, C., Alcami J., Penades S., "Multivalent Manno-Glyconanoparticles inhibit DC-SIGN-mediated HIV-1 trans-infection of human T cells", *ChemBiochem*, 2009, Vol. 10, pp.1806-1809.

[121] Nguyen D.G., Hildreth J.E., "Involvement of macrophage mannose receptor in the binding and transmission of HIV by macrophages", *European Journal of Immunology*, 2003, Vol. 33, No.2, pp. 483-493.

[122] Lai J., Bernhard O.K., Turville S.G., Harman A.N., Wilkinson J., Cunningham A.L., "Oligomerization of the macrophage mannose receptor enhances gp120-mediated binding of HIV-1", *Journal of Biological Chemistry*, 2009, Vol. 284, No.17, pp.11027-11038.

[123] Lambert A.A., Gilbert C., Richard M., Beaulieu A.D., Tremblay M.J., "The C-type lectin surface receptor DCIR acts as a new attachment factor for HIV-1 in dendritic cells and contributes to trans- and *cis*-infection pathways", *Blood*, Vol. 112, No.4, pp.1299-1307.

[124] de Witte L., Nabatov A., Geijtenbeek T.B.H., "Distinct roles for DC-SIGN+-dendritic cells and Langerhans cells in HIV-1 transmission", *Trends in Molecular Medicine*, 2008, Vol.14, No.1, pp.12-19.

[125] de Witte L., Nabatov A., Pion M., Fluitsma D., de Jong M.A., de Gruijl T., Piguet V., van Kooyk Y., Geijtenbeek T.B.H., "Langerin is a natural barrier to HIV-1 transmission by Langerhans cells", *Nature Medicine*, 2007, Vol. 13, No.3, pp. 367-371.

[126] van der Merwe P.A., "Surface plasmon resonance", users.path.ox.ac.uk/~vdmerwe/internal/spr.pdf., pp1-50.

[127] Tabarani G., Thépaut M., Stroebel D., Ebel C., Vives C., Vachette P., Durand D., Fieschi F., "DC-SIGN neck domain is a pH sensor controlling oligomerization: SAXS and hydrodynamic studies of extracellular domain", *Journal of Biological Chemistry*, 2009, Vol. 284, No. 32, pp. 21229–21240.

[128] Luczkowiak J., Sattin S., Sutkevičiūtė I., Reina J.J., Sánchez-Navarro M., Thépaut M., Martínez-Prats L., Daghetti A., Fieschi F., Delgado R., Bernardi A., Rojo J., "Pseudosaccharide functionalized dendrimers as potent inhibitors of DC-SIGN dependent Ebola pseudotyped viral infection", *Bioconjugate Chemistry*, 2011, Vol. 22, pp. 1354–1365.

[129] Thépaut M., Valladeau J., Nurisso A., Kahn R., Arnou B., Vivès C., Saeland S, Ebel C, Monnier C, Dezutter-Dambuyant C, Imberty A, Fieschi F. "Structural studies of Langerin and Birbeck granule: a macromolecular organization model", *Biochemistry*, 2009, Vol. 48, No.12, pp. 2684-2698.

[130] Andreini M., Doknic D., Sutkeviciute I., Reina J.J., Duan J, Chabrol E., Thepaut M., Moroni ., Doro F., Belvisi L., Weiser J., Rojo J., Fieschi F., Bernardi A., "Second generation of Fucose-based DC-SIGN ligands: affinity improvement and specificity versus to Langerin", *Organic & Biomolecular Chemistry*, 2011, Vol. 9, No. 16, pp. 5778-5786.

[131] Timpano G., Tabarani G., Anderluh M., Invernizzi D., Vasile F., Potenza D., Nieto P. M., Rojo J., Fieschi F., Bernardi A., "Synthesis of novel DC-SIGN Ligands with an α-fucosylamide anchor", *ChemBioChem*, 2008, Vol. 9, No. 12, pp. 1921–1930.

[132] Mitchell D.A., Jones N.A., Hunter, S.J., Cook J.M.D., Jenkinson S.F., Wormald M.R., Dwek R.A., Fleet G.W.J., "Synthesis of 2-C-branched derivatives of D-mannose: 2-C-aminomethyl-Dmannose binds to the human C-type lectin DC-SIGN with affinity greater than an order of magnitude compared to that of D-mannose", *Tetrahedron: Asymmetry*, 2007, Vol. 18, 1502–1510.

[133] Wu L., Martin T.D., Carrington M., KewalRamani V.N., "Raji B cells, misidentified as THP-1 cells, stimulate DC-SIGN-mediated HIV transmission", *Virology*, 2004, Vol. 318, No. 1, pp. 17-23.

[134] Eckstein P., Jackson M.C., Millman N., Sobrero A.J., "Comparison of vaginal tolerance tests of spermicidal preparations in rabbits and monkeys", *Journal of Reproduction and Fertility,* 1969, Vol. 20: 85-93.